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Cellular Immortality, Clonogenicity, Tumorigenicity and the Metastatic Phenotype

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IN RECENT years, considerable progress has been made towards resolving the genetic events underlying neoplastic behavior. As a consequence, it is now generally believed that tumorigenesis and the development of metastases are multi-step phenomena requiring the activation or repression of a number of genes either by mutation or epigenetic modification and/or the insertion of viral transforming gene(s) into the cellular genome [1–14]. This work has also produced evidence which clarifies the significance of some properties commonly associated with tumor cells, and investigations on the genetic basis of the metastatic phenotype need to be cognisant of this new information.

According to Hayflick, normal cells are programmed for only a limited number of divisions *in vitro* [15], whereas transformed or tumor cells are capable of infinite multiplication [16]. Thus, tumor progression probably requires, as an early and fundamental step, the generation of immortal tumor stem cells. These stem cells are then believed to undergo proliferation and diversification to produce a heterogeneous population of transformed cells that are capable of anchorage-independent growth, as assessed for example by their ability to form multilayered foci on culture dish surfaces or colonies in semi-solid media, and are tumorigenic on inoculation *in vivo*. Acquisition of metastatic capability would then require the emergence of cells with further properties which permit them to (a) be released from their primary site, (b) successfully negotiate the circulatory system, (c) attach at a new site, (d) invade at the new site and (e) be vascularised. Recent experimental work has considerably clarified issues relating to cellular immortality, transformation, tumorigenicity, stem cells and the metastatic phenotype, and it is therefore pertinent at this juncture to re-assess the relationships between these phenomena. Furthermore, it is useful to re-examine traditionally accepted markers of neoplasia such as clonogenicity in soft agar and define whether these can still be considered valid.

IMMORTALITY

Since normal cells usually only undergo a limited number of divisions, a cell which has apparently unlimited potential for self-renewal can be considered immortal, and if it can give rise to a number of different specialised cells within a particular tissue or cellular system, it can be regarded as a pluripotent stem cell. In this respect, some tumor cells, which are immortal *in vitro*, have characteristics of stem cells found in normal differentiation and development, as for example, in the haemopoietic system. While immortality might be a prerequisite for tumorigenicity, it is not however necessarily equated directly with the neoplastic state. It is true that many cell lines are both immortal *in vitro* and tumorigenic *in vivo* but there are examples of cells which, although immortal *in vitro*, never produce tumors even in immunosuppressed hosts (Table 1). The NIH 3T3 embryonic fibroblast cell line derived from NIH Swiss mice, for example, is immortal but not tumorigenic if passaged under appropriate culture conditions and provides direct evidence that these two phenomena are not inescapably linked [26].

TUMORIGENICITY *IN VIVO*

Serial transplantability of tumors in appropriate hosts is *de facto* evidence of immortality of at least part of the tumor cell population. Thus, it is assumed that within a progressively growing tumor there is a population of tumor stem cells. However, tumorigenicity does not necessarily equate to im-

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Table 1. *Tumorigenicity and transformation in immortal cells*

Immortality	Morphological transformation	Growth in soft agar	Membrane changes	Tumorigenic state	Examples
+	+	+	+	+	Tumour-derived cells [17]
					Ras transfected 3T3 [18]
+	+	+	−	+	SV40 transformed cells [19]
					Chemically transformed SHC [20]
+	+	+	−	−	Te-85 [21]
+	+	−	−	−	SV-W138 [22]
+	−	+	−	−	Chemically transformed embryonic cells [23]
+	−	−	+	Intracranial only	Eb virus immortalised lymphocytes [24,25]
+	−	−	−	−	NIH-3T3 (cells grown at low density [26]

mortality *in vitro*, in that spontaneous mammary tumors do not, under standard culture conditions, readily produce established cell lines [27]. While it is possible that some essential component required by these cells is missing *in vitro*, it is clear that occasional mammary cell lines with unlimited growth potential are available and these can survive under identical culture conditions. Hence, an important adaptation step to *in vitro* conditions might be required to secure immortal cell proliferation of the spontaneous mammary tumor cells.

