Origin of Cells Cultured *In Vitro* from Human Breast Carcinomas Traced by Cyclin D1 and HER2/neu FISH Signal Numbers

EVA MATOUSKOVA[1], IVA KUDLACKOVA[1], ALENA CHALOUPKOVA[1],
MARKETA BROZOVA[1], IRENA NETIKOVA[2] and PAVEL VESELY[1]

[1]*Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, 166 37 Prague;*
[2]*Department of Cytostatics Preparation, General Faculty Hospital, 121 08 Prague, Czech Republic*

Abstract. *Background: We have developed and optimized a feeder layer method for cultivation of normal human mammary luminal cells and found it suitable for establishing more than 150 primary cultures from individual human breast carcinomas. Here, we investigated if the malignant cells that are in situ additionally characterized by increased numbers of proto-oncogenes can be traced by the FISH method in the ex vivo-derived cultures. Materials and Methods: Paraffin sections from 9 tumors with derived cell cultures kept frozen in our cell bank were screened by FISH for cyclin D1 (CCND1) and c-erb-B2 (HER2/neu) proto-oncogene signal numbers. Results: In 6 tumors (5 primary tumors, 1 cutaneous metastasis), increased numbers of FISH signals were found in 55-99% of cells. Then, the relevant cell cultures were FISH screened; in cell populations maintained for up to 2-6 passages in vitro the incidence of cells with increased FISH signals was found to be low (2-16%). Moreover, the cells with multiplied signals that survived more than one passage in vitro were evidently unable to divide further. However, in all 6 tumors at least a small fraction of cells displaying only two signals of CCND1 or HER2/neu genes was identified directly in invasive tumor structures in the vicinity of cells with multiple signals. Conclusion: Our findings suggest that these invasive tumor cells displaying only two proto-oncogene signals were most probably involved in the initiation and propagation of ex vivo tumor-derived primary cell cultures.*

Correspondence to: Eva Matouskova, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Flemingovo n. 2, 166 37 Prague 6, Czech Republic. Tel: +4202 2018 3537, Fax: +4202 2431 0955, e-mail: matous@img.cas.cz

One of the main problems in the study of neoplastic changes in human breast cancer has been the difficulties encountered in routine cultivation of malignant cells from original primary tumors and metastases (1-3). The normal human mammary gland is composed of two types of epithelial cells: luminal (secreting) and myoepithelial (basal). Cultivation of myoepithelial cells has been solved satisfactorily with complex serum-free or low-serum media (4). However, it is the luminal lineage (positive for cytokeratin 19) that is believed to be the target of malignant transformation (2, 5, 6). Therefore, it was assumed that a successful method for cultivation of normal luminal cells would also support *in vitro* growth of malignant cells (2). Recently, we developed a culture system that allowed for serial propagation of normal luminal epithelial cells on a feeder layer of lethally irradiated 3T3 cells (7). By an analogical approach, we also succeeded in the cultivation of cell populations from small tissue samples of individual primary breast tumors and cutaneous metastases (8). In this way, we successfully established more than 150 primary cultures. More than one third of them could be preserved frozen. In the current study, we investigated if this method also supported the *in vitro* propagation of demonstrably malignant cells from human breast tumors. Chromosomal abnormalities have long been recognized as a distinguishing feature of cancer cells. The HER2/neu and CCND1 genes, that play a key role in the regulation of cell growth, have been considered proto-oncogenes (9-11). They were shown to be amplified and/or overexpressed (by gene amplification or aneuploidy/polyploidy) in about one third of human breast cancers (9-11). Increased numbers of cyclin D1 (CCND1) and/or c-erb-B2 (HER2/neu) signals, as revealed by the FISH method, were therefore considered markers of malignant tumor cells. Nine tumors, with relevant cell cultures kept frozen, were selected for screening by FISH for cyclin D1 (CCND1) and c-erb-B2 (HER2/neu) proto-oncogene signal numbers. In paraffin-embedded tumor tissue, we looked for

Table I. *FISH for cyclin D1 and HER2/neu in tumor paraffin sections and in the derived cell cultures in the 3rd passage.*

