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Research article

Reactions to benign and malignant human cell implants by the epiblast-hypoblast layers of the primitive streak stage chick embryoblast

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Abstract

The reactions to benign and malignant human cell implants between the epiblast-hypoblast layers of the primitive streak stage chick embryo were studied. Benign human breast, human colonic tissues as well as samples from malignant breast, colonic and rectal tissues were used. Specimens of human colonic cell line (HT-29) and a nasal squamous cell carcinoma cell line (RPMI-2650) were included. The chick primitive streak embryoblasts, each with two implants inserted, were cultivated to the 14 somite stage. After fixation and processing, ectodermal, mesodermal and endodermal proliferative reactions were studied. No difference between benign and malignant implant movements within the embryo were observed. Benign implants evoked a mesodermal response while the malignant cell implants elicited significant proliferative ectodermal and endodermal responses. We concluded that the chick embryoblast model can distinguish between human benign and malignant cell implants on the basis of endodermal and ectodermal responses.

Keywords: chick embryoblast, implantation, human tumour cells, epiblast-hypoblast proliferative reactions, primitive streak chick embryo

Abbreviations

MD: morphogenetic displacement; ECTPR: host ectodermal proliferative response; HMR: host mesodermal proliferative response; ENDPR: endodermal proliferative response; EMA: epithelial membrane antigen

Introduction

The behavior of neoplastic cells placed in an embryonic environment might give information on their properties of organization, motility and cell surface reactions. The specificity of their interactions with embryonic tissues, particularly induction of proliferative responses is an important epigenetic process [1].

Cancer cells, implanted into the chick embryoblast, moved within and induced specific changes in chick embryonic tissues as described by Lakshmi and Sherbet [2]. These were described as (a) the apparent movement of implants in the embryo, which they termed morphogenetic displacement (MD), (b) the host mesodermal response (HMR), (c) the ectodermal proliferative response (ECT PR) and (d) the endodermal proliferative response (END PR). Sherbet summarised his views in an exhaustive work on the biology of tumour malignancy and included his work on implantation of tumour cells into chick embryoblasts [3]. He devised a tumour grading system based on the observations of (a), (b), (c) and (d) above. He coined the acronym EGG for this epigenetic grading system. He concluded that it could distinguish benign from malignant implants and could grade the degree of malignancy in implants.

Palayoor and Batra supported Sherbet's findings using mouse mammary carcinoma and normal kidney [4]. Mulherkar, using mouse mammary carcinoma, found no inductive reactions [5]. Mareel et al. found an epiblast reaction to He La cells [6]. They also implanted a series of different types of malignant cells (He La, Hepatoma, polyoma transformed baby hamster kidney and mouse mammary carcinoma) to show that they inhibited endodermal repair at the implant site [7,8]. They did not observe ECT PR to implants. Kawamura and Ito implanted Morris Hepatoma and normal rat liver and concluded that their findings were inconsistent with Sherbet's [9].

With these conflicting reports we tested human tissues in the chick embryonic system, to try to reproduce Sherbet's findings. We found no difference in the MD between benign and malignant cell implants. Benign implants evoked mainly HMR. Malignant implants evoked mainly ECT PR and END PR. The responses to malignant implants could not be quantified or graded. We concluded that the EGG system was not feasible but that the chick embryonic system can distinguish between benign and malignant implants.

Aims of the Study

We aimed to see the reactions of the primitive-streak stage chick embryo epiblast-hypoblast layers, to benign and malignant human tumour cell pellets implanted between them.

Materials and Methods

A standard bacteriological incubator was used for incubating the eggs and explanted embryoblasts and tap water provided humidity at 60%. Heat resistant Pyrex glassware and an aluminium egg separator were sterilised at 200°C overnight. An ultra violet light cabinet provided a sterile environment for the cooling glassware, in particular a large open Pyrex[R] dish.

