SHORT COMMUNICATION

*In vitro* culture of human foetal colonic epithelial cells and their transformation with origin minus SV40 DNA

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Colorectal carcinoma is the second most common cancer in the Western World and its geographical variation in incidence implicates environmental factors as major causative agents. This raises the possibility of identifying the carcinogens and/or tumour promoters concerned and by neutralising or eliminating them from the diet, reduce the cancer incidence. Human colorectal carcinoma cell lines, which are tumorigenic in athymic nude mice can be readily established in *vitro* (Fogh et al., 1977; Brattain et al., 1983), but the normal and pre-malignant epithelium of the human colon, from which the carcinomas develop has proved much harder to grow (Franks, 1976; Moyer, 1983; Paraskeva et al., 1984). This has considerably restricted studies into the complex multi-stage process of human colorectal epithelial cell transformation, with few reports of transformations *in vitro* (Moyer & Aust, 1984, 1987). In this paper we describe a method for the routine culture of human foetal colonic epithelial cells and their subsequent transformation with origin minus SV40 DNA, to generate altered cell lines with considerably extended *in vitro* growth capacities. SV40 DNA was chosen as the transforming agent since SV40 has been reported to transform a wide variety of human epithelial cells, including those of the colon (for a review see Chang, 1986) and because many aspects of SV40 genetics and biology are well defined (Tooze, 1980). The isolation and characterisation of SV40 transformed human foetal colonic epithelial cell lines will prove invaluable for studying the biology of tumour promotion and progression in a major human cancer and the effects of transformation on the differentiation pathway of colonic epithelium.

Standard growth medium and culture conditions have been described in detail previously (Paraskeva et al., 1984). Briefly, cells were routinely grown on collagen-coated petri dishes in the presence of Swiss 3T3 feeder cells (1-2 x 10⁴ cells cm⁻²), at 37°C in 5% CO₂ in air incubator. Plastic petri dishes were coated with a film of collagen type 4 (Sigma, human placental collagen) at 0.4 mg collagen per 5 cm dish by placing a thin layer of collagen solution (prepared in 1 part glacial acetic acid in 1,000 parts distilled water) on the dish and allowing it to dry overnight at 37°C. The 3T3 feeder cells had previously been treated with mitomycin C at a concentration of 10 μg/ml⁻¹ for 2 h. Standard growth medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% foetal bovine serum (FBS), hydrocortisone sodium succinate 1 μg ml⁻¹, insulin 0.2 units ml⁻¹, glutamine 2 mM, penicillin 100 units ml⁻¹ and streptomycin 100 μg ml⁻¹. The culture medium was changed twice weekly. Specimens of descending colon were obtained from therapeutically aborted human foetuses (8-24 weeks gestation). Following washing in ice cold medium and PBS, specimens were cut into 5 mm pieces, transferred to a universal containing 20 ml of EDTA solution (0.75 mM EDTA in PBS) and rotated for 1.5 h at 37°C. Epithelial organoids were collected from the supernatant, washed twice in growth medium and plated under standard conditions. Rapidly growing colonies of cells with a typical cuboidal epithelial cell morphology were observed after 48 h and these continued to proliferate until confluent (Figure 1a). Their epithelial nature was confirmed by positive staining with the monoclonal antibody LE61 (Lane, 1982) which reacts with keratin 18 filaments of simple epithelia and by ultrastructural analysis which revealed the presence of desmosomes (results not shown). Routine fluorescent staining of these cells with Hoechst 33258 (Chen, 1977) found them negative for mycoplasma contamination.

Primary cultures of foetal colonic epithelial cells grew rapidly to confluence, but could not proliferate following

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Figure 1 (a) Phase contrast photograph of a primary culture of foetal colonic epithelial cells (x 150); (b) Immunofluorescence of FC/A a pSVori transformed foetal colonic epithelial cell line showing SV40 specific intranuclear T antigen (x 600); (c) Immunofluorescence of FC/A showing tonofilaments characteristic of epithelial cells (x 600).
trypsin/EDTA (0.1% w/v) dispersion to single cells. Consequently, passage was achieved by using the neutral protease, dispase, and replating the epithelial cells as small clumps at high density (split ratio 1:2), as previously described for pre-malignant colonic adenoma cells (Parakova et al., 1984). Using this protocol, foetal colon epithelial cells could be passaged approximately three times in vitro before senescing.