TRANSFORMATION

A further step considered to be important in tumor progression, is the loss of either contact inhibition or anchorage-dependent growth. These parameters are often defined *in vitro* by the appearance of heaped multi-layered foci of cells. The transition to this state is referred to as transformation, which has loosely been used synonymously with tumorigenicity. Although the former term is generally used to describe the change from anchorage-dependent to anchorage-independent growth as, for example, following transfection with transforming virus DNAs or treatment with chemical mutagens, it is clear from recent work that this step does not necessarily confer on these cells the ability to form tumors (Table 1). This statement is reinforced by the work of Stanbridge [28] who has shown that normal (i.e. non-neoplastic) human cells can be anchorage-independent and clonogenic in agar and by the observation that normal bone marrow stem cells are not dependent on attachment to a solid substrate for growth and multiplication when cultured with colony-stimulating factors [29]. Equally, our own work described below demonstrates that colonies of anchorage-independent tumor cells in agar are not necessarily tumorigenic when injected *in vivo*. In this context, it is necessary to define precisely what is meant by transformation. Our contention is that the term be reserved for focus formation *in vitro*, and/or colony formation in semi-solid medium, i.e. anchorage-independent growth, *accompanied* by tumorigenicity *in vivo*. Transformation defined in this way appropriately describes the transition of non-tumorigenic NIH 3T3 cells to highly tumorigenic cells *in vivo* following successful transfection with activated ras or sis oncogenes [13, 14].

CLONOGENICITY

Clonogenicity is an operationally defined capability of cell populations to form self-expanding colonies. It is currently assayed by inoculation of the cell population to be studied into soft agar or into animals intravenously. The number of colonies formed is widely accepted as an indication of the stem cell content of the cell population [30]. The conditions of these assays however are clearly artificial and may not be an accurate reflection of the circumstances in a normal tissue or a tumor.

Assays in soft-agar/agarose

Normal cells, with few exceptions, require a solid substrate for *in vitro* proliferation, that is they are anchorage dependent. Conversely, the capacity for colony formation in semi-solid medium indicates that the cell population has become independent of anchorage to a substratum and this is generally recognised as a characteristic property of neoplastic cell populations. Anchorage independence [31] has been correlated with tumorigenicity for many animal and human cells [19, 23, 32, 33] by parallel *in vitro* and *in vivo* assays in which aliquots of cells

are injected into suitable test animals, and cultured in semi-soft medium. Other reports have refuted an absolute correlation of anchorage independent growth of tumor cells with tumorigenicity [34] and claimed that the two are distinctly different phenotypes [35]. In addition, hybrids produced by the fusion of HeLa cells derived from a human cervical carcinoma with normal human fibroblasts were found to be capable of anchorage independent growth, yet were non-tumorigenic [36] suggesting that the clonogenic and tumorigenic phenotypes were under separate genetic control. It is appropriate therefore to re-examine the significance of clonogenic capability *in vitro* for tumorigenicity and metastatic capability.

Relationship between clonogenicity in vitro *and tumorigenicity*

An alternative and perhaps more appropriate means of assessing the association between clonogenicity *in vitro* and tumorigenicity is to recover colonies from the test cultures and then directly assess their tumorigenic potential *in vivo* (see below). This approach has only recently been attempted and has shown that agar colonies derived from suspensions of tumor cells do not always produce tumors when transplanted into the mammary fat pads of syngeneic mice [37]. Numerous colonies from 16 primary mammary tumors were implanted and the proportion that developed into neoplasms varied greatly between individual prim-

Table 2. In vivo *and* in vitro *colony formation of primary mammary tumour cells*

	(a) Lung colonies	(b) Agarose colonies	(c) In vitro CFE%	(d) Tumours/ implanted colonies
		(e) LCP TUMOURS		
1	1 (0–2)	46 (41–62)	0.04	2/120
2	9 (2–14)	87 (69–103)	0.08	1/20
3	4 (0–8)	62.5 (50–69)	0.06	5/40
4	6 (5–7)	94 (82–128)	0.09	2/40
5	18 (2–43)	221 (166–276)	0.22	4/40
6	3 (0–7)	67 (60–81)	0.06	2/40
7	11 (6–22)	55.3 (48–58)	0.05	2/40
8	14 (1–21)	89 (77–94)	0.09	8/40
9	24 (17–98)	202 (173–216)	0.20	6/170
10	7 (3–40)	26 (21–29)	0.02	ND
				Total 32/550 5.8%
		(f) HCP TUMOURS		
11	85 (44–110)	156 (132–190)	0.15	7/140
12	140 (60–160)	156 (134–202)	0.15	15/20
13	34 (0–49)	220 (209–252)	0.22	3/140
14	84 (36–106)	272 (248–296)	0.27	11/170
15	69 (49–759)	165 (156–190)	0.16	6/40
16	73 (45–110)	136 (122–144)	0.13	3/20
17	84 (72–120)	152 (137–170)	0.15	14/40
18	130 (100–270)	166 (156–185)	0.16	ND
19	130 (60–160)	145.5 (142–154)	0.14	ND
20	65 (11–150)	262 (242–283)	0.26	ND
				Total 59/570 10.4%

(a) Lung colonies in syngeneic mice injected with 5×10^5 cells: (median range).
(b) Agarose colonies (>10 cells)/dish: (median range).