A Nikon SMZ10 [R] dissecting microscope facilitated implantation of cells in the embryoblast. Steriised J cloth was cut into rings to fit inside the Petri dish underneath the watch glass (Johnson & Johnson[R]). Freshly fertilised broiler hens' eggs (Gallus Domesticus) of Arbour Acres breed were obtained twice weekly and stored at room temperature until placed in the incubator. Eggs were used within three days of receipt and the fertility rate varied between 80 to 90%. The use of chick embryoblasts for research was in accordance with national and international guidelines.

Pyrex glass tubing with 1mm wall thickness and 10mm inside diameter, was cut in 5mm. lengths to give glass rings to hold the vitelline membrane in place on a watch glass. A fine stainless steel probe was made by cutting a 3 cm. length of 0.035mm wire and placed in a glass Pasteur pipette and flamed into position. The protruding wire, cut obliquely to a length of 0.5cm, was used to incise the epiblast.

Defined Medium 199 was used for transport of the cells or tissue under study. (Flow Laboratories England, supplied by Medlabs Ltd. Ireland). Dulbecco 'A' solution was used to float the egg yolk and harvest the vitelline membrane with the attached primitive streak (Oxoid Ltd).

Pannett-Compton solution, the supernatant left over after mixing Dulbecco 'A' and 'B ' (Ca++,Mg++ salt solution), was used to cover the embryoblast in culture. Natural medium thin egg white albumen, the nutrient medium of the embryoblasts, was recovered after separation of albumen and the egg yolk in the sterilised aluminium separator. An essential growth stimulator, its colloid osmotic pressure is of great physiological importance for the exchange of water in the embryo [10].

Methylene blue chroma IB 429 1 mg/100 ml (MB) in Pannett-Compton solution was the vital dye used to typify fibroadenoma breast nodules (Chromagesellschaft,Stuggart-Unterpurkheim, West Germany).

Benign Cells for Implantation

Breast tissue

Benign human breast biopsies of fibroadenomas and fibrocystic disease were harvested under sterile conditions. Placed in Medium 199, chopped in 3mm pieces they were stored at 40°C until required within 1-2 hours. Pieces of 0.25 mm sides were used for implantation. Some were stained with MB to aid dissection of breast nodules.

Normal Colonic cells

Normal colonic cells from biopsy specimens at colonoscopy were collected in a sterile container with an antibiotic cocktail. They were washed at least 4 times by vigorous shaking in fresh antibiotic cocktail (500ml. Earle's balanced salt solution (EBSS) (IX), 10 ml fungizone(4ug/ml), 15 ml gentamycin (150ug/ml), 20 ml Penicllin (200 u/ml), streptomycin (200 ug/ml)).

Malignant cells

Malignant cells from human breast carcinoma biopsies, human tumour colonic biopsies, human rectal biopsies were obtained under the same conditions as for normal colonic cells. Tissue culture cells of human colonic tumour HT-29 and of a human nasal carcinoma RPMI-2650 were also used.

Single cells and aggregates, dislodged from solid tumours like carcinomas were aspirated and centrifuged at 400g for 4 minutes. The pellet was resuspended in 50% EBSS and 50% antibiotic cocktail and washed several times. It had approxi-

Table 1. Benign breast biopsy implants.

Type of tissue	A A B C C C C D E
Emb. implanted	8 8 5 7 7 5 8 11 9
Emb. survived	7 4 4 7 4 5 6 8 8
% survival	89 50 80 100 5 100 75 73 89
Number of implants	14 8 8 14 8 10 12 16 16
MD +	2 6 2 4 2 1 8 13 7
%	14 76 25 29 25 10 67 88 44
HMR	14 8 7 12 8 6 6 14 16
%	100 100 88 86 60 60 50 88 100
ECT PR	0 0 0 0 0 0 0 0 0
END PR i	0 0 0 0 0 0 0 0 0

A. Fibrocystic disease, B. Normal breast C. Fibroadenoma, D. Fibro-adenosis, E. Gynaecomastia Implanted embryo mean survival rate was 70%. MD ranged between 12-75%, mean 42. HMR was 50-100% (mean 86%). No ECT PR nor END PR was observed (Figures 1,2).