Tumor signature		MBC1	BT18	BT1	BT116	BT118	BT122
Histopathology		Cutaneous metastases of infilt. duct. carcinoma	Atypical medullar carcinoma	Infiltrating ductal carcinoma	Infiltrating ductal carcinoma	Infiltrating ductal carcinoma	Anaplastic carcinoma
Age of patient		49	52	45	54	75	76
Paraffin sections	ER	++	–	–	+++	+ –	–
	PR	+	–	–	+++	–	–
	c-erb-B2[a]	++	+	+++	+++	+++	–
	FISH cyclin D1[b]	normal	>99% 3-10 sign.	NS	normal	NS	60% 3-4 sign.
	FISH HER2/neu[b]	55% 3-4 sign.	NS	>99% 3-20 sign.	55% 3-4 sign.	>99% 3-20 sign.	normal
Cultures	FISH cyclin D1[b]	normal	9% 4 sign. 7% 3 or >4 sign.	normal	14% 4 sign.	4% 4 sign. 7% 3 or >4 sign.	8% 4 sign.
	FISH HER2/neu[b]	2% >4 sign.	7% 3 or >4 sign.	normal	3% >4 sign.	8% 4 sign. 4% 3 or >4 sign.	3% 4 sign.

ER, estrogen receptor status; PR, progesterone receptor status; NS, not successful FISH (exact numbers of signals could not be determined)
[a]c-erb-B2 expression revealed by immunohistochemistry
[b]% of cells with increased number of oncogene copies determined by FISH and number of signals in these cells
– negative staining, + to +++ positive staining

malignant cells defined by histopathological evaluation and simultaneously demonstrating increased numbers of FISH signals as an additional marker of malignancy. From the nine prescreened tumors, six suitable pairs of tumors with cultured cells were selected and used for the analysis presented.

Materials and Methods

Preparation of epithelial cells. Mammary epithelial cells were isolated, with the informed consent of patients, from biopsies of 6 human primary breast carcinomas (3 infiltrating ductal carcinomas, 2 anaplastic carcinomas, 1 atypical medullar carcinoma) and 3 cutaneous metastases of infiltrating ductal carcinomas, as described previously (7, 8). Briefly, the tumor tissue was incubated overnight at 37°C in 0.05%-0.1% collagenase A (Roche, Basel, Switzerland) and the cells were seeded on the feeder layer of lethally irradiated 3T3 cells (100 Gy; 2.5×10^4 cells/cm^2). The culture medium was based on Eagle's MEM supplemented with non-essential amino acids, sodium pyruvate, hydrocortisone, insulin, choleratoxin, epidermal growth factor, bovine serum and foetal bovine serum. For FISH, the cells were cultured on glass slides placed in a tissue culture dish containing feeder cells. The growth features of several cell populations used in this work were described in Krasna *et al.* (8). The BT18 cells used here as a representative case (Table I; Figures 1, 2) were obtained from pre-operative biopsy of an atypical medullar breast carcinoma of a 48-year-old patient (8).

Immunostaining of tissues and cultures. Paraffin sections and cell cultures were immunostained by standard immunocytochemical methods, as described previously (7, 8).

Fluorescence in situ hybridization. FISH was performed using PathVysion DNA probe kits (Abbott-Vysis, Downers Grove, Illinois, USA) for cyclin D1 and HER2/neu.

Paraffin sections. The sections, 4-5 μm thick, were cut from paraffin blocks and mounted on silane-coated slides. One section was stained with hematoxylin-eosin and used for microscopic confirmation of the invasive part of the carcinoma tissue. For FISH the paraffin sections were baked overnight at 56°C on a slide warmer, deparaffinized, dehydrated and bathed for 20 min in 0.2 N HCl. Then the specimens were pretreated with Target Retrieval Solution (DAKO, Glostrup, Denmark) at 96°C for 40 min and FISH was performed, according to the manufacturer's instructions.

Cell cultures. The cells cultured on the glass slide were fixed for 15 min in 96% ethanol, bathed for 10 min in 0.075 M KCl, fixed again for 10 min in methanol : acetic acid (3 : 1), washed in 2xSSC and dehydrated in ethanol. Then FISH was performed according to the manufacturer's instructions.

