THE DEVELOPMENTAL GENETICS OF MOUSE TERATOCARCINOMA CELLS

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ABSTRACT

Starting from embryonal carcinoma (ec) cells capable of extensive differentiation in culture, the technique of 'thioguanine kiss-of death' has been used to select four independent series of metabolic cooperation defective (mec^-) variants. Ec cells which communicate in high levels of retinoic acid, which is shown to eliminate co- operation in other ec cell- lines, have also been isolated. A novel selective technique was used involving junction- mediated rescue from ouabain-induced ionic imbalances in the presence of retinoic acid.

The communication ability of these variant cell lines has been quantified by autoradiographic measurement of the transfer of uridine nucleotides, and also by an assay of the extent of junction- mediated rescue from ouabain toxicity by resistant fibroblasts. A correlation is observed between the abilities of the cell lines to transfer nucleotides, as measured by the uridine nucleotide transfer assay, and the ability of the cells to differentiate into parietal endoderm and to form the cavitated 'embryoid bodies' which are produced by the parental cell line when grown in suspension culture.

A clone which is rescued from ouabain-toxicity by resistant cells, but which does not transfer nucleotides, has also been isolated from one of the mec lines. It is not yet clear whether this clone is a ouabain resistant mutant, a pore size mutant, or a clone with altered junctional specificity. Embryoid bodies formed from this clone also show very little differentiation. A method of lineage marking of mosaic embryoid bodies has also been developed, and the optimisation of a technique for enriching cultures in mec^- variants using a fluorescence activated cell sorter is also reported here. A series of studies on the junctional communication phenotype of a mec^- ec line isolated previously, including an analysis of the kinetics of junction formation, are also presented.

Finally, work is described which demonstrates that the differentiation of ec cells, as induced by two different stimuli, can be inhibited by medium conditioned by non-dividing STO feeder cells.

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Abbreviations

AFP	alpha-foetoprotein
APRT	adenine phosphoribosyltransferase (EC 2.4.2.7)
cAMP	cyclic adenosine 5´-monophosphate
CFDA	carboxy-fluorescein diacetate
cRABP	cytoplasmic retinoic acid binding protein
dCK	deoxycytidine kinase (EC 2.7.1.74)
dif	differentiation
DMA	dimethyl acetamide
DMSO	dimethylsulphoxide
ec	embryonal carcinoma
EDTA	ethylene diamino tetra acetic acid
FACS	fluorescence activated cell sorter
GPI	glucose phosphate isomerase (EC 5.3.1.9)
HGPRT	hypoxanthine guanine phosphoribosyl transferase (EC 2.4.2.8)
HMBA	hexamethylene bis-acetamide
ICM	inner cell mass
mec	metabolic cooperation
MNNG	N-methyl N´-nitro N-nitroso guanidine
PBS	phosphate buffered saline
RER	rough endoplasmic reticulum
RM	Reichert´s Membrane
SNBTS	Scottish National Blood Transfusion Service
TCA	trichloro acetic acid
TK	thymidine kinase (EC 2.7.1.75)
TPA	12-O-tetradecanoyl phorbol-13-acetate
ZPA	zone of polarising activity

Enzymes not mentioned above

Alkaline phosphatase	EC 3.1.3.1
Plasminogen activator	EC 3.4.21.31

Chapter 1

INTRODUCTION

1.1 General Introduction

This thesis addresses itself to the enigma of how differentiating cells select a particular pathway along which to differentiate: in particular what part if any does junctional transfer of small molecules between cells play in this cell determination. Since the discovery of junctions in developing systems [1,2] there has been much speculation regarding possible connections between junctional communication and cell determination, but the experimental techniques needed to investigate the subject have not been available .

Information is now available on many aspects of the morphology and molecular biology of differentiating cells, but the intercellular interactions involved in cell determination remain largely obscure. One useful model of early mammalian embryogenesis is the differentiation of mouse embryonal carcinoma (ec) cells [3]. This study considers in particular the cellular interactions involved in the determination of endoderm and in related events which occur in ec-derived systems *in vitro*. In this chapter, the normal development of the mouse is discussed, and current research on ec cells reviewed; the general problem of pattern formation in mammalian systems is considered, and the subject of junctional communication

is then introduced with reference to this. Following a review of research on the control of early mammalian development, the introductory section is completed with a summary of previous work on junctional communication in ec derived cells and a description of the rationale of this project.

The bulk of the experimental work reported here consisted of the isolation and characterisation of ec cell lines with altered junctional communication phenotypes. The characterisation of these phenotypes contributes information on the nature of the determination events in ec derived systems. A study on the interactions of ec cells and fibroblasts is also reported. It is hoped that this work will prove a valuable addition to the understanding of early events in the differentiation of mammalian cells.

1.2 Early development of the mouse

Fertilisation in the mouse occurs in the ampulla tubae, the uppermost loop of oviduct. The single cells then move down the oviduct, where the residual follicle cells which surrounded and nourished the ovum are lost over the next 20 hours or so [4]. The first cleavage division then occurs, and by two days post-fertilisation the embryos consist of balls of 2-16 cells termed morulae (the variability is a

consequence of asynchrony at earlier stages), enveloped by a mucoprotein membrane, the zona pellucida [4] (see fig. 1).

The cells of the morula, termed blastomeres, are roughly spherical, and mutual contact is therefore minimal up to the 8-cell stage [5]. Thereafter, the relationship between them changes dramatically, contact becoming so intimate and extensive that cell boundaries can no longer be readily resolved . This process, called compaction, which persists throughout the remainder of cleavage is accompanied by formation of tight junctions between outside cells [5]. These are intercellular contacts which block diffusion between cells. Numerous other changes are initiated at this stage, including the organisation of the cortical cytoskeleton, and the distribution of mitochondria and microvilli [5].

On the third day (considering the middle of the night during which copulation occurred as day 0) the morulae are in the uterus , and the blastomeres are of slightly unequal sizes [4]. By the time the embryo reaches the 35-cell stage, a large eccentrically placed lumen has developed, and the cells are differentiating into a distinct trophoblast around the outside of this, and the ´inner cell mass´ (ICM) internally [4] (see fig. 1). The lumen, initially termed the blastocoel, later

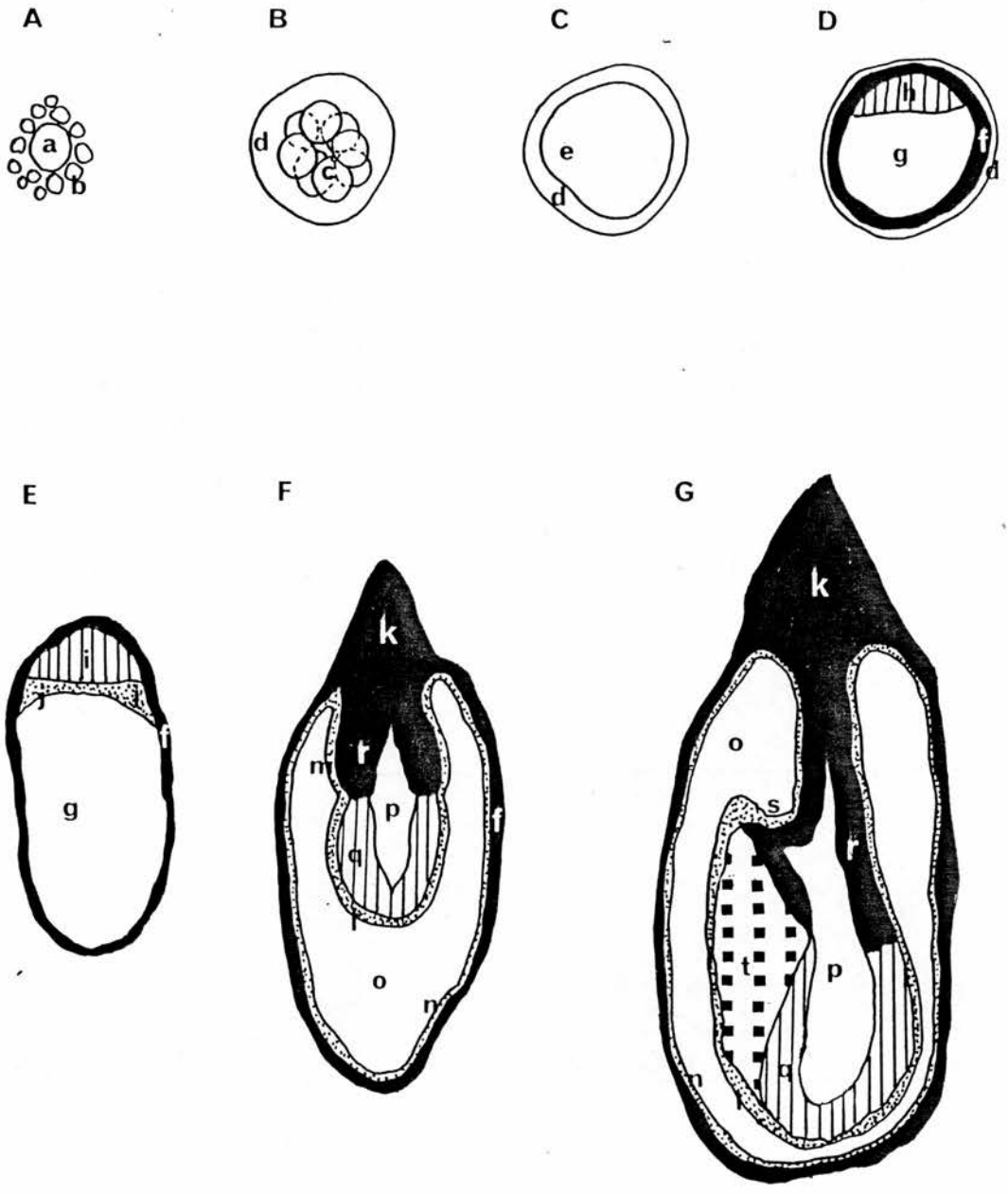
Figure 1

Early development of the mouse embryo

Adapted from Adamson and Gardner [5], and Theiler [4].

A: 0 days, one-celled egg
B: 2.5 days, eight cell morula
C: 3 days, compacted morula
D: 4 days, blastocyst
E: 4.5 days, enlarged blastocyst (implantation stage)
F: 6 days, differentiation of the egg-cylinder
G: 7 days, formation of the mesoderm

a: ovum
b: granulosa cells
c: 1/8 blastomere
d: zona pellucida
e: compacted blastomeres
f: trophectoderm
g: blastocoel
h: inner cell mass
i: primitive ectoderm
j: primitive endoderm
k: ectoplacental cone
l: embryonic endoderm
m: visceral extra-embryonic endoderm
n: parietal endoderm
o: yolk-sac cavity
p: pro-amniotic cavity
q: embryonic ectoderm
r: extra-embryonic ectoderm
s: transverse fold
t: mesoderm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becomes the yolk-sac.

The four-day embryo, in which this differentiation is completed, is called the blastocyst. The trophoblastic cells are flattened and form a single- layered epithelium, the trophectoderm, whilst the cuboidal ICM cells are clustered at one end [4]. By this time the blastocyst has 'hatched' from the zona pellucida and the trophoblast is in close contact with the uterine wall [4].

4-5 days after fertilization, whilst the blastocyst begins to implant into the wall of the uterus, the ICM differentiates into a distinct outer layer with strongly eosinophilic cytoplasm called primary endoderm, and an 'undifferentiated' ectoderm [4] (see fig. 1). The principal ultrastructural change is the development of characteristic cisternae of extensive and branched endoplasmic reticulum in the primary endoderm. The mitochondria also become more rounded [6]. Meanwhile the whole ICM enlarges considerably and bulges into the blastocoel cavity.

At the same time the trophoblast forms an outgrowth called the ectoplacental cone projecting above the embryonic pole, and this dissolves the adjacent uterine epithelium. The ectoplacental cone is later invaded by blood vessels which provide the embryo with nutrients [4], whilst the trophectoderm away from the embryonic

region, the mural trophectoderm, transforms into the primary trophoblast giant cells [7].

From a compact cluster of cells with close associations both with each other and with the trophoblast, the ICM changes into a conical group of cells which are much more loosely associated [6]. Both prior to and during elongation of the blastocyst, there are simple junctional specialisations between ICM cells and between the ICM and the overlying trophoblast [6]. These junctions consist of areas where cell membranes are approximately 20nm apart , and have characteristics of gap junctions (see section 1.52) .

The elongation of the egg-cylinder is followed by its differentiation (on day 6) into embryonic and extra-embryonic areas [4] (see fig. 1). Most of the cells of the embryonic ectoderm are cylindrical in shape. They are often separated by a small furrow from the extraembryonic, irregular cuboidal cells [4].

The endoderm also differentiates (see fig. 1). Those endoderm cells that are situated laterally show only limited areas in contact with other endodermal cells and are consequently less epithelial in appearance. These individual stellate cells are characterised by multiple branching fine filipodia and resemble fibroblast cells <u>in vitro</u> [6]. They are called parietal endoderm, and subsequently migrate around the

outside of the yolk sac cavity to form its outer lining where they are separated from the trophoblast cells by a thick basement membrane known as Reichert's membrane (RM). Acellular in nature, this increases in size with the growth of the embryo and can be distinctly recognised during later development [4]. Although not much is known about the function of Reichert's membrane, there is some evidence that it may act as a passively selective filter for substrates passing from the maternal to the foetal environment [8].

The parietal endoderm possesses distended profiles of rough endoplasmic reticulum [9] identifiable by thin section electron microscopy and produces very large amounts of type IV collagen [10], which is secreted into the RM. In addition, laminin, aa large non-collagenous glycoprotein characteristically found in basement membranes [11] is synthesised by parietal endoderm and found in RM [12,13]. RM also contains heparan sulphate and other sulphated glycoproteins [14].

The cells overlying the embryonic cell mass are cuboidal and form a continuous epithelium, relatively lacking in intercellular space, have numerous short microvilli on their apical surfaces facing the cavity of the blastocyst and are joined by apical junctional complexes [6]. These cells are visceral endoderm.

Ultrastructurally they are further distinguished by narrow profiles of RER, and numerous apical electron-lucent vacuoles [9].

Whereas parietal endoderm cells do not synthesise the antigen alpha- fetoprotein (AFP) some of the visceral endoderm cells do [15]. The visceral endoderm can be divided into two regions: the visceral embryonic endoderm, overlying the embryonic region of the egg-cylinder, and the visceral extra- embryonic endoderm which overlies the extra-embryonic region. A study by immunoperoxidase staining and by immunoprecipitation after radioactive labelling, of the localisation and synthesis of AFP during mouse embryogenesis indicated that AFP is first detectable in embryos in the 7th day of gestation. In 7 and 8 day old embryos, it is confined to visceral endoderm cells around the embryonic region of the egg cylinder but by the 9th day it is also present in extra embryonic ectoderm, mesoderm and embryonic ectoderm which do not synthesise AFP but can absorb it [15].

Transferrin synthesis (identified by immunoperoxidase) also appears to be a marker of visceral endoderm in early embryos [16] and in contrast to parietal endoderm visceral endoderm produces predominantly type I collagen, and little type IV [10].

A central lumen, the proamniotic cavity, appears, at

first in the embryonic ectoderm, and later in the extraembryonic area [4] (see fig. 1). This expands during day 7 of gestation [4] and simultaneously the mesoderm appears. The region of mesoderm proliferation is known as the primitive streak, and its appearance establishes the anterior-posterior axis of the embryo. It originates from the posterior embryonic ectoderm and spreads anteriorly between the ectoderm and the endoderm [7] (see fig. 1). At about the time that primitive streak formation occurs, the amniotic folds begin to cut off the embryonic region from the ectoplacental cavity [7], and the posterior end of the primitive streak bulges into the proamniotic cavity , where another cavity (the exocoelom) forms within it.

The germ-line does not become apparant as a distinct lineage until much later in development. Germ cells can be recognised in haemotoxylin eosin-stained sections for the first time on about day 9, within the epithelium of the hind gut. These then migrate to a structure called the genital ridge, which subsequently differentiates to give rise to the gonads. Sexual differentiation is apparent on about day 12 of gestation [4].

1.3 Embryonal carcinoma cells

Teratocarcinomas are malignant tumours characterised by the presence of a variety of differentiated cell types, such as nerve, skin, muscle and cartilage [17]. The name teratocarcinoma, is derived from the Greek root 'teraton' meaning monster [17], which conveys the idea of malformed development that is characteristic of them (the term 'teratoma' is used for benign tumours of this type, or as an umbrella term for both the benign and malignant tumours). While the composition of differentiating tumours is highly variable, the most typical contain derivatives of all three embryonic germ layers: ectoderm (for example, neural tissue, skin), mesoderm (for example, muscle, bone, cartilage), and endoderm (glandular structures, gut) [17]. In some cases, extra- embryonic tissues such as trophoblast and yolk- sac are also present. In general the differentiated tissues appear to be chaotically arranged [17].

Embryonal carcinoma (ec) cells are rapidly dividing cells which are always present in these tumours, and which give rise, by differentiation, to the other cell types. This differentiation parallels early stages of embryogenesis in certain ways (see sections 1.33, 1.341). It is possible to grow large numbers of ec

cells in culture and this makes this system an attractive source of undifferentiated cells to use as models of early stages of development. Ec like cells can be isolated from mutant mice (section 1.36); chimaeric animals can be formed with contributions from both normal embryos and ec cells (section 1.35); and *in vitro* differentiation can be studied (section 1.34). In addition, the growth of large numbers of ec cells in culture allows the isolation of low frequency varients on the basis of cellular phenotype. It is possible to select for functional changes in culture and to study the implications of these changes for other aspects of the cell phenotype, or for the phenotype of chimaeric animals in which the cells participate (see fig. 2).

1.31 Origin of teratocarcinomas

Whilst teratocarcinomas occur spontaneously only very rarely , they can be induced at low frequencies by injecting metal salts into the testes of domestic fowls or of rats (reviewed in [18]). Stevens and Little were the first to report testicular teratomas in mouse , when they found that nearly 1% of autopsied male strain 129 mice bore them [19]. Subsequently mouse strains of even greater susceptibility were isolated, as was a strain (LT) in which ovarian teratomas occur in about 50% of 90 day old females as a result of parthenogenetic development of oocytes [20].

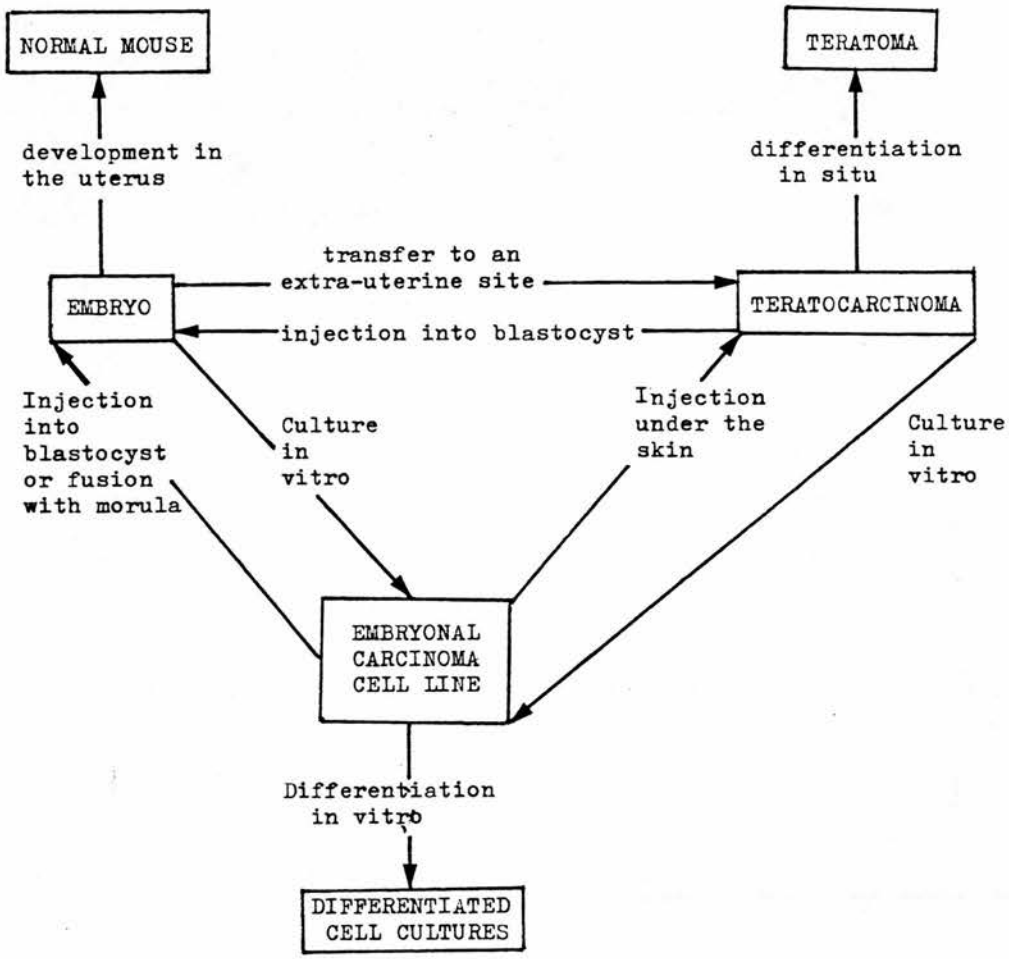


Figure 2

Interconversions of embryos and teratocarcinomas

Adapted from Martin [76].

Teratocarcinomas can also be obtained at high frequencies by implantation of early embryos in extrauterine sites, (reviewed in [17]). Alternatively, experimental transplantation of male foetal genital ridges to an adult host testis results in foetal testis in which nests of germ cells proliferate to become ec cells (reviewed in [18]). Genital ridges transplanted before the germ-cells were present, or taken from germ-cell defective animals did not give teratocarcinomas. Stevens also observed that in most early testicular teratomas there is direct continuity between the germinal epithelium and the tumour cells, suggesting that the tumours arise within the tubules of the testis [21].

Accordingly, there was some controversy over the whether teratomas arise from misplaced pluripotent cells, corresponding to a rapidly dividing cell type of the early embryo , or from parthenogenetic germ cells (see, e.g. [22]). The latter possibility , of course, insofar as parthenogenesis will subsequently give rise to embryonic cells in ectopic sites, is a special case of the former.

Ultrastructural research has confirmed the similarities between ec cells, primordial germ cells, and certain cells of the early embryo. Ec cells have large, irregularly shaped nuclei that contain two or three

large nucleoli. Euchromatin predominates over heterochromatin, and the cytoplasm contains many free ribosomes, a few small spherical mitochondria, occasional profiles of nondistended rough endoplasmic reticulum, moderately developed Golgi, and a few membrane-bound dense bodies [9,23,24]. However, these characteristics are shared not only by the embryonic ectoderm, but also by primordial germ cells [23].

The functional analogy between ec cells and embryo cells is based on the pluripotency of the former which can differentiate into a wide range of cell types, representing derivatives of all three embryonic germ layers [17], even in tumours derived from single cells only [22,25]. The observation that teratocarcinomas can be obtained by implantation of early embryos in extrauterine sites, serves to reenforce this idea [17], as does the demonstration that it is only the embryonic portion of the egg-cylinder stage embryo which is capable of giving rise to tumours [26].

It appears that the malignant behaviour of the embryonal carcinoma cells is also a function of their similarity to early embryo cells. The many non-malignant contributions which ec cells have made to chimaeric animals (see section 1.35) illustrate the fact that the transition from normal pluripotent embryonic cells to malignant embryonal carcinoma cells

is readily reversible. The ineffectiveness of conventional mutagens (reviewed in [18]) in inducing teratocarcinomas reenforces the view that ec cells may be malignant because of their environment rather than because of a cell autonomous defect.

Furthermore, although cells of other established mouse tumour cell lines usually have grossly abnormal karyotypes, most embryonal carcinoma cell lines in culture have chromosome numbers which though slightly aneuploid, are approximately diploid. Small chromosomal re- arrangements are found and many lines are XO, but recently new lines of cells from teratocarcinomas have been made which are apparently euploid [27].

In view of results such as these it is not surprising that ec cells can participate in the formation of chimaeric embryos, and that it has proved possible to grow embryonic cells in vitro in a similar way to ec cells.

1.32 Biochemical properties of ec cells

A considerable number of specific antigens on ec cell surfaces have been described. These are typically also expressed on the surface of primordial germ cells and on some early embryonic cells. "F9 antigen" for instance has been shown to be present up to the 7th day

of development as well as on the surface of primordial germ cells and spermatazoa, whilst it is lost during differentiation [28]. Evans et al (reviewed in [27]) obtained an antibody which recognises determinants on the embryo up to 6.5 days post-coitum as well as on ec cells.

Two dimensional gel electrophoresis was used to show that the spectrum of proteins synthesised by ec cells is similar to that of 5 day embryos [27]. ec cells also characteristically possess high activity of the enzyme alkaline phophatase [29,30]. This activity increases as cultures age [Bernstine E.G. and Hooper M.L., personal communication].

Although they do not appear to secrete a matrix, they produce and secrete large amounts of matrix components. Ec cells of the multipotential line OC 15S1 produce large amounts of a collagenous polypeptide whose mobility on both reducing and non- reducing polyacrylamide gels is identical to that produced by parietal endoderm [31]. The correspondence of the two is supported by examining the cyanogen bromide cleavage products [31]. Similarly, monolayers of both pluripotent (A6) and nullipotent (SCC1) ec cells synthesise fibronectin and release it into the medium. Fibronectin forms an extracellular matrix around many cells in culture and may play a role in the maintenance

of cell morphology and cell-substrate adhesion in cultured fibroblasts [32]. It is suggested that fibronectin may have a role in mediating cell interactions during morphogenesis in normal embryos.[32]

1.33 Differentiation of ec cells in tumours

The histology indicates that when they are first detectable, all teratoma tumours contain ec cells [17]. Only those tumours in which these cells continue to multiply as undifferentiated cells are malignant. These tumours, known as teratocarcinomas, are characterised by fast growth and, depending on the part of the tumour selected, they are retransplantable; those portions which contain ec cells will grow in a new host [17]. The embryonal carcinoma cells are found haphazardly distributed throughout the tumour in the form of small groups or nests.

Many solid tumours of somatic tissues can be converted to growth in the ascitic form. When disaggregated teratocarcinomas are injected into the peritoneum of syngeneic mice, aggregates of differentiating cells are found in the ascitic fluid [17].These bear a superficial resemblance to mouse blastocysts of approximately 3.5 days gestation and are hence called ´embryoid bodies´. However, in the blastocyst, the

outer cell layer is trophectoderm surrounding an inner cell mass (ICM), while the embryoid bodies have an outer layer of endoderm surrounding an embryonal carcinoma core and do not contain any trophoblast derivatives. Such embryoid bodies are therefore more directly comparable to the inner cell mass derivatives of a normal embryo of approximately 5 days gestation, when the ICM has developed an outer layer of endoderm.

These two-layered teratocarcinoma derivatives, known as "simple" embryoid bodies, will sometimes develop in the peritoneal cavity into more complex "cystic" embryoid bodies, [17]. The outer endodermal cells can be distinguished from the inner ec cells by their ultrastructural appearance [28]. They have plentiful rough endoplasmic reticulum which is swollen with a material that is also found between the outer cell layer and the ec core. Cystic embryoid bodies, which apparantly arise from simple ones [22] are more complex, many of them containing ec cells , a variety of differentiated tissues, and a fluid-filled cyst. These structures bear striking similarities to the embryonic portion of older mouse embryos but are clearly disorganised in comparison with them [17]. Simple embryoid bodies removed from the peritoneal cavity can differentiate into cystic embryoid bodies <u>in vitro</u> [17].

1.34 Differentiation in vitro

In studies of differentiated cells which are not descended from single characterised clones, heterogeneities present in the starting material cannot be excluded as sources of variation in the products. The usefulness of transplantable tumour lines is limited by such uncertainties and by the exposure of the cells to indeterminate influences in the host environment. Both these limitations have been overcome since undifferentiated ec cells have been cloned and cultured *in vitro* [17,18]. A number of substances capable of stimulating particular types of differentiation *in vitro* have been discovered (section 1.343). The fact that the maintenance of embryonic ectoderm cells in an undifferentiated state *in vitro* proved so problematical [27,33] suggests that the provision of stimuli for differentiation by the culture milieu may be the norm rather than the exception.

Feeder layers of non-dividing mouse embryo fibroblasts are needed for the maintenance of cultures of certain ec lines (here termed feeder- dependent) in an undifferentiated state [34]. Other cell lines, (here termed feeder- independent), with a more restricted capacity for *in vitro* differentiation can be grown in their absence [30].

1.341 Differentiation of embryoid bodies in vitro

When purified embryoid bodies obtained from the ascites cavity of a mouse were plated in suspension culture they were found to undergo further differentiation to give yolk sac membranes, mesoderm and blood islands [28]. Over the first 5-7 days of culture after the embryoid bodies were allowed to settle out of suspension and attach to the surface of a petri dish, cells were observed either migrating out of them or dividing to produce nests of cells around the attached body [28]. Over the next 30-120 days a large number of new cell-types differentiated [28].

Martin and Evans [34,35] extended this work by forming embryoid bodies from clonal feeder-dependent ec cultures in vitro. Aggregates of cells were seeded over a substrate to which they could not attach. After 24 hours in suspension, clumps of some cell lines became embryoid bodies consisting of an outer layer of endodermal cells surrounding an inner ec cell core.

After several days in culture, the outer layer of such embryoid bodies contains both parietal and visceral endoderm cells. Thin section electron microscopy confirms these assignations, despite the rounding up of the parietal endoderm (a general feature of parietal endoderm in culture [6]). Both types of endoderm were reported to be present in roughly equal proportions,

although one or the other type often predominates on an individual aggregate. In embryoid bodies derived from the cell- line PSA1, for instance, about 30% of the endoderm cells in the outer rind produce transferrin [16]. Embryoid bodies also produce a significant amount of type IV collagen [36]. The distinction between the two cell types however, is not always clear-cut and occasional intermediate forms are observed [9].

As in the embryo, in the embryoid bodies large amounts of basement membrane material are laid down between the parietal endoderm and the ec cells [12,13, 37,38] and this material can be seen prior to secretion in the dilated RER of the parietal endoderm cells [9]. Its composition is similar to that of the Reichert´s membrane laid down in vivo. Ec cells which form well-differentiated embryoid bodies with thick basement membranes synthesise considerable amounts of laminin-like material [38] as does the mouse teratocarcinoma derived parietal yolk-sac carcinoma cell line PYS [12,13].

Differentiation of ec cells into parietal endoderm is also accompanied by changes in cell surface saccharides as detected by radioactively labelled lectins [39]. This work was done by isolating parietal yolk-sac lines from tumours and comparing them with cultured ec cells.

The line SCC-S2 of clonal ec cells stops its differentiation at this stage and remains as two-layered simple embryoid bodies when kept in suspension [40]. In other lines, however, accompanying the formation of this endoderm layer, about 4 to 5 days after detachment, internal changes in the cores of the embryoid bodies became apparent. Focal necrosis [Boyd, S., Hooper, M.L., and Wyllie A.H. in preparation] is associated with the formation of one or more small cavities full of cellular debris [9]. These usually form two to three cell diameters from the outer surface: since the embryoid bodies are often more than 10 cells in diameter, the cavity therefore arises eccentrically. At the time that the cavity forms, or shortly thereafter, the ec cells surrounding it elongate to create a simple epithelium of columnar cells. This process may be analogous to the formation of the proamniotic cavity and embryonic ectoderm in the normal mouse embryo [9]. SCC-S2 embryoid bodies do not contain any visceral endoderm and so it was thought that visceral endoderm might be essential for cavitation. However, cavitation will also occur in aggregates of parietal endoderm cells and ec cells, although in this system visceral endoderm is absent [41].

After 6 days in suspension, some of the embryoid bodies

begin to expand and to show a ball- like swelling on one side. Over the next few days these structures, hollow cysts, continue to enlarge and sometimes reached a diameter of several millimetres [9].

1.342 Differentiation induced by removing ec cells from feeder layers

Many ec cell lines will differentiate if grown in monolayers without feeder cells (see e.g. [42]). The type of cells produced depends upon the cell line of origin. A variety of modifications of culture procedures have been used to bring about differentiation (see e.g. [43]). For instance, one ec line readily changes into "big flat cells" as soon as the cells are plated without feeder cells [44]. These are similar to a cell type often seen in cultures of other lines treated with retinoic acid (section 1.3 3). Like all other ec derived "big flat cells", they synthesise principally type I collagen [36, 44]. Similarly, when ec cells are cultured in medium containing serum extracted with organic solvents to remove lipids, they show a high frequency of "spontaneous" differentiation [45].

1.343 Differentiation induced by chemicals in vitro.

The feeder-independent cells used in this study are derivatives of PC13 (see Table 1) . PC13 cells are

Table 1
Embryonal carcinoma cell lines used in this study

A. Cell lines isolated from tumours

cell line	tumour of origin	source of tumour	isolation procedure	ref.
PC13	OTT 6050 B	6-day embryo	uncloned line isolated from outgrowths of cultured embryoid bodies.	[30]
SCC-PSA4	OTT 5568	3-day embryo		[34]
SCC-S2	OTT 5568	3-day embryo		[34]
Nulli-SCC1	LS 402C-1684	3-day embryo		[34]

B. Clonal cell lines isolated from other cell lines

cell line	cell line of origin	isolation procedure	phenotype	karyotype	ref.
PC13.5	PC13	cloning	HGPRT+ mec+	-	[187]
PC13TG8	PC13	thioguanine resistance	HGPRT- mec+	modal chromosome number of 41 includes 1 meta-centric.	[187]
R5/3	PC13TG8	thioguanine 'kiss of death' (Section 1.52).	HGPRT- mec-	modal chromosome number of 78, includes 2 meta-centrics.	[126]
H2T12	R5/3	HAT 'kiss of life' (Section 1.52).	HGPRT- mec+	as R5/3	[128]
PSA4TG12	SCC-PSA4	thioguanine resistance	HGPRT- mec+	modal chromosome number of 40	[199]
RA EC10	PSA4TG	reduced differentiation retinoic acid	HGPRT- mec+	modal chromosome number of 41, 1 X, no Y, trisomy 8, trisomy 15.	[201]
RA EC12	PSA4TG	reduced differentiation retinoic acid	HGPRT- mec+	modal chromosome number of 40, 1 X, no Y, several rearrangements.	[201]

similar in growth properties to the more extensively studied line F9 [30], though they differ biochemically, notably in the collagens which they produce: PC13 cells produce almost entirely type IV collagen; F9 also produces a small proportion of type I [31].

Although not producing laminin, and originally considered to be nullipotent, F9 cells show some spontaneous differentiation in monolayers which can be increased by forming suspension aggregates [46]. They will also differentiate into endoderm- like cells when treated with retinoic acid [47,48]. Similarly, the line nulli-SCC1, originally thought to be nullipotent on the basis of *in vitro* and *in vivo* studies, differentiates in retinoic acid [48]. In fact all mouse ec cells so far tested differentiate in retinoic acid (though see section 1.345). The outcome of retinoic acid treatment can be modulated by a large number of different factors and a bewildering variety of cell types are produced.

Following retinoid treatment, residual F9 ec cells (defined antigenically) have a considerably higher cloning efficiency than their differentiation products [49]. Ec antigens decrease markedly in amount in differentiating cultures [49] and plasminogen activator and laminin are produced [38]. The differentiated cells share these characteristics with the endoderm of

the embryo. Treatment of PCl3 with retinoic acid enormously enhances production of type I collagen, but up to 28% type IV continues to be synthesised [31] and the cells do not produce transferrin [16]. Therefore they do not correspond closely to either parietal or visceral endoderm.

Retinoic acid treated monolayers of F9 can be converted to parietal endoderm cells (characterised by synthesis of plasminogen activator, laminin and type IV collagen) by treatments which elevate cAMP contents of the cells [50] Visceral endoderm cells are not produced: even well differentiated F9 cells do not produce AFP [37]. In contrast, if F9 ec cultures are treated with retinoic acid when they are in the form of small embryoid bodies, cells differentiate on the outer surface which morphologically resemble visceral rather than parietal endoderm [51]. These cells synthesise and secrete AFP. One interpretation of this result is that ec cells treated with retinoic acid differentiate first into bipotent cells analogous to the primary endoderm of the normal embryo which subsequently express either the mature parietal or visceral phenotype depending on the nature of the intercellular contact signals they receive [51].

In the line 01A1 the differentiated cell types formed from cell aggregates exposed to retinoic acid are

dependent on the concentration of the retinoic acid used [52]. Cultures exposed to low concentrations produce an abundance of cardiac muscle, at higher concentrations skeletal muscle is produced. At the highest concentrations studies, neurons and astroglia appear. It is suggested that the commitment of pluripotent cells to particular developmental avenues may be determined in part by concentration gradients of substances such as retinoids [52].

Retinoic acid-induced differentiation of some ec cells can inhibited by growing the cells on BALB/3T3 A31 feeder layers [53], an observation which supports the notion that intercellular interactions are critically important in determination of differentiating ec cells.

Other substances besides retinoic acid stimulate the differentiation of ec cells. These include hexamethylene bisacetamide [54,55] (HMBA), a potent inducer of erythroid differentiation in murine erythroleukemia cells [56]. N,N dimethylacetamide [55] (DMA), and polybrene [55] act similarly. The type of differentiated cell induced from a subclone of the line PCC4azal by HMBA, DMA or polybrene, also depends on the spatial organisation of the ec cells during drug-treatment [55]. In monolayers, 'big flat' cells are produced exclusively [55]. Treatment of aggregates however yields predominantly 'fibroblast-like' cells

Both differentiated cell types produced large amounts of fibronectin. The 'fibroblastic' cells are similar to those obtained spontaneously from aggregated cultures and resemble glial cells differentaiated from embryoid bodies. These cells continue to divide when isolated into pure culture and may be mesodermal or neurectodermal derivatives [55].

Recently too, it has been found that aggregates of a certain ec line are induced to differentiate into muscle by dimethyl sulphoxide (DMSO). In the presence of retinoic acid, neuronal differentiation occurs [57]. The list of substances known to induce differentiation of ec cells is now quite long: 5- bromodeoxyuridine [58], and sodium butyrate [45] are also recorded as having effects.

1.344 Chemical induction of differentiation of tumours

Strickland and Sawey [37] reported that terato-carcinomas resulting from injection of F9 ec cells into 129 mice could be induced to differentiate by feeding the animals with a diet containing very high concentrations of retinoic acid. This crude experiment was followed up by Speers [59], who found that PCC4azal ec tumours grown in 129 mice by subcutaneous transplantation differentiate when the tumours are injected with a combination of retinoic acid and

dimethylacetamide [59]. There is a reduction in tumour growth rate and the survival time of the host increases [59]. Retinoic acid/ dimethylacetamide is also effective in inducing differentiation when administered systemically [59].

1.345 Mode of action of inducers of differentiation

It has been proposed that interaction with a cellular binding protein mediates many of the activities of retinoic acid [60]. This cytoplasmic retinoic acid binding protein (cRABP) is present in ec cells [61] . The activity of retinoids in inducing differentiation correlates in general with their ability to bind to cRABP [45,61]. The carboxylic acid group is essential for activity but the structure can be modified in the cyclohexenyl ring system with retention of effectiveness [37]. Similarly, certain aromatic compounds termed arotinoids with structural similarities with retinoids are active inducers of ec differentiation [45]. Binding to the protein is followed by translocation of the cRABP- retinoic acid complex to the nucleus in a process with superficial similarities to the suggested mode of action of steroid hormones [61]. Retinol, which does not bind to cRABP, also acts on several ec lines. The fact that a much higher concentration of the alcohol than of the acid is required, supports the idea that the retinol might be

active only as a consequence of oxidation to the acid [45].