Table 2. Malignant breast biopsy implants

Type of tissue	A A A A A A A A A
Emb.Implanted	8 10 6 5 5 8 7 8 5
Emb. Survived	8 5 5 4 5 7 7 5 5
Implants	16 10 10 8 10 14 14 10 10
MD +	3 7 8 6 7 8 3 5 9
%	18 70 80 75 70 74 21 50 90
HMR	NA NA 0 NA 0 0 0 NA NA
ECT PR	NA NA 8 NA 8 10 6 NA NA
END PR	NA NA 3 NA 2 2 4 NA NA

A: Infiltrating breast carcinoma. Two implants per embryo(Emb). NA : Not assessed as implants not found. Post implantation embryonic survival ranged from 50 -100% with a mean of 81%. MD ranged from 21 -90%, (mean 62 %). Because of processing or cutting difficulties in five, embryonic responses were not assessable (NA). In the other 4 there was no HMR observed. ECT PR varied from 43-80% with a mean of 69% in survivors. ECT PR was the predominant reaction noted with little or no mesodermal responses (Figures 3, 4).

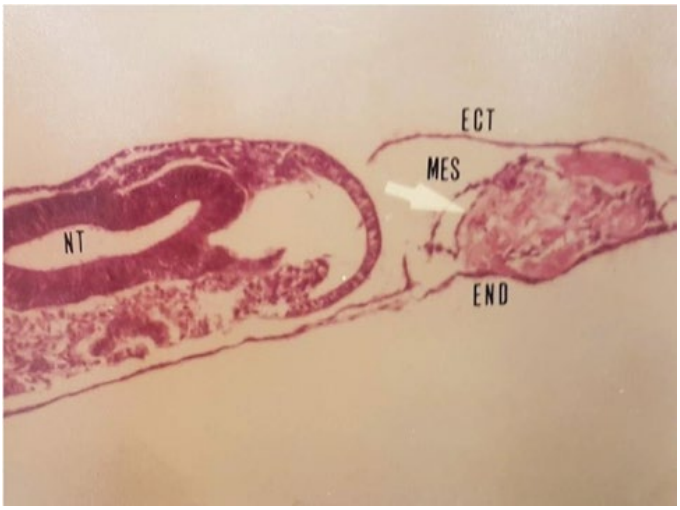

Figure 1. Benign breast implant (arrow) with no ECT PR or END PR Neural tube(NT).

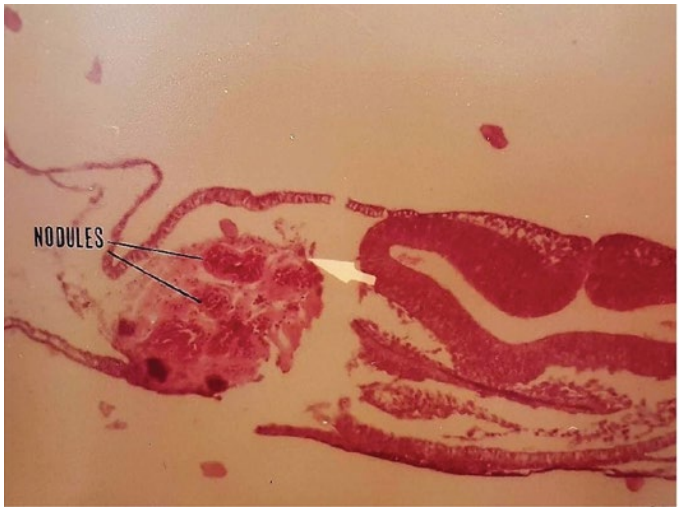

Figure 2. Epithelial nodules of fibroadenoma implant with no ECT PR or END PR.

