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Cilia in cell-cultured fibroblasts

I. On their occurrence and relative frequencies in primary cultures and established cell lines

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INTRODUCTION

The purpose of this report is to present electron microscopic evidence of cilia in fibroblasts grown in conventional monolayer cultures. The ciliary type in question has been described on many occasions in a wide variety of vertebrate and invertebrate tissues (see Barnes, 1961; Sorokin, 1962; Zeigel, 1962; Dahl, 1963; and Wheatley, 1967*a* for further references). It has been referred to as the '9+0' ciliary type but more recently as the 'primary' ciliary type (Sorokin, 1968). Sorokin (1968) has established that its existence is not related to that of a '9+2' cilium nor is it involved in the formation of a ciliated epithelium. While the presence of '9+0' or primary cilia has been repeatedly documented in many tissues, and their persistence has been demonstrated in pieces of chick tissue placed in explant culture (Sorokin, 1962), the only report of their presence in dissociated cells in culture has come from Stubblefield & Brinkley (1966), who noted cilium formation in Chinese hamster fibroblasts which had been previously exposed to Colcemid.

The possibility that fibroblasts grown in cell culture may develop cilia was investigated, since an *in vitro* system would almost certainly facilitate further investigations into their development and functional significance.

MATERIALS AND METHODS

Primary culture and early subcultures of embryo fibroblasts

Primary, secondary and tertiary cultures of fibroblastic cells were grown from rat, mouse, Syrian hamster and chick embryos. After decapitation, the embryos were minced with fine scissors and the cells dissociated by treatment with trypsin. Isolated cells were washed once, seeded into 60 mm Petri dishes containing 5 ml growth medium and gassed with 1 atmosphere of 5 % CO_2/95 % air. The cultures were examined daily and usually subcultured once or twice. Primary, secondary and tertiary cultures in which there were exclusively or almost exclusively fibroblastic cells were selected for electron microscopy; more than 90 % of the cultures were of this kind. Although the designation 'fibroblastic' rests predominantly on the morphological appearance

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of the cells, in a number of the cultures studied electron microscopically both intra- and extracellular evidence of fibre production was observed.

Rat embryo fibroblasts were grown in Hanks's medium supplemented with 0·25 % lactalbumin hydrolysate and 10 % inactivated human serum. Crystamycin was present at 200 units/ml. For comparative purposes cells were also grown in Eagle's medium with 10 % foetal calf serum with Crystamycin as above.

Syrian hamster and chick embryo fibroblasts were cultured in Eagle's medium with 10 % foetal calf serum and Crystamycin.

Mouse embryo fibroblasts were initially grown in Hanks's medium plus 10 % calf serum but were usually subcultured to Eagle's medium containing 10 % calf serum, 10 % tryptose phosphate broth and Crystamycin.

Established fibroblastic cell lines

Except where indicated, the Eagle's medium was used with 10 % calf serum, 10 % tryptose phosphate broth and Crystamycin (200 units/ml). The cell lines used were:

BHK 21/C13—originating from a culture of Syrian hamster cells (Stoker & Macpherson, 1964).

BHK21/C13 PyY—a clone of polyoma-transformed cells (Macpherson & Stoker, 1962).

NIL 2—a line derived from a Syrian hamster culture and which has been through episodes of fibroblastic and epithelioid characteristics (Diamond, 1967).

NIL 2/Py—a clone of polyoma-transformed NIL 2 cells.

Chinese hamster Don/C—obtained from the American Collection of Type Culture, Rockville, Md, U.S.A., and originally described by Hsu & Zenzes (1964).

Chinese hamster CH/3—a subline taking its origin from the Don/C line, produced and maintained for some years at the M.R.C. Virus Research Unit, Carshalton, Surrey. It was grown in Eagle's medium with 10 % foetal calf serum and Crystamycin.

L-929—a long established line of mouse fibroblasts (Sanford, Earle & Likely, 1948). It was cultured in Eagle's medium with 5 % calf serum and Crystamycin.

The established cell lines were grown as monolayers in glass bottles and were collected for electron microscopy before they had become confluent.