The FISH signals were evaluated in non-overlapping nuclei using a fluorescent Olympus BX41 microscope with a UPlanApo 100/1.35 oil immersion objective. The Hitachi camera model KP-M1AP and the LUCIA program (LIM Ltd., Prague, Czech Republic) were used for photography and processing of the signals. In both cultured cells and paraffin sections of the tumor tissue, the total number of signals (CCND1, HER2/neu) in at least 240 cell nuclei per sample were counted.

Results

CCND1 and/or HER2/neu signals in selected tumor tissues and derived cultures. New histological paraffin sections were cut
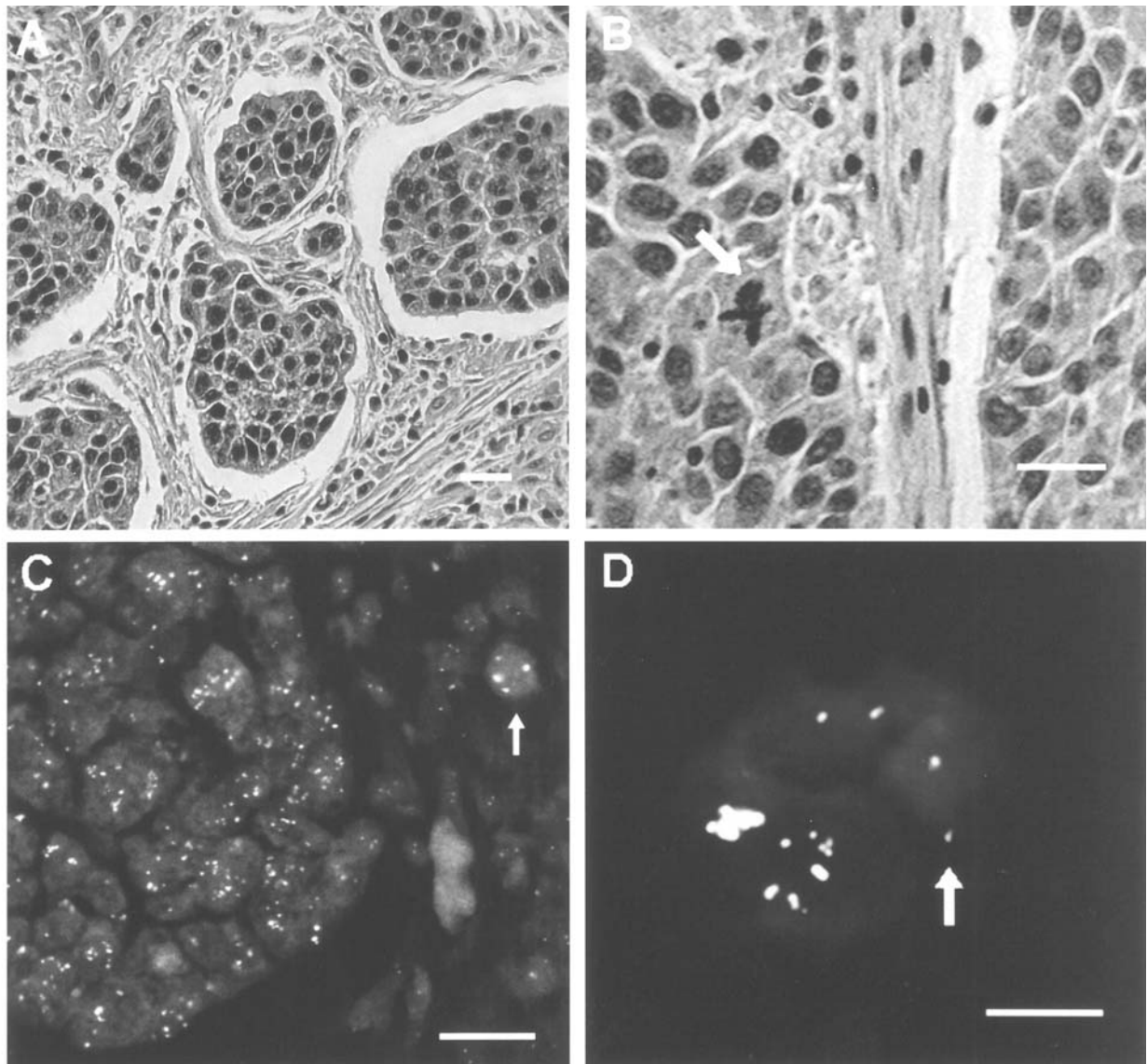
Figure 1. *Paraffin section of the periphery of the BT18 tumor. (A) The tumor consists of rather large cuboid cells (H & E, bar 30 μm). (B) The mitoses in the BT18 tumor were frequently atypical (arrow) (H & E, bar 30 μm). (C) FISH for cyclin D1 in the histological section of the BT18 tumor (bar 30 μm). Note variable numbers of CCND1 signals in individual cells. An increased number of FISH signals also identified the single tumor cell invading the surrounding connective tissue (arrow). (D) CCND1 signals in the island of four invasive cells in the histological section of the BT18 tumor. Two cells display an increased number of the CCND1 gene, while the other two cells only two copies (arrow) of this gene, which is similar to normal cells (bar 10 μm).*