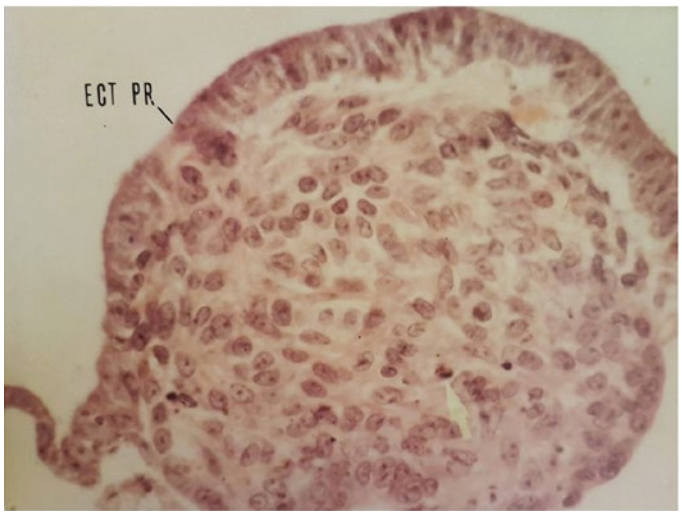

Figure 3. Brest carcinoma implant (Arrow) with ECT PR and pallisading of ECT cells.

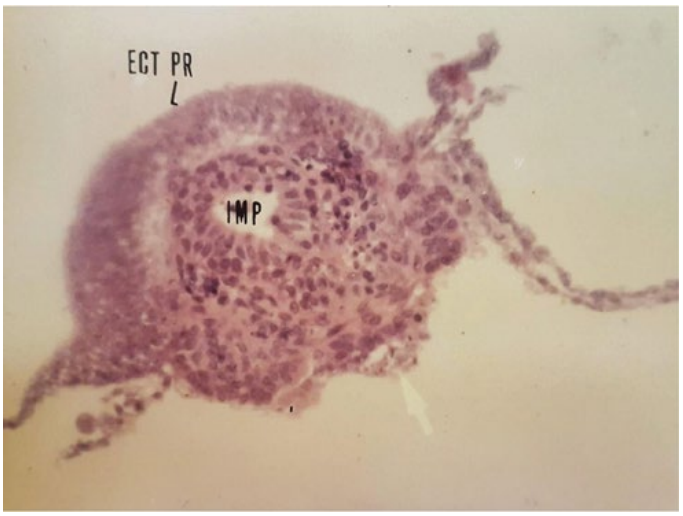

Figure 4. Breast carcinoma implant (IMP) with marked ECT PR x 100.

mately 1 X 10^6 cells when stained and checked on the Coulter Counter.

Cell viability was confirmed on a haemocytometer with 0.25 ml of the cell suspension and 0.05% Trypan Blue together. Human colonic tissue culture HT-29 was originally cultured from a well differentiated colonic adenocarcinoma which originated in the Labaoratory of Dr. Fogh [11]. One million human nasal carcinoma cells came from the RPMI-2650 cell line, passage 53.

Explantation Technique

Egg viability was confirmed by 'candling'

Two dozen eggs were incubated at 37°C. for 20-22 hours at 60 % humidity and then left at room temperature for 1-2 hours before use. The technique of harvesting the embryoblast was described by Sherbet [13]. The vitelline membrane was cut at the equator of the egg yolk and peeled off and floated on to the watch glass. The Pyrex ring was placed on top to steady it with the primitive streak at the centre. The membrane was draped over the edge of the glass ring from outside to in. The watch glass was placed in the Petri dish on top of the J cloth with a hole at its centre. The dish was placed on the stage of the dissecting microscope. Under microscopy an incision was made with the flamed fine wire probe in the epiblast at the edge of the area pellucida above the level of Henson's node. The epiblast and hypoblast were separated to form a pocket on each side in the area pellucida. Pellets of 0.2mm cells were pipetted from their holding solution to the vitelline membrane. Each pellet was pushed with the fine stainless-steel wire probe into the area of the incised epiblast and in turn was pushed into the pocket between epiblast (primitive ectoderm) and hypoblast (primitive endoderm). The covered Petri dish was incubated for 22-24 hours and the embryo grown to 10-14 somite stage (Hamburger and Hamilton stage 10-11). The implant positions were recorded and compared to their initial insertion site (MD).

