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P19 embryonal carcinoma cells

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ABSTRACT P19 cells are a line of pluripotent embryonal carcinoma able to grow continuously in serum-supplemented media. The differentiation of these cells can be controlled by nontoxic drugs. Retinoic acid effectively induces the development of neurons, astroglia and microglia — cell types normally derived from the neuroectoderm. Aggregates of P19 cells exposed to dimethyl sulfoxide differentiate into endodermal and mesodermal derivatives including cardiac and skeletal muscle. P19 cells can be effectively transfected with DNA encoding recombinant genes and stable lines expressing these genes can be readily isolated. These manipulations make P19 cells suitable material for investigating the molecular mechanisms governing developmental decision made by differentiating pluripotent cells.

KEY WORDS: *embryonal carcinoma, differentiation, embryonic development*

Introduction

The eggs of many organisms contain local deposits of macromolecules which provide the developing embryo with landmarks around which the structure of the organism is built. For example, the *Drosophila* zygote contains maternally-derived transcripts whose products are asymmetrically distributed and that play pivotal roles in the early development of that organism (St Johnston and Nüsslein-Volhard, 1992). Unlike *Drosophila*, the mammalian embryo apparently contains no localized signals so the form of the developing organism must be dependent on cues which arise during the early cleavage divisions. Nevertheless, a number of the regulatory *Drosophila* genes appear to have homologues in mammals. One popular strategy to initiate studies on the molecular genetics of mammalian development is to clone these homologues and search for their function in murine embryos. The expression patterns in embryos often give clues to function but experimental tests of function require the capability to modulate expression of the gene product under investigation. It is now fashionable to create mouse null mutations by homologous recombination (Capecchi, 1989; Koller and Smithies, 1992); however, a number of mutations to seemingly important regulatory genes have led to unexpectedly subtle phenotypes (Joyner *et al.*, 1991; Donehower *et al.*, 1992).

Studies on how these regulatory genes function might profit from cell cultures which express or respond to the gene products under investigation. For example, myoblast cell lines have proven valuable in establishing the role of myogenic regulatory genes (myoD, myogenin, Myf5, and Myf6) in the differentiation of these cells (Weintraub *et al.*, 1991). P19 cells are an experimentally tractable culture system with which to identify and/or investigate the roles of gene products in early developmental events, particularly those affecting the determination of cell fate or lineage.

Like other embryonal carcinoma and embryonic stem cells, P19 cells are developmentally pluripotent and appear to differentiate using the same mechanisms as normal embryonic cells. When P19 cells were injected into normal embryos, the P19-derived cells were present in a variety of apparently normal tissues although most of the embryos with large contributions of P19 cells were abnormal in some way (Rossant and McBurney, 1982).

A number of characteristics of P19 cells make them valuable for studies of early developmental events. The cells are immortal allowing for the creation of almost unlimited amounts of material for analysis. P19 cells are easy to grow and maintain in the undifferentiated state but they can also be efficiently induced to differentiate by simple manipulation of the culture conditions. Finally, the genetic composition of the cells can be easily manipulated either by the selection of mutant strains or by selection of clones carrying transfected genes stably integrated into their genomes.

Origin of P19 cells

Teratocarcinomas can develop in some mouse strains from early embryos transplanted from the uterus into ectopic sites (Stevens, 1970). P19 cells were derived from a teratocarcinoma formed following transplantation of a 7.5 day embryo into the testis (McBurney and Rogers, 1982). The tumor which arose from the transplanted embryo grew rapidly and cell cultures containing undifferentiated stem cells were established directly from the

Abbreviations used in this paper: CNS, central nervous system; CREB, cyclic AMP response element binding protein; DMSO, dimethyl sulfoxide; GABA, γ-amino butyric acid; GFAP, glial fibrillar acidic protein; NMDA, N-methyl-D-aspartic acid; RA, retinoic acid; RAR, retinoic acid receptor.

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primary tumor. These stem cells are called embryonal carcinoma and those of the P19 line readily grow in cell culture even in the absence of lethally irradiated feeder cells.

