Metabolic co-operation between embryonic and embryonal carcinoma cells of the mouse

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SUMMARY

Mouse embryonal carcinoma (EC) cells form permeable junctions at their homotypic cell-to-cell contacts which permit intercellular exchange of metabolites (metabolic co-operation). Hooper & Slack (1977) showed how this exchange could be detected by autoradiography as the transfer of [3H]nucleotides between PCI3 (a pluripotential EC line) and PCI3-TG8 (a variant of PCI3 which is deficient in hypoxanthine guanine phosphoribosyltransferase). We now show that cells taken from several different tissues of early mouse embryos, that is, from the morula, the inner cell mass of the blastocyst, and the endoderm, mesoderm and embryonic ectoderm of the 8th day egg cylinder, are able to serve as donors of [3H] nucleotides to PCI3TG8. In contrast, trophodermal cells of cultured blastocysts, and the trophodermal derivatives in the 8th day egg cylinder, that is, extra-embryonic ectoderm and ectoplacental cone cells, showed little or no metabolic co-operation with PCI3TG8. With reference to some common properties of EC and embryonic cells, we suggest how our findings may provide insight into cell-to-cell interactions in the early mouse embryo.

INTRODUCTION

Several authors have postulated that the cell-to-cell interactions of embryogenesis may depend in part upon the passage of substances through permeable, intercellular channels (e.g. Pitts, 1976; Sheridan, 1976; Wolpert, 1978). These channels, which are thought to lie within gap junctions (Pitts, 1976), are freely permeable to molecules of up to 1000 daltons (Loewenstein, 1975). Studies on invertebrate (Potter, Furshpan & Lennox, 1966; Tupper & Saunders, 1972), amphibian (Ito & Loewenstein, 1969; Palmer & Slack, 1970), and avian (Sheridan, 1968) embryos have demonstrated cell-to-cell transfer of ions or fluorescent tracer molecules but, as yet, no data are available concerning passage of molecules between cells of early mammalian embryos.

Permeable junctions between the cells of cultured mammalian cell lines may readily be detected using the procedures devised by Subak-Sharpe, Bürk & Pitts (1966, 1969). These authors observed by autoradiography that cells lacking hypoxanthine guanine phosphoribosyltransferase (HGPRT, EC

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2.4.2.8) failed to incorporate $[^3H]$hypoxanthine into nucleic acid when growing in isolation, but did so when growing in contact with wild-type cells. This effect is now attributed to the passage of $[^3H]$nucleotides through permeable, intercellular junctions (Pitts & Simms, 1977). 'Metabolic co-operation' is the name which has been given to this phenomenon of metabolite exchange between cells in contact.

Cell-to-cell communication in the embryo cannot be studied directly by the method of Subak-Sharpe et al. (1966, 1969) since embryos which carry the mutations necessary for these experiments are not available. More feasible is an examination of metabolic co-operation between embryonic cells and embryonal carcinoma (EC) cells. In several respects, mouse EC cells are equivalent to certain pluripotent cells of early mouse embryos. For example, upon injection into a blastocyst, EC cells, like early embryonic cells, can participate in normal embryogenesis (Papaioannou, McBurney, Gardner & Evans, 1975; Mintz & Illmensee, 1975). A valuable feature of EC cells is that they can be adapted to continuous growth in tissue culture. Under these conditions, the cells may be cloned, so that mutants may be selected, and yet they can retain their property of pluripotentiality (e.g. Dewey, Martin, Martin & Mintz, 1977). Metabolic co-operation has been studied extensively in the embryonal carcinoma line PC13 and its variants. Hooper & Slack (1977) obtained an HGPRT$^-$ variant of PC13, called PC13TG8, and made use of this variant to demonstrate that EC cells participate with each other in metabolic co-operation. Subsequently, Slack, Morgan and Hooper (1978) isolated R5/3, a derivative of PC13TG8, which is severely defective in its capacity for metabolic co-operation (Hooper & Morgan, 1979).

In this communication, PC13TG8 and R5/3 are examined for their ability to participate in metabolic co-operation with cells taken from early mouse embryos. Evidence is presented that EC cells co-operate readily with the cells of morulae, inner cell masses, embryonic ectoderm, endoderm and mesoderm tissues, but not with trophectoderm or its derivatives.

