The isolation and properties 
of a clonal tissue culture strain of pluripotent 
mouse teratoma cells

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SUMMARY

A clonal tissue culture strain of pluripotent cells has been isolated from a transplantable teratoma of inbred strain of mice 129 Sv-Sl CP. This cell strain SIKR when re-inoculated into mice produces teratomas containing at least ten types of tissue. Sub-clones have been isolated and two types distinguished.

(1) 'C-type' with a densely-piled in vitro growth. These are tumourigenetic and pluripotent displaying a comparable range of differentiation to the original SIKR.

(2) 'E-type' spreading, often epithelioid growth. These grow to a lower density in culture than 'C-type'. Mostly non-tumourigenetic; in those cases where a tumour has been obtained it did not display multiple differentiations.

The results are interpreted as demonstrating that the culture consists of equivalently pluripotent cells which may become determined and differentiate spontaneously in vitro into slower growing cell types which are continuously overgrown by the culture.

INTRODUCTION

The ontological history of a differentiated cell may be traced back in development to a point of origin common with other cell types. During embryogenesis groups of cells from any particular cell lineage become restricted in their possible terminal differentiation; and this restriction typically precedes their overt differentiation – although it may be accompanied by ultrastructural changes and is presumably associated with changes at the molecular level. This restriction of a cell's possible fates is referred to as determination. It may be difficult to distinguish this condition from a state of differentiation or proto-differentiation, particularly at a molecular level, but it has an important functional significance for it is at the point when a cell becomes determined that a choice is made between two or more pathways of differentiation.

For an understanding of the molecular basis of the control of differentiation it is this point of choice which must be examined. In all cases this choice occurs in a small mass of tissue in the embryo. Wolpert (1969) has stressed and Crick

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(1970) has calculated the strong theoretical grounds for believing that this is a
general situation. The possibility, therefore, of obtaining a strain of cells in
tissue culture which may become determined to differentiate in a variety of
alternative ways is very attractive. In tissue culture it is possible to grow cells in
relatively large numbers in a controlled environment and they may be subjected
readily to various biological and biochemical manipulations. This paper describes
the isolation and some properties of a clonal strain of such cells from a mouse
teratocarcinoma.

Teratocarcinomas are malignant tumours which contain a diversity of tissue
types with, typically, contributions from all three primary germinal layers
(Willis, 1958). Stevens & Little (1954) developed a strain of 129 mice with a
high spontaneous occurrence of testicular teratomas and Stevens has also
described methods for their experimental induction (Stevens, 1964, 1967,
1968 and 1970). Transplantable lines of tumours have been developed from both
the spontaneous and the induced teratomas, and they may also be converted to
an ascites form in which small spheres of cells resembling early embryos are
found. Kleinsmith & Pierce (1964) were able to demonstrate by in vivo cloning
of cells from these embryoid bodies that the diverse tissues present in these
tumours arose from a single stem line. These pluripotent stem cells may also be
grown in tissue culture. Kahan & Ephrussi (1970) and Rosenthal, Wishnow &
Sato (1970) have reported the isolation of such cells from different teratomas as
clonal lines and in so doing have confirmed the single cell origin of the dif-
ferentiated cell types seen in the in vivo tumours and demonstrated the feasibility
of culturing these cells. Both groups derived the cells from ascites forms of the
tumours which were transplantable lines developed from spontaneous testicular
teratomas. Reported here is the isolation of pluripotent stem cells from a solid
teratocarcinoma derived from the implantation of an early embryo into the adult
testis.