Schindler et al [62] isolated two mutant ec cell lines which failed to differentiate in retinoic acid. They also failed to differentiate in aggregates or in tumours, and lacked measurable levels of cRABP [62]. Ec cells defective in their response to retinoic acid but which possessed cRABP [63] were subsequently isolated. These cells are maintained in retinoic acid, as the phenotype was found to revert to that of the parent in its absence. The significance of this instability is unclear. Other workers [64] have isolated similar cell lines: one mutant does not differentiate and lacks cRABP, another mutant shows reduced differentiation but has normal cRABP activity.

The mechanism by which HMBA induces differentiation is not well understood either. Radioactivity derived from labelled HMBA is taken up into the plasma membrane, nucleus and cytoplasm [56]. Some ec cells selected for lack of response to HMBA retain cRABP and the ability to differentiate in response to retinoic acid [63]. Others possess cRABP but differentiate poorly, if at all, in the presence of either retinoic acid or HMBA. The loss of response to retinoic acid implies that the activities of the two inducers are not independent, though HMBA, which does not bind to cRABP cannot be

acting as a retinoic acid analogue.

1.35 Formation of chimaeras

Brinster [65] demonstrated that ec cells can participate in the formation of chimaeric mice. Teratocarcinoma cells taken from the ascites fluid of 129 mice in which the tumour had been grown intraperitoneally were injected into blastocysts derived from albino mice. 1 out of 60 mice which survived to term in foster mothers had a patch of agouti hair derived from the ec cells .

Similarly, Mintz and Illmensee [66] obtained chimaeras by taking ec cells from the cores of embryoid bodies which had been grown in the ascites for 8 years and injecting these into blastocysts . They then showed that even single cells injected in this way could contribute to nearly all the major tissues of the resultant animal [67]. They subsequently obtained chimaeras in which the germ-line contained teratocarcinoma derived genes. One such chimaera sired 61 offspring, all of which carried genetic markers of the teratocarcinoma cells [67].

This work was extended by other workers who used cells from cultures [14,68]. Normal chimaeric mice were obtained following injection of cells from 3 different ec lines. The chimaerism of the post-implantation

embryos and of the internal tissues of the offspring was demonstrated by electrophoretic analyses of homogenates for GPI in starch gels [68,69]. All tissues tested were shown to be chimaeric in at least some animals [68,69]. External chimaerism in offspring was determined by inspection of coat and eyes.

In two of the three lines, some chimaeras were obtained which developed tumours postnatally. A fourth line colonised the embryo but all the resultant chimaeric mice developed teratocarcinomas . Other workers [70] who aggregated cells from the ec line PSA1 or its HGPRT- derivative NG2 with cleavage stage mouse embryos, and formed chimaeras, observed abnormalities which clearly correlated in amount with the extent of contribution of the ec cells (as measured by GPI analysis) and the degree of abnormality [70]. On the other hand, when some ec lines are injected into blastocysts which are returned to the uterus they colonise only the yolk-sac at low frequency [44] , and injection of euploid lines into blastocysts does not necessarily lead to high levels of participation by the ec cells in developing embryos [44].

Whereas technical problems make blastocyst injection difficult, Stewart [71,72], used the alternative technique of aggregating ec cells with morulae in order to form chimaeric mice. Groups consisting of 3-5 ec

cells were picked out and sandwiched between two 8- 16 cell embryo s, from which zonae pellucidae had been removed [71]. Chimaeras were obtained from aggregates returned to pseudopregnant females. Aggregation techniques seem to give comparable results to those obtained using injection techniques [70,73].

Until recently only ec cells taken directly from tumours, which are apparently euploid have been shown to contribute to the germ line. In vitro cell lines have mostly produced low numbers of chimaeras with limited somatic tissue colonisation. A karyotypically normal female line , METT-1 which grows in culture [74], has proved an exception to this . Mosaicism was widespread in somatic tissues and one chimaera also showed germ- line mosaicism [75].

1.36 Methods of isolating cells from embryos

Evans and Kaufman [33] were the first to establish pluripotential cells in culture from mouse embryos. These cells were isolated from cultures of blastocysts which had been enlarged by inducing a state of diapause just before implantation to increase the size of the population of pluripotential cells. This was done by ovariectomising the mothers, and injecting them with Depo-Provera. Blastocysts were recovered 4-6 days later and seeded into tissue culture dishes, where the

ICMs developed into large egg-cylinder structures, which were picked off the dish, dispersed with trypsin, and passaged onto fibroblastic feeder layersx. Cells grew up which resembled ec cells both morphologically and in their ability to differentiate *in vitro* or to contribute to chimaeras [33].

Martin [76], isolated a similar cell line by culturing immunosurgically removed ICMs in ec conditioned medium. The cells again resembled ec cells morphologically, and in their ability to differentiate in culture [76].

1.4 Pattern formation

Very little progress was made between the 1920s and the late 1960s on the problem of how cellular diversity is generated in organised patterns during development [77]. Developmental biologists concentrated on the process of embryonic induction and inducing substances (see e.g. [78]) and this obscured the problem of pattern formation, by emphasising the importance of the inducing substances [77] .

In order to elucidate the mechanism underlying pattern formation, we need to experimentally intervene in the geometry of developing systems. The original approach, begun in the last century, is to alter the spatial relationships of the developing embryo by transplanting tissue to different sites and observing the resultant disruption of the pattern. More recently, particularly with Drosophila the techniques of developmental genetics have proved an alternative to surgery. Variant organisms are selected on the basis of phenotypic differences in the whole animal and genetic analysis, studies of chimaeras, and correlation of phenotypes have promoted the concept of developmental compartments, which are regions of the developing animal within which particular cell lineages restrict their growth [79,80]. These compartments seem to be

partially independent sub-units of the organism during development. Genes controlling the determination of compartments as particular parts of the animal (leg, antenna, abdomen etc), and the determination of cells within such compartments have also been discovered (see [81]).

More recently this has been extended to the study of the development of the avian wing (see below) . An extensive and increasing number of single genes affecting limb pattern are now known [82]. However, research on vertebrate systems has concentrated on interpreting the effects of surgical manipulations.

The starting point of such research in recent decades was the introduction of the concept of concentration gradients of substances called morphogens which assign positional information within an embryonic field [77]. The term positional information is is defined as spatial position specified with respect to one or more reference points. This idea has been widely applied to the vertebrate limb system, both to the regeneration of amputated limbs of urodele amphibia, and to the development of the limbs of the chick: processes which appear to parallel each other in many ways [83], and in which sequences of digits can readily be manipulated experimentally. In order to explain the geometry of the patterns of digits in experimentally manipulated

chick wings it is suggested that a concentration gradient of diffusible morphogen assigns positional values to components of the developing system. These values are a monotonic function of distance along the antero-posterior axis from the so- called zone of polarising activity (ZPA) [84], which functions as either a source or sink for the morphogen.

This approach appeared to reveal several 'organising' regions controlling differentiation in the animal: the ZPA; the polarising zone [85] (the amphibian analogue of the ZPA); and the apical ectodermal ridge , controlling the proximo-distal axis of the limb [84,86]. The theoretical work of Crick [87] showed that for a small morphogen, the time required to set up a gradient would not be prohibitive for gradients of the lengths postulated. However the techniques of surgical intervention are accompanied by a number of methodological problems, not least the fact that membrane mediated effects, junction mediated effects, and effects of substances diffusing through the extracellular fluids, are likely to be confounded by such gross disruption of cellular interactions.

Furthermore the responses to experimental manipulation of a number of systems (regeneration of the legs of hemimetabolous insects, the development of insect imaginal discs, and the regeneration of amphibian

appendages) have been fitted to the rival polar-coordinate model, by French et al [88]. Surgical removal of tissue in these systems can result in the development of multiple structures. This is interpreted as a consequence of a two- dimensional embryonic field which undergoes pattern regulation (i.e. correction of experimental disruptions) when perturbed by procedures that bring tissues of disparate positional value (as defined by a radial and an angular component) into apposition. The system regulates by the intercalary development of structures which restore the continuity in positional values.

Within the polar co-ordinate model the positional information can be interpreted in terms of phase-shifts in morphogen concentrations, (an idea introduced by Goodwin and Cohen [89]), but not by simple gradients of morphogen emanating from a determined organising zone. In fact the model dispenses with the need to postulate the elusive morphogens at all and the positional values can be held to be characteristics of cell- surface proteins, patterns of intercellular contacts, or of changes in the composition of intercellular matrix material [90]. The role of the cell surface in pattern formation has been considered in other developing systems. In a discussion of compartments in insect development Crick and Lawrence [79] suggested that there might be a gradient of cell surface properties

within each compartment, particularly in view of the fact that anterior and posterior wing disc cells segregate out from each other in mixed cultures.

The requirement for a system of polar co- ordinates can also be relaxed, reducing the problem of pattern formation to the comprehension of a 'Rule of Normal Neighbours', to which the regeneration of the crayfish leg appears to conform [91]. Similarly, Lewis [92] suggests that the polar- co-ordinate model rests on two main rules: a "Rule of Intercalation" - i.e cell proliferation occurs whenever cells with disparate postitional values are juxtaposed, with the cells of the new tissue taking on positional values intermediate between those of the cells on either side of the discontinuity; and the "Complete Circle Rule" which states that whenever a complete circumference is exposed or generated (by amputation, grafting, wound healing, or intercalation) at a given proximo-distal (= radial) level, then growth occurs, and during this growth, all of the more distal phenotypic parts are generated [92].

Other workers [93] have claimed that the geometry of supernumerary components of the developing chick wing produced by surgical manipulations correspond to the predictions of the polar co-ordinate model better than to those of Wolpert et al. Furthermore, appropriately

positioned tissues from morphogenetic fields other than the limb field , can cause the formation of supernumerary wing digits from wing tissue and also direct the polarity of those digits [94]. Such results reduce the credibility of the ZPA as a unique generator of positional information in the limb bud, and lend support to the idea that the polar co-ordinate model more accurately reflects what is going on.

However, Meinhardt [95,96] proposes a further possibility, which incorporates morphogen gradients into a model consistent with this data: that boundaries between differently determined cell types (e.g. compartments) cooperating for the production of morphogens act as organising regions for secondary embryonic fields in which the positional values are assigned by morphogen gradients, leading to peaks of morphogen concentration at compartment boundaries. New organising regions can therefore be created by transplants or amputations. This model allows him to make sense of limb duplications and triplications after heat shock in Drosophila [95], the regeneration of cockroach legs, including the lack of intercalary regeneration after removal of a femur-tibia joint [95], as well as the arrangement of digits in the vertebrate limb [96].

There is no obvious reason why this model building

might not go on indefinitely or why a unitary model should be expected at all. All such schemes are sinply consistent with, rather than positively dictated by, the phenomena which they seek to explain. Moreover, changes in pattern can be totally independent of growth regulation [97], and so the signal for acceleration of mitotic rate that follows the confrontation of tissues of unlike positional value may not be the same as that which initiates pattern respecification . Neither response however, seems to require that a diffusible morphogen be involved [94]. An alternative approach is to search for substances which alter pattern formation in specific ways. Pattern formation can only be studied in this way if spatial heterogeneity already exists in the system. Examples are the effects of lithium on early development [98] or the effects of retinoic acid on limb regeneration.

Maden [97] found that when axolotls with limbs amputated through the forearm are kept in a solution of retinol palmitate (or retinoic acid [99]), instead of regenerating just those parts removed, they regenerate an entire new limb. This gives a limb with the original stump elements, plus the regenerated limb, so that there is a tandem repetition along the proximo-distal axis. This result can be explained on a typical gradient model by the postulate that retinoids stimulate the synthesis of a morphogen (or its

inactivation in a gradient of reversed polarity), resulting in the allocation of more proximal positional values to the stump. Summerbell and Harvey [100] set out to obtain the same results in a developing system, the embryonic chick limb, but instead found that while retinoids do affect development they seem to act primarily on the antero- posterior axis, producing mirror-image reduplications. This result can also be obtained by local application of retinoic acid on filter papers [101] (this also applies to chick leg development [102]). In the case of these local applications of retinoids, the effect parallels that of a polarising region graft. Again, retinoids are altering positional values. Again, the results can be interpreted within an indefinite number of theories of how the positional values are determined in the first place.

1.5 Metabolic cooperation

Subak-Sharpe et al [103] introduced the term metabolic cooperation for the process whereby the metabolism of cells in contact is modified (perhaps controlled) by exchange of material. Metabolic cooperation is a distinct phenomenon from cross-feeding, where material is transferred from one cell to another via the extracellular fluid [104].

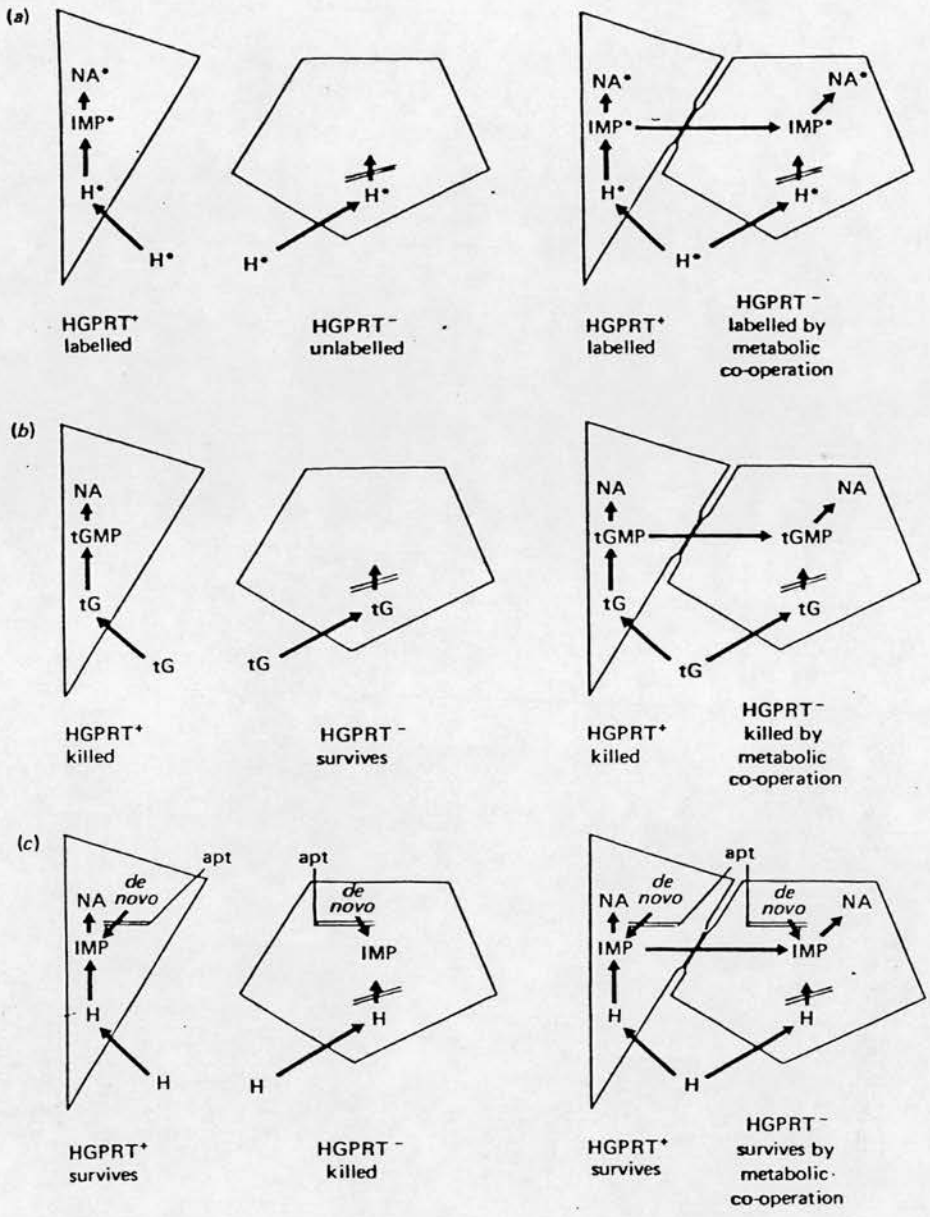
1.51 Transfer of metabolites between cells

The enzyme hypoxanthine guanine phosporibosyl transferase (HGPRT) converts hypoxanthine to the nucleotide inosine monophosphate (IMP). This reaction is the sole means by which the cell can incorporate exogenous hypoxanthine into the pool of nucleotides available for nucleic acid metabolism. The initial demonstration of metabolic cooperation made use of an $HGPRT^-$ cell line, which as a consequence of the genetic lesion, remains unlabelled when incubated in ^3H-hypoxanthine, fixed, and extracted with TCA. In mixtures of $HGPRT^+$ and $HGPRT^-$ cell lines incubated in ^3H- hypoxanthine, autoradiography revealed that many of the cells in contact with strongly labelled cells, were labelled to an intermediate extent [104].

As the period of coculture prior to addition of label was increased, intermediately labelled cells increased too rapidly in number for them to have been derived from strongly labelled cells by division. This suggests that they were $HGPRT^-$ cells cells that had acquired the ability to incorporate hypoxanthine as a result of contact with wild-type cells. The result was interpreted as a consequence of transfer of labelled nucleotides produced by the HGPRT enzyme in the wild-type cells [104] (see fig. 3). Nucleotides themselves cannot cross cell membranes without first being dephosphorylated [105], and hence the labelling could not be due to transfer through the medium of the products of the HGPRT enzyme.

These observations, made on Syrian hamster kidney cells and their polyoma- transformed derivatives, were soon extended to other cell types including human fibroblasts [106]. It was shown that metabolic cooperation could occur between a variety of normal and transformed cells and in heterotypic combinations of cells derived from different mammalian species (e.g. [107,108] reviewed in [104]). It was also demonstrated in cells from human skin biopsies and from amniotic fluid [104].

Transfer of a wide variety of molecules has been demonstrated. Pitts (reviewed in [104]) showed that



Postulated mechanism of metabolic co-operation for nucleotides derived from (^{3}H) hypoxanthine. (*b*) 'Kiss of death' between HGPRT$^+$ and HGPRT$^-$ cells in 6-thioguanine. (*c*) 'Kiss of life' between HGPRT$^+$ and HGPRT$^-$ cells in HAT medium. H, hypoxanthine; apt, aminopterin; tG, 6-thioguanine; NA, nucleic acid. Asterisks denote ^{3}H-labelled compounds.

Figure 3

cells defective in thymidine kinase (TK^-) can incorporate ^{3}H-thymidine when in contact with wild-type cells. Similar experiments have also been made on co-cultures of $APRT^+$ and $APRT^-$ cells labelled with ^{3}H-adenine [109] and of TK^+, dCK^+ and TK^-,dCK^- cells labelled with ^{3}H-deoxycytidine. By combining two of these techniques, it was shown that transfer is bidirectional [109]. Similar experiments demonstrated transfer of phosphorylated derivatives of sugars and choline [110].

In order to eliminate the need for genetic variants in the cells under study, Pitts and Simms [111] modified the technique by prelabelling donor cells with ^{3}H-uridine, then seeding freshly trypsinised unlabelled cells onto them. Once the recipients had been in contact with the donors for some time, autoradiography again revealed cells with intermediate levels of label in contact with the heavily labelled donors.

Intercellular nucleotide transfer may also be detected by the phenomena termed 'kiss of death' and 'kiss of life'[112]. Whilst the purine analogue 6- thioguanine is toxic to wild-type cells by virtue of being metabolised to the nucleotide which is then incorporated into nucleic acid, $HGPRT^-$ cells are resistant to this toxicity because they cannot carry out the first reaction. However, they become sensitive

on forming gap junction with wild-type cells as toxic materials formed in the wild-type cells are passed through gap junctions ('kiss of death') [112] (fig. 3). Similar effects are seen in other salvage pathway variant cells in the presence of the appropriate toxic analogue [104].

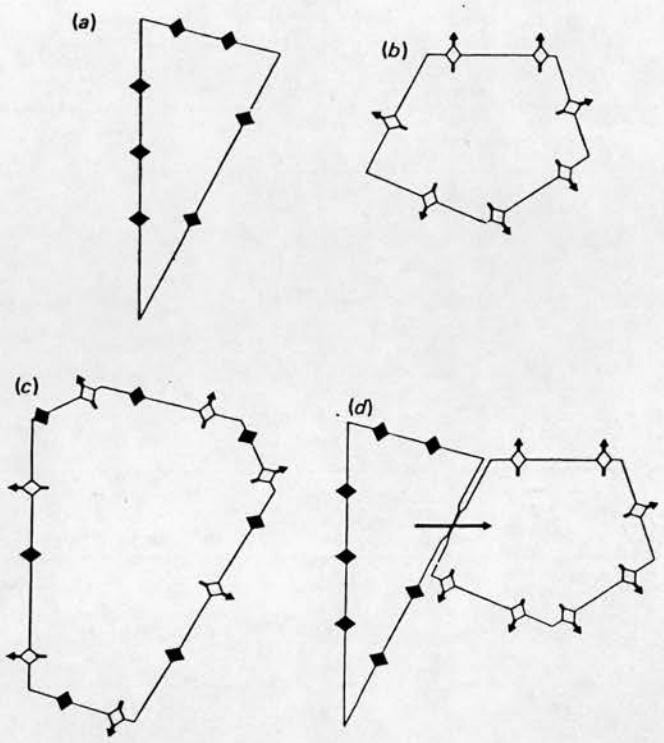
Intercellular transfer of molecules can also rescue cells from environments which are otherwise toxic [112]. Aminopterin blocks de novo purine and pyrimidine synthesis so that the products of both HGPRT and TK are required for growth. $HGPRT^-$ cells will however grow if purine nucleotides are formed in wild-type cells from exogenous hypoxanthine and thymidine and transferred to them through gap junctions [112] (fig. 3). Similarly, Finbow and Pitts [113] demonstrated rescue of proline auxotroph cells from proline deficiency in co- culture with wild- type cells. The rescue was less when the wild-type cells could not form junctions. Similar experiments were carried out with amino- acids of the urea cycle.

Metabolic cooperation can also mediate rescue of cells from the toxic effects of the cardiac glycoside ouabain, an inhibitor of the plasma membrane Na+,K+-ATPase which pumps Na^+ ions out of the cell in exchange for K^+ ions. Ouabain sensitivity is expressed as reduced intracellular K^+ content, reduced rates of

protein synthesis, and cessation of cell multiplication [114]. Synthesis of protein and DNA but not RNA [115] is inhibited. This inhibition is reversible and shows no specificity for any stage in the cell cycle [115].

Corsaro and Migeon [116] demonstrated that ouabain-sensitive (human) cells show increased resistance to ouabain when cocultured with resistant (mouse 3T3) cells. This increased resistance was not seen in co-culture with L-cells (which had previously been shown to be defective in metabolic cooperation, see below, section 1.52) or when the sensitive and resistant cells were not in contact with each other. The intermediate sensitivity of the co-cultures was explained as a consequence of free exchange of Na+ and K+ between the cells through permeable junctions [104] (fig. 4). Using $^{86}Rb^{+}$ as a tracer for K^{+} it has been shown that cocultures of human and mouse cells in ouabain possess higher intracellular $^{86}Rb+$ levels than would be predicted if the two cell lines behaved independently [114]. Rescue from ouabain toxicity can thus be used to detect alkali metal ion transfer between cells [104].

Pitts [117] devised a system in which incorporation of a radioactive precursor can only occur as a result of metabolic cooperation. TK^{-}, ouabain- resistant cells were co-cultured with TK^{+}, ouabain sensitive (oua-S)



Postulated mechanism of rescue from ouabain toxicity of sensitive cell by coculture with resistant cell. (*a*) Ouabain-sensitive (oua^S) cell. In the presence of ouabain the Na^+, K^+-ATPase (◆) is inhibited and the cell dies due to an inability to pump Na^+ out of the cell. (*b*) Ouabain-resistant (oua^R) cell. In ouabain, the Na^+, K^+-ATPase of this cell (◇) remains active. Arrows indicate movement of Na^+, which is accompanied by an equal and opposite flow of K^+. (*c*) Fusion hybrid between oua^S and oua^R cell. Membrane contains ATPase of both parents so that in ouabain only a fraction of the ATPase is active, resulting in an intermediate level of resistance. (*d*) oua^R and oua^S cells connected by a junction permeable to Na^+. Since Na^+ can pass freely from one cell to the other, the situation is formally analogous to (*c*).

Figure 4

Postulated mechanism of rescue from ouabain toxicity of sensitive cell by coculture with resistant cell

Reproduced from Hooper [193].

cells and labelled with H- thymidine in the presence of ouabain. In the absence of cooperation, the oua- S cells would be killed by the ouabain, whilst the TK- cells fail to incorporate radioactivity. Cooperation between the two cell lines results in rescue of the oua-S cells, which therefore incorporate thymidine, and also incorporation of label into the TK^- cells as a consequence of transfer of metabolic intermediates.

Lawrence et al [108] showed that rat-ovarian granulosa cells and mouse myocardial cells in contact with each other both respond to hormonal stimulation designed to produce cAMP in one cell type only. The result is explained as a consequence of junctional transfer of cAMP, or of a related 'second messenger'.

The transfer of metabolites between cells is not the only way in which cell- cell communication can be detected. Microelectrodes have been widely used to inject ions or fluorescent tracer molecules. If communicating channels are present, the passage of ions can be monitored by another electrode in a second cell which detects the voltage deflection caused by the current injected into the first cell [118]. This type of exchange is referred to as ionic coupling or electrical coupling. Similarly the spread of injected fluorescent molecules is used for the direct visualisation of the contact mediated cell-cell

exchange [118].

Whereas the visualisation of dye transfer is a more direct measurement of communication than is autoradiography of nucleotide transfer, microinjection cannot be carried out on a large scale. This problem has been overcome by Goodall and Johnson [119] who incubated cells in the nonfluorescent, non-polar reagent, 6- carboxyfluorescein diacetate (CFDA), which enters the cell freely, where it becomes entrapped following enzymatic conversion to the hydrophilic fluorophore 6-carboxyfluorescein. They then aggregated the cells with unlabelled cells and observed the formation of communicating channels between cells by the passage of dye from labelled to unlabelled cells. Previous attempts to use unmodified fluorescein esters in this way had been less successful due to leakage of the less hydrophilic chromophore from the cells [120,121].

1.52 Cells defective in junctional communication

Mouse L-cells and their derivatives were among the first cell lines found to be defective in transfer of labelled nucleotides. They are defective both in communication with each other and with various other cell types (see [112] for references). L-cells in fact

have a general defect in intercellular transfer of small molecules. This means that they can be used as negative controls in a variety of techniques for the demonstration of metabolic cooperation. However, some L-cell sublines co-operate under certain circumstances (reviewed in [112]). A number of other junction-defective cell types have been reported but are less well-characterised than the L-cell (reviewed in [104]).

In contrast to the use of existing lines, Wright et al [122], selected a co- operation defective line from a polyoma-transformed Syrian hamster cell line defective in TK, dCK and HGPRT. The parental line, resistant by virtue of its TK-deficiency to growth in BrdUdr (bromodeoxyuridine) and subsequent treatment with blue light, became sensitive to this treatment via the 'kiss of death' when co-cultured with TK^+ cells. This was used as a selective procedure to enrich for co-operation deficient cells. After multiple rounds of selection the survivors were cloned and one clone was extensively studied. Co- operation for ^3H-thymidine-derived nucleotides by this clone was undetectable, and only very low levels of cooperation for ^3H-hypoxanthine or ^3H-adenine- derived nucleotides could be detected. The differences between the results for different nucleotides were attributed to differences in pool sizes rather than to selectivity at

the level of the permeable junction [123].

The cells of this clone also differed from those of the parental line in size, morphology, growth rate and karyotype [122]. The fact that addition of dibutyryl cyclic AMP and theophylline to the cultures caused a reversion towards the wild-type morphology, and a return of the ability to cooperate, suggested that the morphology and communication ability are related [123]. A substantial number of differences between polyacrylamide gel profiles of total cell protein from the mutant and its parent were also found [124]. Six of these were abolished by dibutyryl cAMP and theophylline, and may therefore have been involved in the cooperation deficiency.

Communication defective (mec^-) cells have also been selected from a derivative of the chinese hamster DON cell line. A higher kill of mec^+ cells than that achieved by Wright et al [122] was brought about by subjecting mutagenised $HGPRT^-$ cells to four successive cycles of co-cultivation with $HGPRT^+$ cells in the presence of thioguanine. The number of rounds of selection required was reduced, thus making the accumulation of secondary genetic changes less likely. The surviving cells were poor cooperators in a number of assays [125].

Slack et al [126] had previously used the thioguanine

´kiss of death´ method (see fig. 3) to isolate a co-operation- defective variant from the HGPRT$^-$ e.c. line PCl3TG8. After five rounds of selection, a variant, R5/3, was isolated, which showed a reduced level of co-operation as a recipient of ^{3}H- hypoxanthine-derived nucleotides when tested by autoradiography . The possibility that this difference was due to an altered purine nucleotide pool size was eliminated by two approaches: firstly, transfer of the same metabolites, viz. purine nucleotides, was investigated using a colony formation assay based on the ´kiss of life´ (fig. 3)[126]; and secondly, R5/3 cells were shown to be defective in intercellular transfer not only of nucleotides but also of alkali metal ions and amino acids [127]. It was concluded that a difference in junctional membrane properties which has the effect of reducing the probability of junction formation but not abolishing it completely, was responsible. Aside from the cooperation defect R5/3 cells have an higher thioguanine resistance than their parent, PCl3TG8, and have a near tetraploid karyotype, compared with the near diploid karyotype of the parent.

Hooper and Morgan [128] isolated a co-operation competant revertant H2T12 from R5/3 and used this to investigate whether the co-operation deficiency and the other alterations were results of the same lesion. A selective system based on the rescue of HGPRT- cells

from HAT toxicity by metabolic cooperation with wild-type cells was used (see section 1.51). H2T12 communicates for both for nucleotides and alkali metal ions, but it retains the increased level of thioguanine resistance and the increased ploidy present in R5/3, indicating that the genetic basis of these effects is different from that of the observations on communication.

1.53 The nature of the junctional channels

In most cases, whenever ionic coupling has been detected, the spread of injected fluorescent dyes or dye coupling has also been observed [118]. Similarly, nucleotide transfer and ionic coupling are found to occur together. In the Chironomus salivary gland the cells appear to be linked by junctions which behave as molecular sieves such that molecules of up to 1200 daltons pass through the channels with velocities dependent on molecular size. Molecules of 1900 or greater do not pass [129]. Mammalian cell- to-cell channels are similar but discriminate against negatively charged molecules, and have a lower molecular weight cut- off than the channels of insect cell junctions [130].

Electron microscopy has identified a number of specialized regions of contact between adjacent plasma

membranes: these include desmosomes, tight junctions and gap junctions [131]. It is believed that the gap junctions are the sites of contact-dependent transfer, though it is possible that tight junctions may also be involved. A number of lines of evidence support this view. Gap junctions are widely distributed in tissues showing ionic coupling [131] and treatments that disrupt this, such as temporary immersion in low Cl^- or low Ca^{2+} [132], or treatment with hypertonic solutions [133] also interrupt ionic coupling. L-cells, (see above) which are defective in metabolic cooperation and ionic coupling, are also defective in gap junction formation [134], and in somatic cell hybrids of cooperating and non-cooperating cells, the ability to transfer fluorescein is segregated along with the ability to form morphologically normal junctions [135,136,137]. Some segregants failed to transfer fluorescein, but were ionically coupled [136]. These did not possess normal junctions, but small arrays of fibrils of a diameter similar to gap junctional particles (see below), could be seen in freeze fracture preparations of their membranes [137].

Hooper and Parry [138] also showed that R5/3 cells (see section 1.52) have fewer gap junctions and more surface microvilli than PC13TG8, and that both changes are reversed in H2T12, suggesting that the lesion responsible for the cooperation deficiency of R5/3 may

affect the formation of both gap junctions and microvilli. Similarly, the mec- derivative of DON cells isolated by Willecke et al [125], lacks gap junctional plaques evident in freeze-fractured preparations. A major cellular polypeptide of molecular weight 44,000 which is present in PC13TG8 is reduced substantially in amount in R5/3, but reappears in H2T12 with a slightly altered mobility [139]. However, a mechanistic role for this protein in metabolic co- operation seems unlikely as it is absent from some communication- competant clones which are sibs of PC13TG8. An alternative possibility is that it represents a protein whose synthesis requires metabolic co-operation, and indeed there is evidence that cell contact is required for its synthesis [139].

Gap junctions appear in thin-section electron microscopy as specialised parts of cell membranes where the membrane bilayers of the adjacent cells are separated by a 2- to 4-nm gap that can be permeated by colloidal lanthanum which appears to act as a marker of the extracellular space [140]. Freeze fracture shows that these bilayers contain arrays of intramembranous particles packed roughly hexagonally. Purified preparations obtained by subcellular fractionation have been studied by optical diffraction analysis of electron micrographs of negatively stained material and by X-ray diffraction (see e.g. [141]) and this has

given rise to the current view of the gap junction as an array of hydrophilic pores running through the centre of hexameric protein particles or "connexons" [104]. Although there is some controversy over the detailed biochemistry of these particles (reviewed in [142]), all evidence from physiology, electron-microscopy, and X- ray diffraction is consistent with the existence of an aqueous channel extending through the connexons [143], and physiological experiments indicate that these channels are open to passage of small molecules between cytoplasms but that the channels can close in response to changes in the cell environment [144]. Indeed it seems necessary for the survival of the organism that junctions be able to seal in response to changes in cytoplasmic conditions (e.g. the death of one of the adjoining cells) [143].

The lesions in all mec$^-$ cell lines investigated to date are recessive in somatic cell hybrids (see e.g [145] for discussion of these). L-cell hybrids with human mec+ lymphocytes co-operate [135,146] until some of the human chromosomes are lost. A series of hybrid clones isolated following fusion of an L-cell line and R5/3 were restored in their ability to co-operate, indicating the existence of at least two genetically distinct lesions affecting metabolic cooperation, each of which is recessive [145], and hybrids of L-cells and a spontaneous mec- clone of DON cells also co-operate

[147]. Smets et al [146] found that hybrids between an L-cell and an unselected mec- mouse leukemic cell line were still defective in uridine nucleotide transfer, indicating that these two lines were defective at the same locus or loci.

1.54 Chemical regulation of junctional communication

One direct way of identifying functions of the gap junction would be to specifically inhibit junctional communication. A number of inhibitors of metabolic cooperation have been identified. Various studies have supported a close correlation between cell uncoupling and an increase in the cytoplasmic free calcium ion concentration (see [130,148]) or a decrease in intracellular pH (see [112] for a review). High intracellular calcium levels cause the junctional particles to aggregate tightly into crystalline arrays similar to those seen after other uncoupling treatments [148]. This can be brought about by elevation of the external Ca^{2+} load ; treatment of cell cultures (in the presence of high external calcium) with Ca^{2+}-transporting ionophores; treatment with a combination of cyanide and iodoacetate; or with high levels of CO_2 [130]. Cytoplasmic pH and the Ca^{2+} concentration are highly interdependent, so it is difficult to determine whether one is acting via the other or whether both are acting on the gap junctions.

When the extracellular calcium levels are very low, cells also decouple [149] as their adhesion mechanisms are disrupted. Similarly, Nicolas et al [150] showed that an anti e.c. IgG which inhibits aggregation of e.c. cells also inhibits metabolic cooperation.

Junctional communication is inhibited by high levels (10^{-4}M) of retinoic acid. However, the amounts of junctional protein isolable from a rat liver cell line treated with retinoic acid are comparable to those of untreated cells [151] and this is assembled into junctions. Retinoic acid treated human basal cell carcinoma demonstrated an increase in gap junction area in freeze fracture [152]. The observation that the effect is very rapid, and reversible [153] supports the idea that it is acting by modulation of the activity of the junctions rather than by affecting biosynthesis or breakdown of junction components.

In contrast, whilst a number of phorbol esters, notably the tumour-promoter 12-**o**-tetradecanoyl phorbol-13-acetate (TPA), also inhibit metabolic co- operation [154,155], freeze-fracture and quantitative morphological techniques show that treatment with TPA results also in a reduction in the number of junctional plaques [156].

Treatment of cell cultures with cAMP or its derivative dibutyryl cAMP which enters the cell more easily,

produces an increase in junctional permeability [130,157]. A similar channel up- regulation is observed on elevation of the endogenous level of cAMP by serum deprivation or lowering of cell density in 3T3 cells [130,157]. cAMP also causes a number of mec- cell lines to co- operate. These include the mec- derivative selected by Wright et al [123]; R5/3 [Buultjens E., unpublished data]; and an L-cell line (Cl-1D) which fails to make junctions [158]. The mec- DON derivative isolated by Willecke et al shows a small effect [125]. In the case of Cl-1D , cAMP treatment also leads to the appearance of membrane specialisations. These effects of cAMP raise the possibility that a step of phosphorylation intervenes in the assemblage of the channel [130]. Alternatively, as cAMP is known to stimulate the sequestering of Ca^{2+} in the endoplasmic reticulum, it may be acting by promoting the movement of Ca^{2+} from the cytoplasm to the endoplasmic reticulum.

1.55 Role of junctional communication

Gap junctions are found in most animal tissues (exceptions are skeletal muscle, many nerve cells, and blood cells). In excitable tissues they can provide pathways for the intercellular transmission of electrical impulses. In other tissues however, where they are often larger (i.e. more particles per

junction) and more numerous, their functions are ill-defined., and most of the current ideas are predictions or spculations based on observations of junctional permeability made in tissue culture and isolated organs [159].

1.551 Role in Growth Control

There has been much speculation about possible roles for gap junction mediated communication in growth control. Loewenstein and Kanno [160] used intracellular electrical techniques to examine liver cells for the ability to communicate: they found that normal liver cells communicate, but cancerous liver cells showed no communication at all. Communication among normal liver cells was also markedly reduced when cancer cells were grown near to them and it is suggested that normal tissue growth and differentiation depend on the flow of materials from one cell interior to another through junctions [160]. Whilst the corresponding normal tissues cooperate, rat liver tumours, rat and hamster thyroid tumours and human stomach carcinoma have all been found which are defective in this respect. In cells isolated into tissue culture, the same results were obtained (reviewed in [161]). More recently, Morgan et al [162] found that whilst normal brain cells communicate well , two glioma cell lines showed reduced levels of

communication. Based on such data, Loewenstein has developed a coherent theory of how junctions might in fact be crucial in the control of growth [161].

In principle, barriers to communication could equally well act to control growth. Many cell types in culture communicate preferentially with cells similar to themselves (reviewed in [159]) and though this may be an artifact of culture conditions it is observed that primary cultures of normal breast fibroblast and epithelial cells do not communicate with each other in mixed cultures although they do communicate with like cells [163]. Although the two cell types are separated by a basement membrane in the normal adult breast, the invasion of the basement membrane, characteristic of malignant cells, allows contacts to occur between epithelial cells and fibroblasts [163], and it is speculated that breakdown of the communication barrier might be a stage in tumourigenesis [163]. This does not necessarily conflict with Loewenstein´s theory, which does not predict that transformed cells necessarily communicate badly (see [161,164]). The ´wild-type´ embryonal carcinoma lines used in this study possess the capacity for unlimited continual growth and division, in vitro, yet communicate well.

