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Changes in protein synthesis during differentiation of embryonal carcinoma cells, and a comparison with embryo cells

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SUMMARY

Two-dimensional electrophoresis was used to find changes in protein synthesis occurring as pluripotent embryonal carcinoma (EC) cells differentiate to give embryoid bodies *in vitro*. 2-D patterns from other embryonic cell lines, and from the inner cell mass (ICM) cells of mouse embryos, were also analysed for the expression of those proteins showing some change during embryoid body formation and for overall differences between these and the EC cells.

Most changes in protein synthesis occurred before 12 h but endoderm was not discerned morphologically on the outside of EC cell clumps until at least 18 h after their suspension. The number of changes occurring is small compared with the number of polypeptides resolved, but is in line with similar studies. Comparisons with nullipotent EC cells and an endodermal cell line have allowed these changes to be assigned, tentatively, to the different cell types within embryoid bodies, and may allow them to be used as markers of differentiation.

Comparisons between the 2-D patterns derived from ICMs and EC cells reveal substantial differences between the two that might not have been expected from their developmental homology. The importance of these differences to their pluripotentiality is discussed.

INTRODUCTION

The pluripotent stem cells of teratocarcinomas, termed embryonal carcinoma (EC) cells, are capable of participating in normal embryogenesis. This is exemplified by their contributions to chimaeric mice formed by the injection of EC cells into blastocysts (Brinster, 1974; Papaioannou, McBurney, Gardner & Evans, 1975; Mintz & Illmensee, 1975; Papaioannou, Gardner, McBurney, Babinet & Evans, 1978; Cronmiller & Mintz, 1978). Also, although prolonged differentiation of EC cells in culture or in ectopic sites is somewhat chaotic, the first events would appear to mirror closely those found in the embryo, and the types of tissue interaction occurring are likely to be very similar if not identical. Thus when clumps of EC cells are allowed to detach from their substratum, or if they pile up excessively, a layer of endoderm-like cells forms

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on their free surfaces in an apparently analogous way to the differentiation of inner cell mass (ICM) cells in the 4½ d mouse embryo (Martin & Evans, 1975*a*, *b*; Rossant, 1975; Evans, Lovell-Badge, Magrane & Stern, 1976). In conditions where free-floating clumps of EC cells are allowed to form and differentiate they give rise to structures recognizable as 'embryoid bodies' which are characteristic of the ascites form of teratocarcinomas *in vivo* (Martin & Evans, 1975*a*, *b*). If we can find out the nature of the signal which starts the differentiation of the endodermal cells from the embryonic cell cluster, then this would provide a valuable clue to the way in which embryo cells generally are directed down a particular developmental path.

It is necessary, however, to have a reliable means of recognising the cell types involved. Protein markers are often less equivocal than morphological ones. The technique of two-dimensional electrophoresis, because it is capable of resolving many hundreds of proteins, offers an ideal way of finding such markers, as well as following their production, without either prior knowledge of the proteins or specific means of detecting them. Therefore, the changes in protein synthesis during the formation of embryoid bodies from EC cells have been studied by 2-D electrophoresis. A number of polypeptides characteristic of the differentiated state have been found and their time of appearance, with reference to the initial stimulus to differentiate, investigated.

Studies have also been undertaken on a selection of other cell lines and on ICM cells of mouse embryos in order to find cell-type specific markers and to investigate their relationships to the differentiating teratocarcinoma cells.

MATERIALS AND METHODS

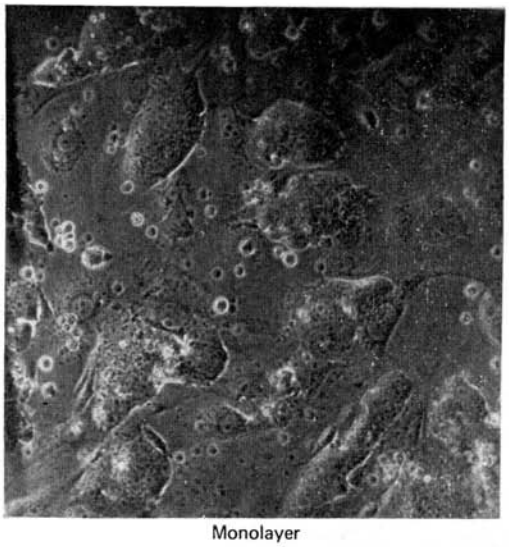
Pluripotent EC cells

The pluripotent EC cells used were OTT5568 S PSMB, a cystic embryoid-body-forming clone similar to those previously described (Martin & Evans, 1975*b*). They were maintained as pure homogeneous EC cells by culturing on a non-dividing fibroblast feeder layer (mitomycin-treated STO cells) from which they were separated by two passages on gelatin-coated petri dishes before labelling. This procedure provides a pure population of undifferentiated stem cells with no remaining feeder cells. Suspended cell clumps were obtained by scraping with a 'policeman'. This method should not select from predetermined clumps and gives small aggregates that differentiate with considerable synchrony.

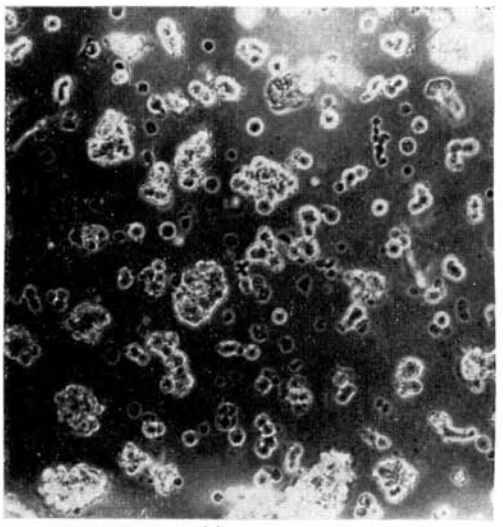
Other cell lines

Nulli SCCl is a 'nullipotent' cell line (Martin & Evans, 1975*a*) derived

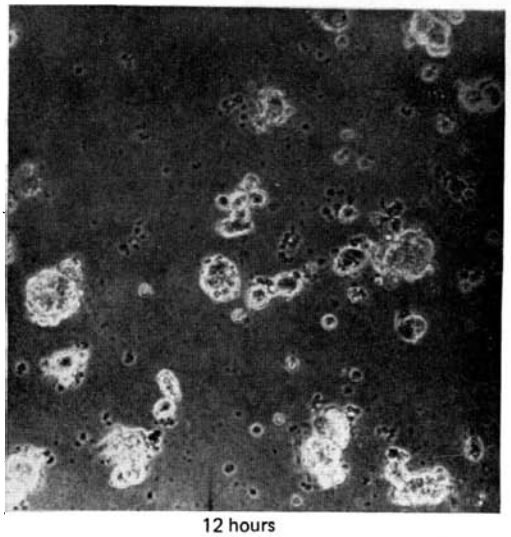
Fig. 1. Phase-contrast micrographs of PSMB embryonal carcinoma cells at various times during the formation of embryoid bodies.