MATERIALS AND METHODS

The mice used were strain 129/Sv-SlCP which were kindly provided in
January 1969 by Dr Leroy Stevens of the Jackson Laboratory, Bar Harbor,
Maine, USA, and which have since been kept as a strictly inbred stock. Two
subcutaneously transplantable tumours were received from Dr Stevens in May
1969 – OTT 5938 and OTT 5568; these were both tumours derived from tera-
tomases induced by the implantation of a 3-day embryo into an adult testis.
Stevens (1970) has described their origin. The tumours were maintained by solid
subcutaneous transplantation for seven transplant generations; the more slowly
growing tumours were chosen as donors. These sub-lines were designated
5938S and 5568S respectively. The latter line gave rise to the cell cultures
described here.
Culture of pluripotent mouse teratoma cells

Primary culture

The tumour was dissected out and finely chopped with scissors in Medium 199 with Hanks's salts (Flow Laboratories), after washing by decantation a solution of collagenase at 1 mg/ml (Sigma crude grade, freshly made solution in Medium 199, Millipore filter sterilized) was added to the tissue and left at room temperature. After 30 min the supernatant solution was replaced with fresh 199 and vigorous vortex mixing provided the first cell extract. A further 15 min digestion of the remaining tissue in fresh collagenase solution provided the second extract. The cells were counted in a haemocytometer and plated at 2 x 10^7 cells per 10 cm Falcon plastic tissue culture Petri dish. Dulbecco modified Eagles medium supplemented with 10 % calf serum (carefully selected) was used throughout and the cultures incubated at 37 °C in a 10 % CO_2 in air, humidified atmosphere. 0.25 % trypsin solution in Tris-buffered saline EDTA (0.025 M Tris (hydroxy-methyl) methylamine; 0.12 M-NaCl; 0.005 M-KCl; 0.0055 M glucose; 0.0024 M-KH_2PO_4; 0.00084 M- Na_2HPO_4; 0.0001 M EDTA pH 7.4) was used to dis-aggregate the cultures for passage.

Cloning

Cloning was performed on an X-irradiated chick fibroblast feeder layer. Chick fibroblasts were prepared from virus-free Brown Leghorn 9-day-old chick embryos (eggs supplied by Poultry Research Institute, Edinburgh) and irradiated with 6000 r (approx. 1.56 C/kg air) X-rays at 43 kV. 5 x 10^5 cells were seeded in each 5 cm Falcon Petri dish in 5 ml culture medium and incubated for one day before the teratoma cells were added. A single cell suspension (contains < 1 % pairs of cells) of 10^4 or less teratoma cells was added to the pre-incubated dish and a cloning efficiency of up to 1 % was obtained. The colonies were picked from the plate with a fine drawn Pasteur pipette after a very light trypsinization and mechanically dispersed on to further X-irradiated feeder layers. For subcloning when the plating efficiency of the cells was higher the colony was surrounded with a glass ring and trypsinized for transfer to new feeder layers. When more than 10^6 teratoma cells were available the use of a feeder layer was discontinued. At first the cells did not grow well at low density and at least 10^6 cells were inoculated into each 5 cm Petri dish.

Inoculation of mice

The cells were disaggregated and re-suspended in culture medium. One to five million cells were injected in a volume of 0.1-0.2 ml subcutaneously into the right flank of a mouse.

Organ culture

The methods described by Wolff & Wolff (1966) of organ culture of tumour cells were followed. The teratoma cells were pelleted by centrifugation after harvest from tissue culture, and a portion of the cell pellet used for organ culture. With chick mesonephros as a feeder a ‘double membrane’ technique was used;
the tumour cells were separated from the mesonephros by a sheet of vitelline membrane from an unincubated chick egg. Cultures were also prepared in the absence of any heterologous tissue on a medium in which Ham's F12 nutrient solution replaced the embryo extract in Wolff & Wolff's medium. In this case the tumour cells were wrapped in a single fold of vitelline membrane.

Histology

The tumours were excised when they were 1–2 cm in diameter and portions were fixed in Bouin's fixative. Paraffin sections were prepared and stained in a variety of ways. Particularly useful was a combination of Alcian blue at pH 2.5 followed by a Masson's trichrome stain. This method has the advantage of staining the cartilage matrix blue and the hypertrophied basement membrane material laid down as a matrix by the parietal yolk sac cells green, thus providing a clear distinction between these two extracellular eosinophilic matrix materials. Tissue types were identified according to the descriptions of Stevens & Hummel (1957) and in accord with those described by Kahan & Ephrussi (1970) and Rosenthal et al. (1970). The percentage area of the histological sections occupied by each tissue was estimated by random sampling. A ruled eyepiece graticule provided a projection on the slide at the magnification used (×400) of a rectangle 100 μm × 110 μm. This was repeatedly placed at random over the sections of the teratoma and the tissue types observed within it recorded. Areas of necrosis (which are not at all extensive in these tumours) and areas immediately adjacent to the surface of the tumour were not scored. Approximately 500 observations were made, distributed over the serial sections of a single tumour. The results were calculated as a percentage of the total of the observed tissue types.