The experiment from which P19 cells were isolated was designed to establish cell lines from female embryos which were heterozygous for X-linked alleles (McBurney and Strutt, 1980). Thus, the embryos for transplantation were derived from matings between C3H/He strain females and male animals carrying an X chromosome derived from a feral mouse (Mus musculus) which carried a number of variant alleles (Neilsen and Chapman, 1977). This male was not inbred. He was from the fifth generation of backcrosses to C3H/He animals so would be expected to carry a number of genes (3% of all genes) not of C3H/He origin. Consistent with this idea, P19 cells do not readily form tumors in C3H/He adult animals although they do develop rapidly-growing tumors following injection into neonatal animals. This suggests that P19 cells differ from the C3H/He genotype at at least one histocompatibility locus. In addition, a variant allele at the *aprt* locus (encoding adenine phosphoribosyl transferase) is present in P19 cells (Cooper *et al.*, 1991) and is probably derived from the feral animal.

Differentiation of P19 cells

P19 cells have a euploid male karyotype (McBurney and Rogers, 1982). This chromosomal composition is remarkably stable provided the cells are maintained in exponential growth.

The cells of many embryonal carcinoma lines differentiate if cultured at high density (Nicolas *et al.*, 1975; McBurney, 1976) or as aggregates (Martin and Evans, 1975). Our initial experiments with P19 cells indicated that they differentiated very inefficiently under these conditions. However, differentiation of P19 cells can be induced if aggregates are exposed to non-toxic concentrations of a number of drugs. The drugs most effective in inducing differentiation of P19 cells are retinoic acid (RA) (Jones-Villeneuve *et al.*, 1982) and dimethyl sulfoxide (DMSO) (McBurney *et al.*, 1982). Both drugs are not demonstrably toxic to P19 cells at the doses effective in inducing differentiation, indicating that their effect is true induction not selection of differentiated cells pre-existing in the cultures.

The two drugs induced differentiation by affecting different cellular targets because mutants have been selected which fail to respond to one drug but continue to differentiate normally in response to the other (Edwards *et al.*, 1983; Jones-Villeneuve *et al.*, 1983) (see Table 1). Both mutants were selected in multiple steps and at least for the RA non-responsive mutant, RAC65, intermediate stages of responsiveness were obtained (Jones-Villeneuve *et al.*, 1983). Thus, it seems likely that these mutant cells carry multiple mutations. The RA non-responsive character of the RAC65 cells behaves as a dominant trait in cell hybrids (Pratt *et al.*, 1990).

Retinoic Acid

The cell types formed in RA- and DMSO-treated cultures are distinct. At the dose of RA normally used ($3x10^{-7}$M), neurons are the first and most abundant cell type evident (Jones-Villeneuve *et al.*, 1982). By six days after initial RA exposure, up to 85% of the cells express neuronal markers such as tetanus toxin binding sites, the 68 and 160 kD neurofilament proteins and the HNK-1 antigen (McBurney *et al.*, 1988). The proportion of neuronal cells in the population declines with time after six days because the neurons are post-mitotic and the non-neuronal cells continue to proliferate.

TABLE 1

CHARACTERISTICS OF MUTANT P19 CELLS

	RA	DMSO
P19	+	+
RAC65	+	
D3	+	-

The non-neuronal cells are probably the progenitors of various other lineages. By day 10 astroglial cells mature and are recognized by their intermediate filaments, which are comprised of glial fibrillar acidic protein (GFAP) (Jones-Villeneuve *et al.*, 1982). The other abundant cell type appears fibroblast-like and expresses the alpha smooth muscle actin (Rudnicki *et al.*, 1990). After prolonged periods of incubation, cells resembling microglia appear (Aizawa *et al.*, 1991). Oligodendrocytes can also develop from RA-treated cells, although these have only been observed in P19 cells grafted into the brains of neonatal rats (Staines *et al.*, in preparation). No oligodendrocytes appear in cell culture or in grafts into adult brain, suggesting that the maturation of this cell type occurs only under conditions in which normal myelination occurs — that is, in the neonatal rodent brain.