from preserved breast cancer specimens of 9 patients, from which the cultured cells were stored frozen in our cell bank. The paraffin sections were screened by FISH for the number of CCND1 and/or HER2/neu proto-oncogene signals, each gene on a separate slide. Increased signal numbers were found in 5 primary tumors and 1 metastasis, which were then selected for comparison with the *in vitro*-derived cell populations. The main histopathological characteristics of the tumor tissues and the results of FISH are summarized in Table I.

The number of cyclin D1 signals was increased in 2 primary tumors: the BT18 tumor showed 3-10 CCND1 signals in more than 99% of invasive cells (in the majority 6-10 signals/cell); the BT122 tumor showed 3-4 signals in about 60% of cells. The number of HER2/neu signals was increased in 4 tumors: 2 primary tumors (BT1 and BT118) showed 3-20 HER2/neu signals in >99% of cells; 1 primary tumor (BT116) and 1 cutaneous metastasis (MBC1) showed 3-4 signals in about 55% of cells. In 3 cases (2 tumors, 1
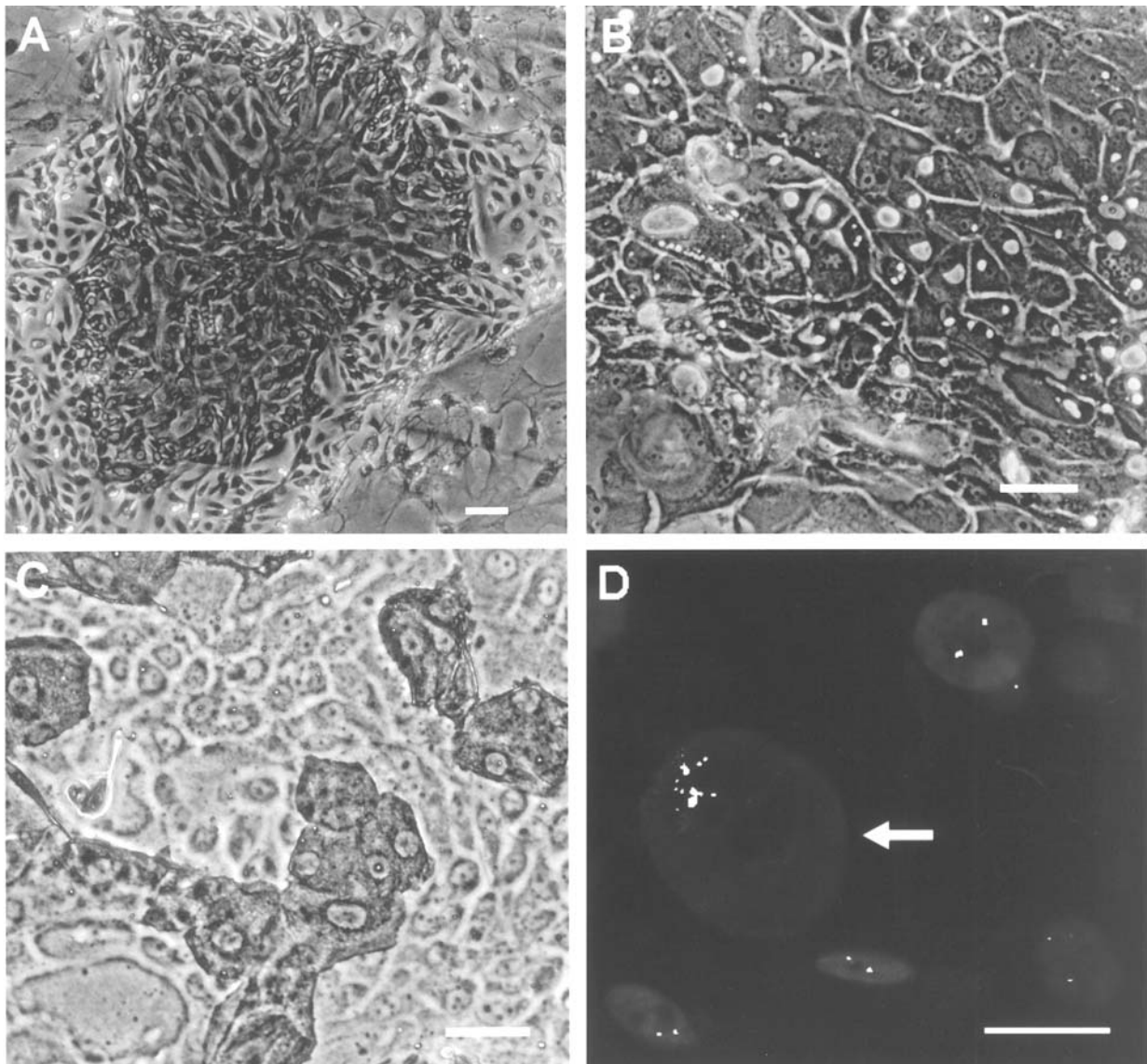
Figure 2. *Human mammary epithelial cells cultured from BT18 tumor tissue. (A) The first passage: 7-day-old colony composed of luminal cells in the centre of the colony (dark cells stained positively for K19) and myoepithelial cells (K19-) growing at the periphery (bar 30 μm). (B) The third passage: BT18 cells were of cuboid shape, frequently with large vacuoles (phase contrast of living cells, bar 30 μm). (C) In the third passage more than 25% of cells were still positive for K19 (bar 30 μm). (D) Cyclin D1 signals (FISH) in cultured cells derived from the BT18 tumor. The majority of cells displayed two CCND1 signals. Cells with more than four signals (arrow) appeared only exceptionally. Such cells were atypically enlarged and did not divide (bar 15 μm).*