Preparation of cells for electron microscopy

Cells were removed from the culture vessel by treatment with 0·25 % trypsin dissolved in basal medium. The cells were spun down at 1000 rev/min for 2 min and the supernatant discarded. An excess of cold (4 °C) fixative was poured down the side of the centrifuge tube in such a way as to dislodge the pellet and allow it to be fixed from all sides without breaking up. Two fixatives were regularly employed, veronal-buffered OsO_4 (Palade, 1952), and glutaraldehyde followed by osmification (Sabatini, Bensch & Barrnett, 1963). Fixation was continued for 1–2 h at 4 °C before dehydration in absolute ethanol. The fixed material was embedded in Epon 812 (Luft, 1961) and sectioned with glass knives on either an LKB or a Cambridge Huxley ultramicrotome at 60–90 nm. Sections were mounted on uncoated copper grids and stained with lead solutions (Karnovsky, 1961) with or without prior staining with 2 % aqueous uranyl acetate. Glutaraldehyde-fixed cells were invariably double-stained. The sections were examined in a Siemens Elmiskop I electron microscope at an accelerating voltage of 80 kV.

OBSERVATIONS

Primary culture and early subculture fibroblasts

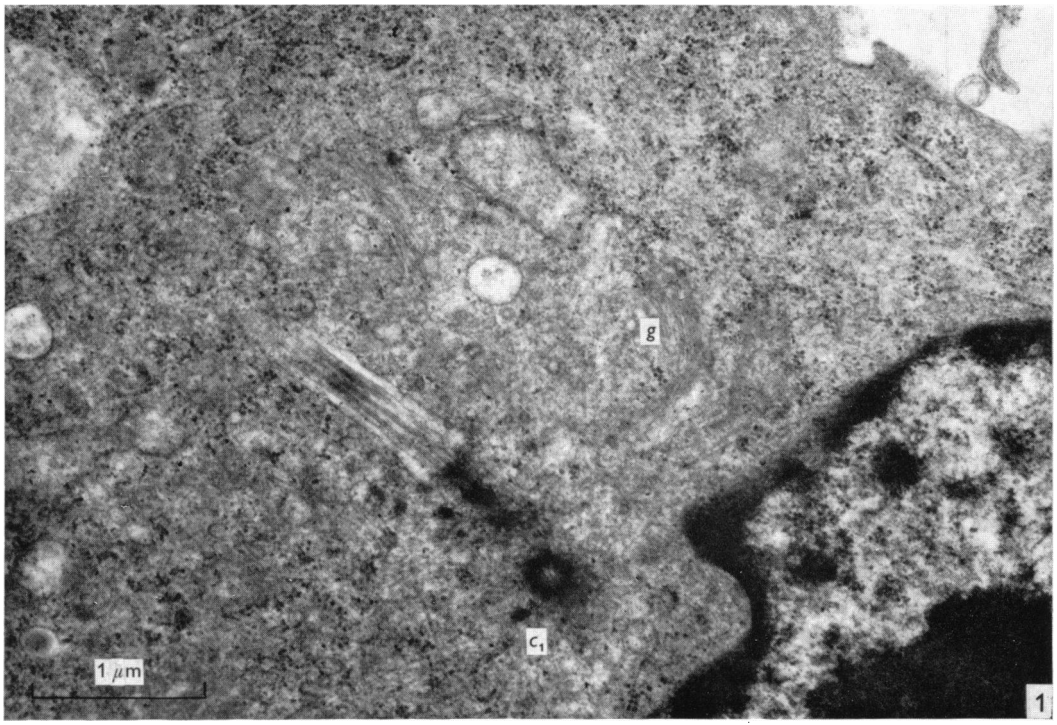
Rat. Cilia were frequently found in ultrathin sections of rat embryo fibroblasts (Fig. 1). They were similar in morphology to those described elsewhere (Barnes, 1961; Zeigel, 1962; Dahl, 1963; Allen, 1965; Wheatley, 1967*a*, *b*; Sorokin, 1968). In some sections the length and degree of differentiation which cilia can attain became apparent (Fig. 2), ciliary shafts of 3–4 μm in length having been observed. Most cilia were enclosed in a deep invagination of the cell membrane since they usually arose from centrioles lying in a juxtanuclear position. From here they sometimes extended beyond the invagination into the surrounding medium; seldom were cilia situated in an entirely superficial position. In transverse section it was established that the cilia lacked central fibres (Fig. 3) and therefore they had a 9+0 formula. As reported in cilia of other body tissues (see Discussion), one peripheral pair of fibres tended to move into a more central position some distance along the ciliary shaft and in the more distal regions, misalignment and discontinuity of the fibres led to greater disorganization of the 9+0 pattern.