MATERIALS AND METHODS

Cell lines and cell culture

PC13TG8 is an HGPRT-deficient EC line. Cells of this line usually retain EC morphology whilst growing in vitro, but they may differentiate to give a variety of cell types when cultured as solid tumours in strain 129 mice (Hooper & Slack, 1977). R5/3 is a metabolic co-operation-defective variant of PC13TG8 (Slack et al. 1978; Hooper & Morgan, 1979). Two HGPRT-deficient fibroblast lines were also used: PyYTG.TGR, a Syrian hamster derivative (Subak-Sharpe, 1965), and STO, a mouse derivative (Martin & Evans, 1975).

Cells were cultured in Alpha FC10 (Alpha medium of Flow Laboratories, Irvine, Scotland, with 10% heat-inactivated foetal calf serum) supplemented
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with nucleosides, as previously described (Adamson, Gaunt & Graham, 1979). The cultures were maintained on gelatin-coated, plastic tissue culture dishes (Sterilin Ltd., Surrey) and were dissociated for sub-culture with TVP (0.025 % Trypsin, 1 mM-Na$_2$ EDTA, 1 % (v/v) chick serum in phosphate-buffered saline lacking Ca$^{2+}$ and Mg$^{2+}$) (Bernstine, Hooper, Grandchamp & Ephrussi, 1973).

**Embryos and embryonic tissue preparation**

The embryos were from natural matings and were either inbred C3H or outbred CFLP stock (Anglia Laboratories Ltd.). The observations made upon metabolic co-operation were the same for embryos of both strains, and the results have been pooled in this communication.

Morulae and blastocysts were obtained on the 3rd and 4th day of pregnancy respectively (the day of the plug being taken as the first day of pregnancy) and the zonae pellucidae were removed using either pronase (Mintz, 1967) or acid Tyrodes solution (Handyside, 1978). Inner cell masses were isolated from blastocysts by immunosurgery (Solter & Knowles, 1975).
Eight different tissues were dissected from 8th day egg cylinders (portrayed in Fig. 1). The germ layers were separated following a 10 min treatment in cold 2-5% pancreatin, 0.5% trypsin solution (Levak-Svajger, Svajger & Skreb, 1969; Dziadek & Adamson, 1978). After isolation, each tissue was suspended for 5 min in TVP, and then dissociated by gentle pipetting to give a mixture of single cells and small clumps.

Co-culture and labelling of embryonic and embryonal carcinoma cells for the detection of metabolic co-operation

[G-3H]Hypoxanthine was purchased from the Radiochemical Centre, Amersham.

Gelatin-coated, 50 mm Petri dishes were seeded with 3·10⁵ EC cells (alternatively, PyYTG.TGR or STO fibroblasts were used in some experiments) in 4 ml of Alpha FC10 with added nucleosides. On the following day, the medium was renewed and embryonic material (morulae, inner cell masses, or cells obtained from the 8th day egg cylinder) was added. After a further 12–16 h, the medium was changed to 2 ml of Alpha FC10 with addition of [3H]hypoxanthine (2 μCi/ml; 1.5 Ci/m-mole). The co-cultures were fixed after a labelling period of 4 h.

For blastocyst/EC cell co-cultures, a different procedure was adopted. Zona-free blastocysts were first cultured until such time (usually the 2nd or 3rd day of culture) as they had formed an outgrowth of trophoblast cells around the central inner cell mass. EC cells (5·10⁴ per dish) were then added. After a further 12–16 h, the co-cultures were labelled with [3H]hypoxanthine as described above.

Fixation, TCA extraction, autoradiography and staining were carried out as described by Slack et al. (1976).

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**Figures 2-5**

Figs. 2, 3. Metabolic co-operation between EC cells and morulae. Morula cells are seen to be heavily labelled with silver grains. PC13TG8 cells in contact with morulae have become labelled by metabolic co-operation (Fig. 2). The gradient of silver grains seen to extend over several EC cell diameters is a consequence of metabolic co-operation between the EC cells themselves. In contrast, R5/3 cells in contact with morulae have not become labelled (Fig. 3); R5/3 is a metabolic co-operation-defective variant of PC13TG8. Bars, 100 μm.