Karyotype analysis

After 4–6 h treatment of a culture with colchicine the cells in mitosis were harvested by a light trypsinization, swollen in hypotonic saline, fixed in 3:1 methanol:acetic acid and spread on an ice cold slide. The chromosomes were stained with aceto-orcein and the metaphase figures examined. A histogram was made of the number of chromosomes observed per cell.

RESULTS

In primary cultures a great diversity of cell morphology was observed. The cells tended to aggregate into clumps no matter how carefully they were originally dispersed, and fibroblast-like and epithelial cells were found together with contractile striated muscle fibres and neurone-like cells with long processes. From some primary cultures the derived mass cell cultures grew only slowly and produced only fibroblast-like or epithelial-like cells which grew to a low saturation density. Others were obtained, however, which grew rapidly and had islands of small cells which piled up in multilayered colonies. On cloning such a culture,
colonies of these piled-up cells developed and later proved to be the pluripotent stem cells.

Nineteen such clones were isolated, 12 failed to grow progressively *in vitro*, one was lost through infection, six were re-injected and of these four gave tumours. From these one was selected (OTT 5568S/1/KR subsequently termed SIKR) which grew best *in vitro* and displayed good *in vivo* differentiation. The cells were first tested by re-inoculation after approximately 25 cell generations *in vitro*. The tumours formed consisted mainly of differentiated tissues together with small areas of embryonal carcinoma (Fig. 1). The more commonly occurring types of tissue are listed in Table 2. At this stage they grew only moderately well in culture, they spread poorly and formed tightly packed cell masses.

The karyotype was examined after approximately 30 cell generations *in vitro* and found to be near diploid with a modal chromosome number of 41. A small number of tetraploid cells were found (Fig. 2). Further examination of the cells after they had become more adapted to tissue culture (see below) produced the same result.

A quantitative approach to the analysis of the histology of these tumours is being made and will be reported in detail elsewhere. Tumours develop reliably
Fig. 2. Frequency of observation of various numbers of chromosomes in arrested mitotic figures of SIKR cells.

Table 1. The % composition of 5 tumours of SIKR

<table>
<thead>
<tr>
<th></th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesenchyme</td>
<td>15-6</td>
<td>27-4</td>
<td>28-2</td>
<td>27-8</td>
<td>22-7</td>
</tr>
<tr>
<td>Neural</td>
<td>53</td>
<td>38-8</td>
<td>30-2</td>
<td>13-7</td>
<td>4-7</td>
</tr>
<tr>
<td>Epithelial</td>
<td>5-3</td>
<td>4-8</td>
<td>7-5</td>
<td>5-0</td>
<td>7-2</td>
</tr>
<tr>
<td>Keratinizing epithelium</td>
<td>0-6</td>
<td>3-0</td>
<td>1-1</td>
<td>2-4</td>
<td>1-8</td>
</tr>
<tr>
<td>Muscle</td>
<td>2-2</td>
<td>1-9</td>
<td>8-9</td>
<td>13-7</td>
<td>21-8</td>
</tr>
<tr>
<td>Cartilage</td>
<td>1-4</td>
<td>7-8</td>
<td>11-0</td>
<td>18-5</td>
<td>10-0</td>
</tr>
<tr>
<td>Embryonic yolk sac</td>
<td>3-5</td>
<td>0-4</td>
<td>1-5</td>
<td>0-3</td>
<td>0-9</td>
</tr>
<tr>
<td>Embryonal carcinoma</td>
<td>10</td>
<td>10-7</td>
<td>6-7</td>
<td>11-6</td>
<td>13-6</td>
</tr>
<tr>
<td>Other</td>
<td>8-1</td>
<td>5-2</td>
<td>4-5</td>
<td>6-9</td>
<td>17-3</td>
</tr>
</tbody>
</table>