1.552 Junctional communication in development

Furshpan and Potter [2,165] were among the first to suggest that junction mediated intercellular communication may be an important regulatory requirement in differentiation and morphogenesis. Since then, several investigators have suggested that gap junctions which may be assembled between contacting cell membranes may remain in an impermeable state but have the potential to serve as regulators in the mediation of intercellular signalling (reviewed in [104]). However, in the absence of suitable inhibitors, much research on junctional communication in development has concentrated on a search for morphological evidence of cooperation (junctional particles). In many studies it has not been possible to look for transfer of dyes, or of electrical impulses in the same system. The demonstration of co-operation, or the lack of it, by such methods merely tells us whether it could be involved in the determination process, and the results must be interpreted carefully. The presence of functional junctions does not mean that they must be transmitting signal molecules. On the basis of morphological evidence only, it is not even possible to know if communication is occurring.

For instance, the limb buds of vertebrates are capped

apically by a thickened epithelium termed the apical ectodermal ridge, which may be viewed as an inducer of limb development (see section 1.4). Whilst, using techniques of freeze fracture, and both scanning and transmission electron microscopy, numerous large gap junctions are demonstrable in this tissue [166], which distinguish it from the adjacent dorsal and ventral ectoderm, it would not be easy to carry out functional assays of communication in this system. Incidentally [167] mesoderm cells throughout the chick wing bud also have the structural capability for electronic and metabolic coupling.

The identification of semi-autonomous compartments in insect development led to speculation that these might correspond to "co-operation compartments". Initial experiments implied, however, that ionic coupling does not respect the segmental boundaries of insect larvae. However, there are a number of reports of invertebrate systems showing ionic coupling but no dye transfer (reviewed in [168]). When cell-cell communication between epidermal cells of fifth instar larvae of Oncopeltus and Calliphora [168] was studied by transfer of injected Lucifer Yellow (MW 450) and lead EDTA (MW 374) Lucifer was transferred freely between cells in the same segment but not across the segmental boundary. Lead EDTA, however, did not respect the boundary [168], and all the cells were ionically coupled. Examination

for junctional plaques by thin sectioning at the segmental boundary in Oncopeltus did not reveal variation which could account for the dye transfer result [169]. Cell-cell passage of Lucifer Yellow is restricted at the boundary between the anterior and posterior compartments in the Drosophila wing disc: indicating that co- operation compartments exist during development [170].

1.6 Junctional communication and the control of early mammalian embryogenesis

Work on some non-mammalian species has led to the cytoplasm of the unfertilized egg being commonly regarded as a mosaic of determinative molecules. The generation of cellular diversity in early development is thus attributed to action of these determinants on nuclei parcelled into their respective territories during cleavage . This is not the case in the mouse embryo.

1.61 Control of polarisation of the cleavage stage embryo

The polarisation evident during compaction is the first manifestation of differences between the inside and outside of the embryo, and the first opportunity for the cells of the embryo to interact in the determination of cell fate. The induction of an apical pole of microvilli can be observed by location of binding sites for the lectin Con-A by immunofluorescence [171]. The ability to induce this polarity develops during the 2-cell stage evidently as a consequence of cell- cell contact [171].

Lo and Gilula [172] examined the extent of junctional communication in such early cleavage stage embryos. Intact, zona-free mouse embryos were monitored for the presence of ionic coupling, the transfer of injected fluorescein, and of injected horseradish peroxidase

(molecular weight 40,000) [172] which is too large to pass through gap junctions. In the 2- cell, 4- cell and precompaction 8-cell embryos, cytoplasmic bridges allow transfer even of the horseradish peroxidase between sister blastomeres [172], whilst no transfer or ionic coupling at all is observed between blastomeres from different sister pairs. Goodall and Johnson [119] confirmed the observations on dye- transfer using CFDA.

Contact with cells from 4- 8-, and 16- cell stages also induces polarity in cells of 8 cell embryos [171]. 4-cells stage blastomeres cannot transmit dye to them [119]. Gap junctions are therefore clearly not involved in controlling early cleavage divisions, nor with the initiation of polarisation.

At the 8-cell stage injected horseradish peroxidase is still passed to only one other cell, again indicating the presence of cytoplasmic bridges between sister blastomeres [172] but ionic coupling is now present throughout the embryo and fluorochromes introduced into one cell spread to all the blastomeres. Indeed, junctional communication as detected by both ionic coupling and dye transfer occurs between all the cells throughout compaction [172].(This result contrasts with that in the Xenopus embryo in which there is ionic coupling at a stage where there is still no spread to adjacent blastomeres of injected fluorescein [173]).

Freeze- fracture data and observations of the obstruction of diffusion of large molecules into the intercellular spaces of viable embryos show that tight junctions also form at this stage [174] and eventually form a complete permeability barrier around the entire apical surfaces of the cells . Experiments with inhibitors show however that compaction does not require the presence of tight junctions [175].

1.62 Control of the differentiation of the blastocyst

Junction mediated intercellular communication might be involved in the determination of the ICM and the trophectoderm, as this event is clearly distinct from the earlier polarisation. Individual cells isolated from 8-cell mouse embryos can contribute to both ICM and trophectoderm when combined with groups of genetically dissimilar blastomeres [176]. Furthermore, [177] isolated blastomeres that continue to cleave can regulate their development to form miniature blastocysts; and multiple embryos, arranged in spatial configurations that militate against extensive sorting out of cells before blastulation, are capable of forming integrated unitary blastocysts [5], implying that the blastomeres are co- ordinately regulating their differentiation.

Whereas trophectodermal vesicles may be formed from

isolated blastomeres, development of only ICM has not been observed [5]. Conversely blastocysts formed by aggregated pairs of cleaving eggs exhibit a disproportionate increase in number of ICM cells and absence of any consistent pattern of distribution of constituent cells between ICM and trophectoderm [5]. The proportion of trophectoderm appears to depend upon the size of the embryo, and hence upon the surface area: volume ratio. These results support an "inside-outside" hypothesis according to which commitment of cells to trophectodermal or to ICM differentiation depends on whether they occupy an external or internal position respectively [5].

Although unitary blastocysts are normally no longer formed by aggregation if one or both members of a pair of morulae are within approximately 8 hours of blastocyst formation , fusion of late morulae and pairs of early blastocysts after partial dissociation can result in morphologically integrated embryos [5]. Hence loss of ability to regulate by the inside cells may be due to prevention of cell mixing by the development of tight junctions externally , rather than to cell determination, and commitment to the ICM or the trophoblast occurs very late.

Indeed, ICMs isolated immunosurgically from early blastocysts can make trophoblast, suggesting that

inside cells are not committed until close to the 60 cells (3.5 day) stage [5]. When ICMs were dissected from 3.5 and 4.5 day blastocysts and injected into empty zonae before transfer to oviducts of pseudopregnant mice there was no evidence of trophoblast formation, although some cells were exposed to the outside [7,178]. Conversely, vesicles of mural trophectoderm microsurgically isolated from blastocysts never produce ICM derivatives when transferred to the uterus [7]. Nor do mural trophectoderm cells integrate with the ICM or contribute to its derivatives, when injected into the cavity of intact blastocysts [7]. Therefore ICM and trophectoderm lineages are apparantly determined at around 3.5 days.

The observation that immunological blockage of compaction [179] which does not affect cell division or disrupt cell- cell contacts but which prevents development of junctions, can result in an increased proportion of trophectoderm [179], argues for a role of junctions in determination of the two lineages. However, there is no segregation of "communication compartments" at this stage: ionic coupling is found in both trophoblast and cells of the ICM of embryos spreading on a substratum in vitro [180], and dye injections in either trophoblast or ICM spread to the entire embryo [172,180]. Nor can the creation of a

special microenvironment in the incipient blastocoel by the tight junctions by itself explain the inside-outside signalling involved , since normal development from a morula to the blastocyst stage can take place inside the cavity of a giant blastocyst formed by fusion of several embryos (providing the zona pellucida is present to prevent direct contact between the morula and the trophoblast cells of the giant blastocyst) [181].

Johnson and Ziomek [207] argue that the two lineages are not determined by a simple 'inside-outside' signalling mechanism. When single 1/8 blastomeres in culture divide, the two 1/16 cells produced differ. The fluorescent ligand binding and microvilli used as markers of polarisation at the 8 cell stage, segregate at division. They suggest that segregation of cytoplasmic determinants at this stage is subsequently manifested in the differentitation of the ICM and the trophoblast.

Other workers have seen the determination of the two lineages as a result of other effects on cell surfaces, which are altered radically during cleavage [5]. Particular interest has centred on the complex T locus. Many mutations at this locus are lethal from the morula stage onwards (for review see [182]), and it is suggested that the T region specifies cell- surface

components that mediate critical cell interactions at various steps in differentiation [5]. Bennett and Jacob and their colleagues postulate that the T region genes are embryonic equivalents of H-2, the major histocompatability complex . They have obtained evidence that a gene product controlled by or closely linked to T is similar to F9 antigen . Consistent with a role for F9 antigen in cell- surface interactions is the finding that compaction is inhibited if cleaving embryos are cultured in the presence of anti-F9 Fab fragments [183] .

However, there is also evidence that the primary lesion in T mutants is in intermediary metabolism rather than altered cell-surface components [5]. Changes in the lipid composition of the cell membrane are also evident during cleavage [5].

1.63 Determination of cell lineages in the early post-implantation embryo

The experimental work described in this thesis relates primarily to the determination of the embryonic ectoderm and the endoderm (see fig 1). When ICMs are dissected from 3.5 and 4.5 day blastocysts and injected into empty zonae before transfer to oviducts of pseudopregnant mice an outer endoderm layer arises [178], and therefore the primitive endoderm must originate from the ICM rather than by ingrowth of trophectoderm [7,178]. Such observations have been interpreted to suggest that the position of cells may be important in this initial differentiation within the ICM- just as it is thought to be in the morula [178]. The ICM begins as a compact cell mass before the differentiation into outer endoderm and inner ectoderm [5,6]. which appears to mark the divergence of stable cell lineages [5]. When enzymically separated primitive endoderm and primitive ectoderm cells from 4.5 day ICMs are injected into the blastocoelic cavity of host blastocysts , the endoderm cells colonise the endoderm of the yolk-sac only, whilst ectoderm cells

colonised the the entire fetal soma and germ line, as well as the extra embryonic mesoderm of the allantois and the visceral yolk sac [184].

Dye spread at this stage has also been examined in vitro. As the embryo becomes older the spread of injected dye becomes progressively more limited [180]. In embryos grown in vitro until differentiation corresponding to that of later post-implantation stages occurs, dye injected into cells of the ICM does not pass into the surrounding trophoblast cells even though they are in direct cell-cell contact [118]. As the cells of the ICM further differentiate (in vitro) into embryonic ectoderm and extraembryonic endoderm, they undergo further segregation in dye spread, so that dye injected into one region of the ICM results in spread to some of the adjacent cells , while other regions of the ICM are impervious to the dye spread [118]. These communication compartments which correspond to the differentiation of the two cell types [180], are characterised by extremely sharp and distinct boundaries beyond which there is no dye spread [118]. Interestingly, although dye spread is confined to specific regions of the ICM, electrical coupling is still detected across the boundary beyond which no dye spread is observed. Thus the trophoblast cells are electrically coupled to the ICM cells although no dye spread occurs between them [118]. The presence of

specialised membrane junctions in these embryos has also been analysed in freeze fracture replicas. The results correlate with the observations obtained from the electrophysiological studies [180].

The segregation of dye spread within the ICM may reflect the formation of semi-independent communication compartments (where small ions can cross the compartment boundary) necessary for the generation of morphogenetic gradients involved in the differentiation of the embryonic ectoderm [180]. If this were the case it would be critical for successful development of the animal to isolate the ICM from the trophoblast which is making intimate contact with the uterine epithelium including the formation of gap junctions [180].

The subsequent differentiation of the egg cylinder certainly involves cell- cell interactions. Egg-cylinders from 6.5 day embryos were dissected into embryonic and extraembryonic halves and cultured in suspension. After 4 days, the endoderm of the extraembryonic part resembled parietal endoderm, morphologically and biochemically, whereas the embryonic fragments did not change like this, nor did isolated visceral endoderm [185]. This implies that contact with the ectoderm adjacent to the trophoblast causes the endoderm to differentiate into the parietal

form [185]. In other experiments, complete egg cylinders were dissected free of the visceral endoderm overlying the extraembryonic ectoderm and cultured in vitro. The endoderm which recolonises develops a parietal endoderm phenotype [185]. Similarly, when embryonic and extraembryonic ectoderm and visceral endoderm tissues are enzymically separated, and reassociated in different combinations, immuno-peroxidase staining of sections of cultured tissues shows that both extraembryonic and embryonic visceral endoderm synthesise high levels of AFP when cultured in isolation or in association with embryonic ectoderm, but do not synthesise it when in close association with extraembryonic ectoderm [186].

The differentiation of the visceral endoderm, also seems to be modulated by the mesoderm which arises subsequently. Incorporation of labelled amino-acids into transferrin could be detected in the egg cylinder from day 7 onwards. However the ability to synthesise transferrin after isolation from the embryo was either much reduced or absent [16].

1.7 Junctional communication in ec systems

Embryonal carcinoma and teratoma derived cells generally communicate well [114,187,188,189]. Various authors (e.g [138,190,191]) have found gap junctions by

using freeze- fracture and/or thin section electron microscopy. Lo and Gilula reported that on differentiation to "endodermal" cells, the gap junctions were maintained and additional tight junctional elements appeared [178]. In their study, both the ec and differentiated cells communicated by nucleotide transfer, fluorescent dye transfer, and ionic coupling [178]. Similarly, Hooper et al [personal communication], found that co- operation for uridine nucleotides occurs between PC13TG8 cells and their retinoic acid induced differentiation products. A wide variety of lines isolated from outgrowths derived from PSA4TG12 cells co- operate both with each other and with ec cells in this assay though ec cells generally cooperate better with other ec cells than with their differentiation products [Morgan R.H.M., unpublished data]. Nicolas [188] using an indirect assay of metabolic cooperation dependent on rescue from HAT toxicity, reported a lack of communication between ec cells and the cell type produced by treating them with retinoic acid. This result is likely to be a consequence of such selectivity.

Cooperation between PC13TG8 cells and the cells of early mouse embryos has also been studied [189]. Cells of 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

^{3}H-nucleotides to this cell line. Trophectodermal cells of cultured blastocysts, and the trophectodermal derivatives of the 8th day egg cylinder (i.e. extra-embryonic ectoderm and ectoplacental cone cells) however, do not co-operate with PC13TG8 [189]. Co-operation between ec cells and a number of established differentiated cell lines is also poorer than cooperation with other ec cells [192].

1.8 Rationale of this project

The difficulties of specifically inhibiting metabolic co-operation in a developing system have meant that the evidence available on possible roles of junctional communication in development has been somewhat circumstantial. By comparing the properties of PC13TG8, R5/3, and H2T12, (see above, section 1.52) it was hoped to derive more direct evidence. PC13TG8 cells grow in monolayers without overt differentiation but on injection into 129 mice give differentiated teratocarcinomas [187] containing principally nervous tissue and neuroepithelium, with small quantities of other epithelia and cartilage. They respond to retinoic acid *in vitro* like PC13 cells (see section 1.343). R5/3, on the other hand, does not differentiate in tumours [193]. H2T12, and its sib clones do not differentiate in tumours either [193] so the lack of differentiation of R5/3 is not necessarily

a consequence of the lesion in metabolic cooperation. PC13TG8, R5/3 and H2T12 respond to retinoic acid in identical fashion *in vitro* [193]. It therefore appears that co- operation deficiency does not interfere with at least this response.

As has been observed (section 1.341), the differentiation of embryoid bodies must involve cell-cell interactions, involving as it does, a certain amount of co-ordinated spatial variation in cell determination. The main concern of this thesis is the development of a series of independent co-operation-defective variants from the feeder-dependent ec line PSA4TG12, with which a start has been made in investigating the differentiation of embryoid bodies. Starting from one of these mec^- clones, a line which has apparantly regained the mec^+ phenotype on the basis of one assay (that of rescue from ouabain toxicity), but which fails to transfer uridine nucleotides normally, has also been isolated (Chapter 3).

Variant cells other than straightforward mec- variants, with alterations in their junctional communication ability might also be of value in understanding metabolic cooperation. Chapter 3 also describes the isolation and properties of one such variant, a line resistant to the effect of retinoic acid on junctional communication. The effect of retinoic acid on co-

operation, not previously demonstrated in embryonal carcinoma cells, is also shown to provide a method of lineage marking in embryoid bodies (Chapter 4), a result which may well be of value in the further investigation of cellular interactions in this system.

The selection of mec⁻ cells by 'kiss of death' techniques has a number of undesirable consequences. Fluorescence activated cell sorting can in principle provide another way of selecting mec- cells. In mixtures of cells pre- loaded with a small molecular weight fluorescent tracer, and unlabelled cells, transfer of the tracer from cell to cell via gap junctions can occur. A cell- sorter can then be used to isolate variant cells which fail to receive tracer. In Chapter 5 the results of work on the optimisation of this technique using CFDA (see Section 1.51) to label the donor cells, are described.

The lesion in R5/3 is not a clearcut total failure to communicate. In Chapter 6, the effects of a number of manipulations of the culture conditions on the level of co-operation are investigated with a view to gaining further insight into the nature of the lesion.

Finally, as a reminder that junctional communication is not the only means by which cells interact, Chapter 7 describes work which demonstrates that the differentiation of ec cells, as induced by two

different stimuli, can be inhibited by medium conditioned by non-dividing STO feeder cells. The implications of all these findings are discussed in Chapter 8.

Chapter 2

MATERIALS AND METHODS

2.1 Cell lines

STO mouse fibroblasts [34] are a thioguanine-resistant and ouabain resistant derivative of a continuous line derived from SIM embryos [194].

The origins and phenotypes of the ec lines used in this study are tabulated in Table 1.

2.2 Chemicals

2-amino-2-methylpropanol, bovine thrombin, colchicine, dibutyryl cyclic AMP , dimethyl sulphoxide, 2-mercaptoethanol, MNNG, ouabain, p-nitrophenyl phosphate, swine-skin gelatin, theophylline, thioguanine and thymidine were all obtained from Sigma. Coomassie Brilliant Blue G-250 dye reagent was obtained from Bio-Rad. Giemsa stain, Kenacid blue R, and Leishman´s stain were obtained from BDH; scintillator 299TM from Packard; 6- carboxy fluorescein diacetate from Molecular Probes, Oregon; and radiochemicals from Amersham. mitomycin-C and retinoic acid were provided by Calbiochem. Phosphate buffered saline tablets, Glasgow- modified Eagle´s medium concentrate, and medium supplements, and in addition trypsin and chicken serum were obtained from Flow. Calf serum came from Flow, Gibco, Sera- labs, or Northumbria Biologicals.

Human plasminogen was a gift from Dr. I. MacGregor of

the Scottish National Blood Transfusion Service (SNBTS). Bovine fibrinogen (plasminogen and thrombin-free) was obtained from Calbiochem. For routine assays of PC13TG8- derived cells a crude human fibrinogen preparation containing endogenous plasminogen (provided by Dr. D. Pepper, SNBTS, Edinburgh) was used.

K2 Nuclear Research emulsion was obtained from Ilford. D19 Developer was obtained from Kodak. Amfix fixer was obtained from Hamilton Tait (Photographers, Edinburgh).

2.3 Cell culture

Cell culture was carried out using either Falcon or Nunc disposable plastic bottles or dishes. Media and cells were transferred using Volac serological pipettes.

Pipettes were soaked overnight in Calgonite (100g/l), washed in water and then 0.01M HCl, rinsed thoroughly in tap water and then distilled water, dried in a hot air oven at 180 °C, and sterilized in canisters by heating to 160 °C for two hours before use.

Cells were grown in Glasgow-modified Eagle's medium [195] supplemented with non- essential amino-acids (0.1mM each), 1mM sodium pyruvate, 10% foetal or newborn calf serum (selected batches), and 0.1mM 2-

mercaptoethanol [196] . Cultures were maintained at 37 °C in a humidified atmosphere of 95% air, 5% carbon dioxide and were checked periodically for mycoplasma contamination using the method of Chen [197]. Any cultures suspected of being contaminated were discarded. Stocks of PC13 and of its derivatives were grown on plastic surfaces pre- coated with gelatin [30]. The plastic was covered with a 0.1% gelatin solution, then stored at 4 °C to allow adsorption on to the surface of the plastic. The excess gelatin was then aspirated away . Stocks of SCC- PSA4, of SCC-PSA4 derivatives and of the lines SCC-S2 and Nulli-SCC1 were maintained on STO feeder layers prepared as described below (Section 2.33).

2.31 Passaging of cells

Cells to be passaged were first washed with with phosphate-buffered saline (PBSA). This is an aqueous solution containing 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4 and 0.2 g/l KH_2PO_4. They were then disaggregated with TVP, a mixture of trypsin (0.25 g/l), disodium EDTA (0.37 g/l) and chicken serum (10ml/l) in PBSA. The trypsin was neutralised with medium containing serum. Split ratios were generally of the order of 1:20.

2.32 Freezing and thawing of cells

Frozen stocks of cells were prepared by suspending cells in medium supplemented with 10% serum and 10% DMSO (freezing mixture). The cells were then transferred to serum test tubes in a volume of between 0.5 ml and 1 ml of freezing mixture, and stored overnight in racks in a -70 ^{o}C freezer, such that they were cooled at a rate of about 1 ^{o}C per minute. The tubes of cells were then transferred to the vapour above liquid nitrogen. In contrast to the freezing process, cells were thawed rapidly by placing the ampoules direcly into a 37 ^{o}C water bath. The contents were removed into a universal tube, and 9mls of medium containing 10% serum were added. The DMSO was then removed completely by spinning down the cells and resuspending in fresh medium.

2.33 Preparation of feeder layers

Feeder layers were prepared from STO cells unless otherwise indicated. Confluent cultures were treated with medium containing 0.01mg/ml mitomycin-C for two hours at 37 ^{o}C [34]. The cells were washed three times with phosphate buffered saline, trypsinised and seeded out at a density of $4x10^{4}$ cells/cm^{2}. The integrity of STO feeder layers depends critically on the condition of the STO cells on mitomycin treatment. For the formation of good feeder layers it is important to use

STO cells which are subject to density dependent growth inhibition. High passage STO stocks (above passage 27), and stocks which had been overgrown with cells which continued to divide even at confluence, were discarded. Feeder layers were generally considered to be suitable for use for approximately one week after mitomycin treatment.

2.34 Preparation of conditioned medium

Conditioned medium was made by incubating 10ml of medium for 24 hours on a feeder layer in a 25cm^2 flask. After conditioning, the medium was filtered through a millipore membrane, (pore size 0.2- 0.22um). Control, unmodified medium was stored for 24 hours at 37 ^{o}C prior to use.

2.35 Establishment of clonal lines

Clonal lines were established by dilution plating of single cell suspensions into multi-well plates. Except where indicated otherwise, feeder-layers had previously been established in the wells. Only where a single colony grew up in the well, was this trypsinised and expanded into a cell line. This methodology cannot exclude the possibility of colonies growing up from small clumps of cells which were inadequately trypsinised. It was impossible to scan the wells

optically to exclude such colonies immediately after the ec cells had been seeded out, as single ec cells could not be distinguished against a background of feeder cells.

2.36 Mutagenesis

Near-confluent monolayers of cells on STO feeders were treated with 5 x 10^{-5} M MNNG at 37 °C for 2 hours. The cells were then washed twice with unmodified medium, and incubated for a further 24 hours. They were trypsinised to remove dead and dying cells and reseeded onto the same surface area. The cells were then grown up to their original density, aliquots were frozen down, and the remainder used as the starting material of selections (see Section 3 for details).

2.4 Toxicity testing

2.41 Toxicity of DMSO and retinoic acid

Cultures were treated for 12 hours with 0.1 µCi/ml ^{14}C-Thymidine , then washed twice with PBS and incubated in fresh medium for 2 hours to allow incorporation of the soluble nucleotide pool into nucleic acid. The cultures were then trypsinised, and seeded onto STO feeder layers at a density of 10^5 cells per 25cm^2. After overnight incubation, the cultures were treated for 3 days with the medium under test, supplemented

with 2 x 10^{-5} M unlabelled thymidine, intended to reduce reincorporation of label from dying cells. Cells remaining after this treatment were removed from the dishes with 250mM sodium hydroxide, neutralised with 250mM hydrochloric acid, added to scintillant and scintillation counted. Background counts from samples of an equivalent mixture of aqueous solution and scintillant were subtracted. These counts were close to those obtained by treating the cells with 10% DMSO (Figure 5). Refeeding of cultures did not markedly affect recovery of label. The toxicity of retinoic acid was measured at a concentration of 0.3% DMSO throughout. LD50 values, defined as the concentrations at which recovery of counts was half that of the untreated controls, were estimated from curves of label recovered against concentration of the substance under test.

2.42 Toxicity of thioguanine

10^3 cells were seeded onto each of a number of STO feeder layers (Section 2.33) in 60mm tissue culture dishes. After an overnight incubation, the cells were treated with medium containing thioguanine at the concentration under test. After one week the colonies were stained with Leishman's Stain, and counted. Experiments were carried out in duplicate, and results expressed as a ratio of the colonies counted in the

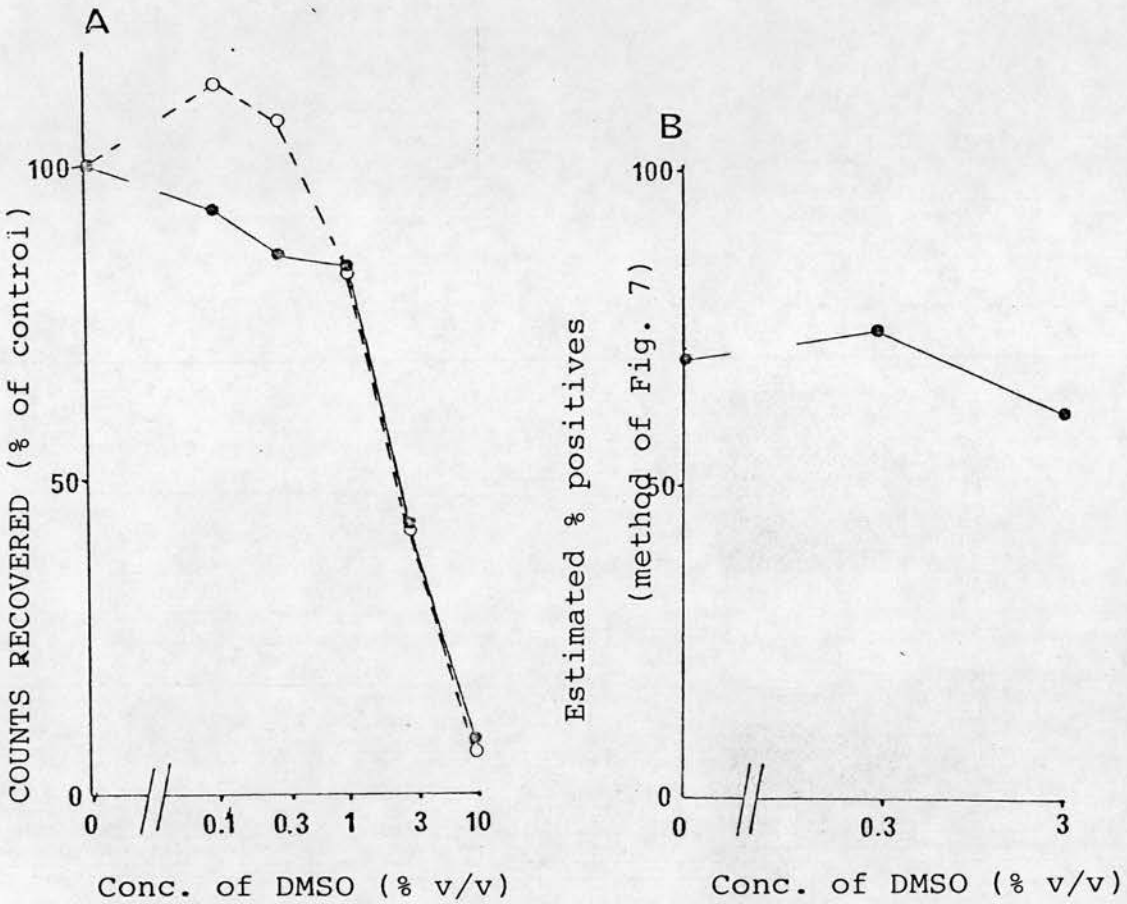


Figure 5

A: Toxic effect of DMSO on PSA4TG12 and PTmr0.

● PSA4TG12; o PTmr0.

B: Effect of DMSO on uridine nucleotide transfer

● PSA4TG12

treated dishes, to the mean number of colonies counted in untreated controls.

2.5 Induction and analysis of ec cell differentiation

2.51 Induction of differentiation of PC13TG8 by retinoic acid

PC13TG8 cells were trypsinised and inoculated into 60mm tissue culture dishes (10^5 cells per dish) either on gelatin-coated surfaces or on STO feeders . Cultures were incubated for 24 hours, and then treated with medium containing retinoic acid for a further 24 hours. They were then returned to unmodified medium for a further 72 hours, trypsinised, counted with a Coulter counter, and reseeded into duplicate gelatinised 60mm dishes (10^3 cells per dish). The resulting colonies were grown up for a further week and assayed for plasminogen activator (Section 2.91).

Colonies derived from control cultures of PC13TG8 not treated with retinoic acid, were uniformly plasminogen activator negative. The number of such colonies was therefore used to estimate the cloning efficiency of ec cells. The proportion (p) of ec cells in the treated population was therefore estimated as c-/C, where c- is the number of plasminogen activator negative colonies derived from treated cultures and C is the mean number of colonies derived from corresponding untreated cultures. The Coulter counts were used to estimate the

total cell number (N) on trypsinisation, and thus the number of ec cells (Np) and committed cell (N(1- p)).

2.52 Induction of differentiation of PSA4TG12 by seeding at high densities on gelatin

After passaging once on gelatin in conditioned medium to remove residual feeder cells, 10^5 cells were inoculated into each 60mm gelatinised tissue culture dish in the medium under test (day 0). This medium was replaced with fresh medium of the same type after 4 days and again on the 7th day after inoculation. Cell pellets were prepared by trypsinising the culture and centrifuging at 500g for 5 minutes. Cells were then resuspended and spun down twice from fresh medium, and twice from isotonic sodium chloride. Pellets were stored at - 70 ^{o}C, then assayed for alkaline phosphatase (see below, Section 2.92).

2.53 Induction of differentiation by formation of aggregates

2.5 x 10^6 cells were seeded into each of a number of 60mm dishes without STO feeders in Eagles medium supplemented with 10% New- born calf serum (selected batches) (EC10 medium). This passage reduces the proportion of feeder cells present. Cultures were fed as necessary until nests of cells were formed. This stage was reached after about 5 days. These aggregates were then detached by blowing fresh medium across the

surface of the dish, transferred to bacteriological dishes to which they could not attach, and grown in suspension for 5-6 days in EC10 medium.

Where the effect of dibutyryl cyclic AMP and theophylline was under investigation, the medium was supplemented with both substances at a concentration of 1mM each during the suspension stage.

After suspension culture, embryoid bodies were transfered to universal containers and allowed to sediment under gravity. They were fixed in Bouin's fluid then spun down in molten bacteriological agar. The agar was allowed to set, and the embryoid bodies were subjected to routine histological processing and embedded in paraffin wax. 4- 5µm sections were cut and then stained with Mayer's haemotoxylin and eosin.

2.54 Differentiation of embryoid body outgrowths

Outgrowths were formed by allowing five day embryoid bodies to attach to plastic coverslips in complete medium. The cultures were incubated for two days before refeeding. They were then re-fed at intervals for two weeks, fixed in Bouin's fluid, embedded in paraffin wax *in situ*, and after routine histological processing, sectioned, and stained with Mayer's haemotoxylin and eosin.

2.6 Techniques for quantification of metabolic cooperation

2.61 Uridine nucleotide transfer

2×10^5 cells were seeded onto each of a number of gelatinised 30mm tissue culture dishes. Some of these dishes were to serve as donor cells, others as recipients. They were then incubated overnight.

In all experiments but those discussed in Chapter 6, donor cells were then treated with medium supplemented with 10µCi/ml of ^{3}H- uridine for a period of 3 hours, washed three times with PBS, trypsinised and seeded onto recipients. Donor- recipient mixtures were then incubated for 4 hours (except where otherwise stated) before washing once with PBS, and treating for autoradiography.

In the work described in Chapter 6, the concentration of ^{3}H-uridine used was only 2.5 µCi/ml, and the recipient cells were seeded onto the donor cells, rather than the converse.

In both cases, following the co-incubation, the dishes were inverted over a tissue soaked in fix (methanol:acetic acid at a ratio of 3:1 v/v, freshly made up), for at least 10 minutes, then extracted twice with ice-cold 10% TCA, washed overnight in running tap water, and air-dried for at least 24 hours.

K2 Nuclear Research emulsion was melted at 50°C under a red safe- light, and mixed with water in a ratio of emulsion:water of 1:1.5 (v/v). Dishes were coated with emulsion by pipetting a small volume of this mixture onto a dish and transfering the excess to other dishes. The dishes were dried for at least 1hr in a stream of cool air in a dark box.

The dishes were stored at 4°C in the presence of silica gel in the dark in airtight tins wrapped in foil. They were exposed for approximately one week, warmed to room temperature, then developed for 3 minutes in D19 developer at room temperature, washed once in water , and fixed for at least 20 minutes in Amfix. The autoradiographs were then washed about 20 minutes in running water, air-dried and stained with freshly filtered Leishman's Stain. The preparations were again air-dried, and mounted in DPX. In the experiments of Chapter 6 the exposure time of one week was inadequate, due to the lower specific activity of ^{3}H-uridine used. Duplicates of the dishes developed initially were therefore exposed for periods of up to 4 weeks, and these were the ones finally scored.

Where applicable, cells were treated with 1mM dibutyryl cAMP and 1mM theophylline four hours after the initial seeding out of the donors and recipients. The labelling of the donors and the co-incubations were

also carried out in this medium. When the experiment was intended to determine the effect of retinoic acid or DMSO on cooperation, recipients were fed with the medium under test only ten minutes prior to the addition of the donors, which did not encounter the substance under test until the co- incubation.

Except in the case of the work described in Chapter 6, dishes were scored by grain counting 100 recipients in contact with donors and 100 isolated recipients per dish. The isolated recipients scored were selected as those closest to the contacting recipients scored, where there was clearly no contact either directly or through chains of cells, with donor cells. In selected cases dishes were set up in which there were no recipients. These were used to indicate the level of heterogeneity of labelling of the donors. Confusion of poorly metabolising donors with recipients was not found to be a serious problem.

The distributions of grain counts are conventionally displayed as histograms in which the counts for recipients in contact are plotted as usual, whilst the data for isolated recipients are inverted underneath (Figure 6). Parameters describing the extent of cooperation have been devised by Slack et al [126], and by Gaunt and Subak-Sharpe [192], but neither is satisfactory when background grain counts and numbers

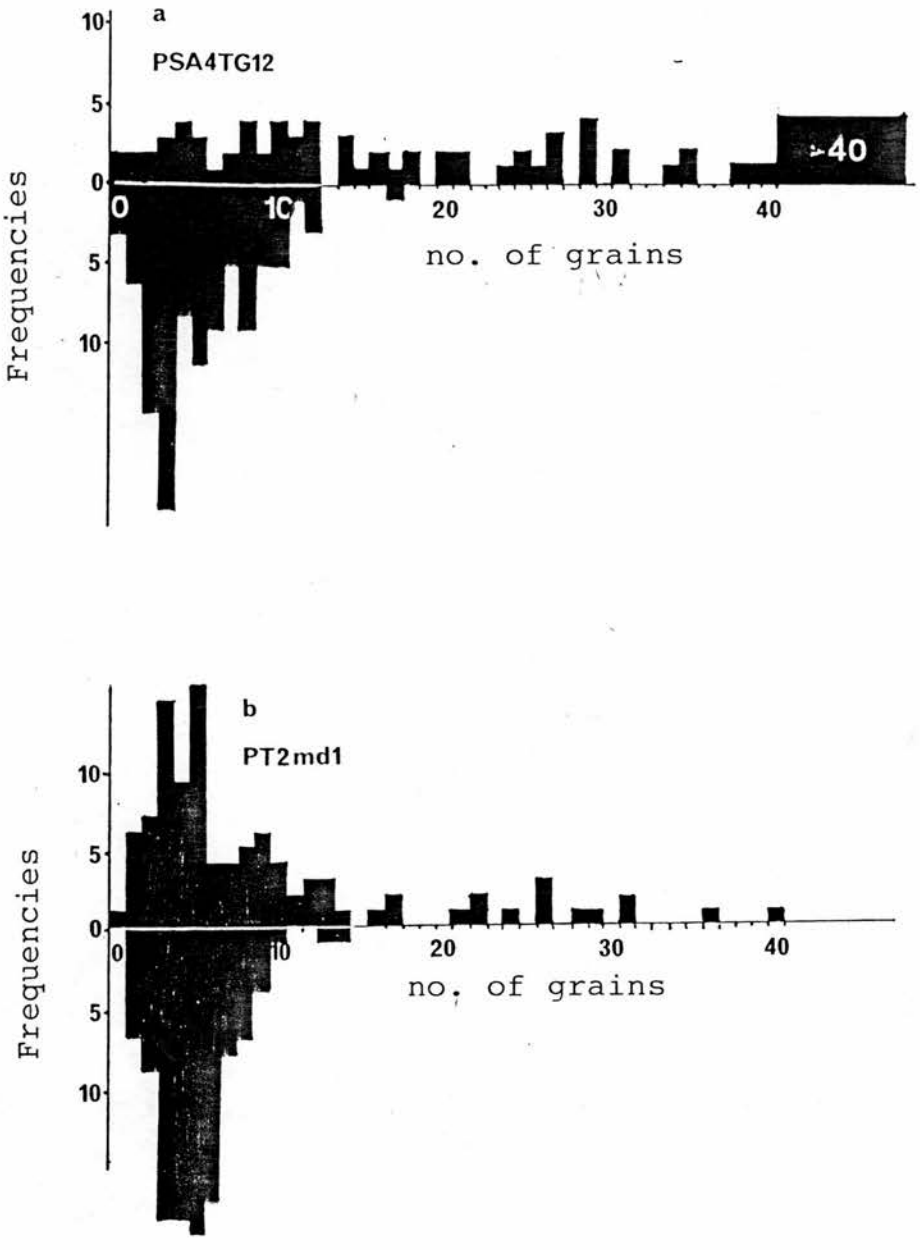
Figure 6

Specimen distributions of grain numbers over recipient cells in uridine nucleotide transfer experiments

Upper histograms: distribution of grain numbers over recipients in direct contact with donors.
Lower, inverted histograms: distribution over isolated recipients.

Summary of measures of extent of co-operation derived from these data

Measure	value for PSA4TG12	value for PT2md1
median of upper distribution:	25	5
median of lower distribution:	4	5
difference :	21	0
Mann-Whitney U :	8654	6058
z :	8.93	2.39
Estimated percent positives (Method of Figure 7) :	73.1	19.5


a
PSA4TG12
Frequencies
10
5
0
5
10
0
10
20
30
40
no. of grains
b
PT2md1
Frequencies
10
5
0
5
10
0
10
20
30
40
no. of grains

of grains overlying donors vary between dishes [104] (see Appendix A) . A novel method of estimating the percentage of contacts showing transfer was therefore derived (see Figure 7, and Appendix A).

2.62 Rescue from ouabain toxicity

10^3 PSA4TG12 or cells under test were seeded onto each of a series of STO feeder-layers in 60mm dishes, or onto dishes coated with gelatine. After overnight incubation, half the dishes were treated with medium containing 3mM ouabain, and half with unmodified medium. After a further 3 days incubation, all the dishes were returned to ouabain-free medium for a further 3 days of growth. Dishes were then stained with Leishman´s stain for colony counting.