The neurons develop rapidly and abundantly. They stain with antibodies to NeuN (Mullen *et al.*, 1992), an antigen present in the nucleus of neurons of the central nervous system (CNS). Early studies indicated the presence of the neurotransmitters acetylcholine (Jones-Villeneuve *et al.*, 1983) and catecholamines (Sharma and Notter, 1988), but more extensive analysis of neurotransmitters (Staines *et al.*, 1993) indicated that about 60% of neurons contain γ-amino butyric acid (GABA), 20% somatostatin, 20% neuropeptide Y, 1% tyrosine hydroxylase and smaller proportions with other neurotransmitters. More recent studies indicate the presence of glutamate in a large proportion of neurons (P. MacPherson *et al.*, unpublished results). Frequently more than one neurotransmitter is present in one neuron. Neuronal processes often appear to form junctions with each other and electron microscopy of these cultures indicates the presence of synapses formed between axons and dendrites (McBurney *et al.*, 1988).

Long-term survival of P19-derived neurons in culture appears to be dependent on the presence of non-neuronal cells (P. MacPherson *et al.*, unpublished results) or growth factors (Schubert *et al.*, 1990). P19-derived neurons thrive when transplanted in the mammalian CNS (Morassutti *et al.*, 1993). Our studies involve grafting RA-treated P19 cells into the striatum of the immunosuppressed adult rat whose striatal neurons have been eliminated by an excitatory neurotoxin. P19 neurons and non-neuronal cells survive for at least 13 weeks in these grafts. We have performed electrophysiological recordings on brain slices prepared from this grafted material using intracellular electrodes. These studies indicate that the electrical properties of the neurons mature with time. Action potentials are induced by membrane depolarization and there is evidence of voltage-dependent sodium and potassium channels. The neurons become responsive to both GABA and glutamate and pharmacologic studies suggest the presence of $GABA_A$ and both NMDA and non-NMDA glutamate

receptors. Excitatory post-synaptic potentials are frequently observed in grafted neurons of more than three weeks of age, and spontaneous activity is evident indicating that the P19-derived neurons are forming networks of synaptically coupled cells (D. Magnuson *et al.*, in preparation).

Mechanism of RA action

RA can be present for as little as 2 to 4 h and still be effective in inducing the irreversible differentiation of virtually all cells in a culture (Berg and McBurney, 1990). Since the first markers of differentiated neurons do not appear until at least three days after RA exposure, it seems clear that RA initiates a cascade of events that culminates in cellular differentiation.

The site of RA action appears to be a group of nuclear retinoic acid receptors (RARs) that are known to be ligand-dependent transcription factors (Evans, 1988). P19 cells express both the RARα and RARγ genes. The RAC65 cell, which does not differentiate in RA, carries a rearrangement affecting one of the RARα genes (Pratt *et al.*, 1990). The mutant allele gives rise to a transcript which encodes a truncated RARα protein lacking part of the domain required for binding RA. This mutant protein is a dominant suppressor of promoters carrying an RA response element. Curiously however, RA-treated RAC65 cells do express some but not all RA responsive genes when exposed to RA (Pratt *et al.*, 1993). It remains to be determined which RA responsive genes play roles in initiating differentiation. RA-induced genes encoding transcription factors such as homeobox genes would seem likely targets.

Dimethyl sulfoxide-induced differentiation

At concentrations of 0.5-1% (v/v) DMSO efficiently induces P19 cell aggregates to develop into a wide variety of mesodermal and endodermal cell types (McBurney *et al.*, 1982; Edwards *et al.*, 1983). Most notable among these cells are cardiac and skeletal muscle, epithelium, and other as yet uncharacterized cells which probably include endothelium and chondrocytes. The first distinct cell type evident in DMSO-treated aggregates appears at 2 to 3 days around the periphery of each aggregate and contains cytokeratins and α smooth muscle actin (Smith *et al.*, 1987). These cells resemble primitive extraembryonic endoderm. Striated cardiac muscle appears after 6 days in the interior of the aggregates. Cardiac muscle can comprise up to 25% of the cells at day 6 to 7 and these cells normally appear in tight clusters which frequently rhythmically contract. These cardiocytes express a variety of sarcomeric proteins which indicate that they resemble embryonic and not adult tissue (Rudnicki *et al.*, 1990). Skeletal muscle develops in these cultures but appears later than cardiac muscle, becoming evident only 9 to 10 days after initiation of differentiation.