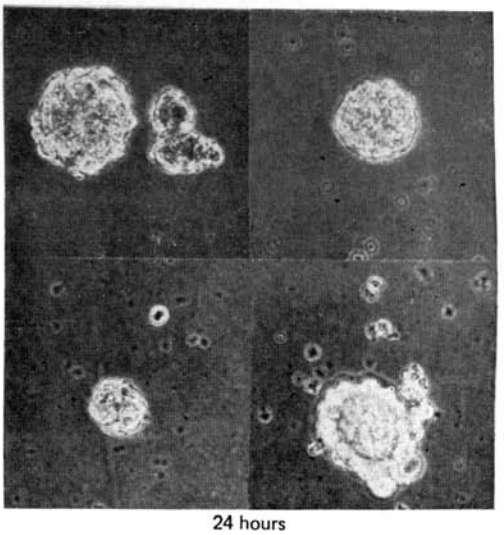
Monolayer



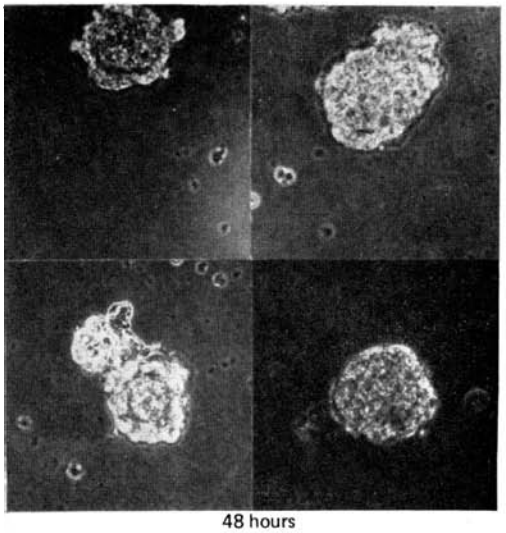
1 hour



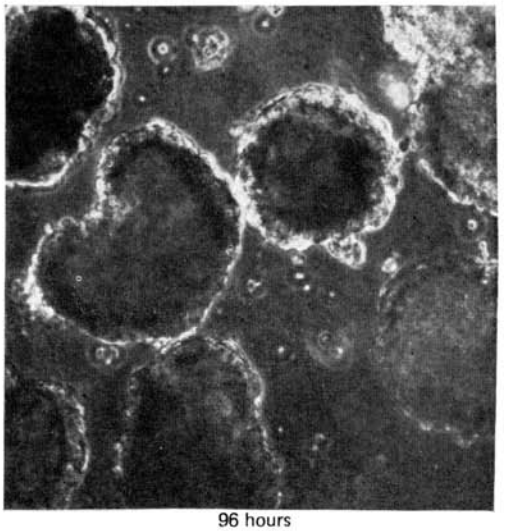
12 hours



24 hours



48 hours



96 hours

Fig. 1. For legend see facing page.

from a spontaneous testicular tumour LS 402C-1684 (Stevens, 1958). This was cultured on gelatin-coated dishes.

PSA5-E was derived from the pluripotent clone PSA5 (similar to PSMB) by plating out without feeder cells and at a low density. It is endodermal in nature.

STO is a fibroblast line (Ware and Axelrad, 1972) routinely employed as feeder cells in this laboratory.

Inner cell masses

Three and a half-day mouse blastocysts (98 h post HCG) were flushed from the uteri of superovulated C3H/He mice that had been mated with 129 S1CP males (detection of vaginal plug indicated day 0) and the ICMs isolated by immunosurgery (Solter & Knowles, 1975).

Cell labelling and sample preparation

Cells were extensively washed in phosphate-buffered saline (PBS) and then labelled for 3 h at 37 °C in methionine-free Eagles medium (Gibco-Biocult Selectamine kit) supplemented with 5 or 10 % foetal calf serum (Flow; the batch used was 50 μM in methionine), and containing 100–200 μCi/ml ^{35}S-L-methionine (Radio-chemical Centre, Amersham; different batches of about 600 Ci/mmol). In the time-course experiments the labelling period was cut to 2 h. Cell aggregates were labelled in 10 ml conical plastic screw-capped tubes (Sterilin) and monolayers *in situ* in gelatin-treated wells of multiwell dishes (Linbro or Falcon).

After labelling the cells were washed with PBS and solubilized with SDS lysis buffer (9·5 M urea, 2 % Ampholines, 0·5 % SDS, 5 % β-mercaptoethanol) to which an equal volume of Triton lysis buffer (9·5 M urea, 2 % Ampholines, 4 % Triton X-100, 5 % β-mercaptoethanol) was added after 5 minutes. This method of lysis was found to solubilize over 97 % of total protein from PSA5-E cells as compared with O'Farrell's (1975) original method (his lysis buffer 'A') which solubilized only about 80 % (Lovell-Badge, 1978). O'Farrell and coworkers (O'Farrell, Goodman & O'Farrell, 1977) have more recently adopted a similar protocol. After lysis cell samples were stored frozen at −70 °C.

For the purposes of the ICM/EC cell comparison a very high specific activity of label was used to increase incorporation. For the ICMs, 10 μl aliquots (50 μCi) of ^{35}S-L-methionine solution were lyophilized in microwells of a microtest plate (Falcon Plastics, type 3034). Batches of 25–50 ICMs were suspended in Hanks buffered salts solution containing 10 % heat-inactivated foetal calf serum, transferred in 5 μl of saline into these microwells and incubated for 3 h at 37 °C. At the end of the incubation excess medium was removed and the ICMs were dissolved in a total of 50 μl lysis buffers. The PSMB EC cells were labelled as monolayers by culturing $2{\cdot}5 \times 10^4$ cells for 48 h in 7 mm

diameter gelatin-treated wells of a multiwell tissue-culture dish. After washing in methionine-free Eagles medium containing 10 % serum, 25 μl of this medium containing 250 μCi of ^{35}S-L-methionine were added. After labelling the cells were washed twice with PBS and harvested by immediate lysis with a total of 100 μl lysis buffers. Batches of about 100 small clumps of PSMB cells were also labelled, immediately after suspension, in an identical manner to the ICMs.