Table 1. *Number of cilia and centrioles detected during investigation of ultrathin sections of cultured fibroblasts*

	Single centrioles sectioned		Diplosomes sectioned		Single cilia sectioned		
Cell type	Total	No. with basal body characteristics	Total	No. with basal body characteristics	Total	No. with their centrioles (basal bodies) sectioned	Biciliates sectioned
Primary cell cultures							
Rat embryo fibroblast	64	23	15	6	33	22	1
Mouse embryo fibroblast	69	17	12	3	27	19	2
Hamster embryo fibroblast (Syrian)	63	18	12	4	26	16	2
Chick embryo fibroblast	65	10	21	4	8	8	0
Established cell lines							
BHK 21/C13	60	20	19	6	35	23	2
BHK 21/C13/PyY	70	24	15	11	29	20	1
NIL 2	50	10	13	5	20	9	2
NIL 2/Py	36	11	10	2	8	6	0
Chinese hamster Don/C	179	48	68	18	31	20	1 (+1)*
Chinese hamster CH/3	102	26	33	7	17	11	0
L-929	89	(1?)	24	(1?)	0	0	0

* In one cell, a collection of centrioles was seen of which at least 3 had ciliary processes (see Fig. 9).

The majority of diplosomes which were associated with cilia showed the typical arrangement of one centriole at right angles to the axis of its partner (Fig. 1). In some instances, however, the centrioles lay parallel and both displayed basal body features, suggesting that more than one cilium may be produced. On one occasion in a rat embryo fibroblast, definite evidence of a biciliate cell was found.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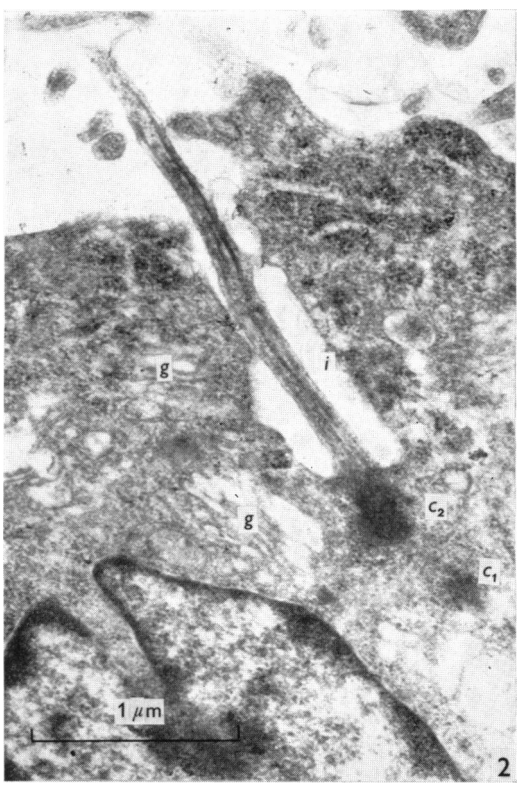
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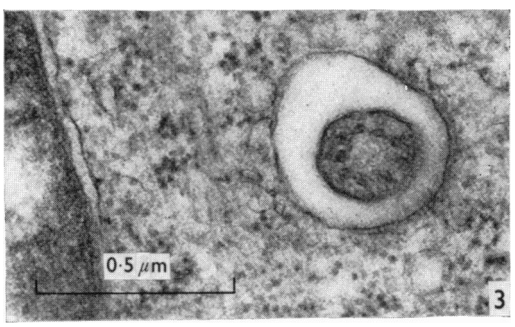
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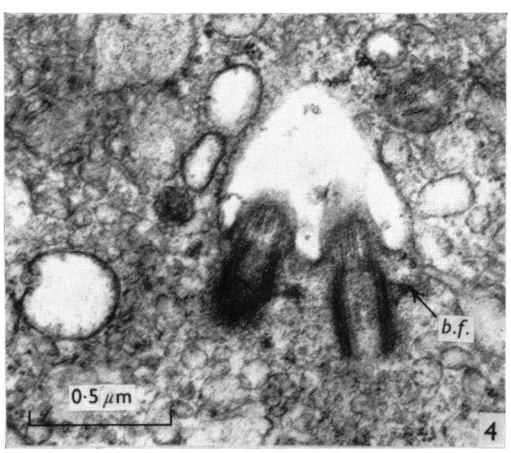
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Table 1 gives details of the actual numbers of cilia and centrioles detected in rat embryo fibroblasts and the other cell types examined. No attempt was made to count the number of cells examined. Essentially the analyses in Table 2 gives an estimate of the expectancy with which one might detect a cilium as compared with a centriolar apparatus during random sectioning. In the rat cells a total of 101 centriolar apparatuses were recorded, 79 examples in which cilia were either not sectioned or genuinely absent and 22 examples in which they were associated with cilia. A total of 34 cilia (33 single, 1 biciliate) were seen, of which 11 were sectioned in planes which