Fig. 4. Absence of metabolic co-operation between EC cells and the trophectoderm of blastocyst outgrowths. After autoradiography, a blastocyst culture appears as a heavily labelled inner cell mass (ICM) surrounded by a more lightly labelled zone of large, flattened trophectoderm cells (troph.). In this field, EC cells are seen to be in contact with trophectoderm cells yet they have failed to become labelled by metabolic co-operation. Bar, 100 μm.

Fig. 5. Metabolic co-operation between EC and inner cell mass cells. The most heavily labelled cells are inner cell mass cells. PC13TG8 cells in direct or indirect contact with the inner cell mass are seen to have become labelled by metabolic co-operation. Bar, 100 μm.
Fig. 6. Metabolic co-operation at (a) embryonic ectoderm/PC13TG8 contacts, (b) extra-embryonic ectoderm/PC13TG8 contacts. In each panel, the upright histogram shows the distribution of grain numbers over HGPRT− (PC13TG8) cells which were seen to be in contact with HGPRT+ (embryo-derived) cells. (Where two or more PC13TG8 cells were in contact with the same wild-type cell, and they were also in contact with each other, a grain count was made upon only the most heavily labelled PC13TG8 cell.) The inverted histogram shows the distribution for isolated PC13TG8 cells in the same culture dish – this distribution thus provides the background range for the autoradiogram. An embryonic/PC13TG8 cell-to-cell contact is regarded as positive for metabolic co-operation if the grain number over the PC13TG8 cell exceeds twice the 90th percentile of the background distribution (arrowed). This value is arbitrary but has been considered to permit a reasonable distinction between co-operation-positive and co-operation-negative contacts (Gaunt & Subak-Sharpe, 1979). Percentage values in brackets are the proportions of HGPRT+/HGPRT− cell-to-cell contacts scored positive for metabolic co-operation.

RESULTS

The morula (3rd day embryo)

Zona-free morulae attached readily to monolayers of PC13TG8 and of R5/3. After incubation of the co-cultures in the presence of [3H]hypoxanthine, followed by autoradiography, the morulae were heavily labelled with silver grains (Figs. 2, 3). PC13TG8 cells in the vicinity of the morulae also became labelled; the pattern of labelling was that of a gradient extending over several cell diameters (Fig. 2). In contrast, R5/3 cells in contact with morulae did not become labelled (Fig. 3). Since R5/3 is known to be defective in permeable junction formation,
we conclude that the spread of radioactivity from morulae to PC13TG8 was due to metabolic co-operation between embryonic and EC cells.

The blastocyst (4th day embryo)

Two distinct tissues were detectable in the blastocyst cultures: a central inner cell mass, and a peripheral zone of trophoblast cells. EC cells attached only rarely to the upper surface of the trophoblast but, adherent to the plastic substrate, they established contact with the outer edge of the trophoblast zone. Metabolic co-operation was not detectable at these trophoblast/EC cell contacts; this was found both for trophoblast/PC13TG8 contacts (Fig. 4) and trophoblast/R5/3 contacts (not shown).

Isolated inner cell masses attached readily to EC cell monolayers. Like morula cells, inner cell mass cells established metabolic co-operation with PC13TG8 (Fig. 5) but not with R5/3 (not shown).

The egg-cylinder stage (8th day embryo)

Embryo-derived (wild-type) cells were distinguishable in autoradiograms from EC (HGPRT−) cells by their different morphology (e.g. Figs. 8, 9, 10) or by their more intense labelling with [3H]hypoxanthine (e.g. Figs. 7, 10).

Cells from each of the six inner cell mass-derived tissues, namely embryonic ectoderm, extra-embryonic and embryonic mesoderm, extra-embryonic and embryonic endoderm, and parietal endoderm, established metabolic co-operation at almost all (> 90%) of their contacts with PC13TG8. The proportions of embryonic/EC cell-to-cell contacts which were positive for metabolic co-operation are given in Table 1. These values were obtained from grain counts as illustrated in Fig. 6. Fields from some of these co-cultures are shown as Figs. 7, 8, 9.