Independent colonic epithelial cell cultures from two 16 week foetuses were grown to confluence, re-plated and after 4 days transfected overnight with an origin defective mutant of SV40 cloned into plasmid p5A18 (pSVori- Gluzman et al., 1980), using the calcium phosphate precipitation method (Spandolis & Wilkie, 1984). Transfected cells were recognised as rapidly growing foci (12-23 transformants per 10 µg pSVori- DNA) which were picked after ~4 weeks. Initially foci were picked with dispase and for the first 5 passages these cells were subcultured with this agent at a split ratio of 1:4 under standard growth conditions and with standard growth medium, described above. Thereafter, cells were passaged with trypsin/EDTA (0.1% w/v) and cultured without feeder support in DMEM supplemented with 5% FBS at a split ratio of 1:20 to 1:100. Under these conditions petri dishes were not coated with collagen. Forty-seven foci were initially picked and three of these, derived from one foetus, were designated FC/A, FC/B and FC/C and chosen for further study. Each of these foci came from different petri dishes and therefore were known to represent independent events. Anchorage independent growth was assayed by a method similar to that of MacPherson and Montagnier (1964). Cells were suspended in 1.5 ml of 0.33% agarose (Sea Plaque, Miles Laboratory) in the appropriate medium and seeded over 5 ml of a base layer of 0.5% agarose using 5 cm Petri dishes. Colonies of >50 cells were scored after 4 weeks.

Clonogenicity in monolayer was tested by plating 200 cells per 25 cm². Cultures were checked immediately for cell aggregates and scored after 4 weeks for epithelial colonies. Cells were tested for tumorigenicity by s.c. injection (4 x 10⁶ cells) into 3 to 4 weeks old athymic ICRF (Imperial Cancer Research Fund) nu/nu nude mice.

Using monoclonal antibody PAB419 (Harlow et al., 1981; Crawford et al., 1982) all 3 independent lines, FC/A, FC/B and FC/C stained positive for SV40 specific intranuclear T antigen (Figure 1b). Furthermore, the epithelial nature of these pSVori- transformants was confirmed by the localisation of keratin 18 filaments (Figure 1c) and the presence of desmosomes at the ultra-structural level (results not shown), their morphology being similar to normal foetal epithelial cells. All 3 transformed cell lines displayed significant anchorage independent growth (Table I). When plated at low density in monolayer cultures FC/A and FC/B formed colonies but no growth was observed with normal foetal colonic epithelium or the FC/C cell line (Table I). FC/C

Table I Clonogenicity of normal and pSVori- transformed foetal colonic epithelial cells

| Cells   | Colony forming efficiency in monolayer (%) | Colony forming efficiency in agarose (%) |
|---------|-------------------------------------------|------------------------------------------|
| Normal  | 0.0                                        | 0.0017±0.0009                            |
| FC/A    | 4.5±1.3                                    | 0.339±0.02                               |
| FC/B    | 2.6±1.1                                    | 0.331±0.054                              |
| FC/C    | 0.0                                        | 0.169±0.038                              |

*Colony forming efficiency in monolayer was calculated at low density as described in the text; Anchorage independent growth was calculated by plating 5 x 10⁶ cells/5cm petri dish as described in the text; Normal colonic epithelial cells derived from 16 week foetuses were used after one in vitro passage; FC/A, FC/B and FC/C represent 3 independently derived pSVori- transformed foetal colonic epithelial cells. Results represent the mean ± s.d. of triplicate cultures.

Figure 2 Histogram showing spread of chromosome numbers in three pSVori- transformed foetal colonic cell lines. All 3 cell lines FC/A, FC/B, FC/C are aneuploid.
formation of human epithelial cells. Generally, SV40 transformed human epithelial cells rarely escape ‘crisis’ and progress to become tumorigenic (Chang, 1986; Brown & Gallimore, 1987), suggesting that they are incompletely transformed and that other events are necessary for the full expression of the malignant phenotype. It is possible in those cells that do become tumorigenic that the continual re-arrangement of chromosomes that begins soon after transformation with SV40 (Chang, 1986) could activate cellular mechanisms that in rare circumstances may result in a unique variant escaping crisis and progressing in vitro. Such mechanisms could include for example the activation of cellular proto-oncogenes and/or the generation of homozgyosity at tumour suppressor loci (Solomon et al., 1987). The non-tumorigenic pSVori- transformed foetal colonic epithelial cells described in this report could be exploited to test whether putative human dietary tumour promoters such as the bile acid, deoxycholic acid, which has been shown to induce mitotic aneuploidy (Ferguson & Parry, 1984), would select for immortal and/or tumorigenic variants. This approach could be extended to determine whether such tumour promotors would select for populations that are more susceptible to malignant transformation by dietary carcinogens. Carcinoma cells have many cellular genes activated that are normally associated with embryogenesis and cellular development (Uriel, 1979). Therefore SV40 transformed human foetal epithelial cells may require fewer subsequent events to produce tumorigenic phenotypes than SV40 transformed adult cells. Thus pSVori- transformed human foetal colonic epithelial cells may provide a sensitive system with which to test for tumour promoters and carcinogens thought to be involved in an important human cancer.

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