Table 2. *Analysis of the relative frequencies of basal bodies and cilia in the fibroblastic cell types examined*

Cell type	Total no. of centriolar apparatuses (with and without cilia)	Total no. with basal body characteristics (with and without cilia)	Ratio of total basal bodies / total centrioles	Total no. cilia*	Ratio of total cilia / total centrioles
Primary cell cultures					
Rat embryo fibroblasts	101	51	0·50	34	0·34
Mouse embryo fibroblasts	100	39	0·39	29	0·29
Hamster embryo fibroblasts	101	38	0·38	28	0·28
Chick embryo fibroblasts	94	22	0·23	8	0·085
Established cell lines					
BHK 21/C13	102	48	0·47	37	0·36
BHK 21/C13/PyY	105	55	0·52	30	0·29
NIL 2	72	24	0·33	22	0·31
NIL 2/Py	52	19	0·37	8	0·15
Chinese hamster Don/C	267	86	0·32	17	0·12
Chinese hamster CH/3	146	44	0·30	33	0·12
L-929	113	2 (?)	—	0	—

*Biciliates count as one.

Key to figures

Abbreviations:

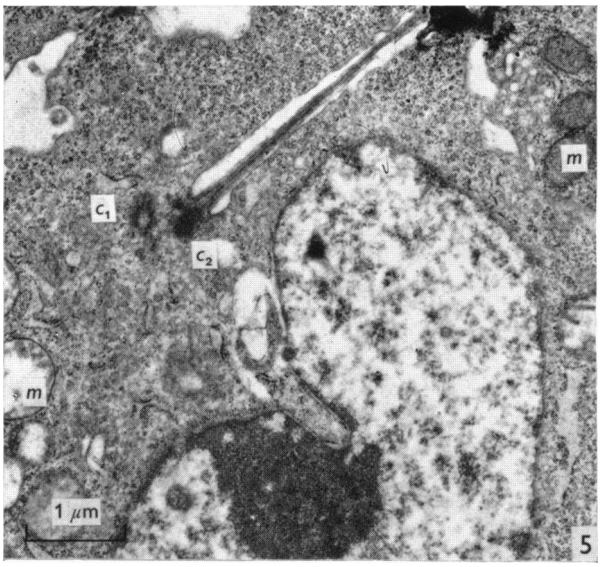
b.f.	basal foot	*i*	invagination of cell membrane
c_1	proximal centriole	*m*	mitochondrion
c_2	distal centriole	*n*	nucleus
d	daughter centriole	*v*	vesicle
g	golgi apparatus		

Fig. 1. Rat embryo fibroblast with a cilium coming from a diplosome lying in a juxtanuclear position. The proximal centriole, c_1, has been sectioned transversely. Glut./U.A.-lead.

Fig. 2. Rat embryo fibroblast from a primary culture. A thick section which includes the diplosome, the membranous invagination and a cilium with a shaft of about 3 μm in length in which fibres can be seen to run nearly to the distal region. Glut./U.A.-lead.

Fig. 3. Transverse section of the proximal region of a cilium in a rat embryo fibroblast showing the 9+0 fibre arrangement. Glut./U.A.-lead.

Fig. 4. Mouse embryo fibroblast. Oblique sections of the bases of two cilia in a mouse embryo fibroblast. Basal foot (*b.f.*). Glut./U.A.-lead.



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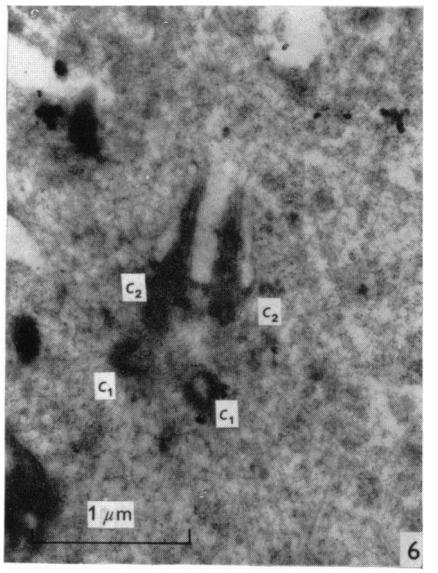