Trophectoderm-derived tissues, that is ectoplacental cone and extra-embryonic ectoderm, behaved quite differently. No metabolic co-operation was observed at ectoplacental cone/EC cell-to-cell contacts (Fig. 10). Similarly, most cells (> 90%) from the extra-embryonic ectoderm region failed to co-operate with EC cells, although a few were seen to co-operate well (Fig. 6). We cannot exclude the possibility that these co-operation-competent cells might be mesoderm or endoderm contaminants which were not removed during separation of extra-embryonic ectoderm from its surrounding tissues.

Extra-embryonic ectoderm cells were tested for their ability to participate in metabolic co-operation with two other HGPRT− cell lines: PyYTGTGR and STO. Although each of these mutant cell lines is capable of forming permeable, intercellular junctions (Gaunt & Subak-Sharpe, 1979; Hooper & Morgan, 1979), both failed to co-operate at the majority of their contacts with cells taken from the extra-embryonic ectoderm region (Table 1).
DISCUSSION

We found that pluripotential EC cells (PC13TG8) were able to establish metabolic co-operation with pluripotential cells of the pre-implantation mouse embryo, that is, with the cells of the morula and of the inner cell mass. In addition, EC cells could co-operate with most cell types present at the egg-cylinder stage of development (Fig. 1): embryonic ectoderm, extra-embryonic and embryonic mesoderm, extra-embryonic and embryonic endoderm, and parietal endoderm. In contrast, EC cells did not co-operate with trophectodermal cells of cultured blastocyst outgrowths. Furthermore, extra-embryonic ectoderm and ectoplacental cone cells of the egg cylinder, which are derived from the trophectoderm of the blastocyst (Gardner & Papaioannou, 1975; Rossant & Ofer, 1977), were found negative for co-operation at most (> 90%) of their contacts with EC cells.

Our observations provide the first demonstration that cells of early mammalian embryos are capable of metabolic co-operation. It is widely assumed (e.g. Pitts, 1976) that co-operation is mediated by permeable, intercellular gap junctions, and so it is of interest that Ducibella, Albertini, Anderson & Biggers (1975) have already demonstrated by electron microscopy the presence of gap junctions between morula cells and between inner cell mass cells of the mouse embryo.

There are two requirements for the establishment of metabolic co-operation between a given pair of cell types. First, it is obvious that both must be capable of forming permeable junctions. A second requirement, however, is that the cell types must be compatible for the formation of permeable junctions. For example, whilst a given pair of cell types may co-operate readily at their homotypic cell-to-cell contacts, they may co-operate rarely (Pitts & Bürk, 1976; Gaunt & Subak-Sharpe, 1979) or not at all (Fentiman, Taylor-Papadamitriou & Stoker, 1976) at their heterotypic cell-to-cell contacts. From our observations, we therefore conclude that EC cells are compatible for metabolic co-operation with cells of the morula, inner cell mass, and tissues of the 8th day embryo which are derived from the inner cell mass. Nicolas, Jakob & Jacob (1978) observed that

Figures 7–10

Figs. 7–9. Metabolic co-operation between PC13TG8 and three different cell types taken from the 8th day egg cylinder. Arrows indicate embryonic (HGPRT+) cells. All other cells are PC13TG8. PC13TG8 in direct or indirect contact with cells from the embryonic ectoderm (Fig. 7), embryonic endoderm (Fig. 8) and embryonic mesoderm (Fig. 9) are seen to have become labelled by metabolic co-operation. Bars, 20 μm.

Fig. 10. Absence of metabolic co-operation between PC13TG8 and ectoplacental cone cells. The two heavily labelled cells are derived from the ectoplacental cone core of an 8th day egg cylinder. PC13TG8 cells in contact with the ectoplacental cone cells are not labelled above background. Bar, 20 μm.
Table 1. Percentage of HGPRT\(^+\) (embryo-derived)/HGPRT\(^-\) (PC13TG8) cell-to-cell contacts positive for metabolic co-operation