Fixation and processing of specimens

After fixation in Bouin's fluid, specimens were floated in formalin on a flat piece of paper and placed in a specimen holder for identification in an automatic tissue processor (ShandonR). The specimens were cut square across at the cephalic end and cut to a point at the tail end for orientation before embedding in wax. This aided recognising the cephalic end as the specimen was very small and showed as a small streak of

Table 3. Homogenate of breast carcinoma in groups A, B, C

	A	B	C
Embryos implanted	6	10	10
Embryos survived	2	10	7
Endoderm Healed	2	10	6
ECT PR	2	0	1
END PR	2	0	1
The ECT PR, END PR reaction to the homogenate is clear in Figure 5.			

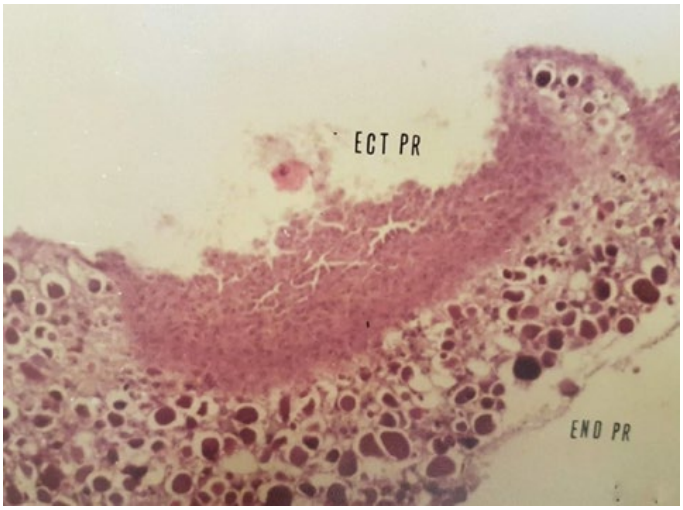

Figure 5. ECT PR and END PR to homogenate of breast carcinoma x 200.

yellow in the block. The wax around it was trimmed carefully so that only a small amount surrounded the specimen. It was sectioned from head to tail by the microtome. Serial ribboned 8u thick sections, placed in sequence in two rows of 25-30 sections on each slide, fitted a 400-500u piece of embryo on each glass slide. Each sectioned embryonic specimen fitted on 5 to 6 slides. Stained with haemotoxylin and eosin, sections were studied in sequence from head to tail under the microscope looking for the cellular implants. To confirm epithelial implants, EMA was shown as a brown colour using an immuno-histochemistry kit supplied by Vector Laboratories, 1429, Rollins Road Burlingome California 94010 USA . (Vecta StainR ABC Kit).

Embryonic responses

The MD of benign and malignant implants were compared using the U-Mann Whitney test with a two tailed P, as the numbers compared were very small.

Comparison of the ectodermal proliferative responses between different malignant implants was made using Student's T test (Armitage 1971).

Embryonic layer reactions and implant movements were recorded according to Sherbet as follows:

1. Host mesodermal proliferative response HMR
2. Ectodermal proliferative response to implants ECT PR
3. Endodermal proliferative response END PR
4. Morphogenetic displacement MD [15]

	Normal colonic cells	colonic polyp cells
Embryos implanted	10	5
Embryos survived	10	1
Number of implants	20	2
MD	0	0
HMR	16	0
ECT PR	0	2
END PR	0	2

HMR was the predominant response with normal colonic cells as seen with benign breast implants with no END PR, ECT PR. Only 1 embryo survived with a colonic polyp implant from polyposis coli and ECT PR and END PR were seen.

Results

Twenty to twenty four hours was sufficient incubation time for primitive streak stage embryoblasts to grow to 10-14 somite stage with a beating heart. (Hamburger and Hamilton stage 10-11). The embryoblast harvesting technique involved very gentle handling of the vitelline membrane in Pannett-Compton solution.