Neither the specific activity of [^{35}S]methionine nor the total amount of methionine have been found to have any effect on 2-D patterns of EC cells (Lovell-Badge, 1978; also see Parker, Pollard, Friesen & Stanners, 1978), so the results from the different labelling regimes should be comparable.

2-D electrophoresis

The proteins were separated in two dimensions by isoelectric focussing followed by SDS polyacrylamide gel electrophoresis using the methods described by O'Farrell (1975), with some modifications (Lovell-Badge, 1978). Isoelectric focussing was carried out in 4 % polyacrylamide rod gels 18 cm long and 0·3 cm in diameter. Eight gels were run at a time at a constant power of 1·75 W for 13·5 h. These first dimension gels were stored frozen at −70 °C. The second dimension gels were $25 \times 25 \times 0{\cdot}12$ cm and contained 9 % acrylamide. A 5 cm stacking gel was used. Equilibration, loading and running were as described by O'Farrell (1975) but with doubled concentrations of Tris/glycine in the electrode buffer. A constant current of 28 mA was passed for about 11 h. The fixed, washed gels were impregnated with PPO for autofluorography, dried under vacuum and exposed to preflashed Fuji RX medical X-ray film at −70 °C (Laskey & Mills, 1975).

$2 \times 10^5 - 10^6$ c.p.m. were loaded onto each IEF gel. Unless stated otherwise the comparisons between different cell types are based on four to eight separate samples of each cell type. Two to four 2-D gels were run with each sample from the time-course experiments, and two such experiments were performed. The results were very comparable between these and so will be discussed together.

The IEF pH range was determined from parallel focussing gels cut into 7·5 mm pieces, each washed and left to soak for 30 min in 0·5 ml degassed distilled water before measuring with a combination glass/reference electrode (Russell pH Ltd) and (EIL) direct reading pH meter. The molecular weight scale was obtained by the use of Combithek (Boehringer Mannheim) calibration proteins run on equivalent SDS gels which were stained with Coomassie blue. Positions of actin (a) and tubulin (α-t and β-t) were identified by running purified protein markers.

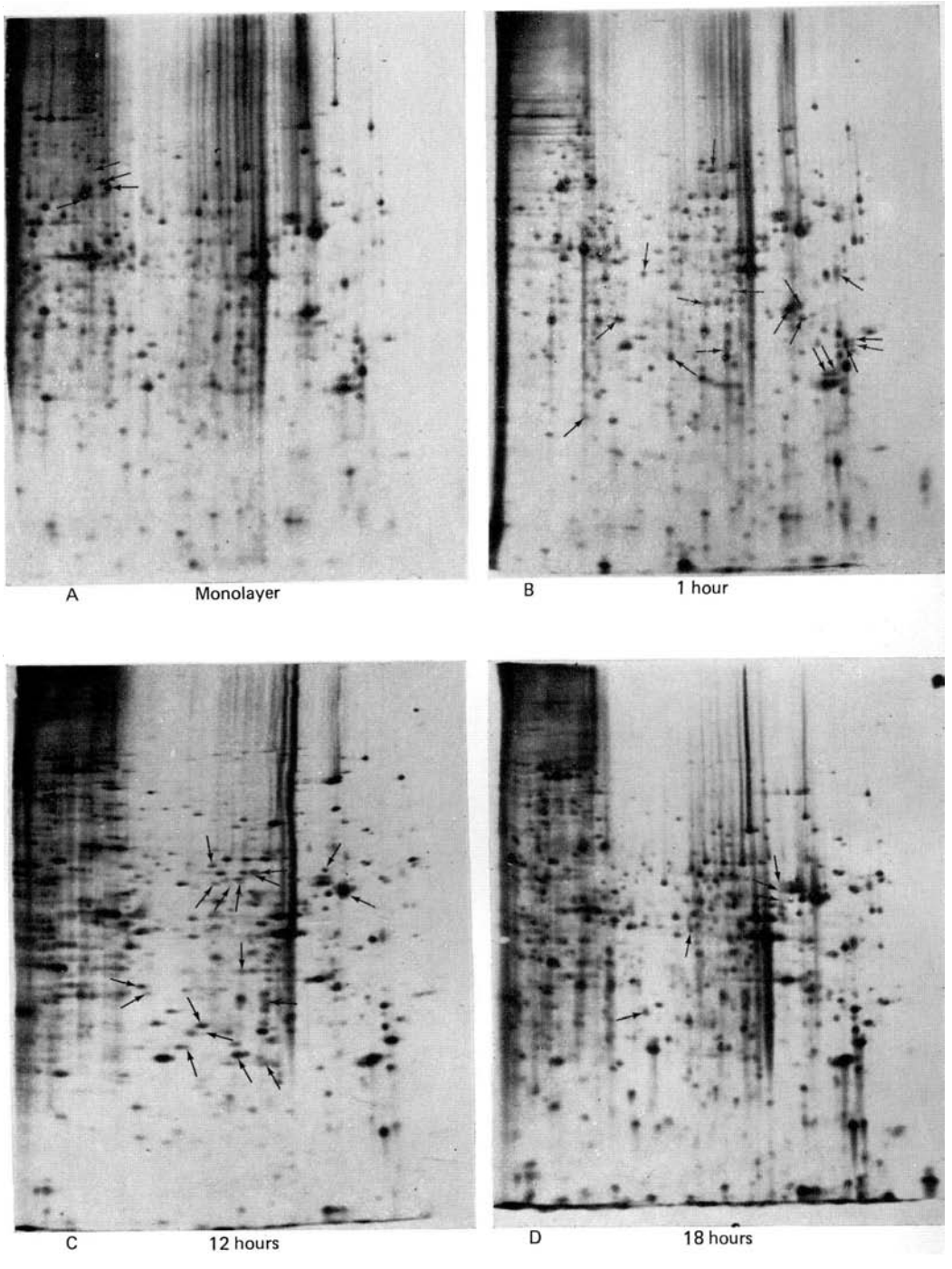


Fig. 2. Two-dimensional protein patterns from PSMB EC cells labelled at various times during the formation of embryoid bodies.