Probability that differences between b, c, d, e are chance

\[
\begin{align*}
\text{Mesenchyme} & \Rightarrow 0-8 \\
\text{Neural} & < 0-001 \\
\text{Epithelial} & > 0-3 \\
\text{Keratinizing epithelium} & > 0-2 \\
\text{Muscle} & < 0-001 \\
\text{Cartilage} & < 0-001 \\
\text{Embryonic yolk sac} & < 0-05 \\
\text{Embryonal carcinoma} & < 0-001 \\
\text{Other} & < 0-001 \\
\text{Total} & < 0-001
\end{align*}
\]

Tumours b, c, d and e were formed from a parallel inoculation of a common cell suspension. The composition of the tumours was found by observing the number of times each type of tissue was found in a randomly placed grid square of 100 × 110 μm. The observed number of times each tissue occurred was compared with the number expected on a null hypothesis that the four distributions (b, c, d, e) are drawn from a common population and χ² test applied. The value of χ² was calculated and the associated probability of the null hypothesis is given above for each type of tissue. The probability that the distribution of frequency of occurrence of different types of tissue in tumours b, c, d and e is the same is < 0-001.
after a cell inoculation of $5 \times 10^5$ or above. The several tumours produced from a common cell inoculum may display varying quantities of different tissue types and the reasons for this are not clear. Table 1 lists the percentage area covered by each type of tissue in five typical tumours of SIKR; $b$, $c$, $d$ and $e$ are from a common cell inoculum. A tumour may, however, be described by the appearance of particular differentiations within it and this specifies a minimum pluripotency of the cells injected. Table 2 lists the occurrence of various types of tissue in tumours of SIKR and of ten sub-clones derived from it. Tissues which cover an area of less than 1% of the histological sections are readily overlooked and hence the presence of any particular type of tissue type is more significant than its absence in these tabulations. More SIKR tumours have been examined than those of the other clones listed and some other, rarely occurring, tissues such as bone and hair follicles have been observed.

After approximately 30 cell generations of growth in vitro sub-clones of SIKR were prepared. Clones of at least two distinct morphologies were recovered either (i) ‘C-type’ similar to the original growth pattern, small densely packed cells forming piled-up colonies (Fig. 3A) or (ii) ‘E-type’ – a more contact-inhibited cell monolayer of epithelial-like cells (Fig. 3B). C-type clones (and the parent SIKR) grow to a population density of about $5 \times 10^5$ cells per square centimeter whereas E-type clones produce a maximum population of between $2.3 \times 10^4$ and $2 \times 10^5$ depending upon the clone. The proportion of C-type to E-type clones recovered was not constant from experiment to experiment and there is some evidence that it is affected by the environment of the cloning. As an indication, however, in one experiment it was found that from 100 cells, ten C-type and two E-type clones were recovered.

Clonal populations of both C-type and E-type morphology were re-injected into mice. Tumours were produced only from C-type cultures although not all
of these produced tumours. All the tumours contained a variety of differentiations (Table 2). This demonstrates the pluripotency of the original cell of each clone. The E-type clones appear to be non-tumourigenetic or at least to have a considerably reduced tumourigenicity. This precludes testing their potentiality by the production of an in vivo tumour. These clones could be either non-tumourigenetic but still pluripotent or they might represent determined cell lines which have lost their tumourigenicity together with their pluripotency.

One E-type clone, SIKR BM1, was chosen for further study. The cells could grow on repeated feeding up to a density of $2 \times 10^5$ cells/sq. cm. Their morphology was clearly different from the original culture or from a C-type clone but they were not so extremely epitheloid and contact inhibited as the cells of some of the E-type clones recovered. Their karyotype was diploid. It was considered that a tumour might be produced by a massive inoculation, but inoculation of $1.6 \times 10^7$ cells of SIKR BM1 gave no tumour. Another approach to the problem of testing their potentiality is to culture the cells in conditions where they may grow organotypically. SIKR BM1 was grown in organ culture following the method of Wolff & Wolff (1966). A single tissue composed of epithelial

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Fig. 3A. A typical C-type colony of teratoma cells in vitro; phase contrast. $\times 125$.