The colony counts for one experiment are tabulated in Table 2. The index of co- operation for the line under test was defined as the mean number of colonies which grew up in ouabain, divided by the mean numbers in the controls, divided again by the corresponding ratio for the control line (PSA4TG12). A high ratio corresponds to rescue of a large proportion of the ec cells by the mechanism discussed in Section 1.51, and a low ratio to poor rescue. Cells co-operating as well as PSA4TG12 should give a ratio of 1.

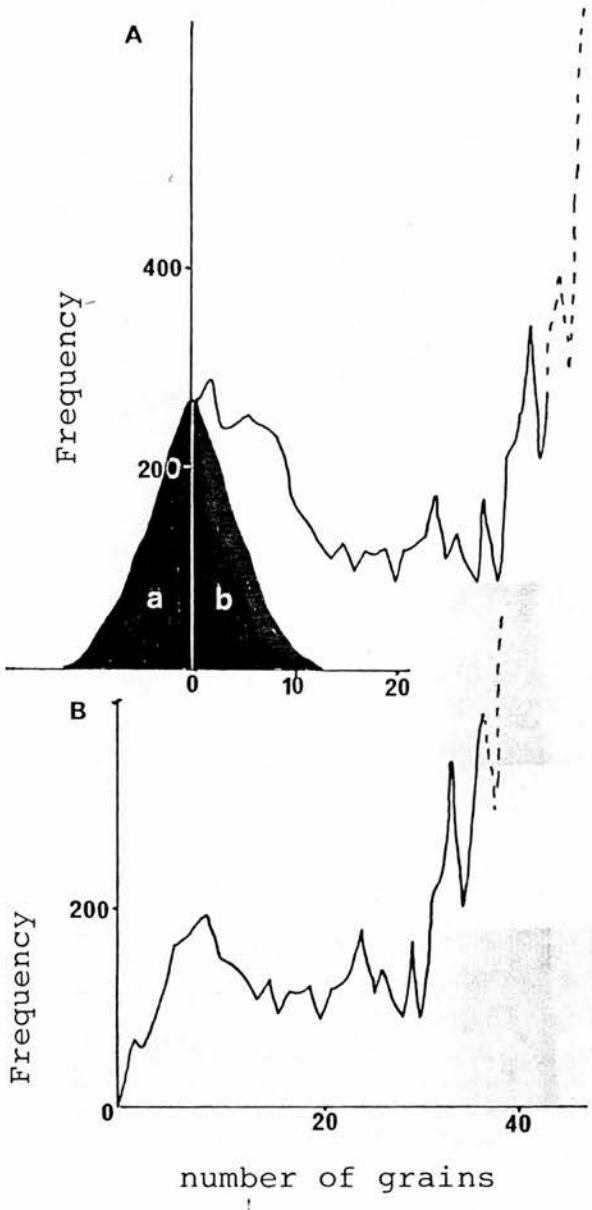
Figure 7

Analysis of uridine nucleotide transfer data

The data analysed is that for PSA4TG12 displayed in Figure 6.

A frequency distribution of the signed differences in the grain counts between all possible pairs of one contacting and one isolated recipient (10,000 pairs in all) was plotted (A); this was then transformed by subtracting the frequencies for negative differences from those observed for positive differences of equal magnitude (B), i.e. reflecting shaded area (a) in the y- axis to give shaded area (b), then taking away both these areas from the curve (A); the fraction of cell- cell contacts showing co-operation was estimated as the ratio of the area beneath the transformed frequency distribution (B) to that beneath the untransformed curve (A). In this example, the estimate obtained is of a percentage positive contacts of 73.08. The median of the transformed frequency distribution (B), is 29.

The rationale for these manipulations is described in Appendix A.


A
400
200
Frequency
a
b
0
10
20
B
200
0
Frequency
20
40
number of grains

2.631 Flow cytometry and fluorescence-activated cell sorting

High cell density cultures of PC13.5 were treated with mitomycin-C as described for STO cells (Section 2.33) 24 hours before use. They were not trysinised and reseeded after treatment however but were used *in situ*.

Donor cells (STO feeder layers or mitomycin-C treated PC13.5 cells) were incubated for 15 minutes in 40µg/ml CFDA in serum-free medium, washed three times in medium containing 10% serum, and recipient cells seeded on, at a density of about 5×10^5 per 25cm flask (except in the cases of the selections, when the density varied between approximately 5×10^5 and 2×10^6 per 25cm flask). The cells were then left for approximately 4 hours, trypsinised, diluted into serum-free medium, and examined using a FACS4 (Becton-Dickinson). Cells were fed through the machine at a rate of approximately 500 per second . The cells were passed through a beam of 488nm light produced by an argon ion laser (Spectra-Physics model 164-05). Fluorescence was detected using optical filters OG530, LP520 and SP540. The signal was amplified logarithmically. Forward light scatter was also measured. Neutral density filters (2.0 O.D. units) were used to reduce the forward scatter intensity. Sorting was carried out with a nozzle of orifice diameter 70µm.

Ec cells which had not been exposed to donors were also examined. These had been seeded onto gelatin at the same time as the other cells were seeded on to the donors.

2.632 Fluorescence-activated cell sorting of cells marked with latex beads

Exponentially growing cultures of ec cells were incubated overnight in medium containing sterile polystyrene beads of diameter either 1.1μm or 0.46μm. The cells were then washed three times with PBSA and incubated in unmodified medium for a further 24 hours. Complementary 50:50 mixtures of R5/3 cells and H2T12 cells were prepared in which one cell line was labelled with the smaller beads and the other with the larger ones. These were then seeded on to labelled STO donors and sorted on the FACS as described above. The donor cells were gated out by rejecting all cells with fluorescence or forward light scatter parameters outside the tight ranges occupied by the ec cells, and the ec population sorted into two fractions: those above the median fluorescence intensity, and those below. These fractions were seeded onto separate gelatinised tissue culture dishes, incubated overnight, fixed in methanol and stained with Leishman's stain. The preparations were examined microscopically for beads. The size of the beads in 20 cells in each

preparation was recorded. There is little transfer of phagocytosed beads between ec cells in culture [Hooper M.L., personal communication]. Consequently the proportions of these samples of cells with either size of bead were assumed to be equal to the proportions of the corresponding cell line in the sample.

2.7 Karyology

Log-phase cultures were treated with 100ug/ml colchicine for 2 hours at 37 °C. Cells were trypsinised, spun down at 1000rpm for 5 minutes and

resuspended gently in 0.075M potassium chloride. After a 4 minute incubation at room temperature, the cells were spun down again. They were then fixed in ice-cold methonal:glacial acetic acid (3:1 v/v) for at least 30 minutes, spun down again, and resuspended in further fix. Drop preparations were made onto clean microscope slides. The chromosomes were then stained in Giemsa stain, and chromosomes in apparantly unbroken spreads counted using either a video monitor or a camera lucida.

2.8 Lineage marking

Embryoid bodies of mixed origins were grown by initiating the protocol above (Section 2.53) with a 1:1 mixture of HGPRT :HGPRT cells. They were then treated with EC10 medium supplemented with 10^{-4} M retinoic acid for 30 minutes. This was intended to ensure that co-operation was blocked well before the H-hypoxanthine was added. ^{3}H-Hypoxanthine was added as a concentrate to a final concentration of 2.5μCi/ml. The retinoic acid was retained. The incubation mixture was then left for 3 hours at 37 °C. The embryoid bodies were allowed to settle in a universal, washed 3 times with PBS, and fixed in Bouin´s fluid.

Paraffin sections were prepared as described above. Sections were deparaffinised in xylene, passed through

an alcohol series, transferred to distilled water, then air-dried. K2 autoradiographic emulsion (see Section 2.61) was melted at 50 °C under a red safe-light, and mixed with water in a ratio of emulsion:water of 2:1. The autoradiographs were then treated as described above for the uridine nucleotide transfer assays until staining. The sections were then stained with eosin, and mounted in DPX.

2.9 Enzyme assays

2.91 Plasminogen activator assays

A modification of the fibrin lysis method of Strickland and Mahdavi [47] was used to assay colonies for enzyme activity. Cultures were washed twice with phosphate buffered saline and overlaid with 1.5ml of serum-free medium containing 3.3mg/ml bovine fibrinogen, 0.4 units/ml human plasminogen (this was freed of low molecular weight material by gel- filtration on Sephadex G25M prior to use), and 0.67 units/ml bovine thrombin . Plasminogen was omitted from control dishes. After the formation of a fibrin gel, dishes were incubated at 37 °C for 4 hours. The preparations were then fixed by overlaying the gel with 5ml methanol, stained with Kenacid blue R (2mg/ml in methanol: glacial acetic acid: water: 45:10:45 v/v), and examined for the presence of a lysis zone around

each colony.

For routine assays of PC13TG8-derived cells a crude human fibrinogen preparation containing endogenous plasminogen (see Section 2.2) was used. The gels were stained with Leishman's stain. Controls established that both retinoic acid treated and untreated cultures gave the same results with the two preparations.

2.92 Alkaline phosphatase assays

Cell pellets were thawed slowly on ice then resuspended in chilled distilled water. Sonicates were prepared by three 10 second bursts in an MSE sonicator at an amplitude just sufficient to produce cavitation. They were then centrifuged at 3000g for 15 minutes. The extract was assayed at pH 10.0 using p-nitrophenyl phosphate as substrate. The reaction mixture (0.5ml) contained p-nitrophenyl phosphate (10mM), magnesium chloride (2mM), 2-amino-2- methylpropanol/HCl buffer (0.5M), and an appropriate amount of extract. Incubation was at 37°C for 30 min, after which time 1.5 ml of 0.25M sodium hydroxide was added to each tube. Absorbancies were measured at 410nm.

Protein was determined by a standard dye-binding assay based on the change in absorbtion of Coomassie Brilliant Blue G-250 in response to protein

concentration variation [198]. Several dilutions of bovine serum albumen solution were prepared as a standard. 5 ml of the dye reagent diluted 1:4 in water was allowed to react with 0.1 ml of each of these standards and with the appropriately diluted samples. Absorbancies were measured at 595nm and the concentration of protein in the samples determined by comparison with the standard curve.

Chapter 3

ISOLATION AND PROPERTIES OF VARIANT CELL LINES

3.1 The selection of cell lines by thioguanine 'kiss of death'

In view of its ability to differentiate extensively *in vitro* and to contribute to chimaeric embryos, the cell line PSA4 was chosen as an appropriate source from which to select mec$^-$ cell lines (see Section 1.8). The thioguanine 'kiss of death' technique, used to isolate the line R5/3 (see Section 1.52) requires an HGPRT- cell line as its starting material. Such a derivative of PSA4, namely the clone PSA4TG12 was available. This was isolated by selection for resistance to 10μg/ml 6- thioguanine [199]. It has a similar developmental capacity to that of PSA4.

In order to achieve a high kill of wild-type cells by the kiss of death technique, the parental cells must be cultured in the presence of 6- thioguanine under conditions where they form junctions at high frequency with HGPRT+ cells. In view of the evidence that ec cells form junctions rather better with other ec cells than with non-ec cells (Section 1.7), either PSA4 cells, or PC13.5 (a subclone of PC13) cells were used in this role. PSA4 cells have the superficial attraction of being as close to the parental cells as possible, thus making it unlikely that the two cell lines will sort out and fail to make good contact with each other. However, PC13 cells proved much easier to grow reproducibly, as they show very little spontaneous

differentiation and do not require feeder layers. The PSA4 cells, vary considerably in growth habit and in the extent of spontaneous differentiation in culture, partially as a consequence of the difficulties of providing them with reproducible feeder- layers.

The feeder layers themselves complicate the selection procedure more directly, as they are necessary for the maintainance of the PSA4TG12 cells in an undifferentiated state (see Section 1.34). STO cells lack the HGPRT enzyme and consequently are not killed directly by treatment with 6- thioguanine. However, in the presence of $HGPRT^+$ cells as in a kiss of death selection, the STO cells detach from the substrate (presumably as a result of incorporation of toxic nucleotide analogues into their nucleic acid as a consequence of metabolic cooperation), just as do other HGPRT- mec+ cells (despite the mitomycin- treatment feeder cells carried through into the donor cell population in uridine nucleotide transfer assays are labelled by the tritiated uridine and are therefore presumably making RNA). The feeder- dependent cells growing upon feeders which detach are also lost.

Theoretically this problem can be avoided in a number of ways. In principle, the use of a mec^- feeder layer would avoid the problem. The feeders would not incorporate toxic nucleotides, just as mec- variants

are resistant to the 'kiss of death'. Unfortunately, such feeder cells are not available. The imposition of a barrier between the feeder cells and the HGPRT+ cells such that metabolic co- operation, but not the cell-cell interactions needed for the feeder effect, was obstructed, might be another way around this problem. Possible barriers include filters and gels of materials such as agar. It was found that ec cells would not readily attach to filters, nor do they grow well on agar gels. In Chapter 7 it is shown that the feeder effect can, at least partially, be reproduced by medium conditioned by the feeder cells. It might therefore have been possible to recover mec^- ec cells from selections carried out in conditioned medium. However, this approach was not used. A further possibility is the use of a parental cell line selected to be feeder independent. This would allow the selections to be carried out without feeders.

In pursuit of the latter strategem, a culture of PSA4TG12 was passaged twice at a high density on gelatine, and then cloned, again on gelatine. These clones were screened for their ability to co-operate, as measured by the ouabain rescue assay (this assay is described in Sections 1.51, 2.62, and Table 2), and the two of them which scored lowest, PT1 and PT3, were chosen as the starting material for 'kiss of death' selections (see Sections 3.11 and 3.13). Two other

clones, PT2 and PT4, chosen at random from PSA4TG12 cells cloned on STO feeder layers, were used as starting material for independent selections (see Sections 3.12 and 3.14). The performance of these clones in the ouabain rescue assay is shown in Figure 9, as are the results of uridine nucleotide transfer assays (see also Section 3.43). All four of these clones differentiated in a similar way to PSA4TG12 when induced to form embryoid bodies (Figures 8 and 10), and were morphologically indistinguishable from their parent. Initial results suggesting that PT1 and PT3 had a slightly higher cloning efficiency on gelatin than PSA4TG12 were not reproducible. The karyotypes of these cell lines were also very similar to that of PSA4TG12 (see Fig. 11). The loss in developmental capacity reported to accompany more rigourous selection for feeder independence [200] was therefore not observed in either PT1 or PT3.

Initial attempts to isolate mec- cells from PSA4TG12 made it apparent that the mode of growth of the cells gave rise to practical problems quite different from the theoretical problem of junction-mediated toxicity killing the feeders, which proved less troublesome than anticipated. The logistic problems of coordinating large-scale stocks of the three cell lines (STO cells, selected stocks, and $HGPRT^+$ ec cells) made it very difficult to carry out successive rounds of selection

Table 2
Specimen results of ouabain rescue assay for metabolic co-operation

Cell line	Substrate	Colonies counted (Duplicate counts, separated by ';').		
		No ouabain	ouabain	ratio
PSA4TG12	STO feeders	585;439	382;406	0.770
PSA4TG12	gelatin	209;230	0; 0	0
PT2md1	STO feeders	442;567	184;146	0.327
PT2md1	gelatin	104; 60	0; 0	0

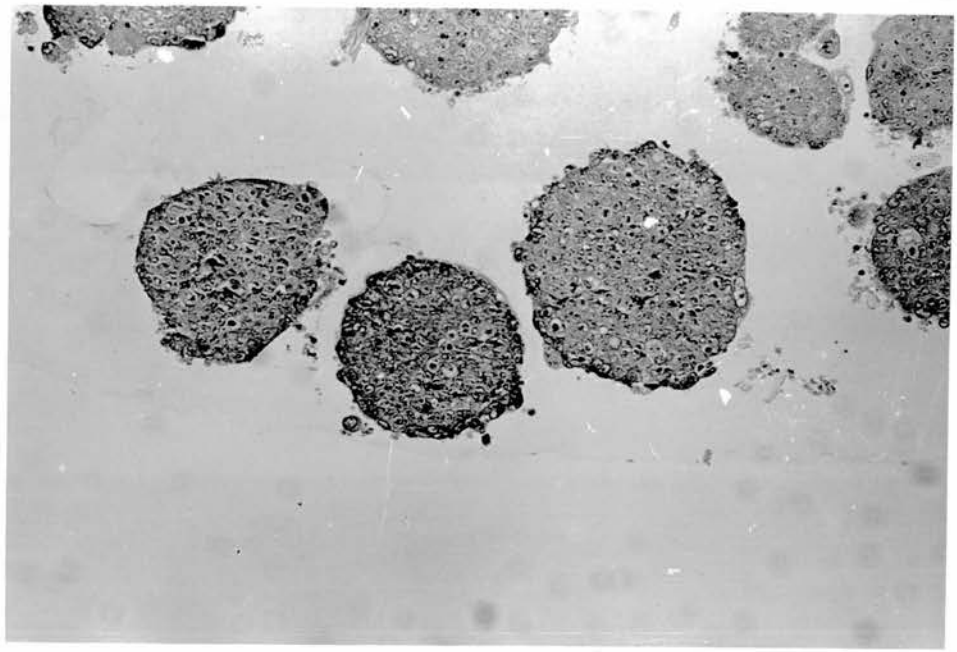
The ratios tabulated are the mean number of colonies which grew up in ouabain, divided by the mean numbers in the controls. The index of co-operation for the line under test is the value of this ratio for that line (in this case PT2md1) divided by the corresponding value for the control line (PSA4TG12). In this case, the value of the index is therefore 0.425.

Figure 8
Photomicrographs of embryoid bodies

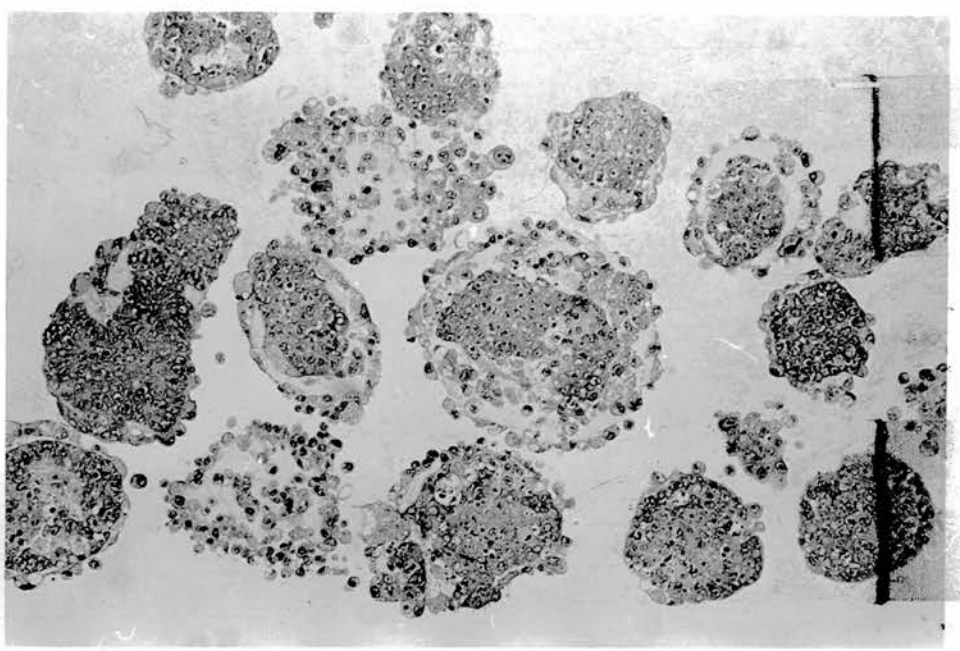
Haemotoxylin and eosin stained paraffin sections of six-day suspension stage aggregates of PT lines.

A: undifferentiated aggregates of PT2md1
B: aggregates of PT1md2, showing an outer layer of parietal endoderm cells and Reichert's membrane.
C: aggregates of PSA4TG12 showing cavitation.

A



B



C

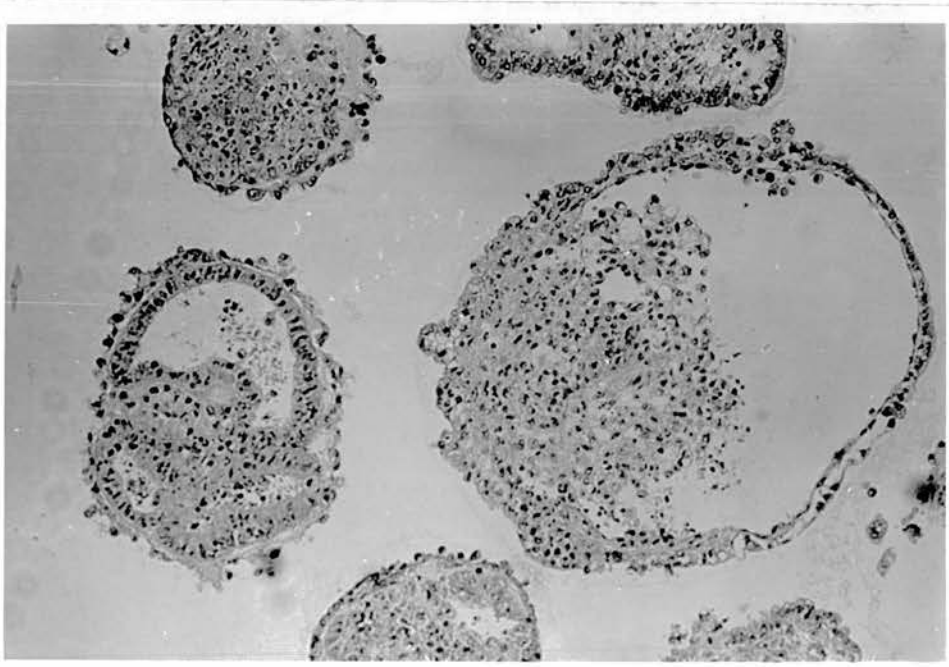


Figure 9

Metabolic Co-operation by PT clones, PSA4TG12 and Nulli-SCC1

- o unselected clones
- ● clones selected by thioguanine 'kiss of death'
- ▲ clone selected in ouabain and retinoic acid on feeder cells

The points plotted are means of all experiments. Error bars are 1 s.e.m. above and below the mean. The data are tabulated below:

Clone	Ouabain Rescue Assay			Uridine Nucleotide Transfer			
	n	mean index	s.e.m.	n	mean % positives		s.e.m.
PSA4TG12				6	71.72		2.47
PTmr0	10	.534	.075	3	63.32		6.72
PT1	4	.765	.128	1	72.87		
PT1md1	2	.707	.051	1	82.49		
PT1md2	4	.461	.093	5	39.73		6.04
PT1md21	1	.292		1	18.20		
PT2	2	.869	.178	1	71.17		
PT2md1	5	.158	.068	4	20.95		2.46
PT2mo1	1	.852		2	17.15		10.08
PT3	3	.952	.210	1	86.06		
PT3md1	2	.511	.166	4	27.91		10.53
PT4	1	.648		1	75.10		
PT4md1	1	.438		3	34.39		10.53
Nulli-SCC1		not done		1	73.46		

The values of n are the total numbers of determinations made. All uridine nucleotide transfer data tabulated here is for 4 hour co- cultures of donors and recipients.

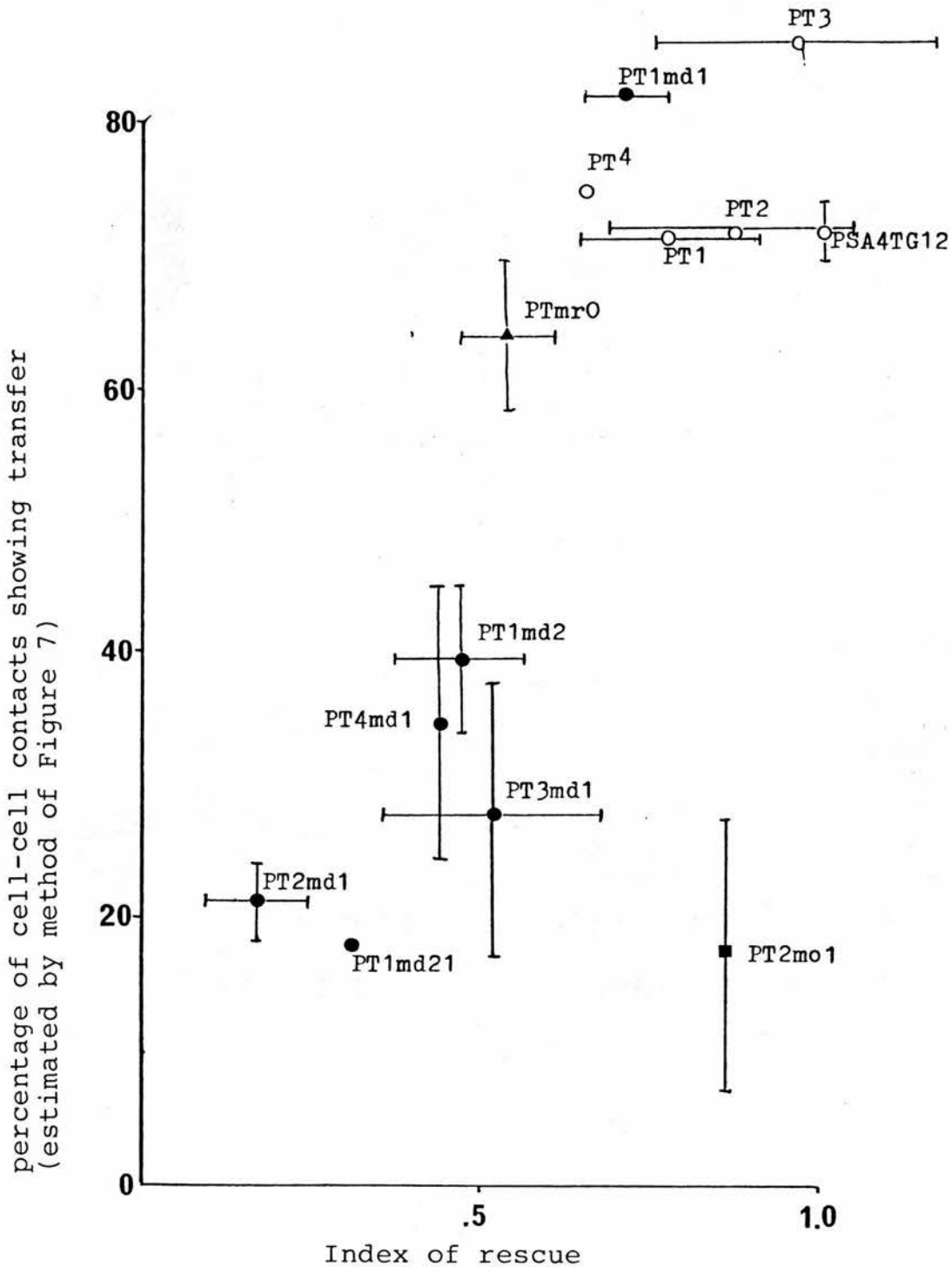

PT3
PT1md1
80
PT4
PT2
PSA4TG12
PT1
PTmr0
60
PT1md2
40
PT4md1
PT3md1
PT2md1
20
PT1md21
PT2mo1
0
.5
1.0
Index of rescue
percentage of cell-cell contacts showing transfer
(estimated by method of Figure 7)

Figure 10

Differentiation of embryoid bodies of PT clones

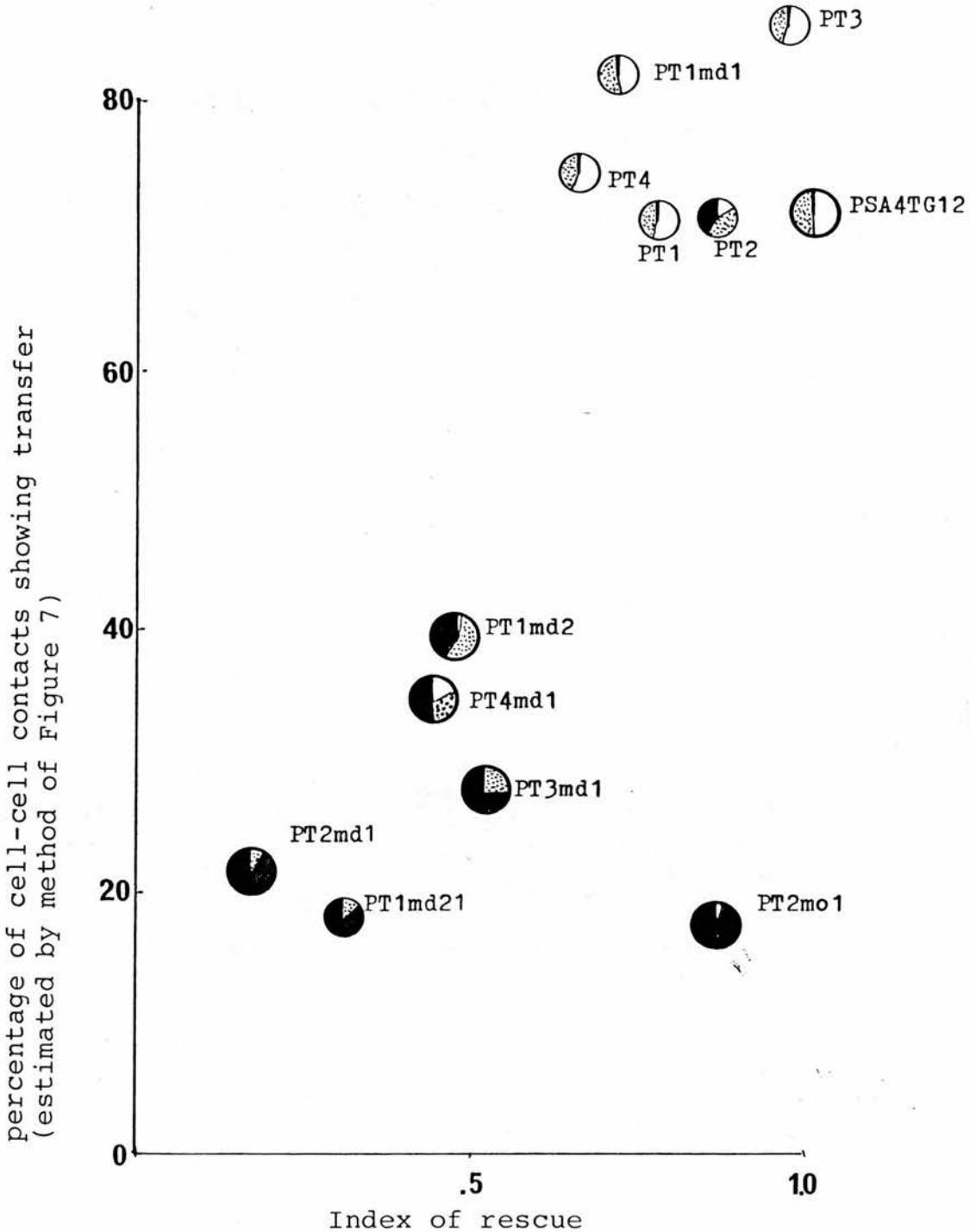
6-day suspension stage embryoid bodies were classified as follows:

A: undifferentiated aggregrates
B: differentiation of endoderm and secretion of matrix
C: formation of cavity or further stages of differentiation evident.

The results of this classification are tabulated below:

Cell-line	Expt.	Frequencies of classes A	B	C	Total
PSA4TG12	2	0	18	27	45
PSA4TG12	3	0	42	48	90
PSA4TG12	4	1	42	27	70
PT1	1	1	21	24	46
PT1md1	1	2	50	37	89
PT1md2	1	2	28	0	30
PT1md2	2	57	11	1	69
PT1md2	3	2	51	3	56
PT1md2	4	23	30	0	53
PT1md2	5	84	19	0	103
PT1md21	5	99	13	0	112
PT2	5	12	16	4	32
PT2md1	2	10	0	0	10
PT2md1	3	11	6	0	17
PT2md1	4	100	0	0	100
PT2md1	5	8	0	0	8
PT2mo1	4	100	0	0	100
PT3	1	3	27	33	63
PT3md1	3	130	5	0	135
PT3md1	4	21	18	0	39
PT4	1	5	47	66	118
PT4md1	2	7	23	42	72
PT4md1	3	85	55	1	141
PT4md1	5	73	16	2	91

These results are displayed on the figure as pie charts. The 'pies' are divided in proportion to the mean fraction of the three categories of embryoid bodies over all experiments. Shaded area: class A; stippled area: class B; open area: class C. The smaller 'pies' correspond to clones where only a single experiment was carried out.The 'pies' are plotted on the same axes as Figure 9 in order to illustrate the relationship with the measures of junctional communication. Note that the apparantly discordant result for PT2 was obtained in only one experiment where differentiation was generally very poor.


PT3
PT1md1
PT4
PSA4TG12
PT1
PT2
PT1md2
PT4md1
PT3md1
PT2md1
PT1md21
PT2mo1
80
60
40
20
0
.5
1.0
percentage of cell-cell contacts showing transfer
(estimated by method of Figure 7)
Index of rescue

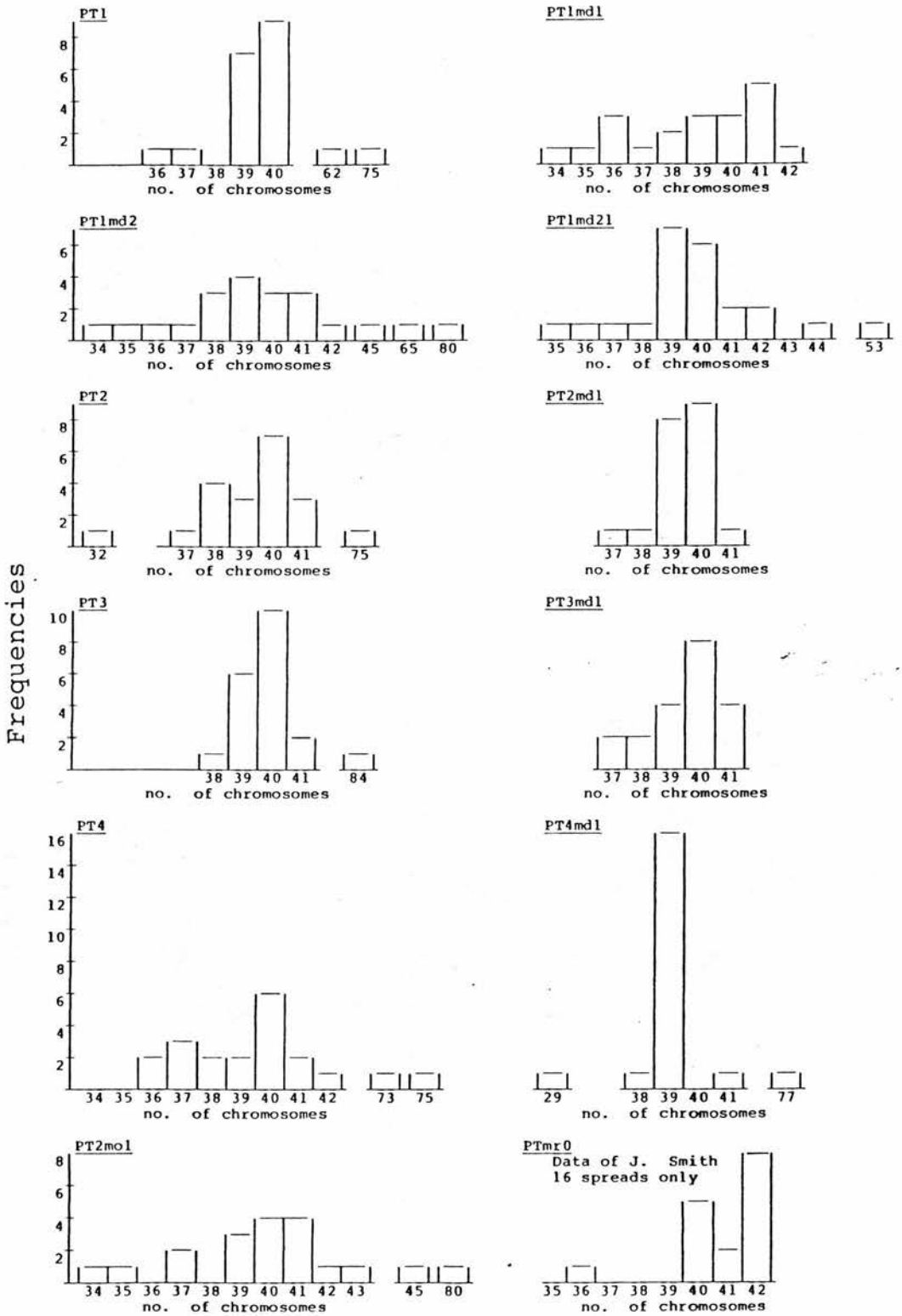


Figure 11

Chromosome counts of PT clones

under the same conditions, particularly as the STO cells are also very unstable in their growth habit (see Section 2.33). A strategy of re- seeding selections with new feeder cells, or of trypsinising cultures when the feeder layers began to detach, was adopted in order to ensure that adequate numbers of feeder cells were always present. Cultures in which the growth of the ec cells appeared to be restricted by layers of thioguanine- killed cells on the outside of the colonies also needed to be trypsinised and re- seeded.

These operations, which had not been required in the selection of previous mec^- variants, made each round of selection unique in detail. They also made it very difficult to estimate the survival frequency of the cells, as the cells were not grown up as independent colonies which could be counted. The only data from which it would be possible to estimate survival is the length of time required for the culture to grow up to confluence (Table 3). Assuming a generation time of approximately one day it is possible to make very rough estimates of survival frequencies.

In order to ensure a good kill in a 'kiss of death selection', it is essential that each $HGPRT^-$ cell makes good junctional contact with an $HGPRT^+$ cell. The overall cell density is therefore critical. A total of about 10^5 cells were therefore seeded out per cm^2 in

Table 3

Length of time for which survivors of 'kiss of death selections were grown up

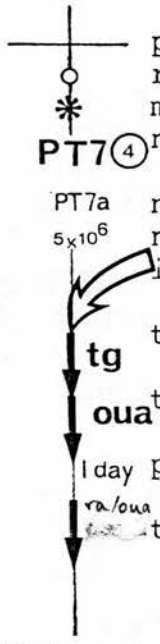
Starting material	Round of selection 1	2	3	4	5	6	7	8
PT1	15	10	10	29	10	10	11	15
PT2	7	9	12	-	-	-	-	-
PT3	16	7	-	-	-	-	-	-
PT4	22	17	5	7	-	-	-	-

The times are the period in days between the initial exposure of the cultures to 6-thioguanine and the recovery of a stock of survivors consisting of approximately 5×10^6 cells. The doubling time of the cells is approximately 1 day at high densities, but they grow more slowly in sparse culture.