The induction of differentiation by DMSO appears to follow cooperative kinetics because within the aggregates differentiation of cells is an all-or-none event and cardiac muscle cells appear in groups or communities rather than individually (Smith *et al.*, 1987). When the DMSO non-responsive mutant cells, D3, are co-aggregated with P19 cells, the D3 cells inhibit P19 cells from differentiating in response to DMSO (Campione-Piccardo *et al.*, 1985). All of these experiments are consistent with the idea that DMSO is affecting a pathway that has an extra-cellular component and is mediated either by a soluble factor or by direct cell-to-cell contact. Consistent with this interpretation is a recent report of a factor from extraembryonic endoderm that induces differentiation of P19 cells (Mummery *et al.*, 1991).

The mechanism of DMSO action remains obscure. There is some evidence from other systems that DMSO may affect calcium metabolism, and we have found that DMSO induces elevated levels of free intracellular calcium (P. Morley *et al.*, unpublished results). Various other drugs, such as 6-thioguanine, butyrate, and dibutyryl cyclic AMP, induce differentiation in a fashion similar to DMSO (Edwards *et al.*, 1983). That cyclic AMP induces P19 differentiation (McBurney, unpublished; Sharma *et al.*, 1990) suggests that the cyclic nucleotide signalling pathways may be important, although embryonal carcinoma cells such as P19 do not regulate cyclic AMP responsive genes through the CREB-mediated pathway characterized in other cell types (Masson *et al.*, 1992). The D3 mutant fails to respond to any of the chemical inducers except RA so the site(s) of the genetic lesion in these cells must be downstream of the targets of all drugs.

Dose dependence of differentiation

The cell types that develop following exposure to any of the inducing drugs are different depending upon the dose of drug used. This was initially established for RA (Edwards and McBurney, 1983). Aggregates exposed to relatively high doses (greater than 10^{-7} M) develop neurons and astroglial cells as described above. At lower doses (around 10^{-8} M) few neurons are present and skeletal muscle is abundant. At still lower doses (around 10^{-9} M) cardiac muscle is formed while skeletal muscle decreases in abundance. The dose relationship between the three cell types (cardiac muscle, skeletal muscle, neurons) is the same for the other drugs (DMSO, butyrate, cyclic AMP) (Edwards *et al.*, 1983), suggesting that the graded doses of all drugs are mimicking in culture a gradient present in the embryo which might participate in specifying the distribution of cells along the rostral-caudal axis.

Cell-to-cell contact

Cell aggregation is essential for the response to DMSO — no differentiation is induced if cells are cultured in the presence of DMSO while growing on solid plastic surfaces (McBurney *et al.*, 1982). High cell density is also necessary for efficient neuronal development in RA-treated cultures. In cells plated at low density and exposed to RA, only non-neuronal cells develop. P19 cells form gap junctions but it is unclear whether the cytoplasmic syncytium formed is essential for differentiation. Disruption of cell contact by reducing the extra-cellular calcium concentration severely hampers neuronal differentiation in RA-treated aggregates (Schmidt *et al.*, 1992).

DNA transfection studies

The cell types which emerge from RA- and DMSO-treated cultures are highly reproducible and appear with kinetics reminiscent of those of the same cell types differentiating in murine embryos. The P19 cells have, therefore, been useful for studies in culture aimed at investigating cellular and molecular events associated with the development of those cell types formed in abundance — notably neurons and cardiac muscle.

The major experimental challenge in studying embryonal carcinoma differentiation is in dealing with the heterodisperse nature of

the differentiated cells which develop. With drug-induced differentiation of P19 cells the spectrum of cell types is more limited than with spontaneously differentiating embryonal carcinoma and embryonic stem cells, but in no case does a single differentiated cell type develop. On the positive side, embryonal carcinoma cells must traverse the intermediate stages of development before acquiring the fully differentiated phenotypes, so gene products which appear only transiently in differentiating tissues do appear in these cultures in the appropriate cell types.