Fig. 3B. An area from a clone of E-type teratoma cells in vitro; phase contrast. $\times 125$. 
vesicles was formed, Fig. 4. This has been observed in a number of cultures both using chick mesonephros in double membrane configuration and without any other tissue and it would seem unlikely that it represents either a mono-specific induction or a chance single differentiation of an essentially pluripotent stem cell. Parallel organ cultures of C-type sub-clones and of SIKR were largely necrotic. Occasional recognizable patches of mesenchyme, embryonal yolk sac, epithelium, neural tissue and keratinizing epithelium have been seen in various cultures but multiply, well-differentiated cultures were not obtained.

At the time when $10^8$ cells of the original SIKR clone had been obtained these were stored frozen. After thawing one ampoule of $10^6$ cells and passaging through another ten generations to provide a large working stock it was found that the cells were growing better in tissue culture than previously and would now grow with small inocula (1000 cells per 5 cm Petri dish). The cells grew more as a monolayer although, if the culture was allowed to become dense, they still piled up into multilayered masses. A cloning efficiency of up to 25% was achieved without the use of either pre-conditioned media or X-irradiated feeder layers. The karyotype remained the same at a modal near-diploid number of 41, and the range of differentiations produced upon re-injection was not diminished. With this much more vigorous growth in vitro it was thought possible that
Fig. 5 A I & II. Microphotograph of a histological section of a tumour produced after injection of an E-type clone which was isolated after prolonged in vitro culture of the SIKR cells. × 50 and × 500.

Fig. 5 B I & II. Microphotograph of a histological section of a tumour produced by an E-type clone. × 50 and × 500.
derived E-type clones might prove to grow sufficiently in vivo to produce a tumour, in other words that they might represent in vitro transformed cell strains. This proved to be the case and two E-type sub-clones have been isolated which have produced in vivo tumours. In both cases the tumours contained a single type of tissue and did not display multiple differentiations (Fig. 5A, I). A C-type clone isolated in parallel with these as a control was pluripotent. Additionally, one other E-type clone has been isolated which has produced an in vivo tumour; this also consists of a single type of tissue Fig. 5B. C-type clones isolated in parallel with this clone displayed the full range of differentiations, Fig. 6.

DISCUSSION

The main purpose of this paper is to report the isolation of a clonal tissue-culture strain of near-diploid pluripotent cells from a mouse teratocarcinoma. Cells of this strain, SIKR, have a high cloning efficiency, grow well in vitro and produce in vivo tumours containing a multiplicity of differentiations. The development of lines of pluripotent cells which will grow readily in vitro opens the possibility of using them to study the processes of cellular determination. Similar lines have previously been isolated by Kahan & Ephrussi (1970) and by
Rosenthal et al. (1970); these were isolated from ascites forms of passaged spontaneous testicular teratoma whereas the line reported here was isolated from the solid form of an embryo-derived tumour.

The histology of the tumours of SIKR shows that the progeny of the cells grown in vitro are able to differentiate into at least ten types of tissue. As tissues such as epithelia or neural tissue contain a number of types of cell, the total number of cell types into which a SIKR cell may develop is in excess of this. Most of the cultures of cells obtained after sub-cloning which produced tumours in mice, produced teratocarcinomas; demonstrating that the original cell of the sub-clone was pluripotent. Although there are differences which may be observed between the tumours produced by the various sub-clones (summarized in Table 2) they are probably not very significant, for only a single tumour of each sub-clone has been critically examined. It may be assumed that the original cells were more or less equivalent in their capabilities for differentiation; a considerably more extensive investigation would be required to unequivocally demonstrate differences between them. Kahan & Ephrussi (1970) considered that the differences which they observed in the differentiation of their various sub-clones were of degree of differentiative ability rather than selective restrictions in pluripotency. Such an explanation is not incompatible with the present results.