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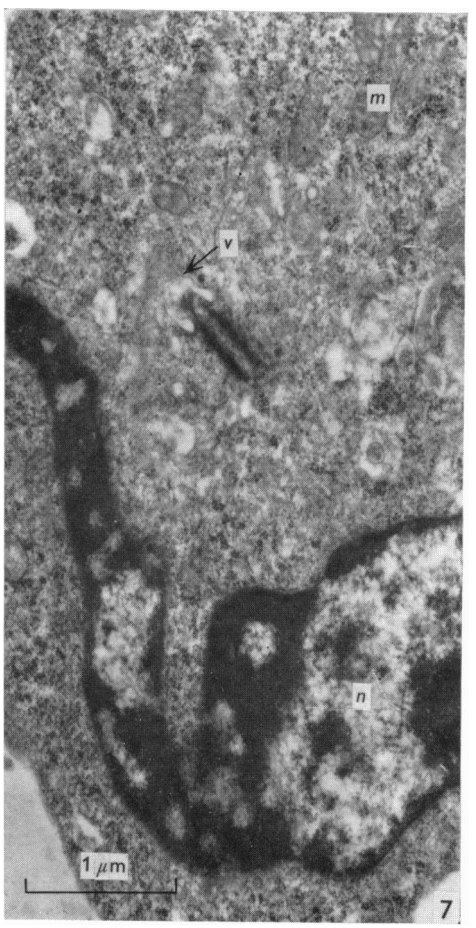

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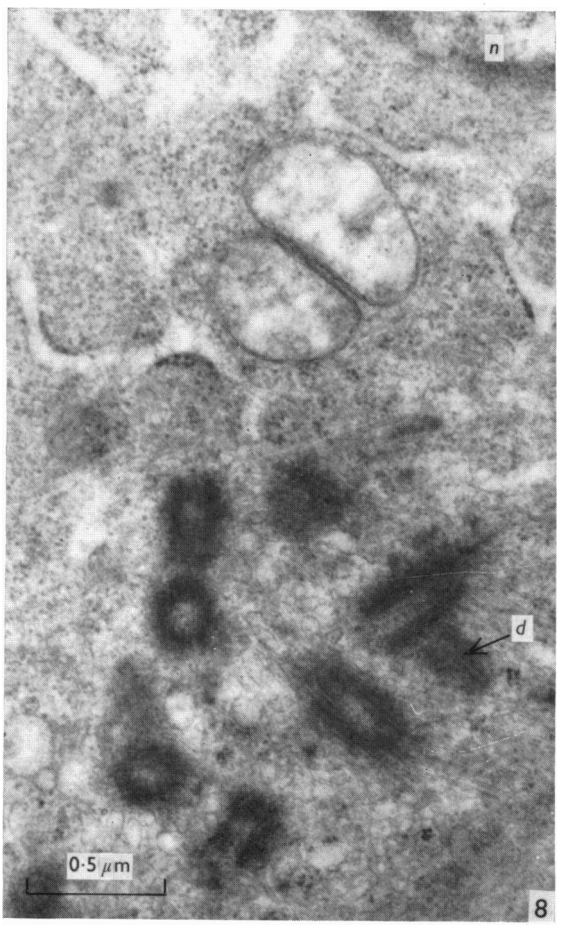

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did not reveal their associated centrioles. The over-all ratio of detection of cilia compared with centrioles was therefore of the order of 1 to 3.

No differences were found in the relative frequencies of cilia in primary, secondary or tertiary cultures of rat embryo fibroblasts; furthermore, cells grown in Hanks medium showed no difference from those grown in Eagle's medium. Cilia were morphologically similar after osmic acid or glutaraldehyde fixation but the latter gave better preservation of cytoplasmic microtubules and filaments in and around the cilium-centriole complex.

Mouse and hamster. The fibroblasts grown from embryos of these two species possessed cilia with a frequency closely comparable to that of rat embryo fibroblasts (see Tables). Morphologically the cilia from all three different cell types were indistinguishable. Biciliate cells were found occasionally (Fig. 4).

Chick. The fibroblasts from chick embryos possessed fewer centrioles with basal body features, and relatively fewer cilia than the primary mammalian fibroblasts ($P < 0{\cdot}0005$). Furthermore, the cilia were less well differentiated than those in the other species, giving the appearance described as rudimentary by Sorokin (1962).