Placing precisely just 0.3 ml of Pannett-Compton solution inside the glass ring onto the embryoblast was crucial to survival of the embryoblast, as we found out at a cost of losing many specimens in the beginning by exceeding that amount.

A total of 216 embryoblasts were implanted, 168 survived to 14 somite stage giving mean survival of 78%. The Figures show histological horizontal cross sections of the embryoblasts with implanted cell clumps. HMR occurred almost exclusively to benign implants of breast and colon. Localised ECT PR and END PR was exclusive to malignant cellular implants. ECT PR was observed with a colonic polyposis implant. MD was no different for benign and malignant implants (Mann-U Whitney two tailed P test, P=0.26). The incidence of localised ECT PR to malignant colorectal tumours and malignant breast implants were compared and no difference could be found. (Student's t test, t =0.92). We recognise that the numbers here were small. Implants of homogenate of breast carcinoma did not inhibit regeneration of endoderm at the site of implantation. On the contrary, marked host ectodermal and endodermal responses were noted at the site. Breast carcinoma implants stained positive for Epithelial Membrane Antigen (EMA).

Discussion

Our results support Sherbet's findings of ECT PR to human breast cancer implants but we found no significant difference in the MD of benign and malignant breast implants. Based our observations, we concluded that the MD of the implants depended on (a) the prospective potency of the epiblast upon which it makes first contact (head, heart or somite), (b) epiblast streaming and (c) the growth of the embryonic axis relative to the site of implantation. We concluded that MD of implants is not a distinguishing factor between benign and malignant cell implants.

The significance of the HMR main response to benign implants is unknown. It is not an immune response as Solomon

Table 5. Human colorectal cell implants.

Type of tissue	A	A	B	B	B	C	C	D
Cells per pellet	1.5	1	2	5.6	1.4	0.25	1.3	1.5
Viability %	81	79	85	40	80	86	79	75
Emb. implanted	7	6	7	7	7	9	8	5
Emb. survived	5	2	5	6	7	5	7	5
Implants	10	4	10	12	14	10	14	10
MD	2	1	5	8	8	7	5	5
HMR	0	0	0	0	0	0	1	0
ECT P R	8	4	4	4	2	6	9	0
%	80	100	40	33	29	60	64	0
END P R	8	4	4	4	2	6	9	0
%	60	20	0	0	0	0	14	0

A. Rectal carcinoma B. Colonic carcinoma C. HT-29 human colonic cell carcinoma D. Metastatic colonic carcinoma from skin. Two implants per embryo (Emb) (Cells per pellet X 10^6). Implantation survival rate was 33-100% (mean 76%). MD was seen in 20-75% (mean 45%). HMR was seen only once. ECT PR rate was 29-80% (mean 38%). A polypoid type of reaction was seen to a rectal malignant tumour implant (Figures 6, 7).

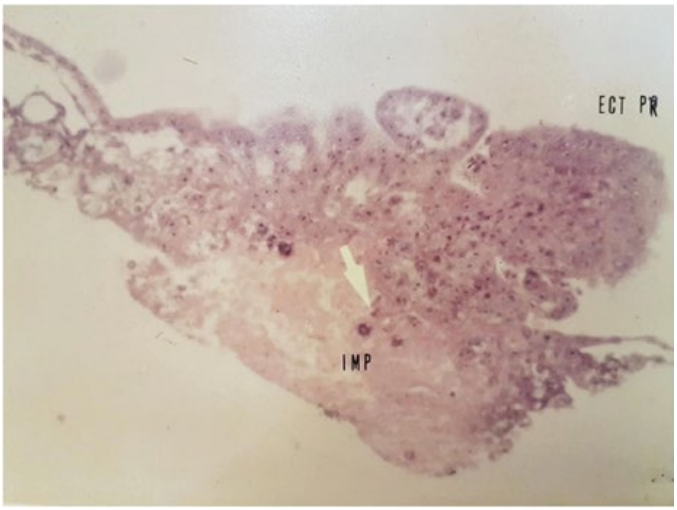

Figure 6. Recta carcinoma implant (IMP) and marked ECT PR X 200.