Figure 12

Selection of PT1 series of cell lines

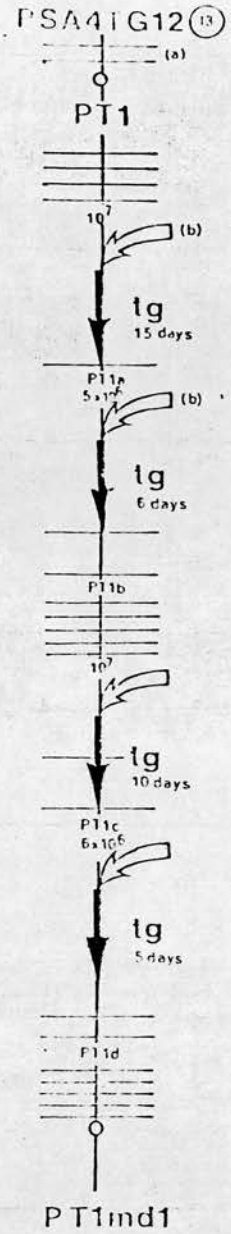
Key to symbols

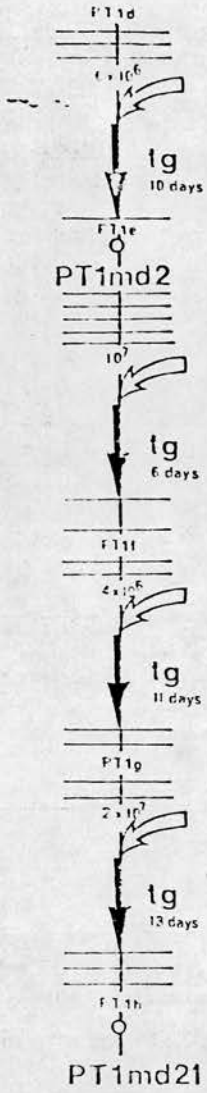


passaging
recloning
mutagenesis
name of clone chosen for further characterisation. (number in brackets indicates passage number)
name of uncloned intermediate
number of cells fed into selection
introduction of HGPRT+ cells into selection (these are PC13.5 cells unless otherwise indicated)
treatment with thioguanine (10μg/ml unless otherwise indicated)
treatment with 3mM ouabain
period for which cells were kept in selective medium
treatment with 3mM ouabain and 10^{-4} M retinoic acid

Notes to Figure 12

a) cells cloned at high density on gelatine
b) PSA4 cells used in this round of selection.


PSA41G12 (13)
(a)
PT1
10^7
(b)
tg
15 days
PT1a
5x10^6
(b)
tg
6 days
PT1b
10^7
tg
10 days
PT1c
6x10^6
tg
5 days
PT1d
PT1md1


PT1d
4x10^6
tg
10 days
PT1e
PT1md2
10^7
tg
6 days
PT1f
4x10^6
tg
11 days
PT1g
2x10^7
tg
13 days
PT1h
PT1md21

order to ensure that an adequate density was maintained. These were incubated for at least 12 hours before treatment with thioguanine, by which time the great majority of the cells present were in close contact with several other cells. The selection of R5/3 (see Section 1.52) had been carried out with the $HGPRT^+$ (Chinese hamster DON or mouse ec PC13) cells in a 5-20 fold excess. Theoretically, at least, the $HGPRT^-$ cells will be killed equally well at low $HGPRT^-$:$HGPRT^+$ cell ratios as at high ratios, providing sufficient $HGPRT^+$ cells are present. Contacts between $HGPRT^-$ cells do not reduce the probability of their elimination. Therefore whilst the overall cell density was kept at an approximately constant level, the ratio of $HGPRT^+$ to $HGPRT^-$ cells was allowed to vary between approximately 1:1 and a 6:1 excess of $HGPRT^+$ with the availability of the cells.

3.11 Isolation of co-operation variants from clone PT1

With these considerations in mind, non-mutagenised PT1 cells were subjected to four successive rounds of 'kiss of death' selection (see Fig. 12 for details). One round of selection was defined as the period between the initial treatment of the cells with thioguanine and the recovery of a stock of sufficient cells (approximately 5×10^6) for characterisation to be considered.

The pooled survivors of each round were assayed for the ability to survive in a ouabain rescue assay, which provided a rapid screen for mec$^-$ cells. The alternative, of using a colony formation assay to screen the cells for the ability to survive a further 'kiss of death', was not adopted as this would have involved co-ordinating the culture of prohibitively large numbers of cell lines, some of them on a very large scale. The results of the ouabain rescue assays are summarised in Figure 13. After four rounds of selection, the stock recovered (PT1d) consistently scored low in the ouabain rescue assay and was cloned . A single clone (PT1md0) , which showed the lowest extent of rescue among the ten clones tested (mean index of .267, s.e.m. .122, 3 determinations) was chosen for further study. This clone however became contaminated with STO cells which had escaped mitomycin blockage of division. In order to remove the STO cells, it was necessary to reclone. A subclone termed PT1md1 was isolated which scored rather lower in the assay than PSA4TG12 (Section 3.4), but which is apparantly not so co- operation defective as the initial clone , PT1md0 (though the two clones have not been compared directly).

The pooled survivors from the fourth round of selection (PT1d) were also fed into a further 'kiss of death'

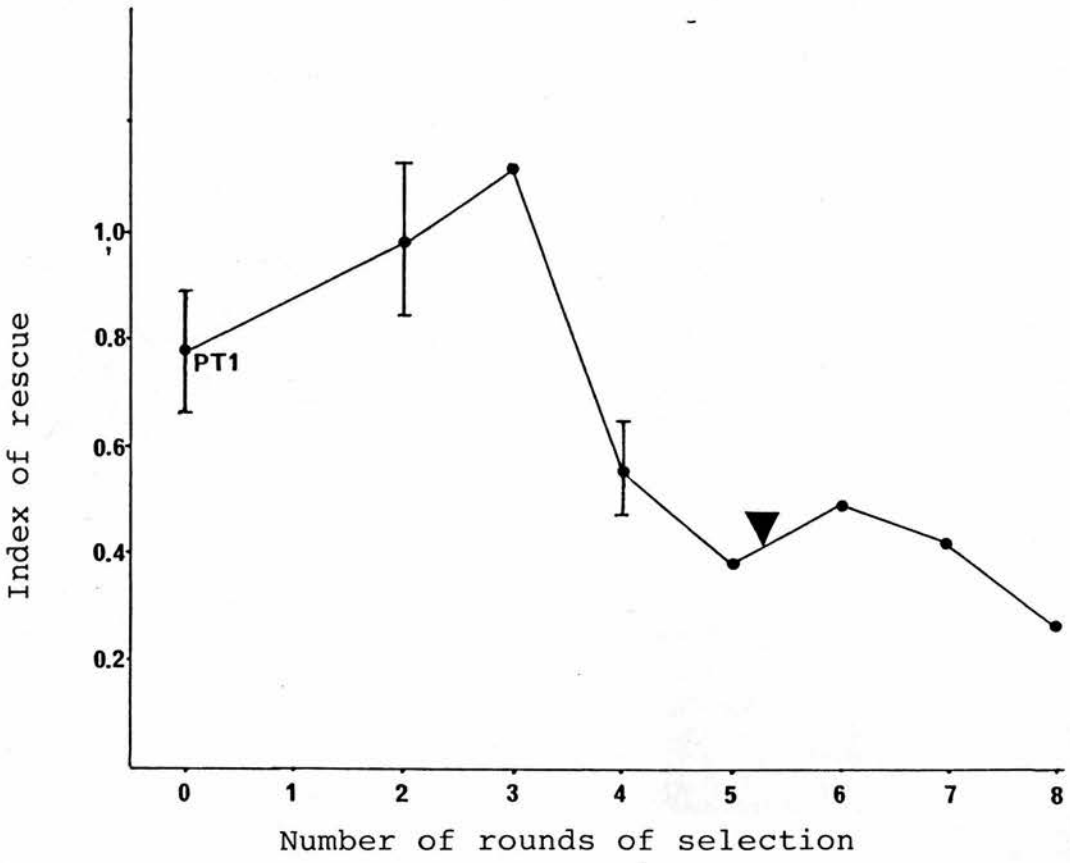


Figure 13

Ouabain rescue indices of intermediates in the selection of the PT1 series of cell lines

error bars indicate means ± 1 s.e.m. where more than one determination was carried out.

▼ indicates recloning.

selection (Fig. 12). The survivors , (named PT1e), again scored low in the ouabain rescue assay, and were again cloned. Of the two clones obtained, one was rescued to approximately the same extent as PSA4TG12 in the ouabain rescue assay (indices of 1.24 and .64 in two seperate experiments), whilst the other clone, PT1md2, scored consistently low though some rescue was nevertheless observed (Section 3.4).

PT1md2 cells were subjected to a further three rounds of selection, and then recloned (Fig. 12). During this selection, the cultures became contaminated with 'escaped' STO cells. In order to remove the STO cells it was necessary to reclone. The clone which scored lowest in the ouabain rescue assay (PT1md21) was further characterised (see Section 3.4).

3.12 Isolation of PT2md1

In view of the long period in culture required for the isolation of mec^- cells from PT1, and the leakiness of the variants obtained, it was decided to carry out a parallel selection on a mutagenised clone of PSA4TG12, independent of PT1 (Fig. 14). After only three rounds of selection, in each case with a very short period required for cells to grow up (Table 3), a stock was isolated which scored low in the ouabain rescue assay (see Fig. 15). The clones isolated from this stock

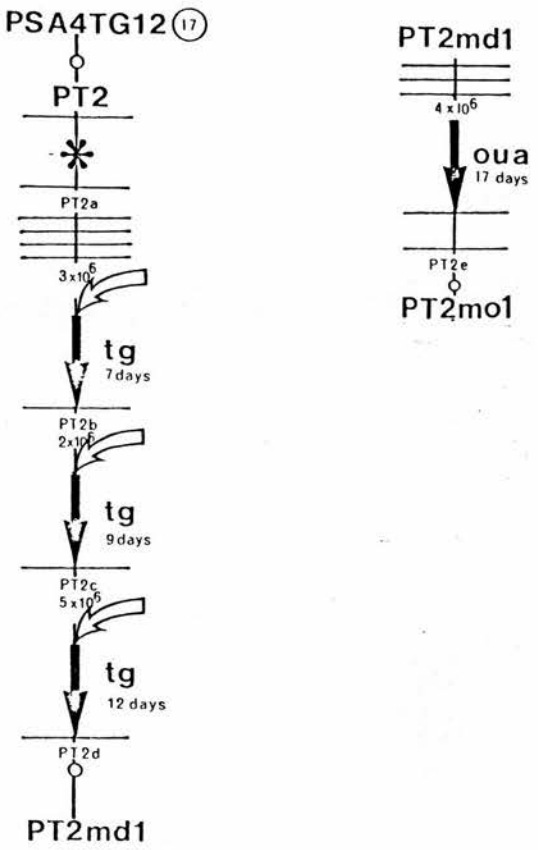


Figure 14

Selection of PT2 series of cell lines

see legend to Fig. 12 for key to symbols

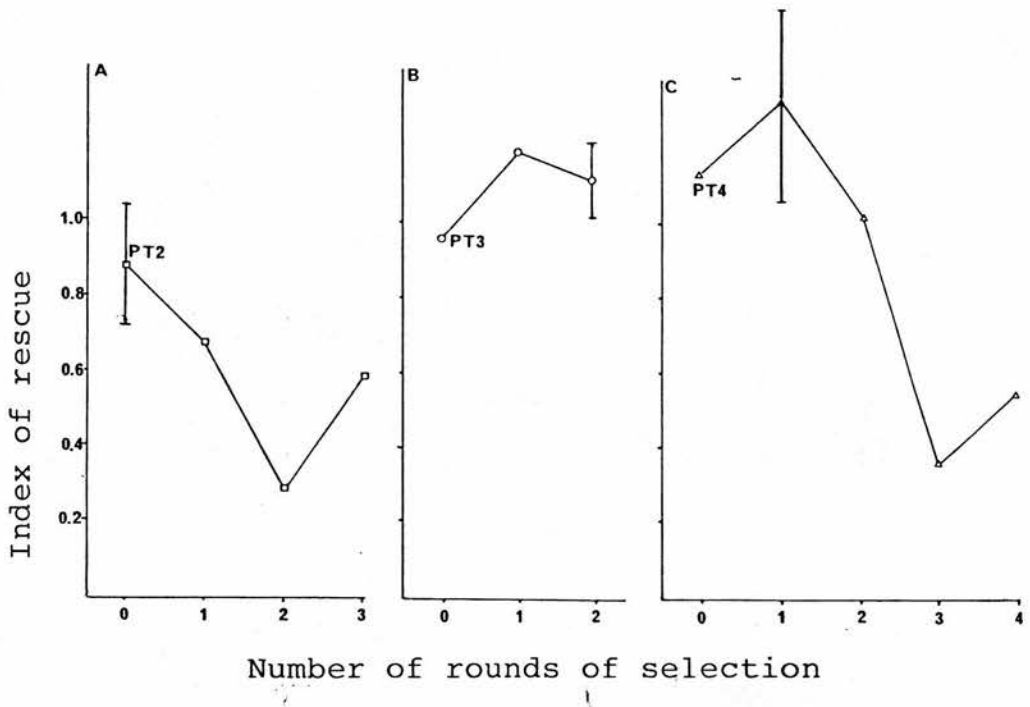


Figure 15

Ouabain rescue indices of intermediates in the selection of the PT2 (A),PT3 (B), and PT4 (C) series of cell lines.

Error bars indicate means ± 1 s.e.m. where more than one determination was carried out.

The index for PT2e, the uncloned survivors from the selection on STO feeders in ouabain of cells from PT2md1, was .936 (single determination only).

included one, PT2md1, which was hardly rescued at all (Section 3.4). This clone had a rather different growth habit from its sibs, and showed very little spontaneous differentiation when plated at low densities on gelatine. The PT2md1 cells formed much flatter colonies, resembling those of PC13. Unlike PC13 cells however, they did not clone well on gelatine coated substrates (see Table 2). The sib clones shared the growth habit of PSA4TG12 with one exception, a clone which also demonstrated low rescue from ouabain toxicity, giving indices of .500 and .323 in two separate experiments . The other three clones gave rescue indices of .701, .848 and 1.14 (single determinations) and formed differentiated colonies on gelatin like those of PSA4TG12.

3.13 Isolation of PT3md1

A sib clone of PT1, PT3 (see above), was mutagenised (Section 2.36) and the cells grown up were fed into a thioguanine kiss of death selection (Fig. 16). After two rounds of selection only, the pooled survivors, PT3c, appeared to show reduced rescue in the ouabain rescue assay, compared with PSA4TG12. These cells were therefore cloned, and the clone which scored lowest in the rescue assay (PT3md1) was further characterised (Section 3.4). Further ouabain rescue assays on the uncloned population however failed to reproduce the

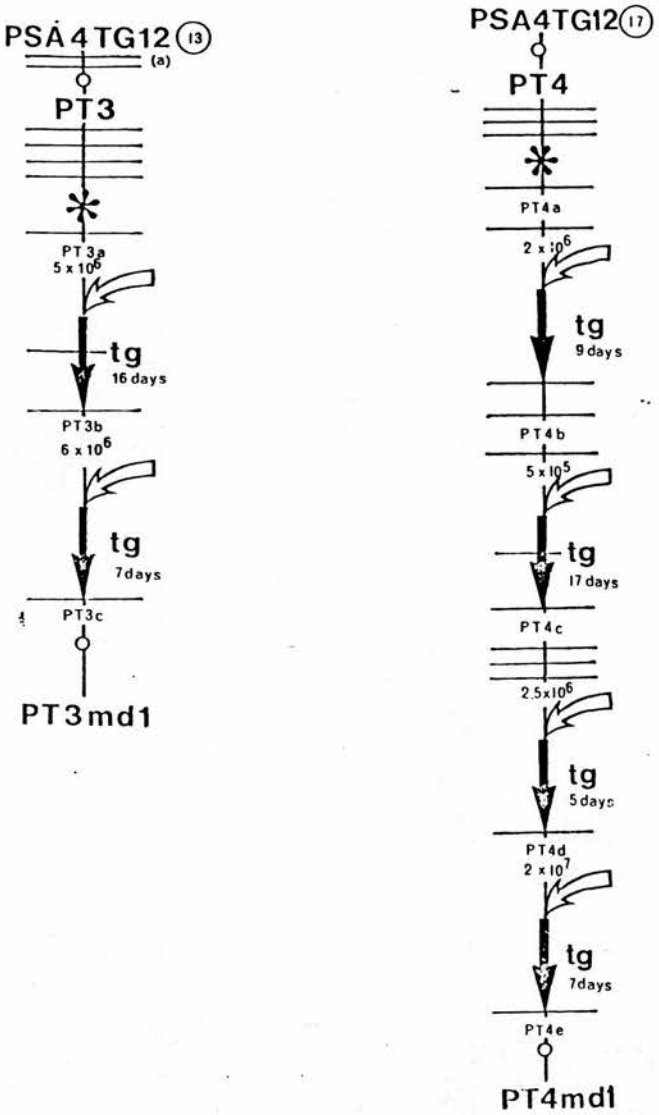


Figure 16

Selection of PT3 and PT4 series of cell lines

see legend to Fig. 12 for key to symbols

initial result (Fig. 15). PT3md1 differed from its sibs in its growth habit, resembling PT2md1 , although its rescue from ouabain toxicity from STO feeders was only slightly reduced compared to the starting material.

3.14 Isolation of PT4md1

A further selection was carried out, starting from mutagenised PT4 cells. After three rounds of selection, using the same approach as before (see Fig. 16), a reduction in the level of ouabain rescue was detected (see Fig. 15). The stock was lost however, although cells which had been fed into a further round of selection were recovered (see Fig. 16). Eight clones were isolated from the survivors of this, the fourth round of selection and were screened in the ouabain rescue assay. Colonies of all these clones showed some differentiation on gelatin. The clone which scored lowest in the ouabain rescue assay, PT4md1, was further characterised (Section 3.4).

3.2 Selection of cells by ouabain rescue

The ouabain rescue assay can be adapted as a method of selecting mec^+ revertants from mec^- cell lines . When mec^-, ouabain sensitive cells are grown on STO feeder cells in the presence of ouabain, only mec+ revertants,

or ouabain resistant mutants are expected to grow (see Section 1.51 and Figure 4). It proved relatively easy, in the case of H2T12, to select mec+ revertants from a mec- line isolated by thioguanine kiss of death (R5/3) (see Section 1.52). Although R5/3 cells in this case were mutagenised, and a different selection procedure was used, this suggested that it might be possible to select mec+ revertants from the lines whose isolation is described above (Section 3.1) by treating cultures on STO feeders with ouabain.

4 x 10^7 PT2md1 cells were seeded onto a total surface area of 300 cm2 of STO feeder layers. The cells were left overnight then treated with ouabain for 17 days (Fig. 14). The survivors were grown up into a stock, which was cloned. None of these clones, nor the uncloned population, showed any morphological differentiation in aggregates. The uncloned population gave an index of .936 in the ouabain rescue assay. The co-operation abilities of three of the clones were studied. None of them co-operated well in the uridine nucleotide transfer assay. One of them, PT2mol was however, rescued as well as PSA4TG12 in the ouabain rescue assay (Section 3.4) and was chosen for further characterisation, whilst a second showed rescue similar to that of PT2md1 (ouabain rescue index of .435). The third gave an index of .840 in a single experiment.

3.3 Isolation of a cell line resistant to the effect of retinoic acid on junctional communication

All substances known to inhibit junctional communication also have profound effects on other cellular functions (Section 1.53). It is often impossible to know whether the other effects are consequences of the defect in communication, whether the communication defect is a consequence of the other effect, or whether there is no link between the two. Variant cells resistant to the effect on junctional communication might well retain wild-type responses in functions which are unconnected with co-operation, and this might be useful in determining how the different effects are related.

The effect of retinoic acid on uridine nucleotide transfer in a number of ec lines was examined (Figs. 17 and 18). Pitts et al [153] found that retinoic acid eliminates metabolic cooperation in various cultured cells at a concentration of 10^{-4} M in the presence of 10% serum and of 10^{-6} M in the absence of serum (retinoic acid is largely bound to serum proteins when these are available) (see Section 1.53). As ec cells are very sensitive to serum- deprivation, the effect was studied only in the presence of serum. Inhibition was observed at a concentration of 10^{-5} M, and co-operation was negligible at 10^{-4} M. There is a suggestion, however, that 10^{-6} M retinoic acid, actually

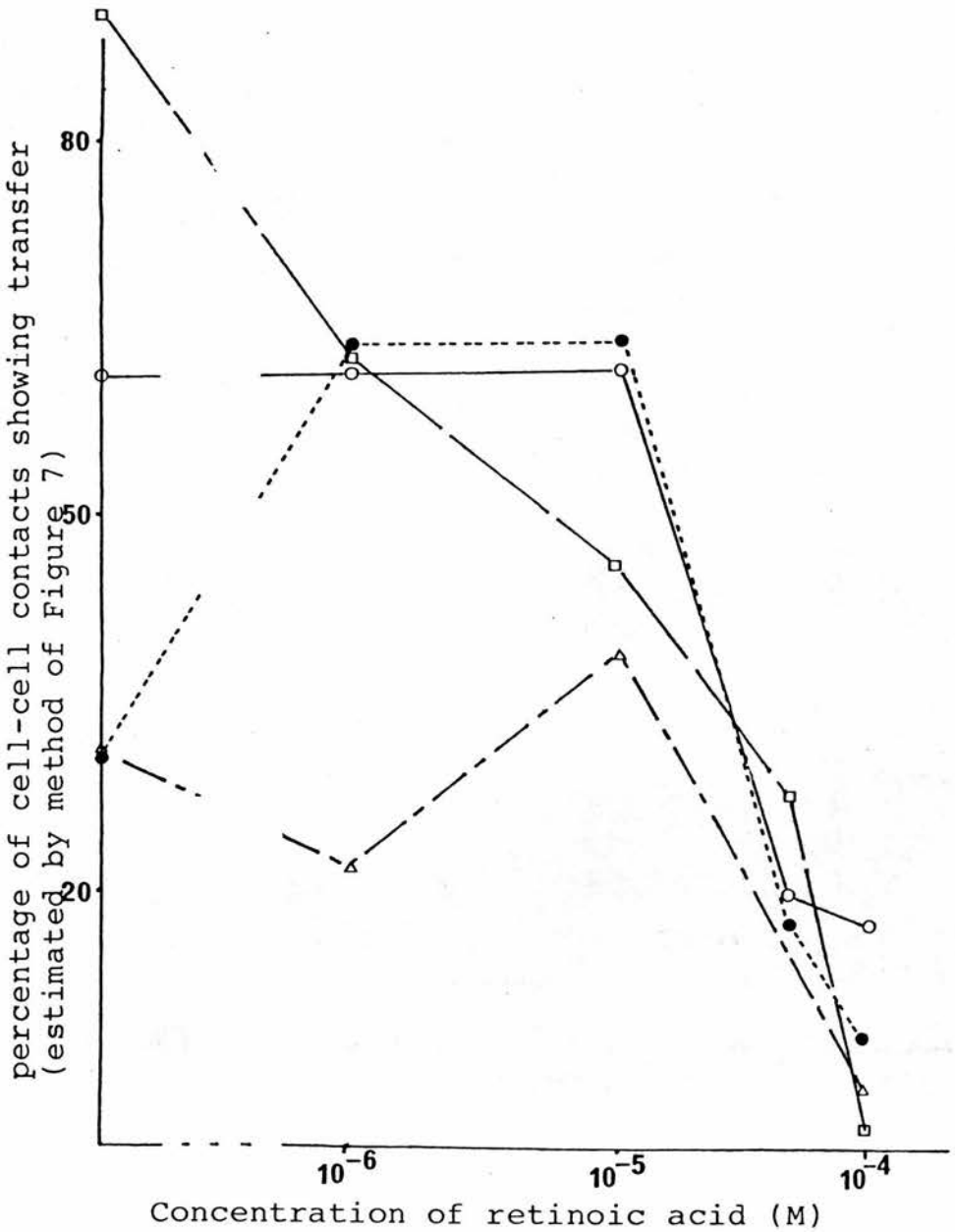


Figure 17

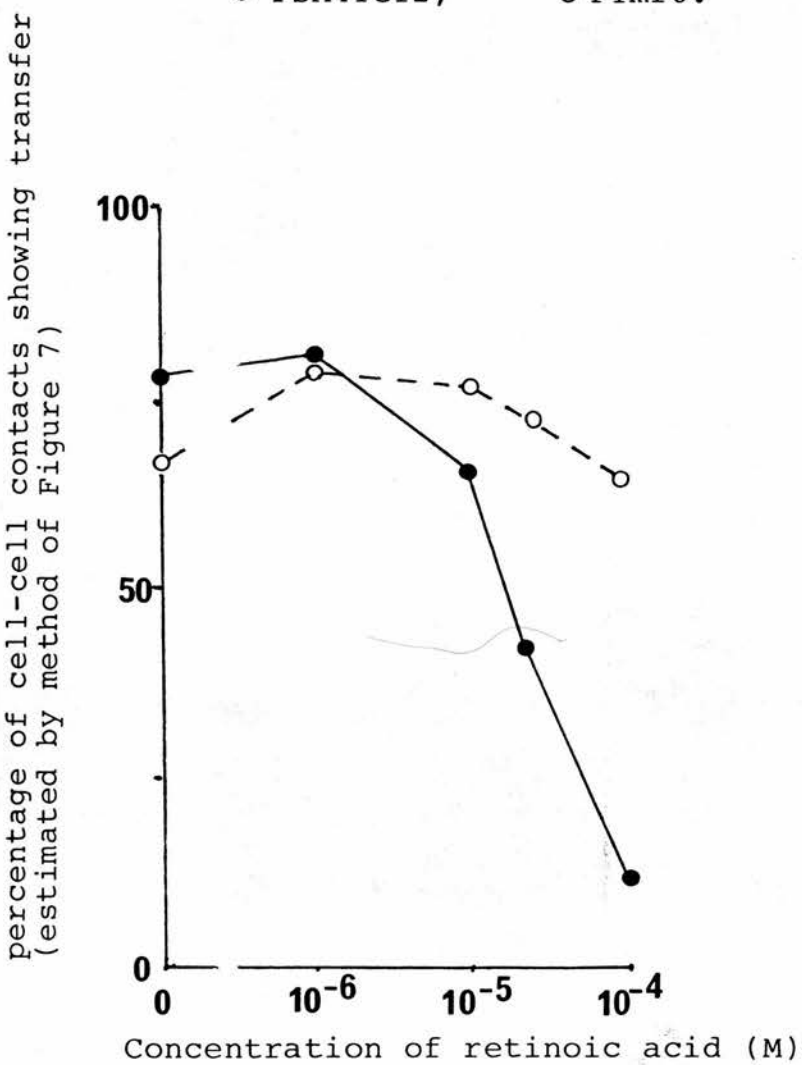
Effect of retinoic acid on uridine nucleotide transfer

△ PC13TG8
□ H2T12
● EC10
○ EC12

Figure 18

Effect of retinoic acid on uridine nucleotide transfer in PSA4TG12 and PTmr0

● PSA4TG12; o PTmr0.



stimulates co- operation slightly. These effects are not due to the DMSO used as a solvent to get the retinoic acid into solution. Cells co-operate even in toxic DMSO concentrations (Fig. 5).

In principle a cell line resistant to the effect of retinoic acid on metabolic co-operation could therefore be selected by treating ouabain-sen sitive ec cells with 10^{-4} M retinoic acid and ouabain on STO feeder layers. The STO feeder layers at least partially inhibit the induction of differentiation by the retinoic acid (see Section 1.343 and Chapter 7), and therefore it should be possible to recover ec cells which co-operate under these circumstances (see Sections 1.51 and 3.2 above for the theory of ouabain rescue selections). At such a high concentration of retinoic acid, it was thought that non- specific toxic effects might present a serious problem.

A colony forming assay for toxicity of retinoic acid on feeder layers could not be used, as it would be impossible to identify colonies which had differentiated into cells indistinguishable from the STO background. Consequently, cells were prelabelled with ^{14}C- thymidine, treated with retinoic acid on feeder layers, and the recovery of the label after washing was used as a measure of survival (see Section 2.41). The majority of the label was retained

following three days in even 10^{-4} M retinoic acid (see Fig. 19). Extensive differentiation of PSA4TG12 does occur in response to this treatment despite the inhibitory effect of the STO cells. (PSA4TG cells (see Table 1) are much more sensitive to retinoic acid in the absence of feeder cells, see [201], but note that in this reference a cell counting assay is used which will confound differentiation to a non-dividing cell type with toxic effects).

A culture of PSA4TG12 was passaged twice on STO feeders in the presence of 10^{-4} M retinoic acid. This stage was intended to enrich for cells which would be resistant to the toxic effect of the retinoic acid: subsequent work suggested that it was unnecessary as retinoic acid is not markedly toxic to the cells under these conditions. Then 2.5×10^7 of the cells grown up from this treatment were subjected to 10^{-4} M retinoic acid together with 3mM ouabain on STO feeders for 5 days. Survivors were grown up for 22 days then cloned (see Fig. 20). Retrypsinisation during this period made it impossible to estimate accurately the extent of survival, and ec cells surviving were mixed with a large proportion of cells of differentiated morphology. The survivors were then cloned on STO feeders. A single clone termed PTmr0, the cells of which are morphologically like PSA4TG12, was chosen for further study.

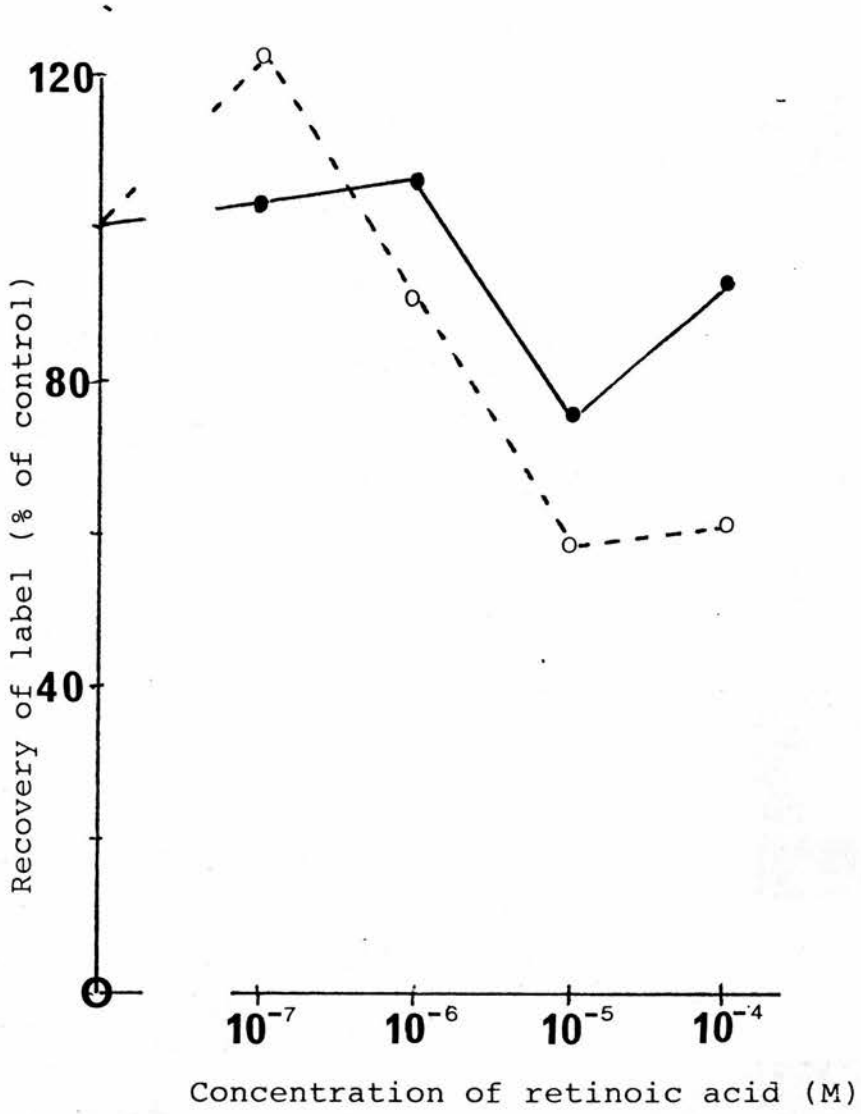


Figure 19

Toxic Effect of Retinoic Acid on PSA4TG12 and PTmr0

• PSA4TG12 o PTmr0

No consistent differences between the two cell-lines were recorded in three experiments carried out at the highest concentration (10^{-4}M).

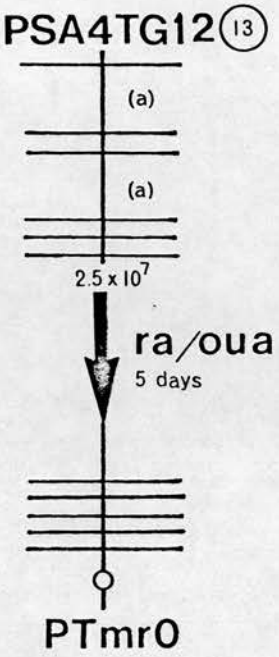


Figure 20

Selection of PTmr0

see legend to Figure 12 for key to symbols

Note:
(a) cells passaged at high density in 10^{-4} M retinoic acid.

3.4 Properties of selected clones

Figure 21 summarises the above selections, and indicates the clones which were chosen for further study. The rest of the chapter discusses their properties. All characterisation was carried out on cells which had been passaged 8 or fewer times since cloning.

3.41 Morphology and growth habit of PT clones

Whereas the morphology and growth habit of the parental clones, PT1-4, and of PTmr0 are indistinguishable from that of PSA4TG12, the clones, PT2md1, PT3md1, PT2mo1, do not appear to throw off differentiating derivatives in mass culture, and the cultures are therefore much more uniform in appearance. These cells form more even monolayers than PSA4TG12, which frequently forms small clumps. The other clones appear to be intermediate in this respect.

3.42 Resistance of PT clones to toxic agents used in the selections

Toxicity tests were carried out in order to find out whether differences between the phenotypes of the selected clones and that of their parent, PSA4TG12, might be consequences of the selection of variants with

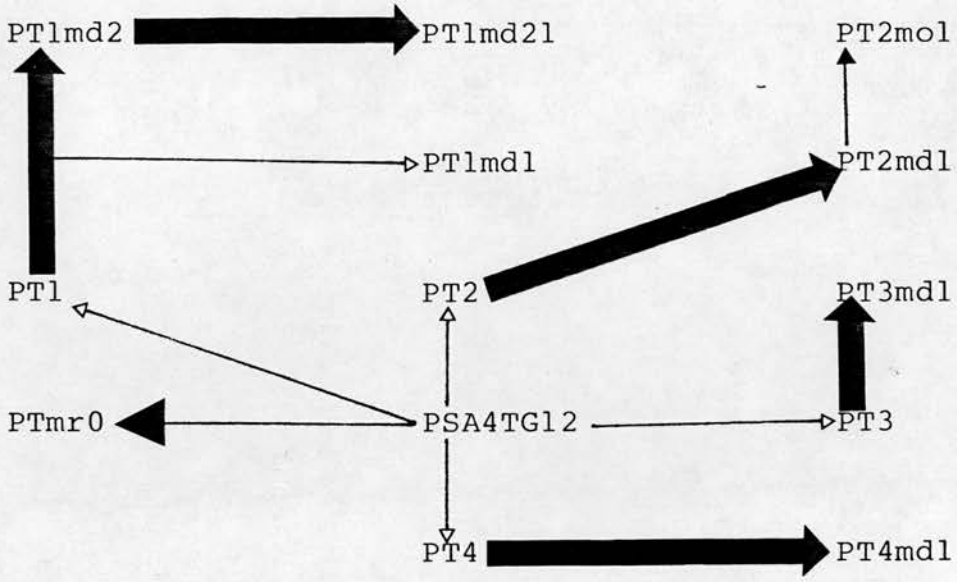


Figure 21

Summary of origins of clones chosen for further study

→ cloning

➡ thioguanine 'kiss of death'

➡ ouabain/retinoic acid on STO feeders

→ ouabain on STO feeders

note that the survivors of each selection were cloned prior to characterisation.

enhanced resistance to toxic effects unrelated to metabolic cooperation of the reagents used during the selections. A colony formation assay was used to examine the thioguanine resistance of the clones recovered from 'kiss of death' selections (for method see Section 2.42). The feeder cells do not incorporate thioguanine as they lack HGPRT and therefore do not contribute to the kill of ec cells by 'kiss of death' effects. The resistance of the clones is much the same as that of PSA4TG12 (Figure 22), a result which contrasts with that obtained for the other mec- ec line selected in this way, R5/3 (Section 1.52), which is more resistant than its parent, PC13TG8.

None of the selected clones, including those selected in ouabain, PT2mol and PTmr0, survived treatment with 3mM ouabain on gelatin. No colonies were recorded as growing up on gelatin in the presence of ouabain (method of Section 2.62), whilst in the absence of ouabain, colonies were formed. Concentration dependence of ouabain toxicity was not studied. It would be most useful to study such concentration dependence, as the possibility that PT2mol survives the ouabain-rescue assay as a consequence of an increased ouabain resistance cannot as yet be excluded.

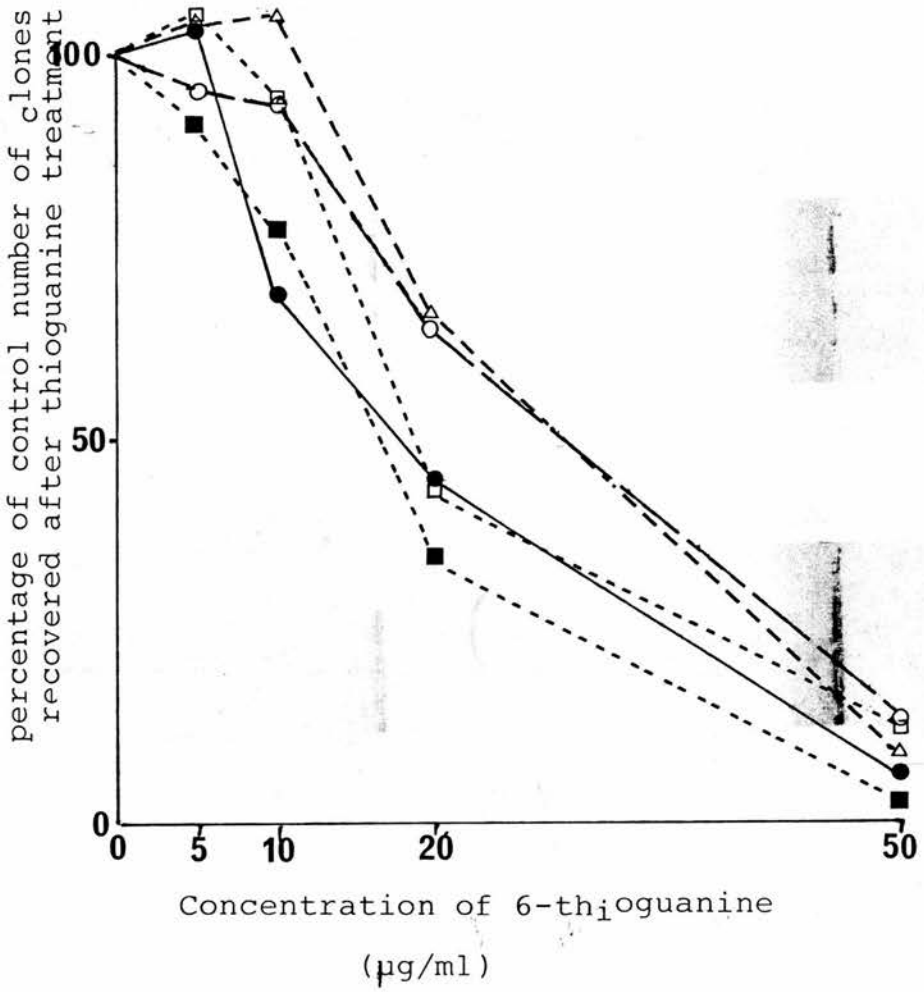
As explained above (Section 3.3) a simple colony formation assay system could not be used to study

Figure 22

Resistance of selected clones to 6-thioguanine toxicity

■ PSA4TG12
● PT1md2
□ PT2md1
○ PT3md1
△ PT4md1

Each point is the mean of the results of two experiments. Each experiment was carried out in duplicate. The method is described in Section 2.42.



retinoic acid toxicity on STO feeder layers. As it was considered necessary to compare the resistance of PTmr0, selected in retinoic acid, with PSA4TG12 the ^{14}C- thymidine prelabelling technique was again used. There was no consistent difference between the sensitivity of PTmr0 to retinoic acid, and that of PSA4TG12 (Fig. 19). The same experimental design was used to measure the sensitivities to the toxic effects of DMSO of the two cell lines, which are also the same (Fig. 5).