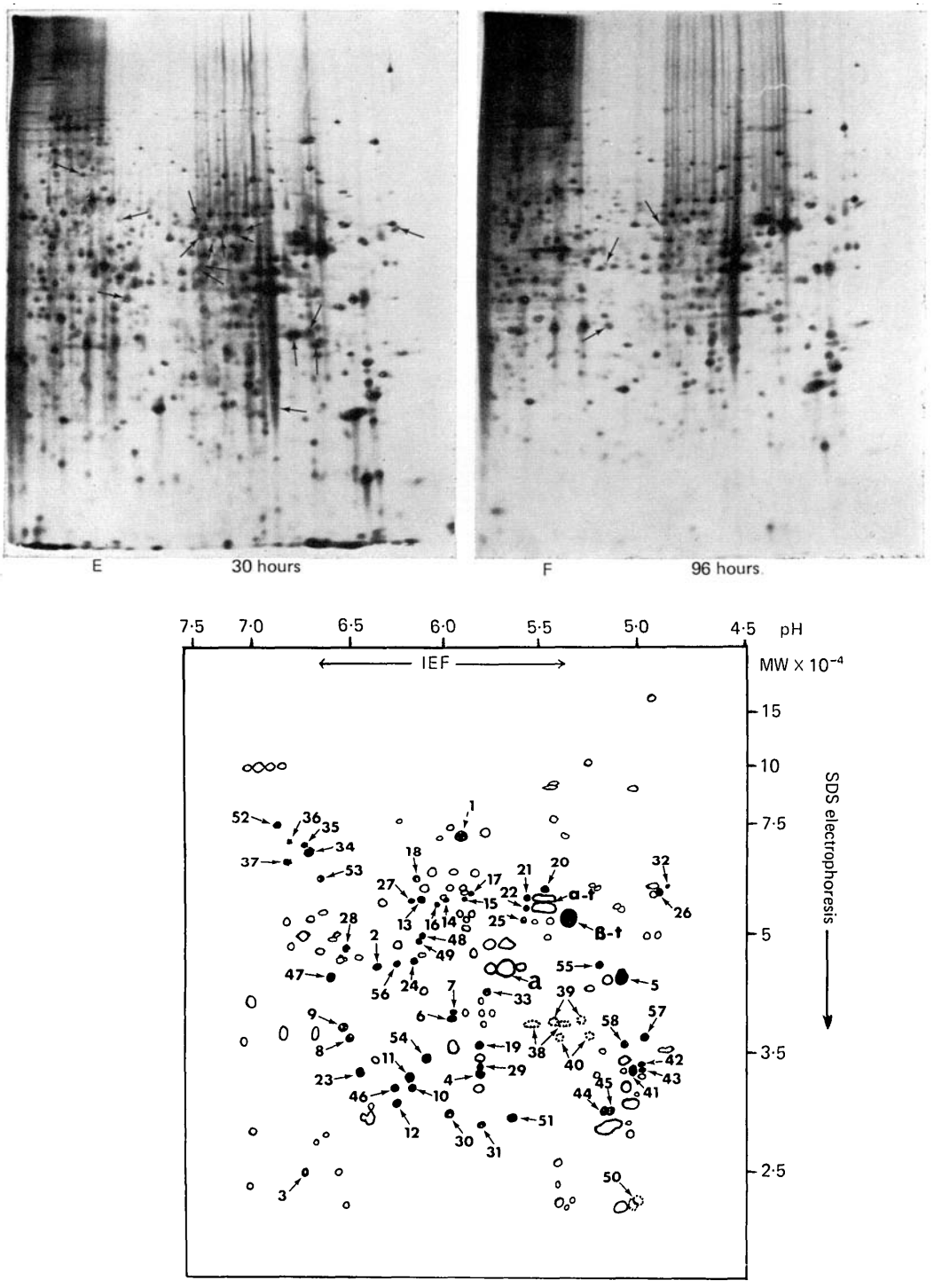


Fig. 3. Key to the numbers assigned to spots showing some change during embryoid body formation: Open spots represent some of the polypeptides common to all stages; those that are dotted show changes in position not related to differentiation (see text).

RESULTS

Differentiation of EC cells in vitro

A trial experiment revealed differences between the 2-D patterns from PSMB cells grown as monolayers and 3-day-old embryoid bodies derived from them. The chronology of these changes in polypeptide synthesis was therefore analysed in more detail by two time-course experiments. In the first the sampling times, i.e. mean number of hours after suspension, were 1, 6, 12, 18, 24, 30, 36, 48, 60 and 72 and in the second they were 1, 12, 24, 36, 48, 72 and 96. Equivalent monolayer samples were also obtained. Figure 1 shows the PSMB cells under phase contrast at various times during the formation of embryoid bodies. One hour after suspension they were seen to be either in small clumps or as single cells. The latter tended to aggregate together and by 12 h most of the cells were in fairly small, compact clumps. Endoderm was first discernible, by phase-contrast microscopy, at about 18 h, and by 24 h most of the clumps had a quite distinct layer on the outside. During the next 48 h the embryoid bodies changed little in appearance, simply increasing in size, but by 96 h some had started to become cystic (Martin & Evans, 1975*b*). Sections of these have shown them to contain ectoderm-like, and possibly mesoderm-like, cells (unpublished; also Martin, Wiley & Damjanov, 1977).

Representative two-dimensional polypeptide patterns from various times during the formation of embryoid bodies from PSMB EC cells are shown in Fig. 2*a–f*. There was considerable streaking in the SDS dimension, which is unfortunately emphasized by photographic reproduction. This streaking may be partly due to freezing the IEF gels before equilibration as we have subsequently found that it is reduced if they are equilibrated first, although O'Farrell (1975) found no difference. However, in as much as there is overall reproducibility the streaks did not unduly affect comparisons. A truly qualitative change is considered to have occurred only when the presence or absence of a spot is consistent between all the samples of the particular stages being compared. Because of differences in loading, exposure times, etc. between the gels, a change was only considered quantitative when a spot varied in intensity relative to its immediate neighbours. It must also be borne in mind that a quantitative change may have a qualitative basis because the embryoid bodies are composed of more than one cell type. Experiments have shown the contribution to labelled proteins by endoderm to be as much as 50 % at 30 h, declining to about 35 % at 96 h (Lovell-Badge, 1978).