<table>
<thead>
<tr>
<th>HGPRT(^-) cells</th>
<th>Ectoplacental cone core</th>
<th>Extra-embryonic ectoderm</th>
<th>Extra-embryonic mesoderm</th>
<th>Extra-embryonic endoderm</th>
<th>Embryonic ectoderm</th>
<th>Embryonic mesoderm</th>
<th>Embryonic endoderm</th>
<th>Parietal endoderm</th>
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<tbody>
<tr>
<td>(A) PC13TG8</td>
<td>nt</td>
<td>8 (50)</td>
<td>96 (25)</td>
<td>100 (25)</td>
<td>96 (25)</td>
<td>96 (25)</td>
<td>100 (25)</td>
<td>94 (25)</td>
</tr>
<tr>
<td>(B) PC13TG8</td>
<td>0 (50)</td>
<td>6 (50)</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>PyYTG.TGR</td>
<td>nt</td>
<td>6 (50)</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>STO</td>
<td>nt</td>
<td>0 (50)</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
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<tr>
<td><em>nt</em> — not tested.</td>
<td></td>
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</table>

Metabolic co-operation between PC13TG8 and cells taken from the 8th day mouse embryo. The percentages of HGPRT\(^+\)/HGPRT\(^-\) cell-to-cell contacts which were positive for metabolic co-operation were calculated from grain counts as described under Fig. 6. Values in parentheses are the numbers of HGPRT\(^+\)/HGPRT\(^-\) contacts which were observed. (A) and (B) were separate experiments.
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whilst PCC4/AzaR1 EC cells could establish metabolic co-operation with cells from each of six different mouse EC cell lines, they failed to co-operate with cells from each of nine differentiated lines. The authors concluded that EC cells co-operate between themselves but not with differentiated cell types, whatever their state of differentiation or origin. This conclusion is not supported by our own findings that PC13TG8 EC cells co-operate readily with mesoderm and endoderm cells taken from the mouse embryo. It seems likely that the technique used by Nicolas et al. (1978) for the detection of metabolic co-operation is less sensitive than the autoradiographic procedure used in our study. However, it remains a possibility that PC13TG8 differs from PCC4/AzaR1 in its capacity for permeable junction formation.

It is unclear as to whether the lack of communication which we have observed between EC and trophoblast cells is due to incompatibility of these cell types, or whether it is due to an inability of trophectoderm cells to form permeable junctions. In addition to EC cells, we tested trophectoderm for its ability to co-operate with two other HGPRT- cell lines but in neither case was there good evidence of metabolic co-operation. However, if gap junctions are sites of permeable, intercellular channels (Pitts, 1976), it appears that trophectoderm cells may indeed be capable of forming permeable junctions, since cells of this type have been found by electron microscopy to form gap junctions both at their homotypic cell-to-cell contacts (Ducibella et al. 1975; Arguello & Martinez-Palomo, 1975; Magnuson, Demsey & Stackpole, 1977), and also at their heterotypic cell-to-cell contacts with cells of the inner cell mass (Ducibella et al. 1975) and uterine epithelium (Tachi & Tachi, 1979).

It is not known to what extent the properties of EC cells can be considered to mimic those of the pluripotential cells of early embryos. It can be assumed that there are many properties in common since EC cells injected into blastocysts can contribute to the formation of a normal chimaeric mouse (Papaioannou et al. 1975; Mintz & Illmensee, 1975). Therefore we feel that our observations on metabolic co-operation between embryonic cells and EC cells might give insight into the interactions between embryonic cells themselves. Thus, pluripotential embryonic ectoderm, like EC cells, might retain its capacity for communication with the adjacent endoderm and mesoderm layers. Further, inner cell mass and embryonic ectoderm cells might be incapable of metabolic co-operation with trophectoderm. This latter suggestion is not supported by those electron microscopy studies (Ducibella et al. 1975) which show gap junctions between the inner cell mass and trophectoderm cells of mouse blastocysts. There is clearly a need for more direct measurements of communication between cells of mammalian embryos.

Although there is no evidence that metabolic co-operation plays a role in the cell-to-cell interactions of embryogenesis, several authors have speculated that permeable junctions may permit passage of morphogenetic substances (e.g. Wolpert, 1978). Our findings raise the further possibility that trophectoderm
cells may provide an important barrier, preventing metabolic co-operation between the embryonic and uterine tissues which they separate.

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*Note added in Proof.* Lo and Gilula (Cell, in the press) observed passage of fluorescent tracer molecules between inner cell mass and trophoblast cells in 24–36 h blastocyst cultures, but not in 36–72 h cultures. This supports a prediction that these two cell types might become incapable of metabolic co-operation with each other.

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