It is concluded that none of the clones tested in these assays (with the possible exception of PT2mol) differ from PSA4TG12 as a consequence of resistance to direct toxicity of the agents present during the selections.

3.43 Junctional communication abilities of PT clones

The clones selected for further study were assayed for junctional communication using the uridine nucleotide transfer assay (Section 2.61) and the ouabain rescue assay (Section 2.62). The results are displayed in Figure 9. The parental clones, PT1, PT2, PT3 and PT4 appear to co-operate normally in the uridine nucleotide transfer assay. Although PT1 and PT3 were chosen as the starting material for selections because they showed poor rescue from ouabain toxicity, repetition of this experiment has suggested that they are little

different from PSA4TG12 in this respect: the initial result was a consequence of experimental variation. PT2 and PT4 also behave much like PSA4TG12 in the ouabain rescue assay.

All the PT clones recovered from the selections discussed here (with the exception of PTmr0) were chosen for further study because of their performance in the ouabain rescue assay. They all (except PTlmd1) scored poorly in the uridine nucleotide transfer assay though some transfer was recorded in all cases. PTmr0 was assayed for uridine nucleotide transfer both in the presence of retinoic acid and in unmodified medium (Fig. 18, Table 4). It showed an enhanced level of metabolic cooperation compared to PSA4TG12 in the presence of high concentrations of retinoic acid but relatively poor ability to cooperate in unmodified medium . The uridine nucleotide transfer assay is intended to measure fairly directly the transfer of uridine and cytidine nucleotides, with molecular weights of the order of 400, similar to that of the substituted guanine nucleotides thought to be the active agents passing through gap junctions in the thioguanine 'kiss of death' selection. This transfer is measured between like cells. The ouabain- rescue assay measures transfer of small cations; the results can potentially be affected by the extent of cell spreading and cell mobility; and it is junctions

Table 4

Summary of results of experiments on the effect of 10^{-4}M retinoic acid on uridine nucleotide transfer in PTmr0

Period of co-culture (hours)	Estimated percent positive contacts			
	no retinoic acid		10^{-4}M retinoic acid	
	PSA4TG12	PTmr0	PSA4TG12	PTmr0
3	69.4	52.1	8.5	62.4
3	91.3	40.0	-4.3	24.5
3*	80.0	50.6	33.7	41.1
6*	56.6	66.1	13.4	56.9
4	77.5	67.0	11.4	63.9

*results obtained within one experiment. Each line of data otherwise refers to an independent experiment.

between dissimilar cell types (ec cells and fibroblasts) which are studied. The fact that 'kiss of death' selections result in enrichment for cells which are rescued poorly from ouabain toxicity , (and conversely that the ouabain-based selection of PTmr0 produced a cell line with altered uridine nucleotide transfer phenotype) confirms that the two phenomena are related, and excludes the possibility that the selected lines merely have altered nucleotide metabolism. It therefore appears highly likely that the selected cell lines have altered junctional communication abilities.

Because of the way in which the clones were chosen, clones which fail to transfer uridine nucleotides, but which have the PSA4TG12 phenotype in the ouabain rescue assay, would have been overlooked in the screening of survivors of the 'kiss of death' selections. The phenotype of PT2mol, implies that such cells can be obtained by other means. Similarly the observation that PTmr0, in the absence of retinoic acid, shows only a small reduction in uridine nucleotide transfer, but scores relatively poorly in the ouabain rescue assay, indicates that when a selective system is available, it is possible to isolate cells which are rescued only poorly from ouabain toxicity although the ability to transfer nucleotides is little impaired.

3.431 Effect of cAMP on metabolic cooperation in PT lines

The effect of dibutyryl cyclic AMP and theophylline on the level of uridine nucleotide transfer was studied in the cell lines PSA4TG12, PT1md2, PT2md1, PT3md1, and PT4md1 (see Section 2.61 for method). Dibutyryl cyclic AMP is more permeant than the unmodified nucleotide, whilst theophylline acts as an inhibitor of phosphodiesterase and thus blocks breakdown of cAMP [202]. The cells were grown overnight in the medium under test: previous reports indicated that effects of enhanced cAMP levels on metabolic cooperation (Section 1.54) were not immediate . In all cases, an apparent increase in transfer in cultures treated with dibutyryl cyclic AMP and theophylline was noted over the levels recorded in the controls (Fig. 23). In the case of PSA4TG12, where the control level of co- operation is high, only a small increase was noted. In the cases of PT1md2, PT3md1, and PT4md1, the level of co-operation returned to that observed in PSA4TG12. In the case of PT2md1, an intermediate level of co- operation in dibutyryl cyclic AMP and theophylline was recorded.

3.44 Differentiation of PT cell lines

3.441 Differentiation of aggregates

Embryoid bodies were derived from the PT clones using the method of Section 2.53. The aggregates were grown

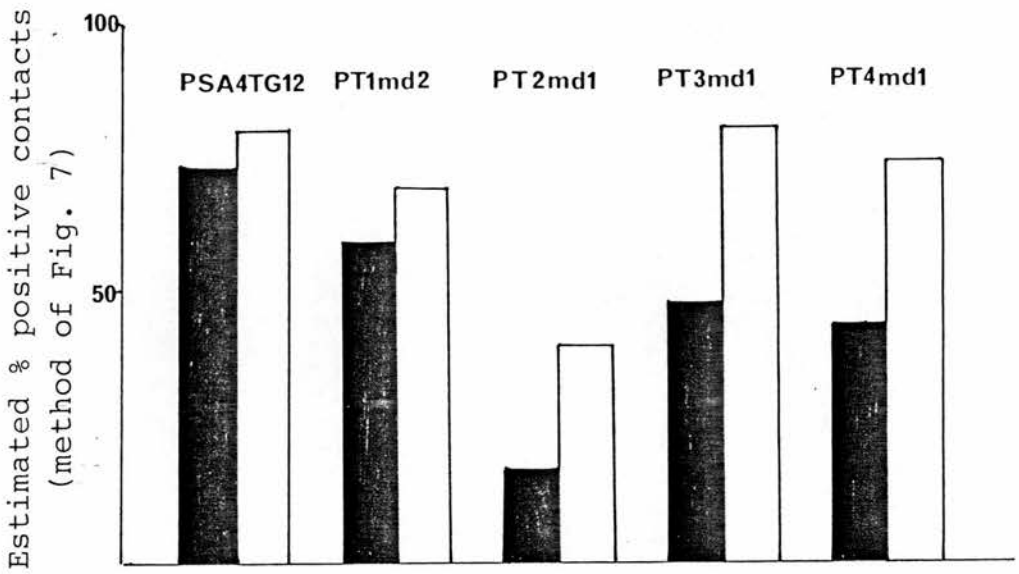


Figure 23

Effect of dibutyryl cAMP and theophylline on uridine nucleotide transfer

shaded bars: controls
open bars: treated with db cAMP and theophylline

for 6 days in suspension. The individual embryoid bodies obtained were categorised -morphologically according to the extent of differentiation evident in the paraffin sections. Three categories were used: embryoid bodies displaying no clear evidence of differentiation; those with endodermal cells on the outside and a layer of matrix present beneath it: and finally, those showing cavitation or in which further stages of differentiation were evident, such as the formation of a distinct epithelium around the cavity or of nests of neural cells. Typical embryoid bodies are illustrated in Figure 8. In only a few cases where cavitation was not observed were more advanced stages of differentiation evident, even though it is obviously possible for a cavity to be present outside the plane of the section. Similarly, cavitation was almost invariably accompanied by endoderm formation. In some sections, where there was otherwise no cellular differentiation and no matrix evident, it was unclear whether the outer layer of the embryoid body was showing signs of incipient primary endoderm formation. Such embryoid bodies were categorised as undifferentiated.

The results of this analysis are shown in Figure 10. It is clear that the cell lines which co-operate normally, also differentiate normally, whilst those showing reduced levels of co-operation are defective in

their differentiation. Cell lines which are intermediate in their ability to co- operate are intermediate in their differentiation ability.

Cultures of PTmr0 were also induced to differentiate via the formation of aggregates. Paraffin sections of these revealed histology qualitatively indistinguishable from that of control outgrowths derived from PSA4TG12 (Fig. 24). The results of these experiments are not directly comparable with those for the other PT clones, as the embryoid bodies were grown for only 5 days in suspension.

When seeded onto thermanox coverslips (method of Section 2.54) aggregates of PTmr0 gave rise to differentiating outgrowths containing beating muscle and neural tissue, indistinguishable from outgrowths produced by PSA4TG12. Paraffin sections of these outgrowths revealed glandular, fibroblastic and epithelial tissues (Fig. 24). Stratified epithelia were also observed. No attempt was made to induce differentiation by formation of outgrowths from embryoid bodies of the other PT lines.

3.442 Effect of cAMP on differentiation of PT lines.

The differentiation of embryoid bodies of PSA4TG12 and of PT3md1 in 1mM dibutyryl cyclic AMP and 1mM

Figure 24

Histology of PTmr0 outgrowths

Haematoxylin and eosin stained paraffin sections of PTmr0 outgrowths and of PTmr0 embryoid body.

Top: low power illustration showing the variety of cell types present in an outgrowth.

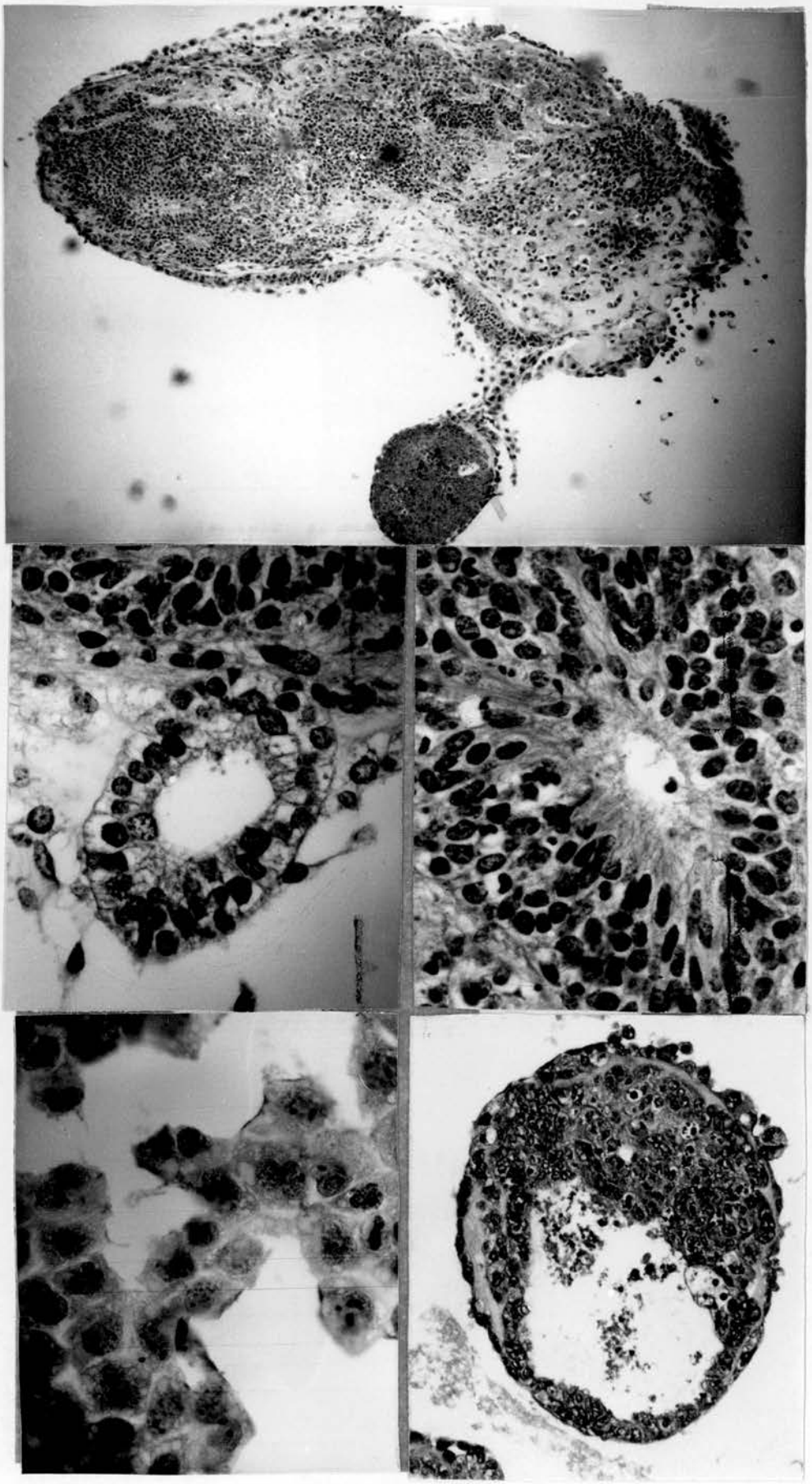
Examples of tissues observed in outgrowths.

Centre left: unidentified tubule

Centre right: neural tubule

Bottom left: squamous epithelium

Bottom right: embryoid body (5 day suspension stage)



theophylline was also studied in a single experiment (see Section 2.53 for methods). In contrast to the extensive differentiation in the controls, these PSA4TG12 embryoid bodies showed little cavitation. However a very large amount of Reichert's membrane was secreted. The PT3md1 embryoid bodies grown in dibutyryl cyclic AMP showed virtually no differentiation at all (see Table 5). In view of the very high levels of additives to which the cells were exposed for much longer than in the uridine nucleotide transfer experiments, and of the variety of effects which cAMP is known to have, it would seem unwise to deduce that these effects on differentiation are closely linked to the effects on junctional transfer discussed in Section 3.431

3.45 Karyotypes of PT clones

Metaphase spreads were prepared as described in Section 2.7. Twenty metaphases were counted for each clone chosen for characterisation. All chromosomes counted were telocentrics, and the modal chromosome numbers were all close to that of PSA4TG12 (see Fig. 11). In some cases the counts gave a frequency distribution with a very small variance whilst the other distributions were not so tight. Only single preparations of each cell line were examined so it is not possible to know whether this is a result of

Table 5

Effect of dibutyryl cyclic AMP and theophylline on differentiation of embryoid bodies

Cell Line	Medium	Morphology of Embryoid bodies		
		A	B	C
PSA4TG12	control	0	42	48
PSA4TG12	db cAMP and theophylline	0	94	7
PT3md1	control	130	5	0
PT3md1	db cAMP and theophylline	56	1	0

A: undifferentiated aggregates
B: differentiation of endoderm and secretion of matrix
C: formation of cavity or further stages of differentiation evident

experimental variation or of differences between the cell lines. No attempts at banding or further characterising the chromosomes were carried out.

3.5 Summary

This chapter describes the isolation and characterisation of four independent series of mec- ec cells from PSA4TG12. Starting from a product of one of these selections, a cell line has been isolated which is rescued from ouabain toxicity by resistant cells, but which does not appear to transfer uridine nucleotides. In addition the inhibition of uridine nucleotide transfer by retinoic acid is shown to occur in ec cells in culture, and the isolation of a clone resistant to this effect, is described.

Toxicity tests indicate that none of these cell lines has become more resistant than PSA4TG12 to straightforward toxic effects of agents present during the selections (with the possible exception of PT2mol), whilst the correlation of measures of metabolic cooperation derived from the two different assays used, which involve different metabolic pathways, confirms that the cells have altered junctional communication abilities. These alterations are not evident as gross changes in the karyotypes of the cells: chromosome counts indicate that these are all close to that of

PSA4TG12.

The ability of the cells to differentiate, either when seeded at low densities on gelatin, or when grown as aggregates in suspension culture, correlates with the results of the uridine nucleotide transfer assay. Very poorly co- operating clones show no signs of differentiation in suspension culture. Intermediate clones fail to form cavities or to engage in later stages of differentiation but produce parietal endoderm which secretes a matrix. However, although treatment with agents which enhance cellular cAMP levels causes the co-operation abilities of the mec⁻ clones to increase, treatment of suspension aggregates with the same agents reduced the extent of differentiation (in one experiment).

Chapter 4

LINEAGE MARKING OF MOSAIC EMBRYOID BODIES

It would be possible to address far more questions than at present using the variant cell lines described in Chapter 3, if a method were available for marking derivatives of specific cell lines in mixtures of lines of different developmental potential. In view of the availability of HGPRT variant cell lines, it would be convenient if HGPRT could be used as a lineage marker. The effect of retinoic acid on metabolic cooperation makes this feasible.

$HGPRT^+$ cells can be labelled by ^3H-hypoxanthine which is incorporated into their nucleic acid, where it can be detected by autoradiography. $HGPRT^-$ cells are not labelled. In mixtures of $HGPRT^+$ and $HGPRT^-$ cells however, transfer of tritiated nucleotides through gap junctions (see Section 1.51) results in labelling of the $HGPRT^-$ cells too and it is difficult to identify dividing lines between marked clones unambiguously. Rosenstraus and Levine [203] nevertheless used this technique to mark cells in embryoid bodies derived from mixtures of $HGPRT^+$ and $HGPRT^-$ cells. The published autoradiographs show very unclear demarcation of the clones.

By treating such mosaic embryoid bodies after five days in suspension culture, with retinoic acid in order to eliminate metabolic cooperation, then labelling with ^3H-hypoxanthine, but still in the presence of retinoic

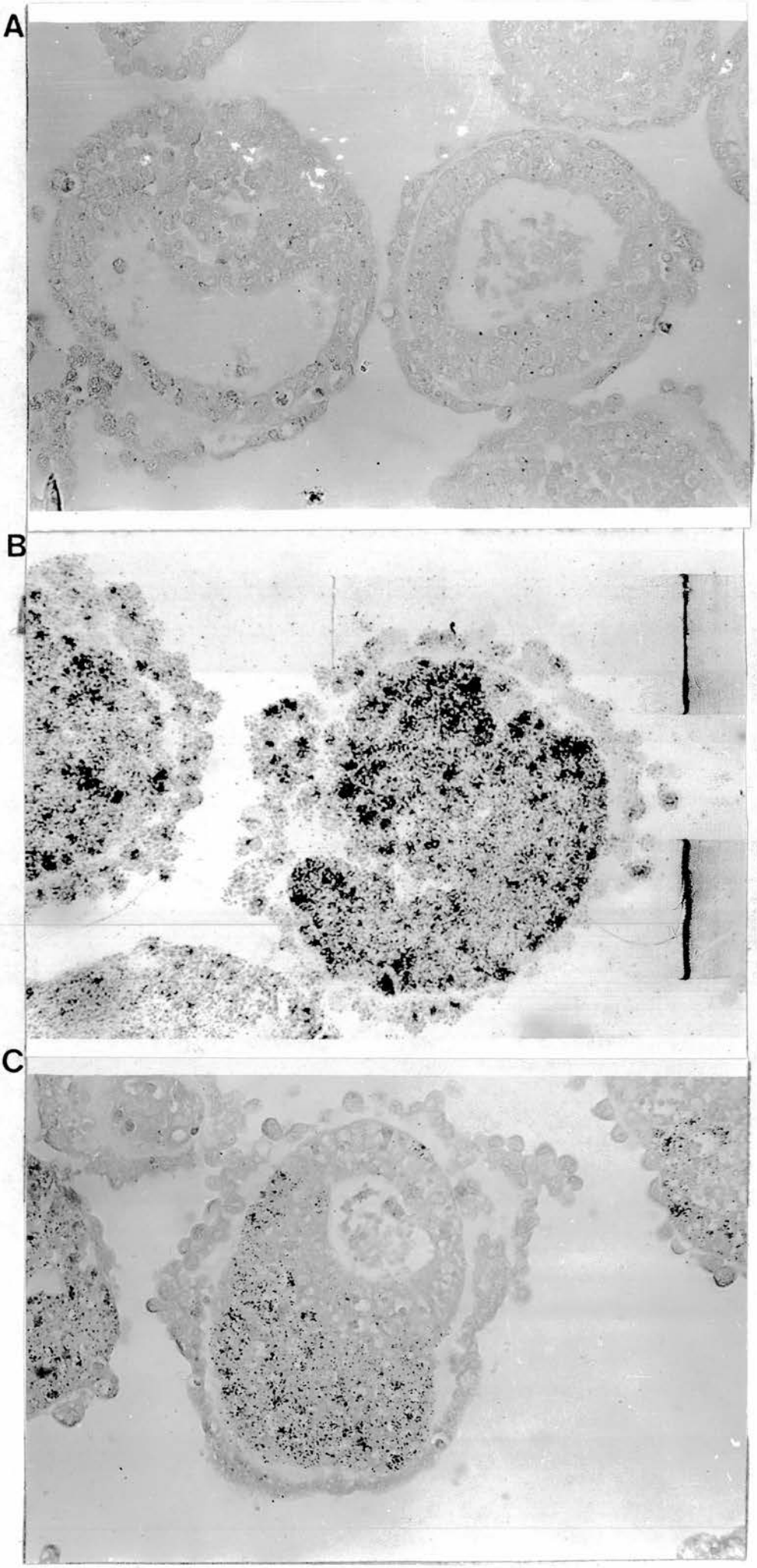
acid, clear demarcation of $HGPRT^+$ and $HGPRT^-$ clones was observed in autoradiographs of paraffin sections (Fig. 25) (method in Section 2.8). The cell lines were PSA4TG12 ($HGPRT^-$) and SCC-S2 ($HGPRT^+$). In the absence of retinoic acid, SCC- S2 cells co-operate normally [204] and clones could not easily be distinguished in autoradiographed paraffin sections of mosaic embryoid bodies which had been treated with ^3H-hypoxanthine but not with retinoic acid (not illustrated).

When outgrowths from embryoid bodies of the line SCC-PSA4, prepared by the method of Section 2.54, were treated with retinoic acid and ^3H-hypoxanthine and then autoradiographed in the same way, tissue variation in HGPRT activity led to very wide variation in levels of labelling among the $HGPRT^+$ cells. Some cell types were hardly labelled at all, even though very large numbers of grains were observed over others. Similarly, the endoderm cells derived from the ec cells in embryoid bodies do not label as strongly as the undifferentiated core (see Fig. 25). In view of this result, the technique does not appear to be applicable to the study of lineages in such outgrowths. It should be noted however, that it is on the basis of such lineage marking, even without the safeguard of eliminating metabolic cooperation with retinoic acid, that Rosenstraus and Spadoro [205] claimed evidence for the autonomy of the nullipotent and pluripotent development

Figure 25
Lineage Marking of Embryoid Bodies

Photomicrographs of labelled aggregates prepared as described in the Section 2.8.

A: PSA4TG12
B: SCC-S2
C: mixture of PSA4TG12 and SCC-S2


A
B
C

phenotypes in mixed cultures.

Despite this serious limitation, this technique should prove very useful in investigating the basis of the loss of developmental capacity of the mec PT clones. A number of interesting questions can be approached by studying the differentiation of lineage marked mosaic embryoid bodies of mec$^-$ and/or dif$^-$ cells (see Chapter 8).

Chapter 5

USE OF FLUORESCENCE ACTIVATED CELL SORTING TO STUDY METABOLIC COOPERATION

Using the observation that junctional communication can be followed microscopically by labelling cells with CFDA (section 1.51), it was thought possible that this might provide a novel way of selecting for mec$^-$ variants. In mixtures of pre-labelled donor cells, and an unlabelled parental cell population, transfer of 6-CF from cell to cell via gap junctions can occur. A cell sorter can in principle then be used to isolate variant cells which fail to receive tracer.

Mixtures of CFDA labelled donor cells and B2B2 embryonic stem cells, obtained from Dr. Martin Evans (see Section 1.36) were subjected to three rounds of selection for poorly co-operating variants. Optimisation experiments were carried out whilst these selections were in progress (see below). Possible improvements to the protocol were thus identified after the sorting had been carried out. The mixtures were prepared as described in Section 2.631. In each case the left hand tail of the fluorescence frequency distribution was collected, after low forward light scatter particles, thought to correspond to dead cells and debris, had been gated out. A total of 4×10^4 cells were isolated from the first round, from a total of 10^6 cells (including PC13.5 donors) fed into the machine. These cells were grown up, and 7.6×10^6 cells fed into a further round of selection. 1.02×10^5 cells were isolated and again grown up. 2.5×10^6

survivors were fed into a further round of selection and 6.7×10^4 cells recovered. These cells were grown up in two separate samples. These were tested for rescue from ouabain toxicity by the method of Section 2.62. Rescue indices of 1.69 and 1.24 were recorded, implying that no variant with reduced metabolic cooperation was present as a high proportion of either population.

The optimisation experiments suggested that STO cells were preferable to PC13.5 as donors in such selections. It had been thought likely that a better separation of the mec^- and mec^+ cells would be observed when the donors were also ec cells. This was found not to be the case: in mixtures of PC13.5 donors with either R5/3 or H2T12, the donor and recipient fluorescence peaks coalesced, and R5/3 and H2T12 recipients also ran together, making it impossible in principle to separate poorly co- operating cells from those cells which co-operated well, or from the donor cells. The forward light scatter of the donors and recipients was also indistinguishable. Furthermore, PC13.5 cells did not form confluent monolayers, and left patches in the cultures where recipient cells could not form contacts with donors. Consequently, some of the recipient cells were not in contact with donor cells and would therefore appear artefactually as mec^- cells in the fluorescence profiles.

Using mitomycin-treated STO cells as the donor cells, the method of Section 2.631 was used to show that transfer of 6-CF to R5/3 cells is markedly less than transfer to H2T12 cells (Figure 26), as is predicted by their abilities to transfer other molecules. Populations of unlabelled R5/3 or H2T12 cells were fed through the machine and their autofluorescence and forward light- scatter examined. The two populations were found to have similar high levels of autofluorescence (Figure 26). This was not noticeably reduced by using cells which had been grown in phenol-red free medium for two passages. Labelled STO feeder cells, to which no recipient cells had been added, were also examined. These had much higher levels of fluorescence and a highly variable (within the cell population) level of forward light scatter. Forward light scatter is closely related to cell size. This result was therefore attributed to variability in cell size. The ec cells could therefore readily be separated from the STO donor cells by the cell sorter (Figure 27). In mixtures of labelled donor cells and recipients which had been co-incubated as described in Section 2.631, the light-scattering properties of the cells were maintained, but the two populations interacted to give a fluorescence profile in which, whilst there was little change in the fluorescence of the STO donor cells, the ec cells were considerably

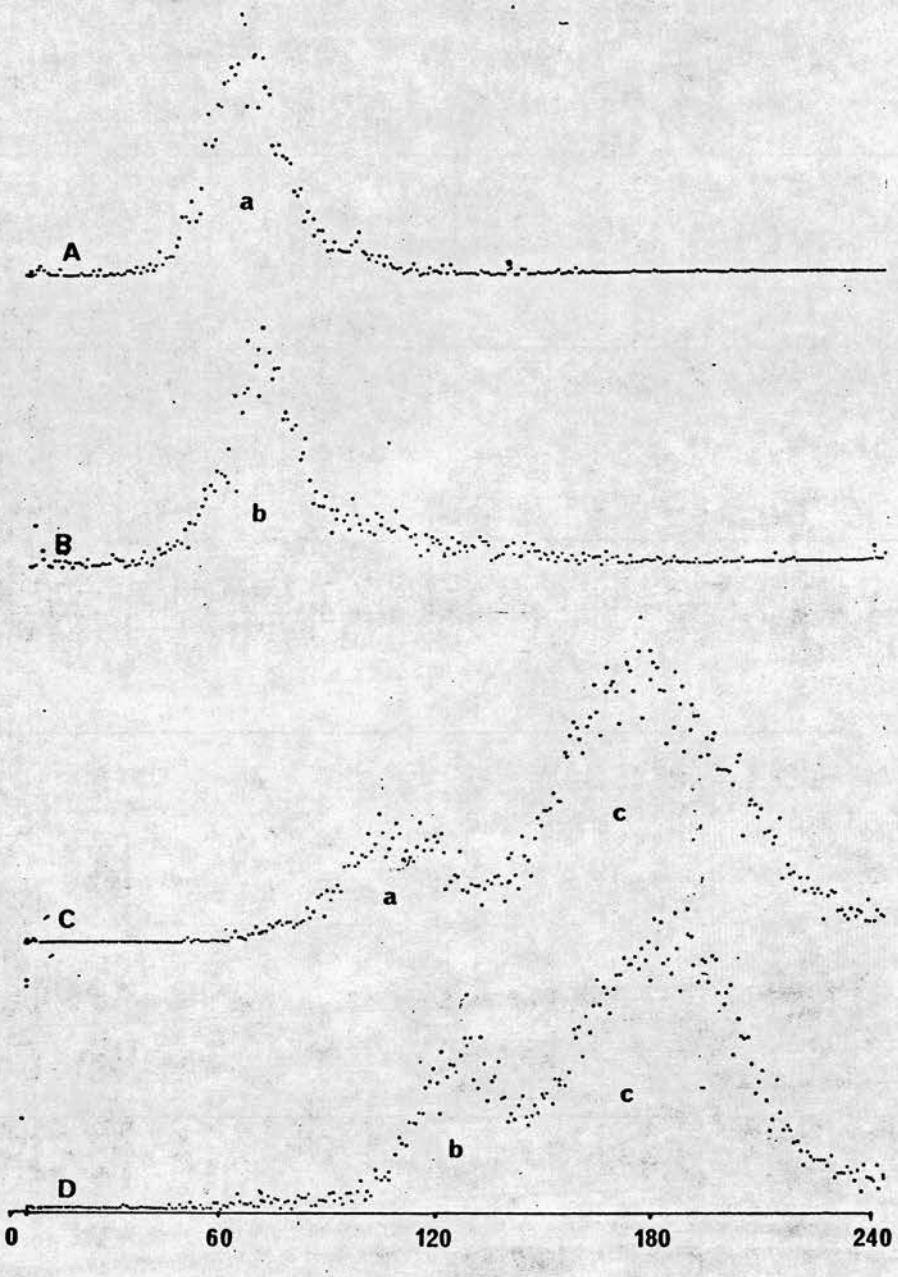
Figure 26

Flow cytometry of R5/3 or H2T12 cells with and without CFDA-labelled donor cells

ordinates: relative frequency
abscissa: channel number. This axis represents a logarithmic scale of fluorescence intensity. One decade change in fluorescence intensity corresponds to a difference of 60 channels.

A: R5/3 cells alone
B: H2T12 cells alone
C: R5/3 cells and mitomycin-treated CFDA-labelled STO cells.
D: H2T12 cells and mitomycin- treated CFDA-labelled STO cells.

a: R5/3 cells; b: H2T12 cells; c: STO donor cells.


A
a
B
b
C
a
c
D
b
c
0
60
120
180
240

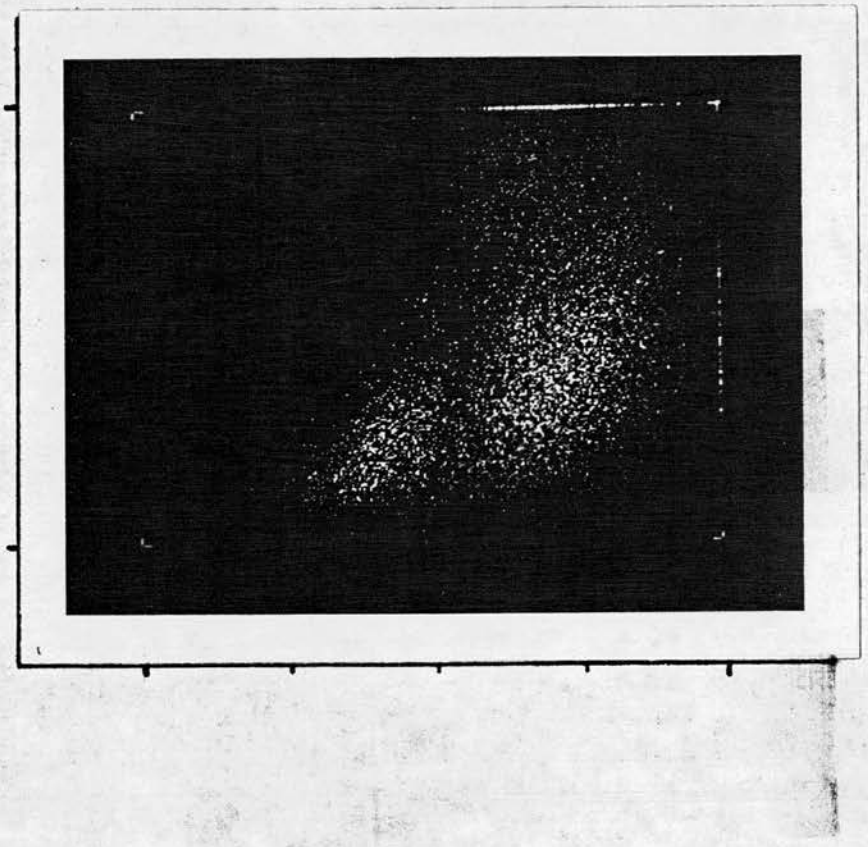


Figure 27

Plot of forward light scatter against fluorescence for R5/3 donor mixture.

ordinate: light scatter: linear scale.
abscissa: fluorescence per cell: logarithmic scale, each mark on the axis represents one decade of fluorescence intensity.

This mixture corresponds to that in Figure 26 (C). The R5/3 recipient cells are the population in the bottom left of the scatter. The STO donor cells are the population to the right. Note the wide variation in donor light scatter and fluorescence (very highly fluorescent cells, or those with very high light scatter appear together at the ends of the scales).

more fluorescent than they had apppeared in the absence of the donors (Figure 26) whilst remaining less fluorescent than the larger STO cells. The shift in fluorescence intensity was greater in H2T12 cells than in R5/3.

H2T12 and R5/3 cells were marked with optically distinguishable latex beads (method of Section 2.632). Complementary 50:50 mixtures of R5/3 cells and H2T12 cells were prepared in which one cell line was labelled with the smaller beads and the other with the larger ones. These were then seeded on to labelled STO donors and sorted on the FACS. Comparison of samples without beads and corresponding labelled samples showed that the beads made no major difference to the forward light scatter or fluorescence of the ec cells, although large numbers of cell-free beads came through the machine and had to be gated out. The ec population was sorted into two fractions: those above the median fluorescence intensity of the aggregate ec cell population (i), and those below (ii). Where the R5/3 cells were labelled with the larger beads, only 5 out of the 20 labelled cells scored from fraction (i) contained the larger beads, whereas 13 out of 20 labelled cells in fraction (ii) contained larger beads. When the R5/3 cells were labelled with small beads, enrichment was again observed: the ratio in fraction (i) was 6:14 small:large; and in fraction (ii), 18:2. In both cases

the low fluorescence fraction was enriched for R5/3 cells (chi^2, 2 degrees of freedom = 14.0, $p<0.01$).

It is concluded that the method of fluorescence activated cell-sorting may be an alternative to 'kiss-of-death' techniques for the isolation of mec cell lines. It should be possible to optimise the enrichment factors in such selections by looking at fluorescence profiles of mixtures in conjunction with observations on enrichment of marked cells. As yet, however, no variant has been isolated by this method.

Chapter 6

FURTHER STUDIES ON METABOLIC COOPERATION IN PC13TG8 AND R5/3

6.1 Kinetics of junction formation in R5/3 and PC13TG8

Gaunt and Subak-Sharpe [192], found selectivity in metabolic cooperation when the transfer of nucleotides between different cell lines was investigated by ^{3}H-hypoxanthine labelling of mixtures of $HGPRT^{+}$ and HGPRT- cells (see Section 1.7). This selectivity was manifested as a reduced rate of appearance of permeable junctions in the heterotypic (transfer between different cell types) co- cultures. Variation in length of labelling time was used to look for breakdown of junctions in such systems . Regardless of the duration of the ^{3}H- hypoxanthine labelling, there appeared to be the same proportion of the heterotypic contacts positive for metabolic cooperation after a given time of co-culture, implying that breakdown of junctions was not occurring. It would be of interest to know whether the reduced level of co-operation in selected mec^{-} cells is also a consequence of a reduced rate of junction formation.

The results of Gaunt and Subak-Sharpe called for a probability model for permeable junction formation . It was proposed that the initial contacts of two cells lead to a state in which they are not proceeding further towards junctional communication but the cells have a certain probability per unit time of leaving this state to initiate permeable junction formation.

This chapter reports an investigation of the kinetics of junction formation in the PC13TG8:R5/3 system. The quantification of the results adopts a statistical approach following the lines suggested by Gaunt and Subak-Sharpe.

By variation of the time between seeding of recipients onto uridine labelled donors, and fixation of the cultures, the rate at which junctions are formed can be studied. A three stage process was modelled viz:

(i)Settling of recipients and formation of contacts.
(ii)Formation of gap junctions
(iii)Transfer and incorporation of nucleotides.

Observations of differences in rates of formation of junctions may therefore be confused by effects operating at the other stages. Previous experiments have however indicated that the differences between the cell lines under study are not due primarily to differences in nucleotide metabolism (see Section 1.52).

Uridine nucleotide transfer experiments were carried out as described in section 2.61, and scored as described in the legend to Figure 28. At least 100 'constellations' were scored on each dish. Using the ratio R^+/R as a measure of the extent of communication, there appeared to be a large difference in the time course of junction formation in R5/3 and PC13TG8 (Fig.