metastasis), we found increased signal numbers of only 1 proto-oncogene, while the number of copies of the second proto-oncogene was normal. In the remaining 3 cases (3 tumors), the increase of 1 proto-oncogene was also found, but FISH for the second proto-oncogene was not successful. The number of FISH tests was in all cases limited by the small amount of tumor tissue available.

Cell populations derived from the tumor tissues with increased cyclin D1 or HER2/neu signals were recovered from previously frozen stocks and analyzed by FISH for signals of the 2 proto-oncogenes (Table I). It was found that at the 2nd - 6th passage (Table I summarizes the results of the 3rd passage) none of the cultured populations contained any substantial fraction of cells with increased numbers of CCND1 or HER2/neu signals. The fraction of cells with more than 2 signals varied between 2-16% per population, frequently showing just 4 signals that could be caused by cell tetraploidy – a frequent artifact of tissue cultures.

Moreover, the cells that *in vitro* manifested increased numbers of CCND1 or HER2/neu signals were usually atypical: they were enlarged and appeared isolated, not forming pairs, clusters or colonies (Figure 2D). All this implied that these cells were evidently unable to divide.

The BT18 tumor - a representative case. In order to illustrate our results, the BT18 carcinoma is presented in detail. It was chosen for highly increased numbers of CCND1 signals and very clear FISH imaging.