Out of about 600 polypeptide spots that could be compared with confidence there were 53 that exhibited some change. Figure 3 provides a key to the spot numbers, and they are indicated with arrows on the relevant autofluorographs. Not all of the changes were necessarily related to embryoid body formation. Thus spots 38–40 showed a shift in pI within a sample that could be attributed

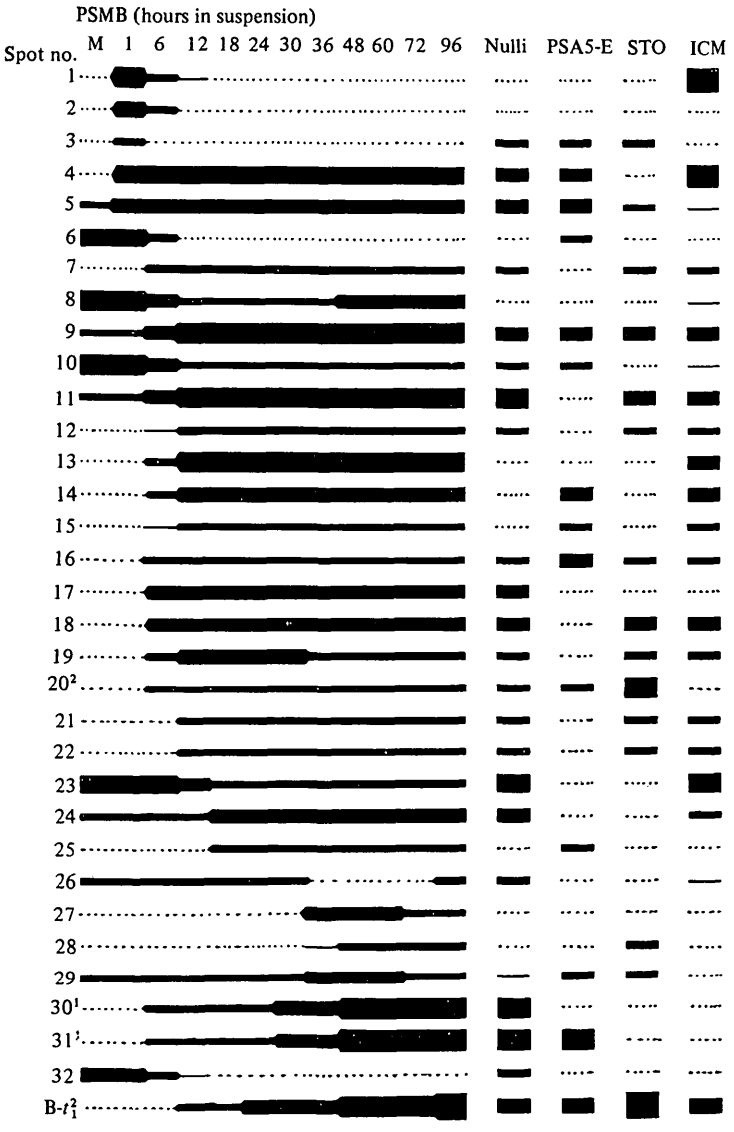


Fig. 4. Diagrammatic representation of the protein changes occurring during EC cell differentiation and the expression of these proteins in other cell types. [1] Refers to lower M.W. form. [2] Refers to higher M.W. form. ....., Undetectable; ——, just detectable.

to length of storage or number of freeze/thaw cycles; spots 41–45 showed coordinated variation within a sample, i.e. were either all expressed or all not expressed; 33, 46 and 47 varied qualitatively and 48–53 varied quantitatively between separate but equivalent samples; 34–37 were grouped in two different arrangements seemingly at random (see Figs. 2*b* and *d*). These variations may have been due to subtle differences in the labelling conditions (e.g. cell

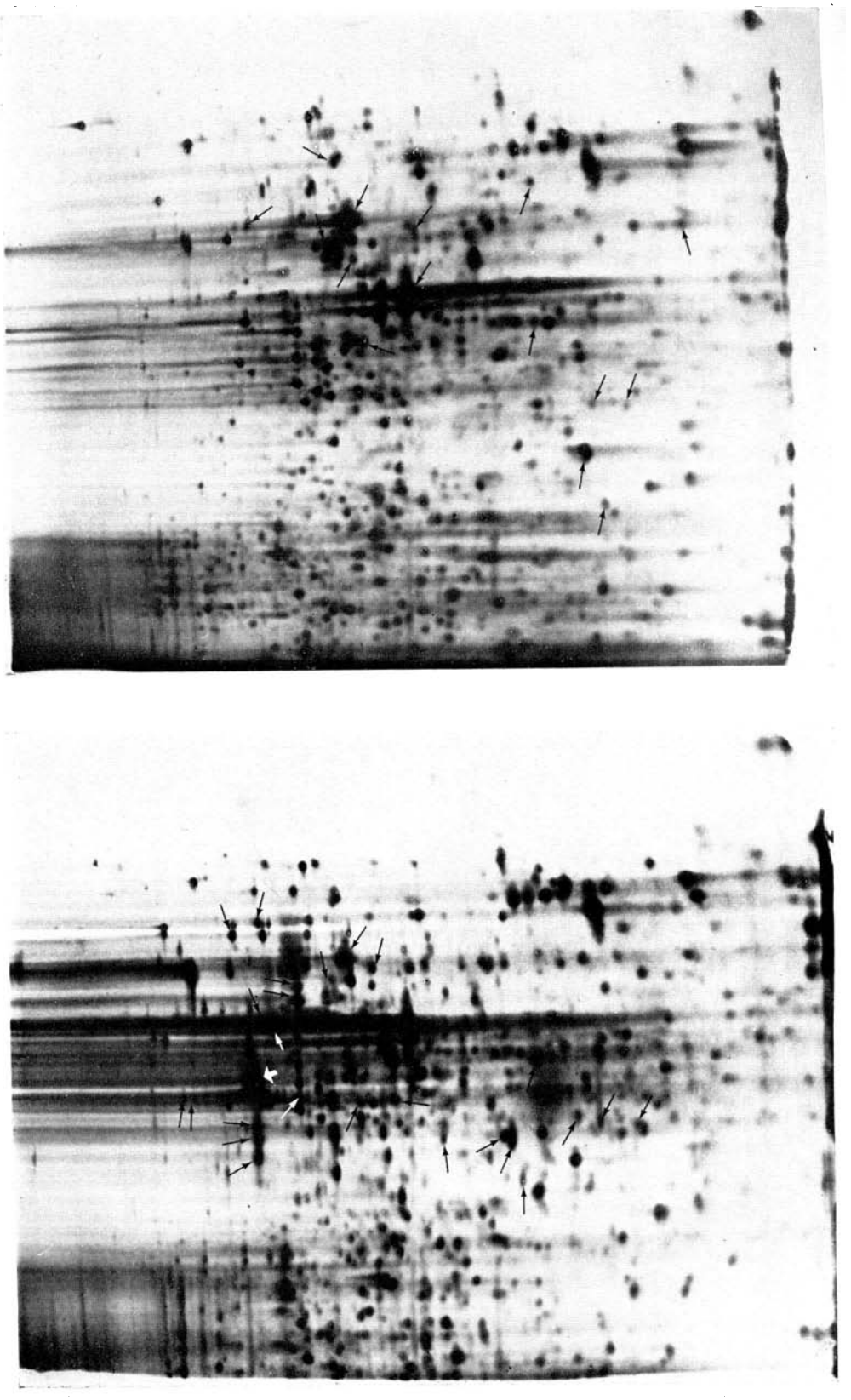


Fig. o dimensional protein patterns from (*a*) immunosurgically isolated ICM's and (*b*) 30 h embryoid bodies. The arrows indicate qualitative and quantitative differences.