Established cell lines

BHK 21/C13 and BHK 21/C13/PyY. Cilia were detected in BHK 21/C13 cells with a frequency which compares closely with that of primary hamster embryo fibroblasts. In form the cilia of BHK 12/C13 cells were often well differentiated and examples reaching over 3 μm in length were not infrequent (Fig. 5). The polyoma-transformed subline BHK 21/C13/PyY proved indistinguishable from its progenitor line with respect to both the frequency and morphology of their cilia. Biciliate cells were found in both lines (Fig. 6) and multiple centrioles, usually 4 or more, were occasionally seen.

NIL 2 and NIL 2/Py. There was a high incidence of cilia in NIL 2 cells, comparing closely with primary hamster embryo fibroblasts and BHK 21/C13 cells (see Table 5). Studies on the polyoma-transformed subline NIL 2/Py were less extensive due to unfavourable material. Although only 8 cilia were detected, the ratio of cilia to centrioles was not significantly lower than in NIL 2 cells ($P < 0{\cdot}2 > 0{\cdot}1$). Biciliate cells were found in the NIL 2 line.

Chinese hamster Don/C. Because of previous studies by Stubblefield & Brinkley (1966) on this cell line, considerable attention was paid to it in the present study. The frequency of detection of cilia compared to centrioles was significantly lower than in primary hamster embryo fibroblasts ($P \ll 0{\cdot}0005$, see Tables) and lower than in established Syrian hamster cell lines. Unfortunately no primary cultures of Chinese

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Fig. 5. BHK 21/C13 fibroblast with a long cilium (over 3 μm), completely ensheathed by an invaginated cell membrane. Glut./U.A.-lead.

Fig. 6. BHK 21/C13 fibroblast with two cilia each with an associated diplosome. The fibroblast appeared to have only one nucleus. Glut./U.A.-lead.

Fig. 7. Chinese hamster fibroblast of the CH/3 line. An elongated centriole which appears to be a basal body with a vesicle at one end (*v*). Glut./U.A.-lead.

Fig. 8. Collection of centrioles in a Chinese hamster fibroblast, Don/C line. The group probably formed by repeated division of centrioles without migration apart since evidence of replication is still apparent (*d*). Glut./U.A.-lead.

embryo fibroblasts were available for comparison. However, morphologically the cilia were similar to those seen in the other cell lines. On several occasions multiple centrioles were found in Don/C fibroblasts (Fig. 8) and in one instance there was evidence of multiple cilium formation (Fig. 9). By serial sectioning, it was established that at least two further centrioles seen in Fig. 9 also has associated cilia. As far as could be assessed from the serial sections, the cell in question was uninucleate.

Chinese hamster CH/3. Cilia were detected less frequently in CH/3 cells than in primary hamster, mouse or rat embryo fibroblasts. They were also less frequent than in BHK or NIL cells. However, the cilium/centriole ratio proved to be almost