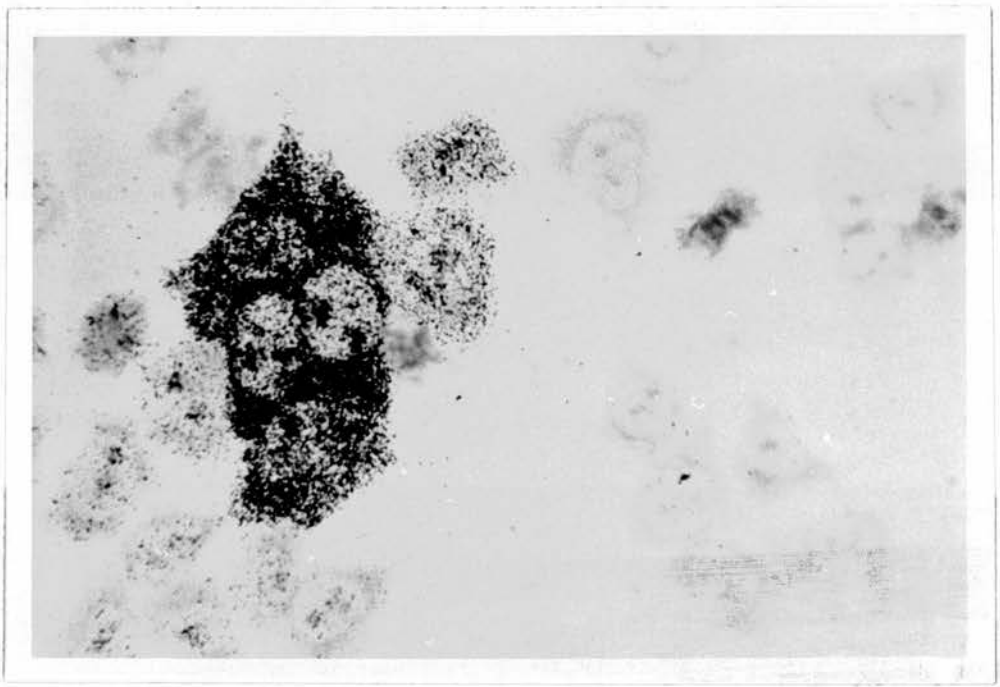
Figure 28
Analytical method and notation (Chapter 6)

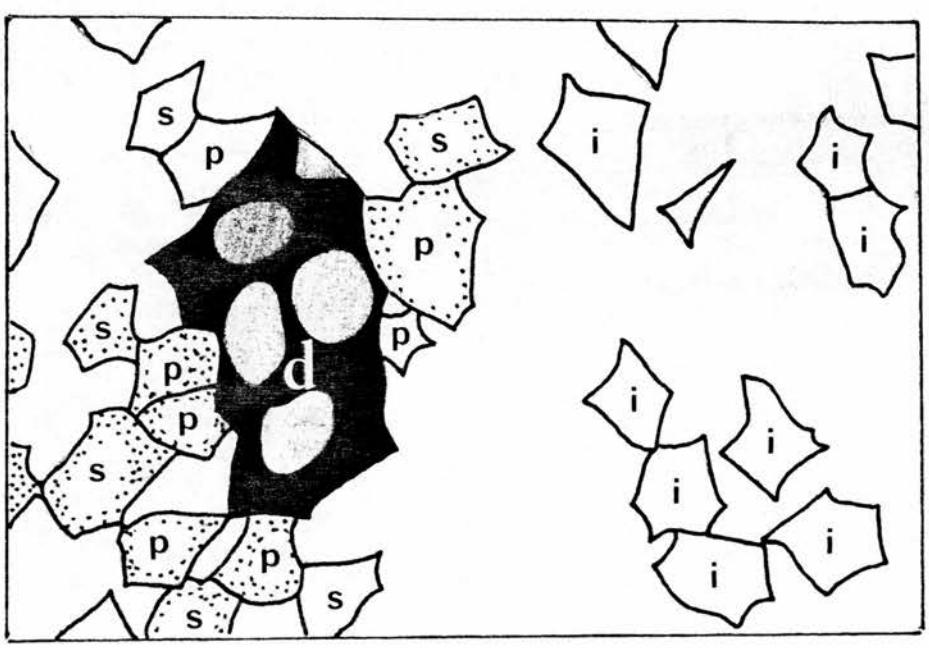
The basic unit of analysis is termed a 'constellation' of cells (all cells excluding those marked 'i') . This consists of a central group of pre- labelled donor cells (d) , and all the recipient cells in immediate contact with these, termed the primary recipients (p) (cells seeded on after labelling are termed recipients irrespective of whether they have co-operated). Recipients in contact with the primary recipients, but not with the donors are termed secondary (s), whilst those not in contact either directly or via a chain of cells, are termed isolated recipients (i). In cases where two or more positive primary recipients are in contact with each other, these are termed positive pairs, positive triplets etc..

For each autoradiograph, the distribution of grain counts over isolated recipients (i) was estimated by scoring a sample of at least 100 cells. Primary and secondary recipients were then scored. All primary recipients with more grains than twice the 90th percentile of this distribution were scored as positive. Those with fewer were scored as negative. The following notation was adopted.

	Individual Constellations	Populations of Constellations
Number of constellations		n
" of donor cells	d	D
" of primary recipients	r	R
" of positive primaries	r^+	R^+
" of negative primaries	r^-	R^-

A measure of the level of cooperation within a population of constellations is the fraction of all contacts between groups of donors and primary recipients through which significant junctional transfer has occurred (p). In Section 6.1, where large variations in p are studied, R^+/R is used as an estimator of p. However, the estimates obtained are slightly biassed as positive pairs, triplets etc. are scored as though junctional communication were known to occur directly between the donors and all primary recipients involved. In fact, some of the primary recipients might be in communication only with other primary recipients, and not at all with the donors. This problem was overcome for the purposes of Section 6.2 by using the analytical method described in Appendix B.

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29). However, presumably as a consequence of the difference in size between the two cell types, there were also large differences in the numbers of recipients observed at equivalent points in the two time courses (Fig. 30). Such variation in the rates of settling and contact formation of the cells could theoretically confound kinetic analysis of the formation of junctions. By examining the fit to a simple mathematical model it is possible in principle to separate the effects we are interested in from variations in attachment rates and/or cell motility, and also to quantify the rates of junction formation.

Let b =rate of breakdown of contacts between primary recipients and donors (contacts broken per cell pair per unit time)

g = rate of formation of junctions between cell pairs in contact but not communicating (initiation events per cell pair per unit time)

It follows that:

$$dR^+/dt = gR^-(t) - bR^+(t) \qquad (1)$$

Using the identity:

$$R^- = R - R^+ \qquad (2)$$

then integrating and rearranging, we obtain the equation:

$$R^+ = g\int_0^t R\,dt - (b+g)\int_0^t R^+\,dt \qquad (3)$$

For each dish on the time course, r and r had been scored over a sample of at least 100 constellations, and R and R estimated for n = 100. Numerical integration was then carried out using the trapezium rule to estimate the integral terms.

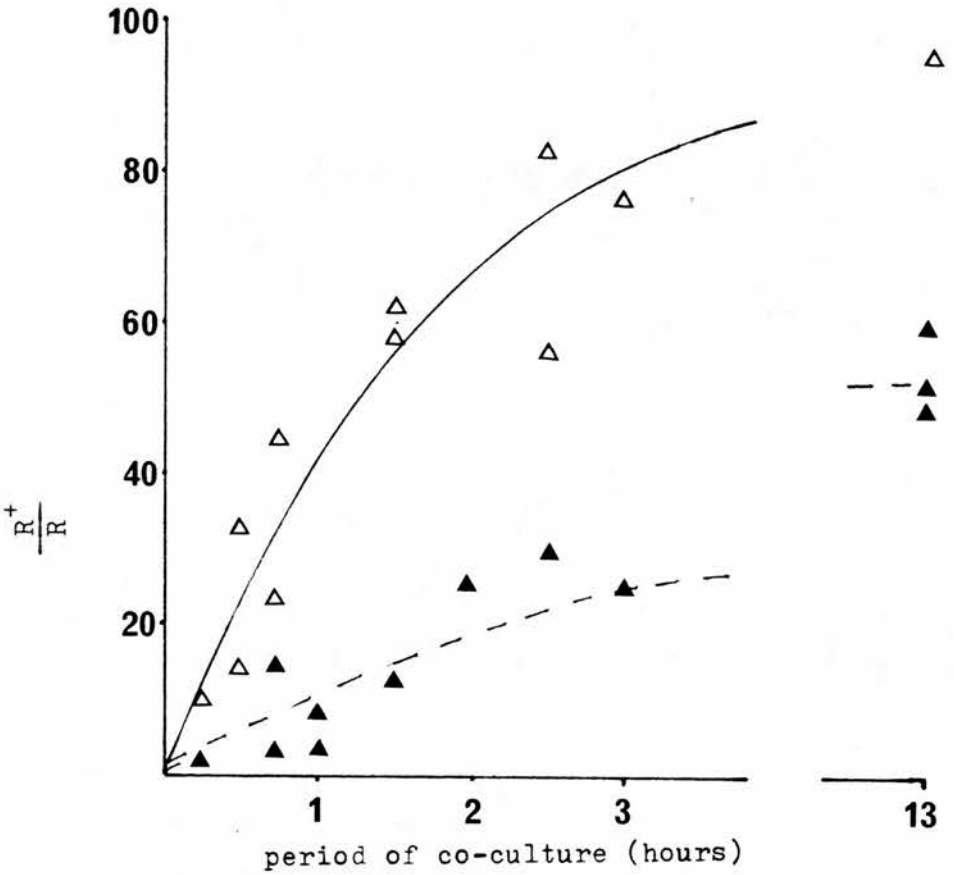


Figure 29

Time course of junction formation

△ PC13TG8
▲ R5/3

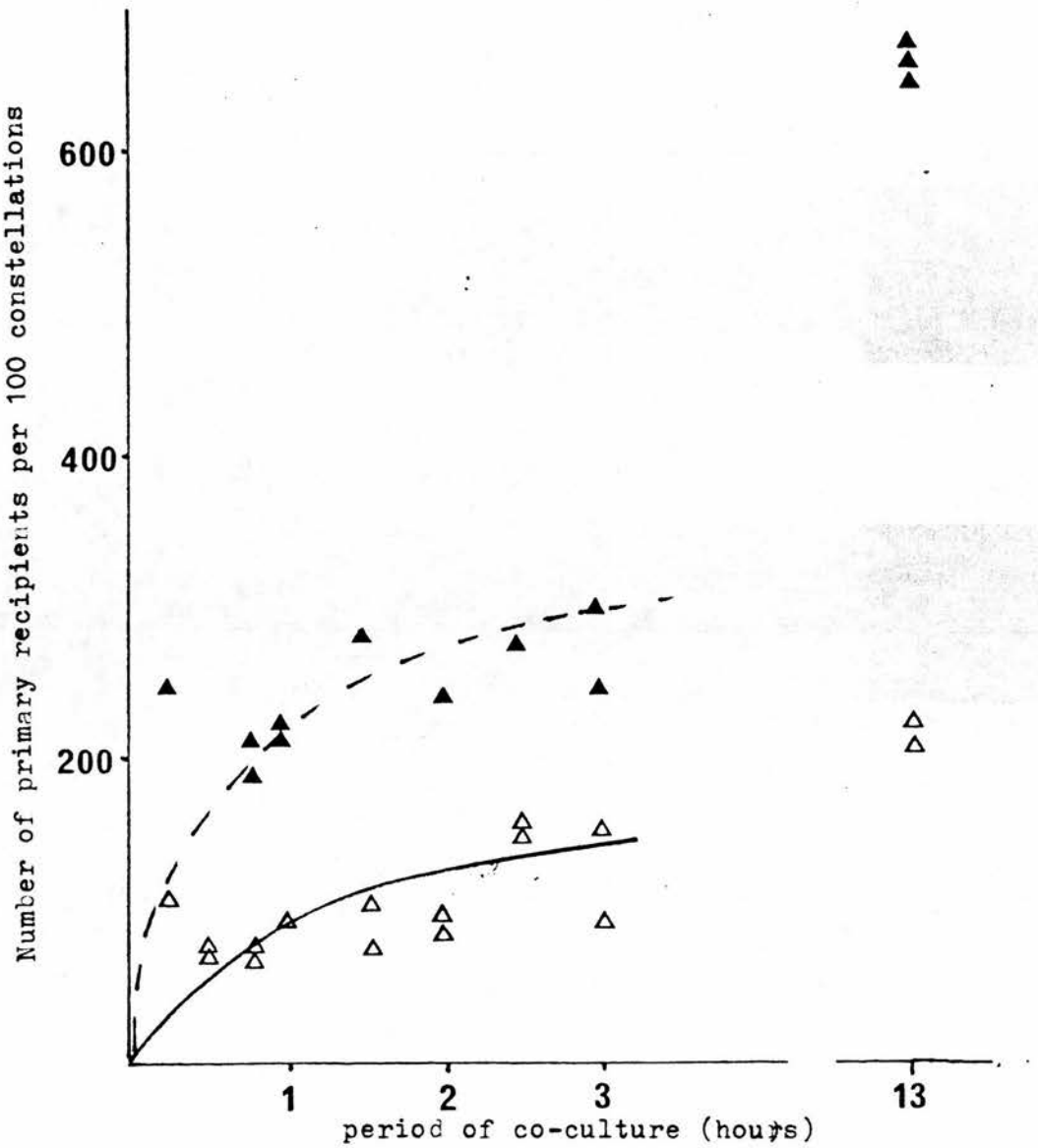


Figure 30

Time course of formation of cell-cell contacts

△ PC13TG8
▲ R5/3

This data is for the same experiment as that displayed in Figure 29.

g and b could be estimated for each cell line by linear regression using the identity (4) below, derived by rarranging (3) above and introducing an error term, e to account for stochastic variation:

$$\frac{R^+}{\int_0^t R^+ \, dt} = g \frac{\int_0^t R \, dt}{\int_0^t R^+ \, dt} - (b+g) + e \qquad (4)$$

This analysis was carried out for the data shown in Figs. 29 and 30 and the results are illustrated in Figure 31. It is concluded that R5/3 forms junctions much slower than PC13TG8, (estimated rate of 0.17×10^{-2} initiation events per eligable cell per minute for R5/3, compared with 1.27×10^{-2} events per eligable PC13TG8 cell). Breakdown of cell contact is very slow compared to the formation of junctions and can be neglected (estimated rates of -0.008×10^{-2} breakdowns per cell pair per minute for R5/3, -1.09×10^{-2} breakdowns per cell pair per minute for PC13TG8, neither rate significantly different from zero).

Figure 31

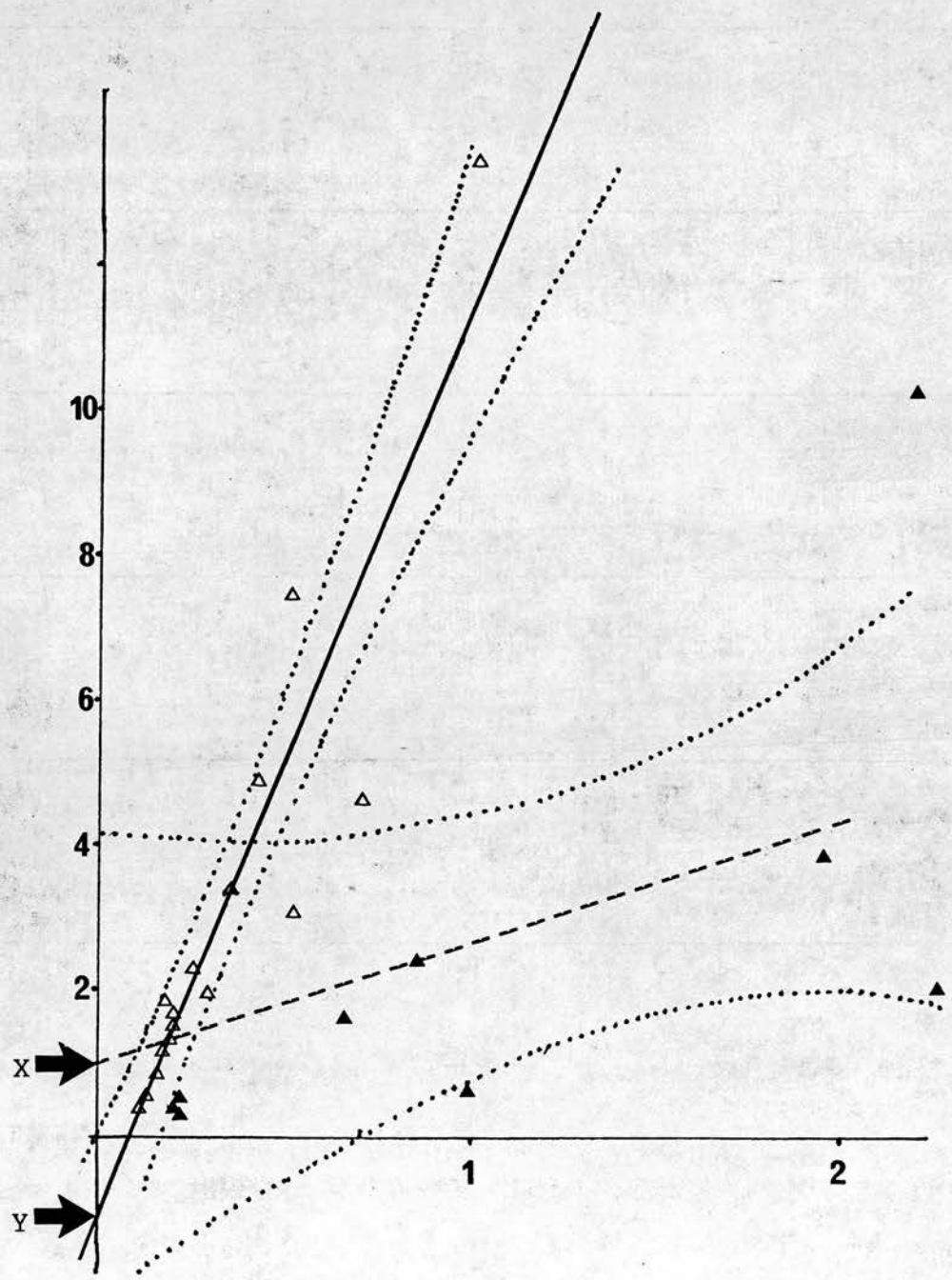
Analysis of kinetics of junction formation in R5/3 and PC13TG8

ordinate: $\frac{R^+}{\int_0^t R^+}$ units: $cell^{-1} min^{-1} \times 10^{-2}$

abscissa: $\frac{\int_0^t R^-}{\int_0^t R^+}$ units: $cell^{-1} min^{-1} \times 10^{-2}$

Data of a single experiment. Open triangles, unbroken regression line: PC13TG8; shaded triangles, broken regression line: R5/3 (note: two points at co-ordinates (5.6,6), and (3.3,13.4) are not shown but were included in the analysis used to fit the regression line). Dotted lines indicate 95% confidence limits on the regressions.

X) Intercept of regression line for R5/3 with the y axis.
Y) Intercept of regression line for PC13TG8 with the y axis.


10
8
6
4
2
X
Y
1
2

6.2 Effects of intercellular interactions on junctional communication

Whereas metabolic cooperation is an obvious way in which cells might mediate their interactions, it is also possible that the level of co-operation might be regulated either by itself, or by other types of regulatory cell interaction. Such effects might confound the interpretation of measurements of cooperation made in cell cultures. Using the uridine nucleotide transfer assay the effects of a number of variables inherent in the cell-culture system were studied. The level of co-operation was described by the parameter p´, the derivation of which is explained in Appendix B.

6.21 Dependence of metabolic co-operation on size of ´constellation´

All primary recipients which could be found on two dishes each of R5/3 cells and of PCl3TG8 cells showing uridine nucleotide transfer were scored as described in legend to Fig. 28. p´ was determined as a function of d and r and the results of this analysis are tabulated in Table 6. Although a very large amount of data was accumulated multiple regressions carried out on this data indicated no significant effects of d or r on the value of p´. It is concluded that the proportion of recipients in a constellation does not affect the probability of a recipient being scored as positive in this assay.

Table 6

Relationship between level of uridine nucleotide transfer and numbers of donor cells and/or recipient cells in the 'constellation'

Maximum likelihood estimates of p (values of p') ± standard errors (determined using the relationship between the second differential of the likelihood function and the variance of the estimate [212]).

Multiple regressions of the values of p' on r and d were carried out. The points were weighted in inverse proportion to the variances of the estimates of p [212]. None of the regression coefficients was significant at the 5% level.

Autoradiograph A: R5/3

r \ d	1	2	3	4	5	6	7
2			.277±.043	.246±.042	.235±.073	.300±.103	
3		.206±.028	.162±.024	.204±.026	.305±.055	.152±.062	
4		.127±.022	.159±.031	.205±.026	.214±.042	.294±.055	
5		.128±.032	.239±.070	.153±.131	.126±.037	.200±.080	
6		.083±.056	.190±.047	.175±.040	.237±.110	.102±.040	
7			.143±.059	.162±.068	.133±.048	.143±.132	

Autoradiograph B: R5/3

r \ d	1	2	3	4	5	6	7
1		.273±.067	.222±.139				
2	.166±.090	.224±.027	.182±.082	.194±.066			
3	.259±.068	.210±.019	.235±.060	.144±.037	.424±.131	.585±.140	
4	.116±.080	.198±.017	.218±.045	.181±.028		.248±.029	.250 ±.097
5	.200±.080	.179±.017	.284±.057	.242±.031	.249±.054	.243±.070	
6		.229±.020	.223±.040	.229±.027	.205±.076	.241±.053	
7		.206±.025	.234±.050	.281±.036	.378±.100	.240±.055	
8	.191±.030		.164±.043	.145±.048	.283±.060		
9				.220±.041		.215±.048	
10						.325±.063	

Autoradiograph C: PC13TG8

r \ d	1	2	3	4	5	6	7
1	.649±.034	.793±.030	.724±.051	.821±.051			
2	.675±.048	.761±.032	.778±.038	.777±.054			
3	.654±.086	.802±.046	.799±.055	.743±.056	.829±.081		
4	.560±.308	.466±.080	.682±.081	.891±.062			

Autoradiograph D: PC13TG8

r \ d	1	2	3	4	5	6	7
1	.679±.062	.806	.786	.846			
2	.808±.049	.800±.032	.764±.058				
3	.857±.051	.804±.030	.747±.052	.836±.033			
4	.842±.073	.930±.017	.687±.064	.811±.082			
5		.915±.030	.854±.050	.876±.027			
6		.939±.029	.923±.041	.974±.017			
7			.887±.035	.892±.041			
8				.889±.036			

6.22 Co-ordination of communication within constellations

If the primary recipients within a constellation behave independently, it is possible to predict the frequency distribution of r^+/r values (after adjustment for positive doublets, triplets etc) by using a simple binomial model. The data accumulated for the analysis in Section 6.21 were re-analysed in order to compare the observed frequencies of r^+/r values with those which would be expected if the recipients within constellations behave independently of each other(Fig.32)

i) Dish D (PC13TG8) showed an excess of intermediate r+/r values over the expected. This could easily be explained if the drain on the labelled nucleotide pool caused by cooperation between one cell pair reduces the probability of detection of cooperation elsewhere. However the results described in Section 6.21 suggest that donor:recipient, and hence source:sink ratios do not have such an effect and the observation was not repeated in dish C. (FIg. 32).

ii)Both R5/3 dishes however showed the converse effect: the whole constellation generally showed no cooperation or an unexpectedly high level. It is tempting to speculate that a component of the junctional transfer machinery which is in short supply is produced co-operatively by the different cells within the

Figure 32

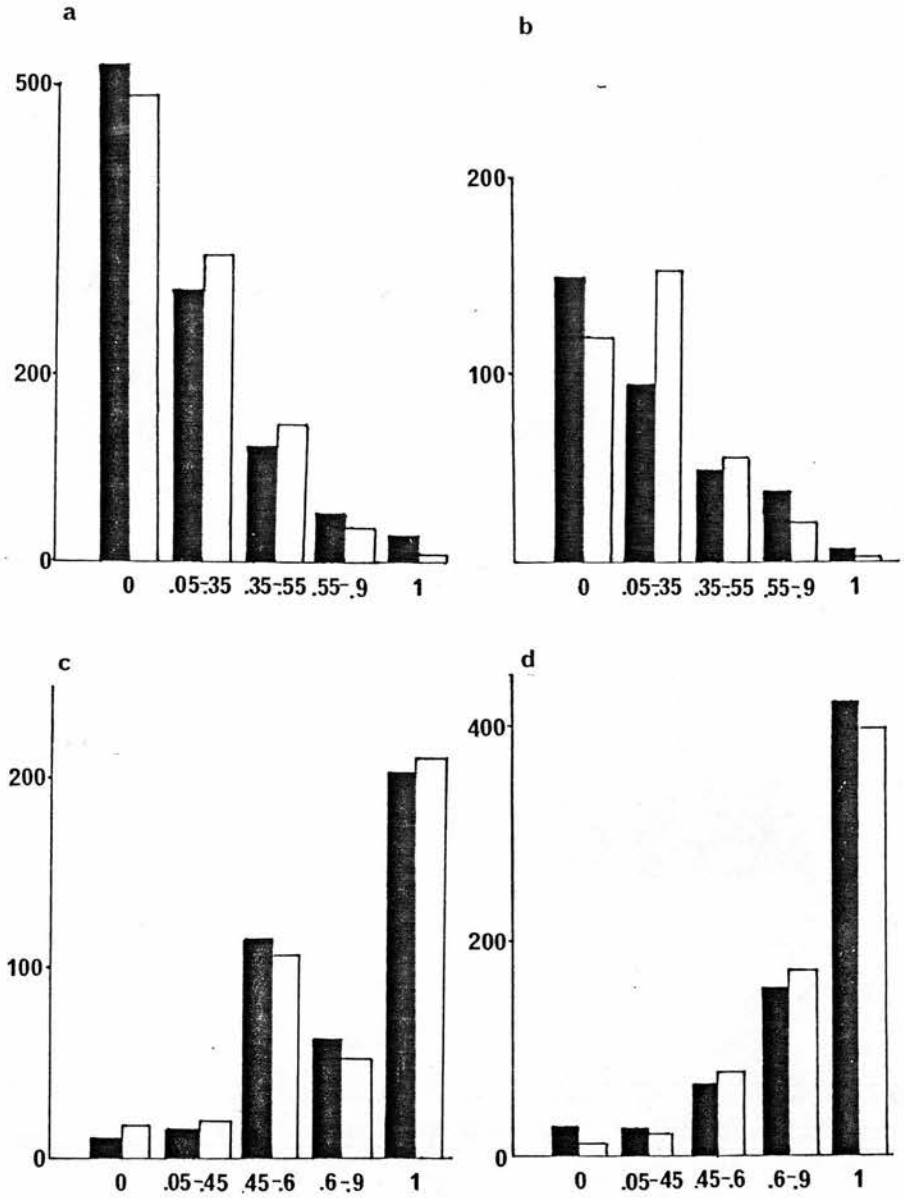
Co-ordination of uridine nucleotide transfer within ´constellations´ of cells

ordinates: frequencies
abscissae: r^+/r
shaded bars: observed frequencies
open bars: frequencies which would be expected if each recipient behaved independently of other recipients within the constellation.

For each value of d and r (see Fig. 28), and each autoradiograph, the value of p´ was determined (see Table 6). The binomial frequency distribution of r^+/r values which would have been obtained had the recipients behaved independently was then calculated. The expected frequencies displayed were obtained by summing these distributions over all d and r values and grouping the data into five classes. The observed frequency distributions of r^+/r values were also determined. Where primary recipients scored as co- operating were in contact with one another, the ´constellations´ were allocated to frequency classes by assuming the validity of this binomial model (i.e. of the null hypothesis). Consequently the results are biassed against rejecting the hypothesis that the recipients behave independently. Each histogram displays the aggregated frequencies for one autoradiograph.

Within each histogram therefore, the grand total of expected frequencies must equal that of the observed frequencies. As there are five classes, the fit with the null hypothesis can therefore be tested with a chi^2 test (4 degrees of freedom). The results are tabulated below:

ref.	cell-line	cell-density	n	chi^2	significance of deviation
A	R5/3	low	927	36.9	$p<0.001$
B	R5/3	high	353	60.0	$p<0.001$
C	PC13TG8	low	410	3.0	$p>0.05$
D	PC13TG8	high	709	20.3	$p<0.001$


a
500
200
0
0
.05-.35
.35-.55
.55-.9
1
b
200
100
0
0
.05-.35
.35-.55
.55-.9
1
c
200
100
0
0
.05-.45
.45-.6
.6-.9
1
d
400
200
0
0
.05-.45
.45-.6
.6-.9
1

constellation and that this co-operation requires junctional communication. In any case this result is valuable as a warning against scoring more than one recipient cell from the same constellation in such assays, where data for independent recipients is required. The outcomes of scoring several recipients from the same constellation are not independent of one another.

6.23 The level of cooperation in adjacent constellations

The level of co-operation in adjacent constellations was compared in order to determine whether constellations could be treated as independent entities, or whether they can act upon each other remotely. Much of the data for the three dishes, A, B, and D, studied in Sections 6.21 and 6.22 above had been accumulated by scanning dishes systematically, and was consequently spatially ordered. All constellations were classified dichotomously according to the level of cooperation recorded and then further classified according to the class of the constellation succeeding them in the ordered data. The resulting class frequencies were tested against a null hypothesis that the two classifications were statistically independent, using chi^2 tests(see Table 7). The data for dish C could not be analysed in this way as it was not ordered.

Correlation of presence or absence of overt uridine nucleotide transfer in adjacent constellations

			Numbers of pairs of adjacent constellations with the given pattern of evident transfer								Significance of chi^2
First constellation -->			mec+				mec-				
Second constellation ->			mec+		mec-		mec+		mec-		
Cell line	auto-radiograph	total no. of pairs	obs.	exp.	obs.	exp.	obs.	exp.	obs.	exp.	
R5/3	A	587	195	189	138	144	138	144	196	190	$p > 0.05$
R5/3	B	393	95	80.6	83	97.4	83	97.4	132	117.6	$p < 0.001$
PC13TG8	D	650	360	357.4	122	124.6	122	124.6	46	43.4	$p > 0.05$

note: R5/3 constellations were scored as positive if there was any transfer evident at all.
PC13TG8 constellations were only scored as positive if all the primary recipients appeared as positive for transfer.

TABLE 7

Only in the case of dish B, was a chi significant at the 5% level obtained. Co-operation was found to be more frequent when adjacent constellations showed transfer. In this dish, the cells were at a very high density and 'bridges' of recipients linked some of the constellations. Consequently it is possible that the effect was related to that observed in Section 6.22 : co-ordination of cells via short range interactions which may be contact dependent.

Chapter 7

EFFECTS OF STO FEEDER- CONDITIONED MEDIUM ON DIFFERENTIATION OF EC CELLS

7.1 Effect on retinoic acid induced differentiation of PC13TG8

When PC13TG8 cells were treated with retinoic acid on STO feeder layers (method of section 2.51), they were observed to retain their ec morphology. Cultures treated on gelatin differentiated. Ogiso et al (Section 1.343,[53]) found independently that in the ec line 311 the effect of retinoic acid can be inhibited by growth on BALB- 3T3/A31 feeder layers. Such artificial combinations of ec cells and embryo fibroblasts therefore provide a simple experimental system in which cellular interactions are clearly implicated in the control of cell determination. It is obviously of interest to find out whether this inhibition is dependent upon junctional communication. If it were so dependent, it is predicted that PC13TG8 cells treated with retinoic acid, in the absence of feeder cells, but in medium conditioned by them would differentiate into endoderm (section 1.343). It is also predicted that the mec^- derivative of PC13TG8, R5/3, would differentiate, when exposed to retinoic acid even on STO feeder layers.

The retinoic acid induced differentiation of PC13TG8 was monitored by trypsinising treated cultures and seeding known numbers of cells at cloning densities. Counts of the total cells present on trypsinisation and of the ec colonies growing up subsequently (identified

by their failure to produce plasminogen activator (see Section 1.343)) were used in conjunction to estimate numbers of ec cells and of committed cells present in the original culture (Fig. 33). The estimated numbers of ec cells present were reduced to 10% of control levels by treatment with 10^{-6} M retinoic acid. Intermediate numbers of ec cells were identified on trypsinisation of cultures treated with retinoic acid in conditioned medium or on STO feeder layers. The effect of the conditioned medium operated irrespective of whether the retinoic acid was added to the medium before or after incubation with the feeder cells. The differences observed between the effects of these two treatments are not statistically significant. The extent of morphological change in the cultures broadly corresponded to the estimated proportion of committed cells.

Neither retinoic acid, nor conditioned medium, greatly influenced the total cell number on trypsinisation, although differentiating cells generally failed to clone and few plasminogen activator positive colonies were counted.

R5/3 maintains the same morphology on feeders as PC13TG8, both in the presence and absence of 10^{-6} M retinoic acid (these experiments were carried out using a modification of the protocol of section 2.51 in which

the cells were seeded initially at a density of only 10^3 cells per $25cm^2$ dish, and were allowed to grow up for one week subsequently without trypsinisation). There is therefore no evidence for any junction-dependent, or even contact-dependent effect of fibroblasts on retinoic acid induced differentiation of feeder-independent ec cells. The observation that retinoic acid induced differentiation can be inhibited both by conditioning medium before the retinoic acid is added and by adding the retinoic acid initially (Fig. 33), implies that binding of retinoids by STO cells cannot explain the effect. However, it is impossible to determine from these data whether an inhibitor is being secreted or a critical medium component is being taken up by the STO cells.

7.2 Inhibition of differentiation of PSA4TG12 with conditioned medium

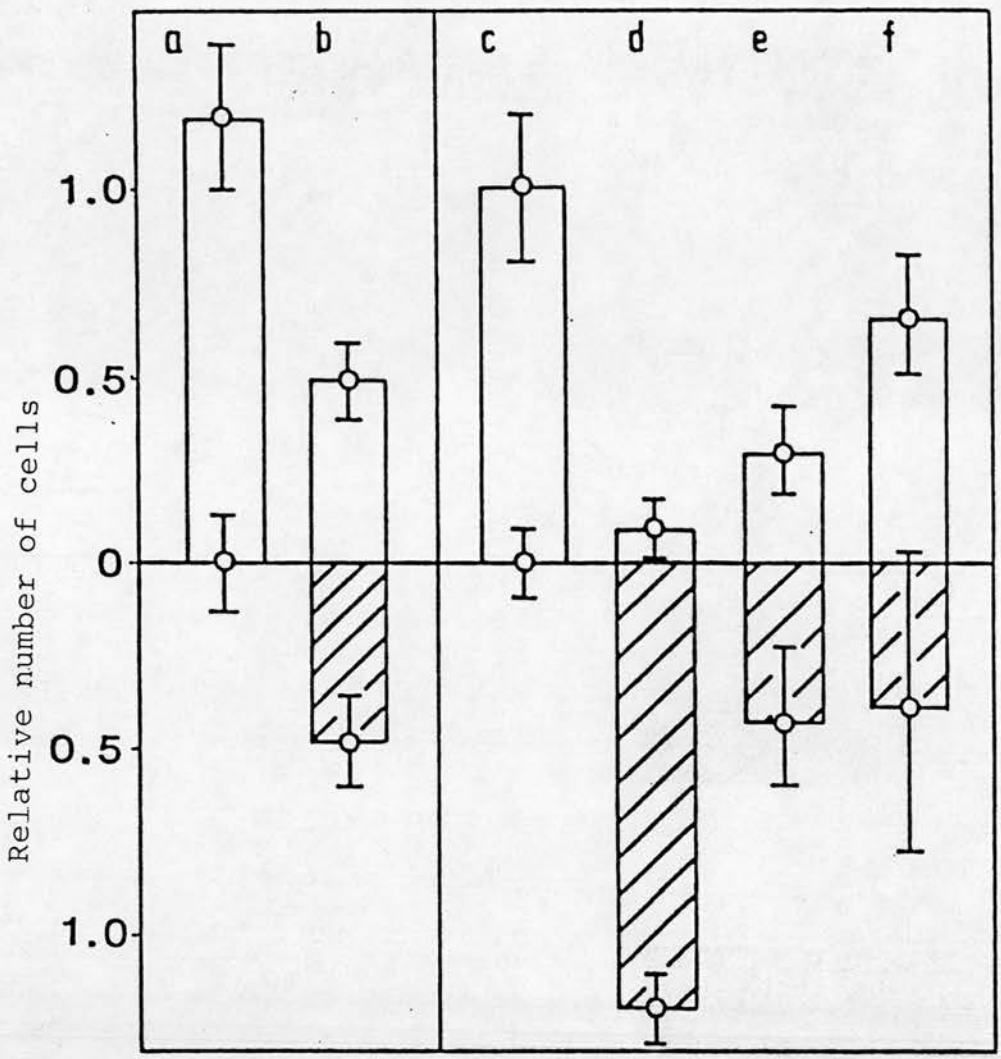
These results suggested that the spontaneous differentiation of cells taken off feeders might be reproduced by conditioned medium. A different methodology was needed to test this hypothesis. Alkaline phosphatase (APase) activity, which is characteristically high in ec cultures (section 1.32), was therefore followed as a marker of undifferentiated cells in cultures derived from PSA4TG12 (Fig. 34). The specific activity of alkaline phosphatase in cultures of PSA4TG12 grown on gelatine in conditioned

Figure 33

Effect of STO feeder cells and STO cell-conditioned medium on retinoic acid induced differentiation of PC1GT 8 cells.

Experiments were carried out in duplicate (for method see Section 2.51) and within each experiment the numbers for each of the two replicate dishes were expressed as a fraction of the mean number of ec cells recorded in column c. The relative number of cells was then defined as the mean value of this fraction determined from five experiments. Error bars indicate 1 SE above and below this mean. Open columns, ec cells: hatched columns; committed cells.

Growth conditions:
(a) STO feeders, unmodified medium
(b) STO feeders, medium containing 10^{-6} M retinoic acid
(c) gelatine, unmodified medium
(d) gelatine, medium containing 10^{-6} M retinoic acid
(e) gelatine, medium conditioned, 10^{-6} M retinoic acid added prior to conditioning by STO cells.
(f) gelatine, medium conditioned, 10^{-6} M retinoic acid added after conditioning by STO cells but before adding PC13TG8 cells.


a
b
c
d
e
f
1.0
0.5
0
0.5
1.0
Relative number of cells

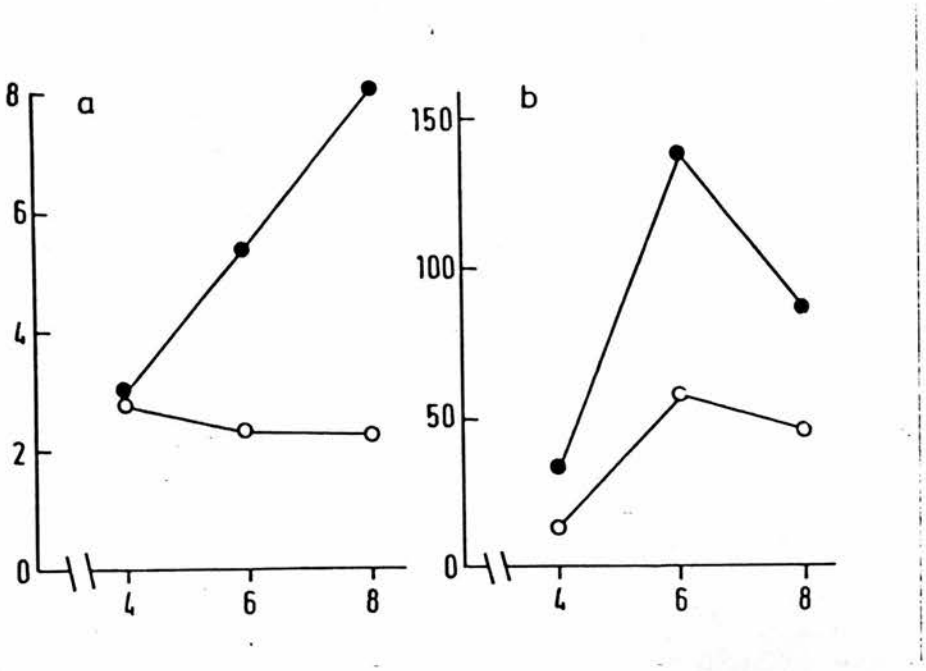


Figure 3.4

Effect of feeder-conditioned medium on PSA4TG12 cells

a) Effect on alkaline phosphatase activity

ordinate: specific activity of alkaline phosphatase (µmol/h/mg cell protein).

abscissa: duration of incubation in medium under test (days).

b) Effect on recovery of protein

ordinate: protein recovered (ug per dish).
abscissa: duration of incubation in medium under test (days).

● conditioned medium.
o unmodified medium.

medium increased with culture age, as has been found in pure cultures of feeder- independent ec cells [Bernstine, E.G. and Hooper M.L., unpublished result], and in contrast to cultures in control medium. When the medium was not conditioned, numerous differentiated cell types, largely of epithelial morphology, appeared (Figure 35). This morphology contrasted with that of the almost pure ec cultures observed growing in conditioned medium (Figure 35). Few surviving feeder cells were observed in these cultures.