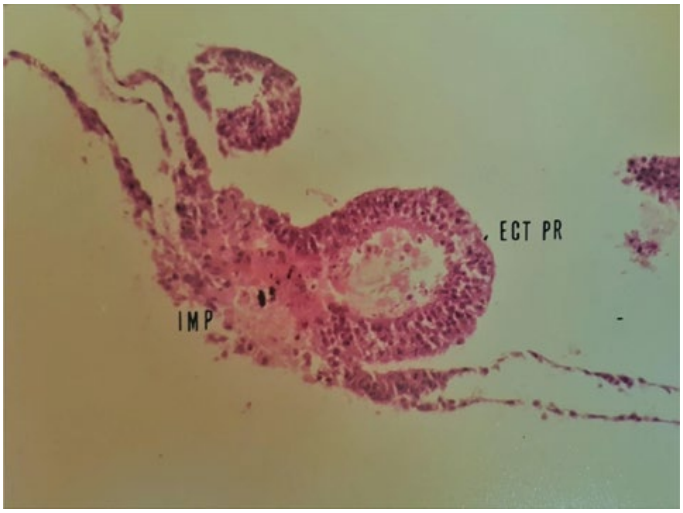

Figure 7. Colonic carcinoma implant (IMP) with ECT, END PR.

demonstrated that immune reactions did not take place in the chick embryo until the 15th day of incubation [15].

The END PR occurred with malignant tumour implants of breast, colorectal, HT-29 and nasal cell squamous tumours but not with benign implants. It nearly always occurred in association with the ECT PR. We disagree with the findings of Mareel et al that the endodermal layer failed to heal over at the site of malignant implants.

ECT PR was the most consistent and exclusive response to the malignant implants of breast, colorectal and nasal cell squamous carcinoma tumours. It was similar to the responses seen to mouse mammary carcinoma implanted by Palayoor and Batra, to He La cells implanted by Mareel and implants of human astrocytoma and human breast by Sherbet and Lakshmi [4,6,2].

This is the first report to replicate Sherbet and Lakshmi's findings with human breast carcinoma cells. It is also the first report chick embryoblast ECT PR to breast cancer homogenate, to polyposis coli, to human colorectal tumour cells, to HT-29 colonic cell line and to human nasal squamous carcinoma RPMI-2650 cell line. ECT PR was not observed with benign implants. Our observation of the ECT pallisading response to breast cancer implants was similar to that reported by Sherbet and Lakshmi [2].

The ECT PR to colorectal tumours was more polypoidal and proliferative than with breast implants. A similar response was seen with implants of breast carcinoma homogenate. It was not possible for us to score the ECT PR and END PR reactions as suggested by Sherbet. Having demonstrated that the MD does not distinguish between benign and malignant implants and that the grading of ECT PR and END PR is arbitrary, we concluded that Sherbet's EGG scoring system has little value in a grading scale of malignant cell implants. A MEDLINE literature review, using the key words in this paper, found no recent research papers on the use of the chick embryoblast implantation technique for human cancer cells. Van Peteghem et al used only the chick hypoblast layer in culture with human malignant cell lines [16].

We speculate that the cancer tumour implants are producing growth factors, such as epidermal growth factor (EGF) or

Table 6. Nasal cell carcinoma from cell line RPMI-2650.

No. cells received	C. X 10^6	
Two implants per embryo		
Cell viability	90%	
Embryos implanted	4	
Embryos survived	3	
Implants 6		
MD	5/6	82%
HMR	0/6	0%
ECT PR	6/6	100%
End P R	0/6	0%

transforming growth factors (TGF) or others, to stimulate the responses observed. This suggests that the primitive epiblast hypoblast layers have receptors for these factors. This could be tested by immunological staining or stimulation with EGF.
This model has great potential in further investigation of cancer cell inductive properties. It raises a question of whether it is possible for cancer cells to induce transformation in mature or stem cells in surrounding tissues.

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