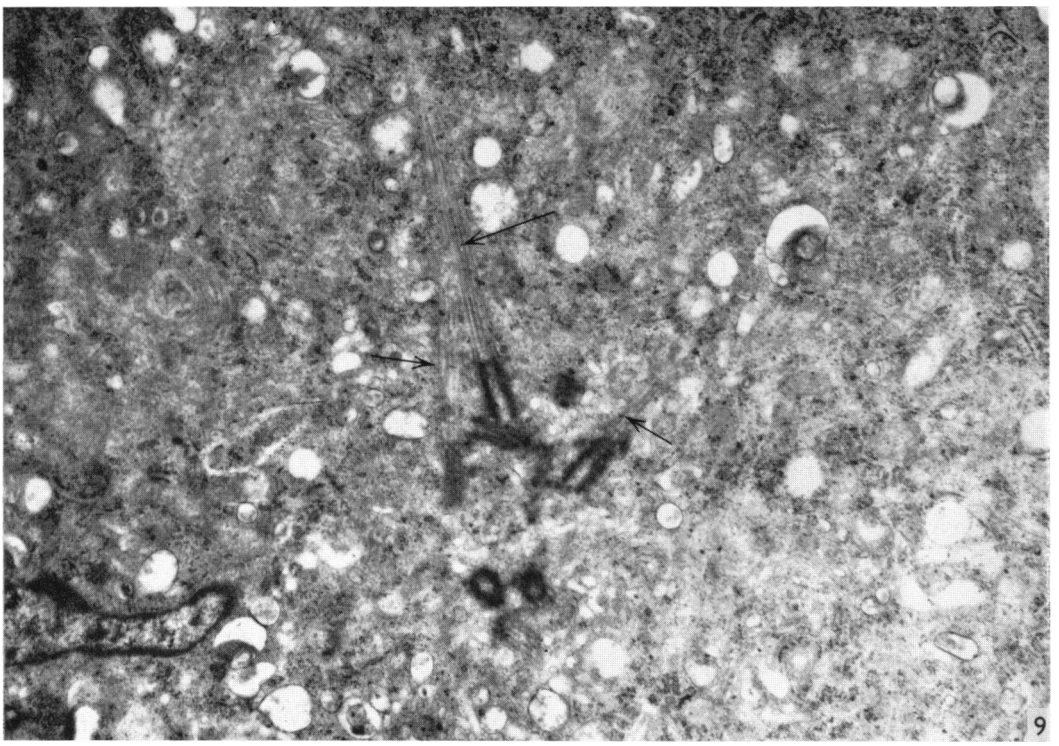

Fig. 9. Collection of centrioles in a Don/C fibroblast with at least 3 or 4 ciliary processes (arrows). Glut./U.A.-lead.

identical with that of the Don/C line (see Table 2). When CH/3 cells were first obtained for study their history was not known. After completion of the work it was discovered that they had taken their origin from the Don/C line (F. Kingsley-Sanders, personal communication). Thus the very close agreement in cilium/centriole ratio between CH/3 and Don/C cells accidentally provided a practical demonstration of the reliability of this method of comparing the relative frequencies of ciliated cells in different cell types.

Cilia in CH/3 usually showed a similar morphology to cilia in other cell lines but occasionally elongated centrioles or basal bodies were observed in them. In some

cases the elongated centrioles were associated with vesicles at one end (Fig. 7), giving the appearance of a cilium forming without proper development of the ensheathing membrane (cf. Dahl, 1967).

L-929. L-strain fibroblasts were obtained for these studies from four different sources. The lack of cilia was a feature common to all four cultured lines although centrioles were observed with a frequency comparable to that in other cell lines (Table 1). Compared with primary cultured mouse embryo fibroblasts, the lack of detection of cilia is a very highly significant difference ($P \ll 0{\cdot}0001$).

DISCUSSION

The presence of cilia in cultured fibroblasts has established that these organelles are not peculiar to differentiated cells in organized tissues, but can be formed by isolated cells, and, more particularly, by cells undergoing rapid proliferation. The presence of cilia in the Chinese hamster fibroblast lines Don/C and Dede has been reported by Stubblefield & Brinkley (1966), who noted that after exposure of the cells to Colcemid cilia developed during the recovery phase. It was their belief that Colcemid had acted as a kind of inducing agent. The present report, however, establishes the ciliated nature of *untreated* fibroblasts from a number of different species, including the Don/C Chinese hamster fibroblast lines, under conventional culture conditions. At present, the reason for the discrepancy between the observations is not understood.

The structure of cilia in fibroblasts grown in culture is the same as that of cilia described in many tissues of the body (Barnes, 1961; Zeigel, 1962; Grillo & Palay, 1963; Dahl, 1963 & 1967; Wilson & McWhorter, 1963; Allen, 1965; Coupland, 1965; Karlsson, 1966; Wheatley, 1967*a*, *b*; Sorokin, 1968). Cilia usually issue from the centrosome region of the cell but seldom occur superficially despite the relatively free surface of cultured cells. From their fibre arrangement they have been referred to as 9+0 cilia in this paper. Others have adopted different formulae, Dahl (1963 & 1967) referring to them as 8+1, Coupland (1965) using 6+2 to 8+2, etc. However, the evidence of Allen (1965), Reese (1965), Karlsson (1966), Dahl (1967), Wheatley (1967*a*, *b*) and others has shown that this is due to the displacement of one of the nine peripheral fibre pairs towards the centre of the ciliary shaft. It is misleading to give formulae of '8+1', '7+2' or '8+2' because the centrally placed fibres in these cilia bear little resemblance to the central fibres of a '9+2' cilium and do not arise from a basal plate. To minimise confusion, the term 'primary' cilia (Sorokin, 1968) may be valuable in future.