Investigations of molecular mechanisms require that cultures be amenable to perturbation by pharmacological agents or modulation of gene expression. Drugs can be added easily to cultures of differentiating P19 cells. These cells are, however, diploid so selection of recessive genetic mutations is difficult. Fortunately, P19 cells are excellent recipients of DNA transfected by calcium phosphate or electroporation procedures.

Recombinant genes transfected and stably integrated into P19 cells appear to offer more rewarding experimental possibilities than transient expression assays. Two kinds of experiments have been performed: (i) the transfected gene may be comprised of a tissue-specific promoter driving a reporter gene whose expression in various cell types is to be assessed, or (ii) the transfected gene may encode a product expected to alter the developmental program, immortalize a subset of cells, or in some other way modulate the normal course of events.

Promoter activity assay

A variety of genes encoding (putative) transcription factors are modulated during differentiation of P19 cells. These include *oct-3* (Okamoto *et al.*, 1990), *egr-1* (Edwards *et al.*, 1991), *Hox 1.6* (Pratt *et al.*, 1993), *c-myc* (St-Arnaud *et al.*, 1988), and *Rb* (Slack *et al.*, 1993). The nature of the regulated expression of these genes can be attempted using run-on transcription assays and measurements of mRNA stabilities. But definitive characterization of the nature of the regulation can be most easily established using transfection assays of mutant versions of the cloned genes.

Transient transfection experiments using the *oct-3* promoter have established that two DNA sequence elements in its promoter cooperate to mediate the transcriptional turn-off of this gene in RA (Okazawa *et al.*, 1991). Both DNA elements bind different nuclear factors.

The *Wnt-1* gene is expressed in a subset of neurons in murine embryos and is also expressed by RA-treated P19 cells (Schuuring *et al.*, 1989; St-Arnaud *et al.*, 1989). The *Wnt-1* promoter is expressed following transfection of RA-treated P19 cells and its expression appears dependent on a DNA sequence element in the *Wnt-1* promoter to which a specific factor is bound (St-Arnaud *et al.*, in preparation). This factor is present in RA-treated P19 cells, which provides a valuable biological source of material from which its isolation can be attempted. Since no other abundant source of this factor is known, the utility of P19 cells cultures is obvious.

The human cardiac muscle α actin gene is regulated in a cell type and developmentally appropriate fashion following transfection and stable integration into P19 cells (Rudnicki *et al.*, 1988). Experiments utilizing various modified promoter constructs (Pari *et al.*, 1991) have established that the DNA sequence elements required for this promoter to be appropriately expressed in P19 cell transformants are more extensive than the sequences necessary for expression in transient transfection assays in skeletal myoblasts (Minty and Kedes, 1986; Miwa and Kedes, 1987) or primary cultures of cardiac myocytes (Sartorelli *et al.*, 1992). This suggests that the relative importance of sequence elements in gene promoters may differ greatly depending on the number of copies of the reporter gene in the cell of interest and whether the gene is naked DNA or chromatin associated.

Although the transgenic mouse is a more powerful system with which to establish the critical sequence elements of a mammalian promoter, stably integrated DNA in P19 cells approximates the transgenic assay in that the gene under investigation is integrated into chromatin, has a low gene copy number, and experiences the environment of the progenitor cell before that of the cell in which expression is turned on.

Transfected genes might alter differentiation

To definitively establish a role for a gene product in differentiation, one needs to experimentally modulate its expression. This can be accomplished in P19 cells by transfection of chimeric genes in which the coding region of the gene under investigation is inserted in a eukaryotic expression vector in the sense or antisense orientations. Antisense expression vectors should reduce the extent of expression from the endogenous gene, while a sense expression vector should augment expression. Modulation of a developmental event should allow one to deduce whether a gene product is necessary and/or sufficient for that event.