Tumor histology: The BT18 tumor showed invasive islands of rather large cells of cuboidal morphology (Figure 1A). Immunohistochemistry revealed negativity for estrogen and progesterone receptors. The cells were in the majority positive for cytokeratin 19 (K19+) typical for luminal cells. The proliferative activity was intermediate as detected by Ki67 and PCNA staining (20-30% positive cells). The HER2/neu protein was immunohistochemically detected in 5% of the tumor cells, while FISH for HER2/neu signals failed because of a limited amount of tumor tissue left available. The observed mitoses were frequently atypical (Figure 1B).

Cyclin D1 in the BT18 paraffin section: Increased numbers of the CCND1 signals in more than 99% of invasive cells were found (Figure 1C, Table I). This amplification was so clearly visible that the distribution of all malignant cells (characterized by increased CCND1 signals) in the surrounding connective tissue, including single invasive cells, could be observed (Figure 1C). The numbers of signals for cyclin D1 varied between individual cells, in the majority 6-10 signals per cell. Less than 1% of cells present directly in the invasive lesions showed only 2 CCND1 signals, which is typical for diploid cells. For example, in one of the smallest invasive islands composed of 4 cells, 2 cells expressed only 2 CCND1 signals and the other 2 cells expressed 8 - 10 signals (Figure 1D).

Growth characteristics of cells in vitro: By collagenase dissociation of a 0.3 cm^3 sample of the BT18 tumor tissue, we obtained about 3.5×10^6 cells. Three days after seeding on the feeder layer, the epithelial cells started to grow from individual cells or small cell clusters forming 730 epithelial colonies (plating efficiency approx. 0.02%). In this early phase, the colonies were composed mostly of luminal cells as revealed by K19 staining. After about one week, the myoepithelial cells (K19-) started to grow at the periphery of colonies (Figure 2A). The first subculture was performed on day 13 and further subcultures followed at 4- to 7-day intervals (Figure 2B). In the second passage, 13% of cells were able to form colonies; in higher passages the plating efficiency decreased to 5%. Altogether, 4 passages were achieved with the yield of 8.4×10^6 cells from the original 730 colonies. In the 2nd-4th passages, 25%-35% of cells were still positive for K19 (Figure 2C).

Cyclin D1 in the cultured cells: Among cells cultured *in vitro* from the BT18 tumor there were 84% cells showing 2 CCND1 signals, 9% 4 signals and 7% different numbers of signals (3 or more than 4). The cells with 3 or 4 signals did not differ in morphology from 2-signal cells, but colonies of such cells were not observed. Cells with more than 4 signals appeared only exceptionally. They were atypically enlarged and did not form pairs or colonies (Figure 2D), which indicated that they were not able to divide *in vitro*.

Discussion

It has been known for many years that the establishment of primary culture from malignant solid breast tumors is rarely successful, especially if only a small amount of the tumor tissue is available. One of the reasons could be unsuitable culture conditions for the malignant cells. It was assumed that development of a culture system supporting proliferation of luminal epithelial cells from the normal mammary gland would also improve *in vitro* propagation of breast carcinoma cells (2, 12). However, another reason for the culture failure may be that the majority of tumor cells are rather non-viable, and only methods that are able to support growth of individual epithelial cells may be successful. The most frequently used approaches for the *in vitro* propagation of mammary tissue are based on outgrowth of cells from organoids in serum-free or low-serum media and require large quantities of tumor tissues as starting material (13). For the selection and cultivation of breast cancer cells, Dairkee *et al.* (14) used a sandwich method mimicking the microenvironment in tumors – low pH, waste metabolites, nutrient and oxygen deprivation. In serum-free medium MCDB170 they achieved limited proliferation of K19+ (luminal) cells expressing several markers of malignancy. The growth of K19+ cells in the sandwich system contrasted with the results of Ethier *et al.* (12), who found that, in serum-free media, only myoepithelial cells grew while luminal cells did not. Ethier *et al.* (12) succeeded in obtaining passageable colonies of luminal cells on collagen-coated dishes using medium enriched with serum and growth factors. However, luminal cells obtained *in vitro* from the tumor tissue did not show the significant mutation present in the parental tumor.