To obtain some indication of the relative frequency of cilia in different cell types the not unreasonable assumption was made that all the cells possess centrioles; the number of cilia detected was then related to the number of centrioles found. Considering that random sectioning of fibroblasts will result in the inclusion of the centrioles and the cilium of a cell only when they have been cut in the same plane, the finding of definite evidence of ciliary processes in every third or fourth centriolar apparatus sectioned indicates that the true percentage of cells with cilia in rat embryo fibroblasts is probably very high. The probability that random sectioning will reveal both cilia of a biciliate fibroblast in the same plane is even smaller. The occasional

detection of biciliate fibroblasts further suggests that an appreciable percentage of the cells could possess more than one cilium. There is now considerable evidence of cells possessing two cilia in a number of different mammalian tissues (Barnes, 1961; Vegge, 1963; Stubblefield & Brinkley, 1966; Dahl, 1967; Wheatley, 1967*b*). It is hoped that more accurate information on the incidence of ciliated cells in fibroblast population and on the proportion of cells with more than one cilium will shortly be available from serial sectioning of monolayers in the plane of the cells.

With the method adopted in this study, it has been possible to show that some cell types possess significantly fewer cilia than others, e.g. chick embryo fibroblast compared with mammalian embryo fibroblast, Chinese hamster cells of the CH/3 and Don/C lines compared with primary cultured mammalian fibroblasts, BHK 21/C13 or NIL 2 cells. It also appears that some lines may be devoid of cilia, e.g. L-strain fibroblasts. The possibility that an abnormal subline of the L-strain was examined is unlikely because cells were examined from four separate sources. The number of centrioles observed in these cells was more than sufficient to establish the high improbability of cilia having been missed. It is possible to conclude from the findings with L-strain cells that cilia are not essential for the life of a cell or, at least, for its continued existence in culture. Furthermore, *in vivo* studies have shown that cilia are not ubiquitous since hepatocytes of rats do not develop cilia from their centrioles (Wheatley, 1968). Differences between cell lines in the incidence of ciliated cells may be of considerable value in establishing genetic and environmental factors which control their development.

Comparison of the ratio of detection of cilia to centrioles has shown that 'malignant transformation' *in vitro* by polyoma-virus infection of BHK 21/C13 and NIL 2 fibroblasts, while changing the behaviour of the cells *in vitro* and *in vivo*, produces no detectable alteration in the frequency of cilia. Malignant tissues of the body, like their normal counterparts, have also been found to possess cilia (Wilson & McWhorter, 1963; Cervós-Navarro & Vazquez, 1966).

Multiple centrioles (four or more) have been seen on a number of occasions in culture fibroblasts, in particular in BHK 21/C13/PyY and Chinese hamster Don/C cells. It was not possible to establish how many nuclei were present in every cell in question, but there was rarely any indication that they were multinucleate, as is the case in osteoclasts or giant cells of granulomatous tissue which possess many centrioles (Matthews, Martin, Race & Collins, 1967). The significance of the phenomenon is not known and merits further investigation.

Future work on 9+0 cilia would be greatly facilitated if they could be detected by light microscopy. Attempts to visualize them, including high power phase-contrast and polarizing microscopy, histochemical staining for ATPase and other enzymes, a number of classical staining techniques for cilia and basal bodies and, in particular, silver impregnation by the Nauta method (see Dahl, 1963), have been unsuccessful. Stubblefield & Brinkley (1966) maintain that they have seen a fibroblast cilium by high power phase-contrast microscopy. However, the only *certain* method of detection of 9+0 cilia is by electron microscopy.

The findings presented here form a useful basis for future studies. Since it is now possible to study cilia in relatively homogeneous populations of cells under carefully regulated conditions, problems such as the fate of the cilia during the cell cycle, their

mode of development and the factors affecting it, and possibly their functional significance can be more easily investigated.

SUMMARY

Cilia lacking a central pair of fibres (i.e. '9+0' or 'primary' cilia) have been observed in fibroblasts grown in monolayer cultures. They were detected by electron microscopy of ultrathin sections of fibroblasts from primary cultures (and early subcultures) of rat, mouse, Syrian hamster and chick embryos. An attempt has been made to estimate the relative frequencies of cilia in different cell populations by comparing the ratio of cilia detected/centrioles detected. Cilia were present in the majority of established fibroblastic cell lines examined, but were not found in L-strain cells. Polyoma-transformed BHK 21/C13 fibroblasts did not differ from untransformed fibroblasts in the morphology or relative frequency of cilia. Biciliate fibroblasts occurred in most of the cell types examined, and on one occasion a Chinese hamster Don/C fibroblast with multiple centrioles and cilia was observed.

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