The precision with which modulation of genetic expression can be achieved with transfected genes offers very powerful methodology for investigations of molecular mechanisms. Apart from the ease of creating transfected P19 cells, this system is also insensitive to the nature of the transgene. A number of constructs one might wish to insert into transgenic animals are likely to be embryonic lethals — a phenotype not compatible with perpetuation of the transgenic line. P19 cells should be able to sustain expression of all constructs except those encoding cell lethals, allowing for a much broader range of potential transgenes to be assayed for their effects on development. There are technical reasons why experiments of this type are difficult even in P19 cells (see below); however, some interesting claims have already been made. For example, an antisense expression vector directed against the neuron-specific microtubule associated protein-2 (MAP2) mRNA may have had an effect not only on the elaboration of neuronal processes but also on the number of neurons formed following RA treatment (Dinsmore and Solomon, 1991).

Sense expression vectors encoding various oncogenes have had effects on P19 cell cultures. The transforming *c-ras* oncogene had no effect on differentiation but resulted in the immortalization and transformation of a subset of cells in both RA- and DMSO-treated cultures (Bell *et al.*, 1986). Some clones of P19 cells expressing transfected *c-fos* (Ruther *et al.*, 1985), *c-jun* (De Groot *et al.*, 1990), *v-src* (Schmidt *et al.*, 1992), and *v-ras* and *v-myc* together (McBurney *et al.*, unpublished results) appear to be «differentiated» in that the cells no longer express embryonal carcinoma antigens and they do not differentiate in RA. These transformed cells are continuous lines whose properties do not resemble any of the cells normally formed by differentiating P19 cells. The significance, if any, of these observations remains obscure.

One of the reasons for transfecting P19 cells with oncogenes is to attempt to arrest differentiation and in the process recover immortalized cells frozen at intermediate stages of differentiation.

Apparently neural cells with various developmental potentials can be recovered following immortalization with oncogenes such as the SV40 T antigen (Whittemore *et al.*, 1991) or by culturing in EGF (Reynolds and Weiss, 1992). No reports of P19 cells arrested by an oncogene at intermediate developmental stages has appeared; however, a bipotential cell capable of differentiating into skeletal muscle and connective tissue was isolated from DMSO-treated P19 cultures where the developmentally «arrested» cells were selected by growth in soft agar (Rudnicki *et al.*, 1989).

Achieving efficient stable expression of transfected genes in P19 cells has proven more problematic than for other continuous cell lines. We find that P19 cells transformed by the *neo* or *hyg* selective genes driven by the mouse *Pgk-1* promoter are recovered at frequencies of about 10^{-3} from the transfected cell population (McBurney *et al.*, 1991). However, the cells must be maintained under continuous selective pressure because even clonal populations of transformed P19 cells segregate non-expressing variants that can rapidly become predominant in the population. This instability appears to be exacerbated by the trauma associated with cryopreservation. Differentiation sometimes results in reduced expression of the transfected gene.

Not all eukaryotic promoters work well in EC cells. In particular, the murine sarcoma virus (MSV) promoter is transcribed very poorly probably due to the presence of a repressor which binds the enhancer (Tsukiyama *et al.*, 1992). In addition, the murine mammary tumor virus (MMTV) promoter is also weak and not inducible because EC cells do not express the glucocorticoid receptor. In our experience, constitutive expression can be effectively achieved in P19 cells with the promoters from the mouse phosphoglycerate kinase *(Pgk-1)* gene, and from the viral promoters from Rous sarcoma virus (RSV) and the SV40 early region. However, many experiments would benefit from the use of inducible expression of the transfected gene. Inducible systems derived from bacterial genes should accomplish this (Baim *et al.*, 1991).

Conclusion

Cultures of embryonal carcinoma or embryonic stem cells can be used as an abundant source of developmentally pluripotent material for studies concerning the cellular and molecular aspects of early differentiation. The major advantages of the P19 cells are that they are easy to maintain in culture, they can be genetically manipulated with ease, and most importantly, their differentiation can be reproducibly controlled with the use of inducers of differentiation such as RA and DMSO. The P19 system should be most useful for investigating the molecular mechanisms by which differentiating cells become allocated to the various lineages, and in this regard it is being used as a source of material for identifying novel components of the embryonic decision-making apparatus, such as novel tyrosine kinases (Howell *et al.*, 1991) and extracellular factors (Mummery *et al.*, 1991). The embryo will be the ultimate test of the validity of new information that emerges, but it seems clear that the relatively simple P19 system offers important technical advantages for certain kinds of studies.

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