We developed a more efficient system for serial cultivation of the luminal epithelial cells from a normal mammary gland (7). By this technique, we also succeeded in cultivation of epithelial cells from very small samples of

the breast tumor tissue (8). The question remained open, however, as to whether the large colonies (and following passages) originating from single cells or small cell clusters isolated from the tumor tissue display the same genetic markers as the majority of cells in the tumor. Solitary tumor cells *in vitro,* deprived of the complex architecture of the tumor tissue, could not be distinguished by staining or immunocytochemical methods from normal cells. Therefore, we attempted to use increased numbers of FISH signals of CCND1 or HER2/neu genes, caused by amplification or aneuploidy/polyploidy, as the identification signature of neoplastic cells. Among 9 neoplastic tumor tissues tested, we found 6 with increased numbers of CCND1 or HER2/neu signals (5 primary tumors, 1 metastasis). The cells derived from them were thawed and cultured *in vitro* for 2-6 passages with a high degree of multiplication (8). Then, the numbers of FISH signals for cyclin D1 and/or HER2/neu were assessed in the cultured cells. The result was that not a single cell population derived from the primary tumors or the metastasis maintained a substantial fraction of cells with increased numbers of the proto-oncogenes. The probability that normal cells had survived was very low because of qualified selection from the biopsy material. It appears that these cells were neoplastic, but genetically mimicking the normal cells and possibly better able to proliferate than cells with large genetic abnormalities.

The reason supporting this working conclusion was that at least a small fraction of cells with only 2 copies of the traced proto-oncogene signals was found to occur side by side with the cells showing multiple signals directly in the invasive areas of all tumor tissues analyzed. These cells could represent the neoplastic cells that were also able to divide *in vitro*. The characteristics of the MCB1 cutaneous metastasis, in which no normal mammary epithelia can be expected, confirmed this view.

There are many lines of evidence, especially cytogenetic, strongly suggesting that carcinomas arise from a well-proliferating progenitor stem cell that has undergone an initial malignant transformation only (15). Such cells have not lost the ability to differentiate and their behavior may be similar to normal cells (16, 17). By proliferation, tumor stem cells gain significant chromosomal rearrangement and imbalances, often resulting in genetically altered non-viable cells. Breast cancers are typically highly heterogeneous. Genetic abnormalities are taken as markers of malignancy, but they can lead to the inability of the cell to further divide. This could explain the observation that cells from malignant breast tumors only seldom grew *in vitro* and a permanent cell line has only rarely been established. Recently, it was found that even diploid cells in breast cancer are not normal because they contain some genetic changes preceding aneuploidization (18).

In the BT18 tumor, chosen as a representative example, the amplification of the CCND1 gene (in the majority 6-10 signals/cell) was observed in nearly all cells in invasive lesions, including small islands and individual migrating cells, whereas cells cultured from the BT18 tumor tissue mainly displayed 2 CCND1 signals. Only 16% of cultured cells displayed a higher number of signals, but in more than half of them it was only 4 signals, probably indicating cell tetraploidy, which is a frequent artifact of tissue cultures. Moreover, cells with more than 4 oncogene signals were usually atypically enlarged and appeared isolated. These cells and other cells displaying more than 2 signals did not form pairs, clusters or colonies, which pointed to only a temporal survival with a lack of growth potential (Figure 2D). Less than 0.1% of cells isolated from the tumor were able to form colonies. This corresponded well with the small fraction (<1%) of "normal-like" (according to 2 signals of CCND1) cells located directly in the tumor (Figure 1D). Cell cultures with increased numbers of CCND1 or HER2 genes exist mainly in aneuploid permanent cell lines, but the majority of temporarily established cultures are close to normal cells. The hypothesis about slowly growing malignant cells being overgrown by faster normal cells is often mentioned. This would imply that we should have been able to see at least some pairs or colonies of aberrant cells particularly at low passage numbers, which did not happen. Our results indicate that demonstrably malignant cells, traceable by increased numbers of cyclin D1 or HER2/neu, did not divide from the second *in vitro* passage on, in spite of the use of a sophisticated method suitable for luminal cell cultivation. Our findings suggest that these invasive tumor cells displaying only 2 proto-oncogene signals were most probably involved in the initiation and propagation of *ex vivo* tumor-derived primary cell cultures. These findings deserve further analysis.

Acknowledgements

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