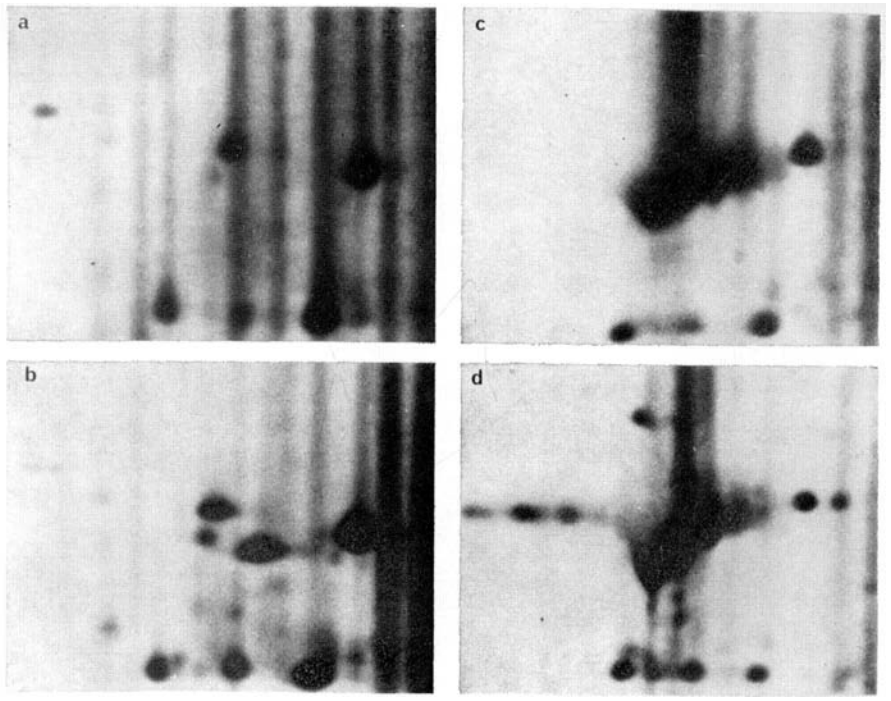


Fig. 7

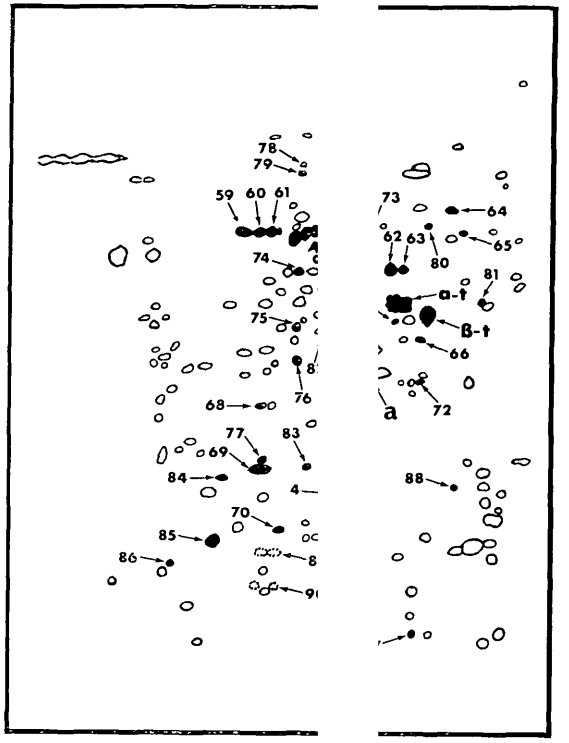


Fig. 6

Fig. 6. Key to the numbers assigned to spots of interest in the ICM/EC cell comparison.

Fig. 7. Details from autofluorographs in the region of pI 6 and M.W. 70000 to demonstrate the appearance of grou the 2-D patterns derived from ICM's and EC cell clumps. The autofluorographs are from (*a*) EC monolayer (PSMB); (*b*) 1 h EC c ımps; (*c*) 1 h EC cell clumps plus medium; (*d*) ICM's.

density, or serum batches), or subsequently during sample handling and electrophoresis.

The more relevant results are summarized in Fig. 4. There are 13 polypeptide spots which exhibit truly qualitative changes in expression, and 8 more that may be similar. Eight show significant quantitative changes, and at least four show shifts in position. Almost all the changes occur before 12 h and the majority of these between 1 and 6 h. Only three new proteins appear after 12 h.

Other cell lines

Gels from Nulli, PSA5-E and STO were scored for the presence or position of the polypeptide spots 1–32, and for β-tubulin. These results are included in Fig. 4. In addition there was one spot more abundant in Nulli than in any other cell type (54) and one that has only been seen in Nulli (55). Also, in PSA5-E, there were two spots absent (56, 57), and one present in increased amount (58), as compared with PSMB and Nulli. See Fig. 3 for the position of these spots.

The STO pattern was characterized by the presence of at least eight novel polypeptide spots, and by the absence of six spots detectable in the gels of all other cell types analysed. There are also numerous quantitative differences.

ICM/EC cell comparison

The autofluorographs from monolayer EC cells labelled with high specific activity [^{35}S]methionine matched closely those from the time-course experiments, so to relate it to that work an ICM autofluorograph is shown together with one from 30 h embryoid bodies in Fig. 5.