Numbers of sub-clones were found, however, which did not produce tumours after re-injection into mice. These non-tumourigenetic clones differed characteristically in their in vitro appearance, growing more as a monolayer than the original cells which tended to grow in piled colonies. Some of these E-type clones are epithelioid whereas others are more fibroblastic in appearance; the class 'E-type clone' probably includes cells of several diverse types but as in vitro cell morphology is a very misleading criterion for classification they have been classed together. Further study of the cells' ultrastructural appearance and of their enzymic constitution is being undertaken as an aid to their identification. Three E-type sub-clones have been obtained which have produced a tumour in vivo and in each case the resulting tumour was found to consist of a homogeneous tissue. The cells giving rise to the tumour were not therefore pluripotent and probably represent cells of a determined type. There is, however, one difficulty of interpretation which, although it is a general difficulty of the in vivo assay of the pluripotency of teratoma cells, is particularly relevant here. A large cell inoculum is required to produce a tumour and it is not known how great a selection of the cells may take place during in vivo growth. Harris (1971) has shown large karyotypic alterations during tumourigenesis from a population of cultured hybrid cells which indicates that a severe selection has taken place. The karyotype of the E-type clones which produced tumours was not followed in these experiments but as, in general, the E-type clones have proved to be non-tumourigenetic the production of these tumours may have involved a selection of an atypical cell from the population injected.
One very interesting feature of teratocarcinomas in vivo is the transformation of cells from malignant to benign, concomitant with their differentiation; this is the converse of the more usually observed cell transformation to malignancy. Pierce (1967) discusses the possible clinical implications of this and Kauffman (1971) has considered the theoretical aspects of malignancy as an epigenetic state. It is of interest that this transformation may also take place in vitro; from a clonal population of tumourigenetic cells sub-clones have been isolated which are non-tumourigenetic. This opens the possibility of further study of this aspect of the teratoma cell's differentiation in vitro which could provide useful information about the reversal of the malignant transformation. The properties of tumourigenicity and pluripotency may be linked as has been suggested for in vivo teratomas where a tumour which fails to grow progressively is found to contain only fully differentiated tissues (Stevens, 1967). Pierce, Dixon & Verney (1960) demonstrated that large embryoid bodies which contained no embryonal carcinoma did not form progressively growing tumours and concluded that the differentiated tissues were non-malignant. In this study, however, some non-pluripotent sub-clones have been found which are tumourigenic if a large cell inoculum is used. It has not been possible to determine whether non-tumourigenetic clones may be pluripotent. This would depend upon some other assay of pluripotency and the only technique available to date – organ culture – has not proved satisfactory for most clones (see below). If the properties of pluripotency and tumourigenicity prove to be reasonably closely associated loss of tumourigenicity may prove to be a useful assay of cell determination.

As differentiation of these cells may be coupled with a loss in tumourigenicity it would be useful to have a system of assay for the cells' range of differentiation not dependent upon their tumourigenicity. It was hoped that a full range of differentiation would be observed after culture in an organotypic environment. The results to date have been disappointing in that most clones have grown poorly or become largely necrotic in the conditions used here. The pluripotent SIKR and other pluripotent cultures have shown occasional patches of histologically recognizable mesenchyme, epithelium, neural tissue and keratinizing epithelium, but these have not been routinely repeatable and the cultures are usually necrotic. SIKR BM1 which was chosen as an example of an E-type clone has produced a series of epithelial tubules in organ culture and this lends support to the idea that this clone may represent a determined cell type. It may be that similar results have not been obtained with the other E-type clones tested because either SIKR BM1 is well suited to organ culture (for instance, its contact inhibition of growth may prevent overgrowth and necrosis) or its specific type of differentiation is favoured under these conditions. The results from organ culture can only be interpreted with caution until a more reproducible method and one which can reliably display the pluripotency of known pluripotent clones can be developed.

The available evidence suggests however, that these cells are able to lose their
pluripotency and become determined during growth in tissue culture although under these conditions the majority of the population maintains its pluripotency over many cell generations and the range of differentiation shown by all clones of pluripotent cells is similar. This suggests that there is a continuous slow spontaneous production of determined cells in the culture which are constantly being overgrown by the stem cells. It would be very useful if this process could be experimentally manipulated. Cell lines of this type should prove extremely useful in studies of the control of cellular determination and differentiation.

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