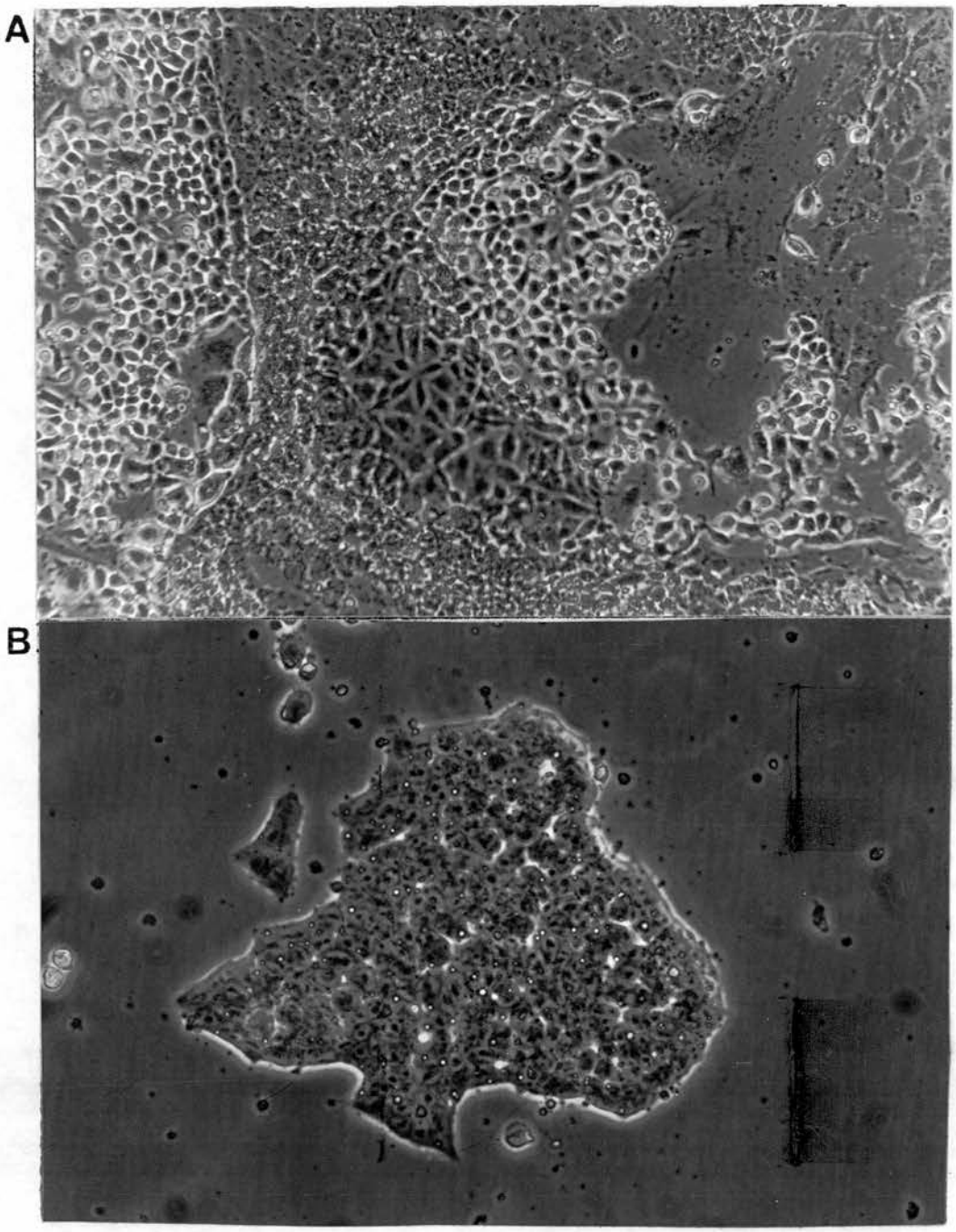
The cloning efficiency of ec cells in the absence of feeders is increased by conditioning the medium with non-dividing mouse fibroblasts [206] and consequently, the mass of protein extractable from cultures plated on gelatine is greater when the cells are seeded out in conditioned medium (Fig. 34). It is clear however, that the effect of conditioned medium on PSA4TG12 is not solely a consequence of increased cell density, as morphological differentiation can be inhibited by conditioned medium even when cells are plated very sparsely (10^3 cells per 60mm dish). The methodology of this study excludes effects on plating efficiency as explanations of the observations on PC13TG8.

Martin and Evans [35] reported a failure to reproduce the feeder effect by feeder-conditioned medium. Their work was carried out in the absence of beta-

Figure 35
Effect of conditioned medium on differentiation of PSA4TG12

Phase contrast photomicrographs of 7-day-old cultures of PSA4TG12 on gelatin. (See section 2.52 for method)

A: unmodified medium
B: conditioned medium


A
B

mercaptoethanol, which has since been shown to enhance growth of ec cells [195] and which is routinely added to our medium. These results show that some at least of the effects of the feeders are transmitted through the medium.

It is concluded that feeder-conditioned medium can inhibit the differentiation of ec cells as induced by two different stimuli.

Chapter 8

DISCUSSION

This study should be considered as a part of a continuing project on the developmental genetics of mouse teratocarcinoma cells. It is hoped that the ec cell lines isolated in this work will be used in further studies in order to expand our knowledge of the genetics and biochemistry of junctional communication and of its role in development. In this context, the firm conclusions which can be drawn merely from the characterisations which have already been carried out are less interesting than the experiments which can now be applied to the cells isolated.

The near-diploid karyotypes of all the PT clones demonstrate that chromosomal duplication, as in R5/3, is not necessary for an ec cell to lose the ability to cooperate. With this exception, no new contributions to the identification or mapping of genes involved in junctional transfer (reviewed in Section 1.53) are reported here. Indeed, no formal proof is presented to show that the alterations in the selected clones represent genetic, as opposed to epigenetic, changes. However, with this proviso the isolation of five new independent series of co- operation variants should make it possible to considerably extend the genetic analyses discussed in Section 1.52.

Similarly, no biochemical characterisation of the cell lines isolated is reported here. Studies of the

biochemistry of the mec⁻ lines may well prove valuable in extending our knowledge of the biochemistry of junctional communication. Biochemical characterisation of the clone PTmr0 is one approach to the study of the mechanism of the effect of retinoic acid on junctional communication. It will be of interest to see if this proves to be linked to the mechanism by which retinoic acid causes ec cells to differentiate (section 1.345).

Fluorescence activated cell sorting (Chapter 5) may prove a valuable technique for measuring co-operation, though as yet it is unreliable. The difficulties would be less prohibitive if a cell-sorter was closer at hand. The work reported here was carried out using a cell-sorter some distance away from the cell-culture laboratory. On the basis of these results it is not possible to claim that this technique could be used successfully as an alternative methodology for the selection of mec⁻ variants. Such a technique would be useful as it would allow effects of mec⁻ lesions to be studied in cells with normal nucleotide metabolism which had not been exposed to toxic nucleotide analogues. The interpretation of the differentiation of such cells <u>in vitro</u> or in chimaeras would not be confounded by the need to consider effects of altered nucleotide metabolism on the phenotype.

It is clear from the results of Chapter 6 that R5/3

differs from PCl3TG8 in the rate of formation of functional junctions. The other results of Chapter 6, whilst indicating that levels of metabolic co-operation by ec cells in culture are not always independent of the behaviour of nearby cells, do not suggest any role for such regulation in cell determination. The effects are not clear-cut. Technical artefacts, such as inconsistencies in the autoradiography, cannot be excluded as explanations for the correlation between levels of co-operation within 'constellations' of R5/3 cells. Therefore, these results are most valuable in illustrating the need for random sampling within uridine nucleotide transfer experiments, when small variations in levels of co-operation between samples are being studied.

The physiological significance of the effect of STO conditioned medium on the differentiation of ec cells (Chapter 7) is also not clear. The production by embryo fibroblasts of factors which help to maintain a population of undifferentiated pluripotent cells, might well be of significance as a component of the no doubt complex cellular interactions regulating later stages of differentiation. It has been found [208] that PCl3 END cells (produced by retinoic acid treatment) and also a number of fibroblastic feeder cells [209] secrete molecules which support the multiplication of ec cells *in vitro*. This raises the possibility that

requirements for ec cell growth, or the growth of the embryonic ectoderm, may be provided in vitro by END cells (or by implication, the feeder cells in feeder dependent lines), and in vivo by the visceral endoderm. It seems reasonable to suggest a further role for these differentiated cells: that of secreting factors which inhibit the differentiation of the ec cells or ectoderm. In vivo this would act as a feedback mechanism to prevent overproduction of visceral endoderm.

Both in this study and in the previous work on R5/3 (Sections 1.52, 1.7 and 1.8), it was found that the isolation of mec^- ec cell lines by thioguanine 'kiss of death' is accompanied by a loss in developmental capacity (dif^- phenotype). In the results presented here, there is a clear correlation between the extent of loss in developmental capacity and the reduction in junctional transfer ability (as measured in two different assays). There are several alternative explanations for this result. Further experiments will be required in order to test them exhaustively .

There is the trivial explanation that independent lesions in a differentiation pathway have co-incidentally been introduced during the extensive period of culture involved in selection of mec cells. The isolation of a single mec^- dif^+ cell line would

confirm this idea, but so far, the four independent selections described here, and that of R5/3, have failed to produce one. The hypothesis becomes less plausible, the more often mec$^-$ cells are found to be defective in their ability to differentiate. Although culture of ec cells for prolonged periods is expected to reduce their ability to differentiate [200], and in the experiments described here the cells were not all at the same passage number, the periods of culture involved in the selections were comparable with periods for which stocks of PSA4TG12 can be maintained without any noticeable loss in developmental capacity and all the characterisation of the survivors was carried out on material which had been passaged 8 or fewer times since cloning. It would be very surprising if a battery of unselected clones cultured for corresponding periods were to prove markedly impaired in their abilities to differentiate.

This does not exclude the possibility that the particular culture conditions used to select for mec lines also select for non- differentiating (dif$^-$) cells by some unknown mechanism unconnected with metabolic co- operation. This somewhat contrived objection to the conclusion that there is a link between junctional communication and differentiation is weakened by the observation that the clone PTlmdl differentiates normally, despite considerable exposure to the

selective conditions. Similarly, the sib clones of PT2mdl and PT3mdl in particular, which appeared to co-operate normally in the ouabain- rescue assay, formed differentiated colonies on gelatin despite exposure to exactly the same selective conditions as their mec$^-$ sibs. It would be of interest to examine embryoid bodies formed from these clones.

Another possibility is that a loss of the ability to co-operate acts as a signal for the cells to differentiate. If this were the case, it would not be possible to recover mec$^-$ ec cells unless there were a prior block in differentiation. Differentiation-competent cells in the original population would differentiate as soon as the co-operation defect arose and all the mec$^-$ cells isolated would also be dif$^-$. Such a theory seems unlikely on several counts. In its simplest form it would predict that isolated ec cells would differentiate. Although growth at low densities does appear to promote differentiation (see Section 1.342), the fact that it is possible to grow single-cell clones of pluripotent cells with high cloning efficiency argues against this. The line PC13TG8 shows little differentiation in culture in the absence of retinoic acid but differentiates in tumours. R5/3 behaves in exactly the same way in culture but does not differentiate in tumours. If the loss of the capacity to differentiate in tumours were necessary to prevent

the differentiation of a mec$^-$ line in culture, it would be very surprising if the in vitro differentiation was not impaired. However, R5/3 differentiates normally in retinoic acid.

A much more elegant explanation of these results is that junctional communciation is necessary for some types of cell determination to occur. It is quite plausible that junction-mediated events are necessary for differentiation in the three- dimensional tumours, whilst these have no such role in monolayers. This general hypothesis also predicts that it should be possible to isolate revertant mec$^+$ cells which differentiate normally. However, the failure of H2T12 to differentiate in tumours (Section 1.8), implies that a seperate lesion from the one affecting communication is at least partly responsible for the loss of developmental capacity in R5/3. Whilst this is still consistent with the view that junctions are needed for cell determination, it is equally consistent with any of the alternative hypotheses. Similarly, the observation that cell lines which have impaired differentiation, may well cooperate normally (see [204], and also the data for Nulli-SCC1 tabulated opposite Figure 9) is consistent with any of the possibilities discussed here.

Clearly, in order to decisively resolve the question ,

further mec$^+$ revertants must be selected. Whilst it appears likely that PTlmdl represents a spontaneous mec$^+$, dif+ revertant, the putative mec$^-$ clone from which it arose was not well characterised. The only product of a revertant selecton discussed in this study is the clone PT2mol and it is not yet clear whether this clone is merely an unusually ouabain-resistant subclone of PT2mdl. Whilst it appears to co-operate normally in the ouabain-rescue assay it is defective both in its ability to transfer uridine nucleotides and to differentiate in suspension. This is consistent with PT2mol being a clone with increased ouabain resistance; a pore-size mutant preferentially transferring small cations rather than nucleotides (because of changes in the charge on the junctional particles); or a mutant with altered specificity, preferentially communicating with fibroblasts rather than with other ec cells.

At present it is therefore impossible to know if the losses in developmental capacity in the PT series of cell lines are a consequence of separate lesions from the ones in co- operation, or of the defects in communication alone. Feeder-layers for PSA4TG12 and its derivatives can be prepared from 3T3 cell lines as well as from STO. It should therefore be possible to select mec$^+$ revertants from mec$^-$ PT clones in HAT medium on such feeder-layers using the same rationale

as that used in the selection of H2T12 (Section 1.52), i.e. the HAT 'kiss of life' (Section 1.51). DON cells, as used in the H2T12 selection, do not make very good feeders for PSA4TG12 (or, presumably, its derivatives), whilst STO feeders cannot be used in such a selection as they do not possess the enzyme HGPRT. It would be of particular interest to select such a mec^+ line from PT2mol. The HAT 'kiss of life' selects for clones which transfer nucleotides. If such clones, selected from PT2md1, PT3md1, or PT4md1, were found to be dif^+, this would be very strong evidence for the theory that junctional communication is necessary for the normal differentiation of embryoid bodies.

Even in the absence of convincing evidence for or against a role of junctions in cell determination in embryoid bodies, it is possible to dismiss some hypotheses about specific ways in which junctions might operate. Whereas junctions are already known not to be involved in determination in early cleavage stages (Section 1.6), there may be parallels between the determination mechanisms operating in the differentiation of the blastocyst and in embryoid bodies: although the cell lineages involved are quite different, the geometry is rather similar. The embryoid body can be treated as a particularly simple model for pattern formation in which, unlike the situation even in the early egg-cylinder, the only

asymmetries in the environment are initially those imposed by the external gravitational field. It is therefore an appropriate system in which to study the generation of radial pattern (perhaps some, at least, of the disorder of later stages of embryoid body differentiation can be attributed to the absence of a point of reference for angular co-ordinates. The ectoplacental cone provides one such point of reference in the embryo, which is not reproduced in embryoid bodies).

The hypothesis that junctional communication within the aggregate is required for the differentiation of the endoderm layer still leaves open the question of what kind of signal is involved. As with the differentiation of the trophoblast and inner cell mass in the embryo (Section 1.6), inside- outside determination occurs in a system in which all the cells cooperate. Again, it is the outside cells which differentiate: a paradoxical observation, as it is the inside cells whose environment changes as they become surrounded. A signal produced by the inside cells in response to their changed environment must be passed to the outside, where the cells respond to, but do not produce, the signal. The hypothesis that it is this transmission which is blocked in the mec^- lines is attractive. The production of such a signal in the inside cells cannot represent an irreversible

differentiation event, as they retain their pluripotency in embryoid body cores (Section 1.6).

In a gradient model for the determination of positional information (Section 1.4), one plausible mechanism whereby the initiation of cavitation is determined implicates the secretion of a diffusible molecule by the cells of the aggregate. Such a substance would diffuse to the outside and be diluted out in the external medium setting up a concentration gradient from the inside to the outside. Let it be assumed that the diffusion of such a molecule might be through gap junctions, and that the secretion into the medium is a specialised role of the outer endoderm layer. The signal for cavitation to commence could then be the accumulation of this substance in a cell above a certain critical concentration.

If such a signal were indeed passing through gap junctions, fairly simple relationships could be observed between the competance of the cells of the embryoid body to cooperate and the stage at which cavitation is initiated, or the relative dimensions of cavity and whole embryoid body. One immediate consequence follows: that if junctional communication is blocked whilst other factors (such as the response of the cells) remain unchanged, a build up of the morphogen occurs in the middle of the aggregate, and

cavitation occurs sooner and to a greater extent. If the model is reversed: the morphogen is assumed to be taken up from the medium, where it is present at a uniform concentration and the signal for cavitation assumed to be a reduction in morphogen concentration caused by absorption by the cells of the aggregate, this observation is still predicted.

This is precisely the converse of the result obtained here. The extent of cavitation is much reduced in embryoid bodies formed from mec⁻ lines. Even in embryoid bodies with substantial parietal endoderm formation derived from PT1md2 PT3md1, or PT4md1, there is very little cavitation evident. On the other hand, well-defined cavity formation is not observed in any PT line in the absence of endoderm differentiation. Therefore the simplest model of a signal molecule being produced uniformly by the core of the embryoid body must be discarded, and the results suggest a role of the endoderm in cavity determination.

In previous work (see Section 1.341), cavitation accompanied the formation of visceral endoderm. Only a very small amount of visceral endoderm (identified by anti-AFP immunoperoxidase staining) is produced under the culture conditions used here, even in PSA4TG12 [Hooper M.L., personal communication]. This is not surprising, as the differentiation of the visceral

endoderm in the embryo seems to be modulated by the mesoderm which arises subsequently [16] (see Section 1.6). The embryoid bodies grown in this study were generally fixed before mesoderm production occurred. On the other hand, despite the absence of trophoblast (see Section 1.6), much parietal endoderm is produced.

Any putative signal is therefore likely to be produced by the parietal or primary endoderm, though the primary endoderm is only present transiently. It is straightforward to devise models whereby such signals, passing through gap junctions, could give rise to the observed pattern of cavitation. However, the lack of junctional communication between extra- embryonic endoderm and embryonic ectoderm (discussed in Section 1.6), makes junctional transfer between the core and the endoderm seem an unlikely route for signal transmission. In any case, the position relative to the parietal endoderm of the embryonic ectoderm in the embryo (Section 1.2) is quite different from that of the core in the embryoid body so such a phenomenon could not be physiological. It is more attractive to see the matrix secreted by the parietal endoderm layer in the embryo as acting as a passive diffusion barrier (see Section 1.2). If this were the case in the embryoid body it is difficult to see how positional information within the aggregate could be determined by a simple gradient and one of the other mechanisms

discussed in Section 1.4 may well be implicated.

The lineage marking technique, the development of which is described in Chapter 4, should prove useful in evaluating these hypotheses. Cavitation and endoderm formation can now be studied in lineage marked aggregates. Consequently, it should be possible to see whether cells which are developmentally competant can confer this competance on dif cells in the same or other aggregates, and whether dif lines might differentiate in mixtures with other dif cells. Different hypotheses, based on those applied to other differentiating systems (Section 1.4), make different predictions regarding the outcomes of such experiments conducted on the various cell lines now available. For instance, if the failure of cells of one line to cavitate were because of a lack of response to a signal, whilst another line failed to cavitate because of a failure to produce the signal, a mixture of the two might well show cavitation, but it would only be the cells which possessed the normal response to the signal which would contribute to the cavity.

In summary, this work has made available a number of new mouse ec cell lines with which to study the effects of alterations in metabolic co-operation ability on other aspects of the cell's phenotype. A beginning has been made on the study of effects on early stages of

differentiation in suspension culture. The results of this work have suggested several hypotheses relating to differentiation in this experimental model of mouse embryogenesis. Experiments to test these hypotheses are also suggested.

APPENDICES

Appendix A

The quantitation of metabolic co-operation

In principle, junctional channels can vary in pore-size, charge, frequency, and distribution in the cell membrane, so it is impossible to quantify all aspects of metabolic cooperation in a system with a single measurement. The nature of the cell- cell contacts must also affect cell-cell communication in a qualitative way. Potentially, therefore, variation exists in the spectrum of molecules transferable, in the rates of transfer through existing channels, in the proportion of cell-cell contacts where junctions are formed, and in the numbers of permeable junctions formed at each contact. The limitations of the assay systems available make it possible to measure such functional variation only very crudely.

The bulk of the measurements of metabolic cooperation made as part of this project involved the scoring and analysis of two assays: the ouabain rescue assay, and the uridine nucleotide transfer assay.

The shapes of the two grain-count distributions obtained from nucleotide transfer experiments are highly variable and do not correspond to any well known family of curves. A non-parametric test, the Mann-Whitney U-test [210] can be employed to test for

significance between the two distributions (often a simple median test which is less powerful, has been used instead). The problem of comparing the levels of metabolic co- operation in different cell combinations or cell lines as in this study, is more difficult. Two approaches have been used previously. Slack et al [126] used a parameter designated the "grain count index" which is obtained by taking the difference between the median grain count over recipients in contact with donors and the median grain count over isolated recipients in the experimental coculture and expressing the value obtained as a percentage of the corresponding value for a control coculture. This makes it possible to compare the results of independent experiments where absolute grain counts may vary considerably - it is obviously much easier to eliminate uncontrolled variables that may influence the result within an experiment than to do so between experiments. Grain count indices are affected by parameters other than junctional permeability and are therefore suitable only for comparisons of the type AB versus AC where one cell type A is common to both cocultures and the others B and C are closely related (e.g. a variant and its parental wild type). Even in these cases, independent evidence is necessary before one can conclude that differences in grain-count index reflect real differences in the permeability of the junctional

membrane [104].

The second approach to comparing different cell combinations (used by Gaunt and Subak-Sharpe [192]) is to score each combination for the percentage of donor-recipient contacts showing evidence of co-operation. The minimum grain number taken to indicate metabolic cooperation is twice the 90th percentile of the background grain count distribution. All recipient cells displaying more than this number of grains are scored as cooperating. Two lines of evidence that contacts scored as negative are in fact not sites of junctional communication were cited. In low background autoradiographs, mutant cells in contact with heavily labelled wild-type cells often showed complete absence of grains. Secondly, the proportions of positives did not increase on raising the specific activity of the label. This method of scoring is not affected by variation in pool size, but, since an arbitrary choice has to be made regarding the number of grains that are considered as evidence of cooperation, the method is suitable only where the background is low. It gives no information about the extent of transfer.

The uridine nucleotide transfer data accumulated in this study was analysed as described in the legend to Figure 7. In the compilation of the frequency

distibution of signed differences between the grain-counts (Figure 7 part A), co- operating recipients overlaid with more than the maximum of the background number of grains, contribute only to the unshaded part of the distribution. Recipients in contact, but not co- operating, should have exactly the same grain count frequency distribution as the background cells. Their contribution to the frequency distribution of signed differences should therefore be symmetrical about the y-axis. and by subtracting the shaded part of the curve (a and b), the contribution of these cells is eliminated. Each experimental grain count makes an equal contribution to the area under curve A, therefore the ratio of the area under the transformed curve B to that under curve A gives an estimate of the proportion of recipients in contact with the donors which are cooperating. The estimate is unfortunately biassed by the contribution of counts of grains over cells which have co- operated slightly, but which still fall within the range of the background distribution. In order to obtain an unbiassed estimate of the percentage positive contacts, the contribution of these counts should not be subtracted when the distribution is transformed. Gaunt and Subak Sharpe treat these counts as though they were equivalent to background, unless they exceed their (high) cut-off and effectively therefore, subtract most of them. The

analysis used here divides them between the two populations such that experimental counts equivalent to the upper part of the background distribution contribute predominantly towards the part of the curve which is retained after transformation (B), whilst those corresponding to the lower part of the curve contribute significantly to the areas which are subtracted (a and b). In this way, the resultant estimate is less affected by the extent of background labelling.

The estimates of percentage positive contacts thus obtained use far more of the information contained in the grain counts than do the parameters used in other studies and are reproduced well between experiments, at least for cell lines which show a high level of co-operation (Figure 9). Where the level of co- operation is reduced, the standard errors of these values are higher (see Fig. 9). The fact that the frequency distributions of differences are compiled from a set of non-independent points unfortunately makes the determination of confidence limits for single estimates difficult.

The estimates correlate well with values of z, i.e. of the normalised deviates derived from Mann-Whitney U tests [210] carried out to test for significance between the background and experimental counts,

providing the sizes of the two populations remain constant. The z values can also be used as measures of the extent of co- operation. However, z values have no obvious physical significance, and the dependence of the values on the size of sample makes this parameter less attractive as a measure of the extent of junctional communication.

Appendix B

Use of the method of maximum likelihood to estimate the proportion of donor - recipient contacts showing transfer, when transfer may also occur between recipients.

In a population of primary recipients which do not co-operate with each other, a function L(R+,R,p) can be constructed expressing the plausibility of different values of p after the observation of R+ positive recipients in a population of R observations . This function is called a likelihood function (see e.g. [211]). The null hypotheses for the analysis in Chapter 6 is that recipients behave independently of one another, and this can therefore be treated as an assumption of the model without increasing the chances of deciding that they do not. Treating the outcome as the result of R independent trials, in which the probability of co-operation being observed is p, it follows, using elementary probability theory, that the likelihood function should be as follows (see e.g [212]).

$$L = p^{R^+}(1-p)^{R^-}$$

By differentiating L with respect to p, setting the derivative to 0, and solving for p, an estimate p′ of p, known as the Maximum likelihood estimate is obtained. In this trivial case, this estimate is exactly equal to the ratio R^+/R.

If a population of pairs of primary recipients is considered in which each cell is in contact with one and only one other primary recipient, namely the other member of the pair, a likelihood function can be constructed by taking the product of three simple functions (f0,f1,and f2 below) corresponding to each of the possible outcomes of scoring a pair for labelling. i.e.

$$f0 = (1-p)^{R0}$$

corresponding to no recipients labelled

$$f1 = [2(1-p)p(1-a)]^{R1}$$

corresponding to one recipient labelled

$$f2 = [2(1-p)pa + p^2]^{R2}$$

corresponding to both recipients labelled

where: R0,R1,and R2 are the observed frequencies of the corresponding outcome and where a is the proportion of recipient pairs where co-operation occurs between the two recipients.

The parameter a can be determined with a high level of confidence by scoring a large number of secondary

recipients in contact with only one primary recipient as positive or negative on the same basis as the primary recipients. The fraction of positives can be used as an estimate of a (the variance of this estimate made only a very small contribution to the variance of the estimate of p´ and was therefore neglected in this study).

p´ is thus estimated by maximising the function L with respect to p as before. Similar likelihood functions are constructed for the different topologies of positive and negative primary recipients observed, and the estimates of p are made by maximising the likelihood functions corresponding to the observed populations of results by equating the derivatives to zero and solving by Newton´s method using a microcomputer.

It was not considered necessary to use this complex analysis other than for the data discussed in Chapter 6, as only in this work were small differences in levels of co-operation under investigation, which might in principle correlate with with the geometry of the ´constellations´.

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Declaration

This thesis has been composed by myself. With the exception of the help and guidance mentioned in the acknowledgements it is entirely my own work.

Publications

Smith T.A., and Hooper M.L. (1983). Medium conditioned by feeder cells inhibits the differentiation of embryonal carcinoma cultures. Exp. Cell Res. 145, 458-461.

SHORT NOTE

Medium Conditioned by Feeder Cells Inhibits the Differentiation of Embryonal Carcinoma Cultures

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Summary. Non-dividing STO mouse fibroblasts have been used for some time as feeder cells for maintaining certain embryonal carcinoma cells in an undifferentiated state. We report here that medium conditioned by these feeders can inhibit embryonal carcinoma (ec) cell differentiation induced either by removal from feeders, or, in the case of cells not normally requiring a feeder layer, by retinoic acid treatment.

The differentiation of mouse embryonal carcinoma (ec) cells into a variety of cell types in vitro has been extensively studied as a model of early mammalian embryogenesis [1], and a number of substances capable of stimulating particular types of differentiation have been discovered. These include retinoic acid [2], hexamethylene *bis*acetamide [3], *N*,*N*,dimethylacetamide [4], Polybrene [4] and DMSO [5]. In fact the maintenance of embryonic ectoderm cells (thought to be analagous to ec cells) in an undifferentiated state in vitro has proved problematic [6–7], suggesting that the provision of stimuli for differentiation by the culture environment may be the norm rather than the exception. The question of how these stimuli act can be appropriately re-cast as one of how at least some of the ec cell population can be protected from premature commitment to a differentiated lineage. We use here the term commitment to mean the process by which a cell becomes programmed to differentiate, without necessarily any immediate phenotype manifestation.

Feeder layers of non-dividing STO mouse embryo fibroblasts have been used routinely for some time for the maintenance of cultures of certain ec lines (here termed feeder-dependent) in an undifferentiated state [8]. Other cell lines (here termed feeder-independent), with a more restricted capacity for in vitro differentiation, can be grown on gelatine substrates [9]. Many of the latter lines will differentiate into endoderm-like cells producing plasminogen activator when treated with retinoic acid [2].

Ogiso et al. [10] have reported that in the ec line 311 the effect of retinoic acid can be inhibited by growth on BALB-3T3/A31 feeder layers. We here report that STO feeder layers can also inhibit retinoic acid-induced differentiation, and further that such inhibition can also be produced by medium conditioned by non-dividing STO cells. Furthermore, we find that the differentiation of a feeder-dependent line can be inhibited by growing the cells in STO feeder-conditioned medium.

We monitored the retinoic acid-induced differentiation of the feeder-independent line PC13TG8 [11] by trypsinizing treated cultures and seeding known numbers of cells at cloning densities. Counts of the total cells present on trypsinization and of the ec colonies growing up subsequently (identified by their failure to

produce plasminogen activator) were used in conjunction to estimate numbers of ec cells and of committed cells present in the original culture (fig. 1). The estimated numbers of ec cells present were reduced to 10% of control levels by treatment with 10^{-6} M retinoic acid. Intermediate numbers of ec cells were identified on trypsinization of cultures treated with retinoic acid in conditioned medium or on STO feeder layers. The effect of the conditioned medium operated irrespective of whether the retinoic acid was added to the medium before or after incubation with the feeder cells (the differences observed between the effects of these two treatments are not statistically significant). The extent of morphological change in the cultures broadly corresponded to the estimated proportion of committed cells.

Neither retinoic acid, nor conditioned medium greatly influenced the total cell number on trypsinization, although differentiating cells generally failed to clone and few plasminogen activator-positive colonies were counted.

Feeder-dependent ec cells differentiate spontaneously into a wide variety of cell types when grown in the absence of feeders [12]. The methodology used for PC13TG8 therefore cannot be used to monitor their state of commitment. Alkaline phosphatase (APase) activity, which is characteristically high in ec cultures [9], was therefore followed as a marker of undifferentiated cells in cultures derived from the feeder-dependent line PSA4TG12 [13] (fig. 2). The specific activity of alkaline phosphatase in cultures of PSA4TG12 grown on gelatine in conditioned medium increased with culture age, as has been found in pure cultures of feeder-independent ec cells (Bernstine, E G & Hooper, M L, unpublished result), and in contrast to cultures in control medium. When the medium was not conditioned, numerous differentiated cell types, largely of epithelial morphology, appeared (fig. 3*a*). This morphology contrasted with that of the almost pure ec cultures observed growing in conditioned medium (fig. 3*b*). Few surviving feeder cells were observed in these cultures.

The cloning efficiency of ec cells is increased by conditioning the medium with non-dividing mouse fibroblasts [14], and consequently, the mass of protein extractable from cultures plated on gelatine is greater when the cells are seeded out in conditioned medium. We are confident, however, that the effect of conditioned medium on PSA4TG12 is not solely a consequence of increased cell density, as morphological differentiation can be inhibited by conditioned medium even when cells are plated very sparsely (10^3 cells/60 mm dish). Our methodology excludes effects on plating efficiency as explanations of our observations on PC13TG8.

Martin & Evans [15] reported a failure to reproduce the feeder effect by feeder-conditioned medium. Their work was carried out in the absence of beta-mercaptoethanol, which has since been shown to enhance growth of ec cells [16] and which is routinely added to our medium. Our results show that some at least of the effects of the feeders are transmitted through the medium. Moreover, we also find that R5/3, a PC13TG8 derivative defective in the formation of functional gap junctions [17], maintains the same morphology on feeders as its parent, both in the presence and absence of 10^{-6} M retinoic acid. We can therefore find no evidence for any junction-dependent, or even contact-dependent effect of fibroblasts on ec cells.

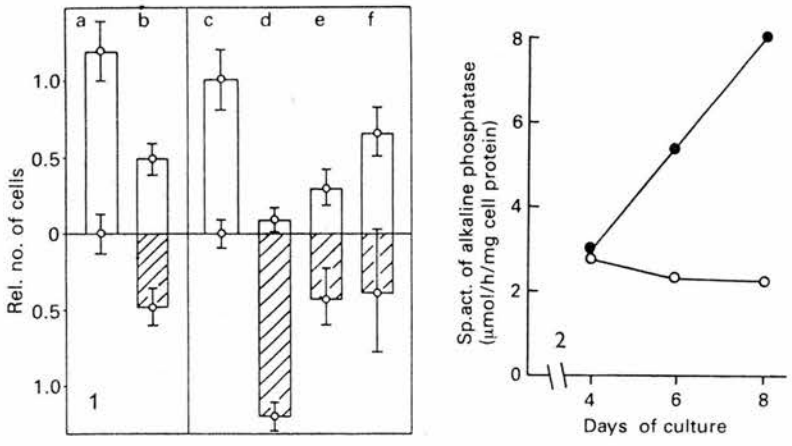

Fig. 1. Effect of STO feeder cells and STO cell-conditioned medium on retinoic acid-induced differentiation of PC13TG8 embryonal carcinoma cells.

Cells were grown in Glasgow-modified Eagle medium supplemented with non-essential amino acids (0.1 mM each), 1 mM sodium pyruvate, 10% fetal or newborn calf serum (selected batches), and 0.1 mM beta-mercaptoethanol [16] in a 95% air/5% carbon dioxide atmosphere at 37°C and were checked periodically for mycoplasma contamination, using the method of Chen [18]. No such contamination was detected during this study. Stocks of PC13TG8 [11] were grown on plastic surfaces precoated with gelatine [9].

Feeder layers were prepared from the STO cell line by the method of Martin & Evans [8]. Conditioned medium was made by incubating 10 ml of medium for 24 h on a feeder layer in a 25 cm^2 flask. After conditioning, the medium was filtered through a Millipore membrane (pore size 0.2–0.22 μm). Control, unmodified medium was stored for 24 h at 37°C prior to use. Filtration of this medium did not significantly alter the results.

PC13TG8 cells were trypsinized and inoculated into 60-mm tissue culture dishes (10^5 cells/dish) either on gelatine-coated surfaces or on STO feeders as indicated below. Cultures were incubated for 24 h, and then treated with the medium under test for a further 24 h. They were then returned to unmodified medium for a further 72 h, trypsinized, counted with a Coulter counter, and reseeded into duplicate gelatinized 60-mm dishes (10^3 cells/dish). The resulting colonies were grown up for a further week and assayed for plasminogen activator.

A modification of the fibrin lysis method of Strickland & Mahdavi [2] was used to assay colonies for enzyme activity. Cultures were washed twice with phosphate-buffered saline and overlaid with 1.5 ml of serum-free medium containing 3.3 mg/ml bovine fibrinogen (plasminogen- and thrombin-free, Calbiochem), 0.4 units/ml human plasminogen (a gift from Dr I. MacGregor of the Scottish National Blood Transfusion Service (SNBTS), Edinburgh; this was freed of low molecular weight material by gel filtration on Sephadex G25M prior to use), and 0.67 units/ml bovine thrombin (Sigma). Plasminogen was omitted from control dishes. After the formation of a fibrin gel, dishes were incubated at 37°C for 4 h. The preparations were then fixed by overlaying the gel with 5 ml methanol, stained with Kenacid blue R (BDH, 2 mg/ml in methanol : glacial acetic acid : water, 45 : 10 : 45 v/v), and examined for the presence of a lysis zone around each colony. For routine assays a crude human fibrinogen preparation containing endogenous plasminogen (provided by Dr D. Pepper, SNBTS, Edinburgh) was used. The gels were stained with Leishman's stain. Controls established that both retinoic acid treated and untreated cultures gave the same results with the two preparations.

Colonies derived from control cultures of PC13TG8 not treated with retinoic acid were uniformly plasminogen activator-negative. The number of such colonies were therefore used to estimate the cloning efficiency of ec cells. The proportion (p) of ec cells in the treated population was therefore estimated as c^-/C, where c^- is the number of plasminogen activator-negative colonies derived from treated cultures and C is the mean number of colonies derived from corresponding untreated cultures.

The Coulter counts were used to estimate the total cell number (N) on trypsinisation, and thus the number of ec cells (Np) and committed cells ($N(1\text{-}p)$).

Within each experiment the numbers for each of the two replicate dishes were expressed as a fraction of the mean number of ec cells recorded in column c. The relative number of cells was then defined as the mean value of this fraction determined from five experiments. Error bars indicate 1 SE above and below this mean. Open columns, ec cells; hatched columns; committed cells.

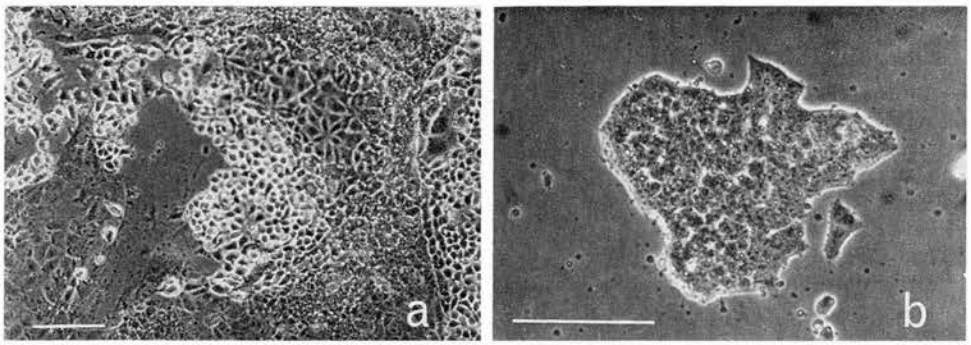

Fig. 3. Phase contrast photomicrographs of 7-day-old cultures of PSA4TG12 on gelatine. (*a*) Unmodified; (*b*) conditioned medium. Method as fig. 2. Bar, 200 μm.

The observation, that retinoic acid-induced differentiation can be inhibited both by conditioning medium before the retinoic acid is added and by adding the retinoic acid initially (fig. 1), implies that binding of retinoids by STO cells cannot explain the effect. However, we cannot determine from these data whether an inhibitor is being secreted or a critical medium component is being taken up by the STO cells. We conclude that feeder-conditioned medium can inhibit the differentiation of ec cells as induced by two different stimuli. The questions of whether a single mechanism underlies the action of both stimuli, and of how the inhibition operates, remain unanswered.

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Growth conditions: (*a*) STO feeders, unmodified medium; (*b*) STO feeders, medium containing 10^{-6} M retinoic acid; (*c*) gelatine, unmodified medium; (*d*) gelatine, medium containing 10^{-6} M retinoic acid; (*e*) gelatine, medium conditioned, 10^{-6} M retinoic acid added prior to conditioning by STO cells; (*f*) gelatine, medium conditioned, 10^{-6} M retinoic acid added to medium after conditioning by STO cells, but before addition to PC13TG8 cells.

Fig. 2. Effect of conditioned medium on the sp. act. of alkaline phosphatase in PSA4TG12 cultures. Stocks of PSA4TG12 ec cells were maintained on STO feeder layers (culture conditions were otherwise as per caption to fig. 1). After passaging once on gelatine in conditioned medium to remove residual feeder cells, 10^5 cells were inoculated into each 60-mm gelatinized tissue culture dish in the medium under test (day 0). This medium was replaced with fresh medium of the same type after 4 days and again on the 7th day after inoculation. Cells were then harvested, extracted and assayed for APase as described by Bernstine et al. [9] except that protein was determined by a standard dye-binding assay (BioRad) based on the colour change of Coomassie Brilliant Blue G-250 in response to protein concentration [19]. Culture grown in ●, conditioned; ○, unmodified medium; means of two determinations.

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