If the ICM autofluorographs are examined for the presence of those polypeptides that showed some changes during the formation of embryoid bodies from EC cells, it would appear that in most respects ICMs resemble EC cell clumps from 6–12 h. These results can be seen diagrammatically in Fig. 4. However, there are at least 16 novel polypeptide spots on the ICM autofluorographs. Some of these are clustered as if they were post-translational modifications of single new proteins, such as the group labelled α which contains at least five spots, the triplet numbered 59, 60 and 61, and the doublet 62 and 63. Other novel spots are numbered 64–70 (see Fig. 6 for key to numbers). Two spots, 71 and 72, present in all EC cell samples were not found in ICMs. In addition, seven spots, 73–79, were present in greater amounts in ICMs than in EC cells, nine more, 80–88, were significantly fainter, and two 89 and 90, showed acidic shifts in pI. The spots representing the various tubulin components, in particular those for α-tubulin, were also substantially fainter in ICM autofluorographs. (N.B. all these spots are different from those that distinguished Nulli, PSA5-E and STO fibroblasts from PSMB cells.)

It was thought that some of the differences between ICMs and the various EC cell samples could be due to the detection of secretion products in the

ICM samples because, from fear of losing material, they were not washed after labelling. Therefore, samples of ICM-sized PSMB cell clumps were labelled shortly after suspension and deliberately not washed in saline afterwards so that a small volume of labelling medium would be solubilized with them. The only real difference from such clumps treated in the usual way (see Fig. 2*b*) is the presence of the polypeptides clustered around pI 6 and M.W. 70000 making up the group α. These were previously seen only in ICM autofluorographs except for polypeptide spot No. 1 which made a transitory appearance in the time-course experiment and, by position, would seem to be one of this group. Details from autofluorographs showing these spots are shown in Fig. 7. The foetal serum component alpha foetoprotein (AFP) is resolved in an identical manner on 2-D gels to the group α, having the same number of components and a distribution attributable to increasing numbers of sialylations (Lovell-Badge, 1978). α would also appear to be mainly a secreted product as is AFP. Therefore the group is tentatively identified as AFP. (The possibility that it is exchange labelled AFP from the foetal calf serum in the medium has not been ruled out.)

DISCUSSION

Differentiation of embryonal carcinoma cells

The results from the PSMB time-course experiments indicate that a progressive change in the polypeptide synthetic profile occurs during the formation of embryoid bodies. Some of these changes may not be due to the synthesis of new proteins but to post-translational modifications, which may still be valid indicators of differentiation.

There are a number of changes that become evident shortly after suspending the EC cells from the substratum (between 0 and 2 h), and of particular interest amongst these is the transitory appearance of spots 1, 2 and 3. Temporary expression of proteins has not been reported by other workers studying preimplantation development by 2-D electrophoresis (e.g. Van Blerkom & McGaughey, 1978*b*, Handyside & Johnson, 1978) and so may not be a common feature of development at this time; although it has been reported to occur during oocyte maturation (Van Blerkom & McGaughey, 1978*a*) and in amphibian development (Ballantine, Woodland & Sturgess, 1979). This could reflect shunting of the E3 cells back onto a normal embryonic pathway at a stage just prior to the termination of the synthesis of these proteins (N.B. spot No. 1 (AFP?) is present in ICMs). On the other hand, the detection of all these early proteins may be a correlate of the cellular reorganization which is presumably occurring at this time, e.g. changes in cell shape and formation of new cell–cell contacts, and not reflect the first signs of differentiation *per se*. It will be of interest to see how soon after suspension these proteins are first

made and whether they are the result of new transcription or the unblocking of mRNA already present.

The majority of changes in polypeptide synthesis occurred before 12 h; at least 6 h before overt morphological differentiation. This situation is somewhat analogous to that found by Handyside & Johnson (1978) for the divergence of trophectoderm and ICM within the developing blastocyst. In this case they have been able to localise the phenotypic changes to each of the presumptive tissues and have concluded that most are occurring before the cells are committed. There is much evidence to suggest that cells within the centre of an embryoid body are unchanged with regard to their developmental potential, at least while they remain as simple embryoid bodies (e.g. Mintz, Illmensee & Gearhart, 1975; Mintz & Illmensee, 1975). The outside cells, on the other hand, are almost certainly irreversibly committed as endoderm, and if the EC cells are roughly equivalent to ICM cells then their commitment probably occurs within 24 h of suspension (Gardner & Papaioannou, 1975). However, this commitment is unlikely to occur before 6 h (unpublished observations). This would suggest that as for the ICM/trophectoderm divergence, most of the detectable changes in polypeptide synthesis are occurring well before the inner cells are committed and at least some are occurring before commitment of the outside cells, (but see Johnson, Handyside & Braude, 1977; and Johnson, 1979 for discussions on commitment).

Comparing the 2-D patterns of the other cell lines with those from the PSMB time-course experiment has provided some interesting results. Thus, Nulli would appear to resemble embryoid bodies far more closely than it does the PSMB monolayer: out of the 30 non-transitory proteins, 20 show similarities with the embryoid bodies. This suggests that Nulli cells are maintained at a later stage and that they may be equivalent to core cells. The 2-D pattern from PSA5-E on the other hand, would be expected to show some proteins attributable to embryoid-body endoderm. Allowing for the likelihood of another endoderm cell type, and that some of the changes represent losses, a scheme for the distribution of tissue-specific proteins can be constructed. This is shown in Table 1. It should be possible to test these predictions and use at least some of these proteins as markers.

It is clear from the results presented here that the vast majority of polypeptides resolved on 2-D gels are present in all the different cell types analysed. Thus, even between STO fibroblasts and EC cells the differences are only about 5 %. Other workers have reached similar conclusions, both for pre-implantation development (e.g. Van Blerkom, Barton & Johnson, 1976; Handyside & Johnson, 1978) and for other developmental systems (e.g. Peterson & McConkey, 1976; Prashad, Wischmeyer, Evans, Baskin & Rosenberg, 1977). It may prove to be a general rule that the differences between precursor and differentiated cell type are in the order of 5 % or less of detectable proteins. It is possible, however, that those tissues analysed so far were not completely differentiating

Table 1. *Distribution of tissue-specific proteins*

	Private proteins	Shared proteins
Monolayer	6, 8, 10	23, 26, 32
Core cells (Nulli)	7, 11, 12, 18, 19, 21, 22, 24, 30[1]	4, 5, 9, 16, (17), 20[2], 31[1], β-t[2]
Visceral endoderm (PSA5E-like) or both endoderm types	14, 15, 25, 29	
Parietal endoderm (only)	13, 27, 28	

[1] Refers to lower molecular weight form.
[2] Refers to higher molecular weight form.

due to the conditions in which they were grown, or because they were analysed too early, as it has been shown that there are more organ-unique soluble polypeptide spots on 2-D gels of adult mouse organs (21–26 %) than of embryonic ones (1–8 %) (Klose & Von Wallenberg-Pachaly, 1976).

One must also consider that not all proteins are being detected on 2-D gels. The polysomal poly(A)$^+$mRNA base-sequence complexity of a mouse EC cell line has been estimated at about 7700 different mRNA sequences (Affara, Jacquet, Jakob, Jacob & Gros, 1977). This is of the same order of magnitude as that found in two other cell lines in the same study, and is comparable to estimates for other mouse tissues from other workers (e.g. Hastie & Bishop, 1976). These 7700 sequences were in three abundance classes, with 56 classified as being 'abundant', 437 as 'intermediate' and 725 as 'rare' (with 601, 82 and 5·5 mean no. of copies per cell, respectively). 2-D electrophoresis of whole cells resolves 600–1000 polypeptide spots (1500 in the best gels). If allowance is made for the fact that many mRNA products are likely to be represented by more than one 2-D gel spot due to post-translational modifications (e.g. see Anderson & Anderson, 1977), and, alternatively, that some can never be resolved due to the limits of the electrophoretic system (O'Farrell *et al.* 1977; Lovell-Badge, 1978) it is likely that 2-D electrophoresis is resolving the abundant and intermediate mRNA class products but very few from the rare classes.

If one can extrapolate the 5 % value from 2-D comparisons to the undetected rare protein classes, then the real number of differences between cell types may be in the order of 400 or more. This would appear to be borne out by mRNA-cDNA cross-hybridization experiments (e.g. Hastie & Bishop, 1976; Affara *et al.* 1977), which also show that the majority of differences between the mRNAs of cell types are indeed attributable to the rare abundance class. Therefore in an analysis of the changes of total cell proteins during differentiation by 2-D electrophoresis, one is restricted to the 10 % or so more abundant

species. These might be uncharacteristic. It will be of interest to carry out comparisons between separated cell fractions.

ICM/EC comparison

Even allowing for the limitations as discussed above, 2-D electrophoresis would seem to be a valid means of establishing homologies amongst different cells. Theoretically, it should be better than techniques that rely on morphology, histochemistry, or the detection of individual cell products, as it is monitoring a significant fraction of the genetic and epigenetic activity of the cells under comparison. With this in mind the 2-D pattern given by ICM cells was compared with those from EC cells to see if their developmental homology was reflected by a phenotypic homology.

The number of differences found between ICMs and EC cells grown as monolayers is considerable, and is of the same degree as that between EC cells and STO fibroblasts. A similar result was obtained by Dewey, Filler and Mintz (1978) using an *in vivo*-maintained line of EC cells, and, although it is difficult to compare their results with ours (e.g. they found 60 differences out of only about 240 polypeptide spots), it could be concluded in both cases that there is little, if any, homology between the two pluripotent cell types.

Some of the differences are resolved if the 2-D patterns of ICMs are looked at with reference to those polypeptides that appear during the formation of embryoid bodies from EC cells. They would seem to correspond most to EC cell clumps of 6–12 h, which could be explained if the ICMs were undergoing endoderm formation. As endoderm should be visible by $4\frac{1}{2}$ days *post coitum* it is quite likely that this process had already begun, especially if allowances are made for the time it takes to isolate and label the ICMs.

Nevertheless, there are still many differences to be accounted for between the 2-D patterns of ICMs and EC cells. This could reflect inherent difficulties in a comparison of this type, due to the nature of the cells and the conditions in which they are grown. Thus the ICM cells are taken from embryos and are examined at one time point during their continuing development. If rapid developmental changes in genetic readout are occurring at this stage it is possible that protein synthesis reflects translation of message from an earlier stage, perhaps even from some long-lived maternal mRNA, as well as translation of current transcriptional products. The EC cells, on the other hand, have been arrested at one developmental point, and so effectively have no past history. Microenvironmental factors might also influence the phenotype of these cells, notwithstanding their epigenotype. The ICMs are from the normal embryonic environment, and will therefore be subject to a different set of external influences, e.g. maternal hormones, levels of metabolites, trophectodermal factors etc., than the EC cells which have been cultured *in vitro*.

On the other hand, the differences in polypeptide synthesis could reflect a

true lack of homology. EC cells from monolayer culture are pluripotential and able to integrate with the ICM of a mouse blastocyst and participate in the development of an adult mouse (Papaioannou *et al.* 1975, 1978) but this is more a test of their pluripotentiality than their homology with ICM cells as embryonic cells from both earlier and later stages can participate in chimaera formation (Gardner & Papaioannou, 1975). Indeed the ability of EC cells to differentiate into trophoblastic cells both *in vivo* (Martin, 1975) and *in vitro* (McBurney, 1976) indicates that they have a wider developmental potency than ICM cells. Teratocarcinomas containing EC cells are formed by the ectopic transplantation of germinal ridges containing undifferentiated primordial germ cells (Stevens, 1964), preimplantation embryos (Stevens, 1968), or the embryonic portion (Solter, Skreb & Damjanov, 1970) (or the embryonic ectoderm alone (Diwan & Stevens, 1976)) of post-implantation embryos. These sources represent a variety of types of pluripotential cells any one of which might be epigenetically homologous and under suitable conditions phenotypically identical to embryonal carcinoma cells.

EC cells have at least partial homology with all of them in their possession of common cell surface antigens (Artzt *et al.* 1973; Stern, Martin & Evans, 1975; Edidin & Gooding, 1975; Stinnakre, Evans, Willison & Stern, 1979) and alkaline phosphatase (Bernstine, Hooper, Grandchamp & Ephrussi, 1973). The level of alkaline phosphatase (Damjanov, Solter & Skreb, 1971) and the pattern of sulphated glycosaminoglycan synthesis (Cantor, Shapiro & Sherman, 1976) as well as the ready formation of endoderm by EC cells suggest, however, that they are not homologous with embryonic morula cells.

Recent evidence would suggest that EC cells may be closer to the 'ectoderm' cells of peri-implantation embryos (Evans, Lovell-Badge, Stern & Stinnakre, 1979), but it may indeed be false to expect any phenotypic homology, as the essential property of pluripotentiality need not necessarily be linked with the entire cell phenotype. A pluripotential cell – whatever its current phenotype – would be expected to differentiate along pathways inherent in the genetic programme of the cell; that is along pathways recognizable as those undergone by embryonic cells. Thus the developmental homology with ICM cells may not necessarily imply a phenotypic homology. The essential features of a pluripotent cell and the mode of its entry into the programme of sequential differential genetic control of development pose interesting problems for investigation.

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