(c) Colony-forming efficiency (CFE): $\frac{\text{agarose colonies}}{10^5 \text{ cells plated}} \times 100$.

(d) Tumours in fat pads × (agarose colonies implanted)$^{-1}$.
(e) LCP = low colonising potential.
(f) HCP = high colonising potential.
ND = Not done.

ary tumors (Table 2). The results suggest a clonogenic heterogeneity within these primary neoplasms. While the majority of the cells were incapable of anchorage-independent growth and died, some were clonogenic *in vitro* but non-tumorigenic, and others were both clonogenic *in vitro* and tumorigenic when transplanted *in vivo*. This last subpopulation may represent the potential stem cells of the mammary tumor. However, since some colonies are not tumorigenic, agarose clonogenicity alone may not accurately measure the true stem cell content of a tumor *in situ*. It is possible for instance that some agarose colonies are formed by limited division of tumor cells which are not stem cells. We therefore consider that clonogenicity needs to be assessed in conjunction with tumorigenicity after *in vivo* transplantation of the colonies in order to evaluate the potential tumor stem cell content.

Pulmonary colony-forming assays

Another method proposed for estimating numbers of colony-forming units, or potential stem cells involves intravenous inoculation of cell suspensions followed by counting of colonies formed in the spleens of sub-lethally irradiated mice injected with bone marrow cells [38], or of the numbers of deposits in the lungs of animals injected with suspensions of solid tumors.

Intravenous inoculation of cell suspensions is a routine technique in cancer metastasis research [39] and has been used in our laboratory to investigate the metastatic colonisation potential of mouse primary mammary tumors. From these experiments it was found that individual tumors differ widely in their ability to form lung deposits after intravenous inoculation of a known number of cells (5×10^5). Approximately half had high colonisation potential (HCP) and seeded numerous pulmonary deposits in all syngeneic recipients, while the remaining neoplasms had nil or low colonisation potential (LCP) [40, 41]. Pulmonary colonisation capability is therefore *de facto* evidence of tumorigenicity of the injected cells, at least in the site concerned.

Direct comparison of agar and pulmonary colonisation assays

To test whether the results from one clonogenicity assay would reflect how cells from the same tumor would perform in the other, cells from LCP and HCP tumors were cultured in 0.3% agarose (10^5 cells/35 mm dish) and colonies counted after 14 days [42]. The results from the 20 tumors studied (Table 2) showed that for a fixed dose of cells individual primary tumors differed greatly in colony-forming efficiency *in vitro*, as well as in lung colony formation, after intravenous inoculation. HCP tumors generally had higher colony-forming efficiency *in vitro* (mean value 0.179%) than the low colonising tumors (mean value 0.091%; $P<0.01$ Student t-test). However the colonisation potential *in vivo* of any given tumor could not be predicted accurately on the basis of an agarose colony-forming assay.

In further studies, the dose responses of cells obtained from individual mammary tumors in both these assays have been examined and compared [42, 43]. The dose responses for clonogenicity in agar plotted as log cell dose against log colonies formed, showed a linear relationship for both HCP and for LCP tumor cells (Fig. 1) with early plateauing of lung colony counts with cells from LCP tumors. For all the tumors tested, computer-generated best fit lines for the *in vivo* (pulmonary colony) response were displaced to the right of the *in vitro* response, i.e. fewer *in vivo* colonies than *in vitro* for the same input of cells. This indicates that cells *in vivo* are subject to many more factors than in the relatively simple *in vitro* assays. Cells released intravenously are exposed, for example, to haemodynamic damage resulting from laminar shear forces and impaction in capillary beds, before reaching an environment which may or may not allow formation of secondary tumor colonies [44]. In general however these dose response studies reinforce the conclusion that differences in pulmonary colony formation between different primary mammary tumors are at least partially the result of different proportions of colony-forming units within each tumor. The graphs (Fig. 1) showed that the minimum dose to obtain any response was less for HCP than for LCP tumors implying they have more stem cells.

Clonogenicity and metastasis

The clonogenicity assays create experimental situations in which cells can express their ability to form expanding colonies and the colonies formed are therefore considered to represent potential tumor stem cells [45]. In the studies described above, potential stem cell content differed from tumor to tumor and represented only a fraction of the total neoplastic population. Also agar colony-forming capability was significantly higher in tumors classified as HCP in the lung colony assay than in LCP counterparts. Metastatic tumor cells must have the properties of potential tumor stem cells as they are capable of extensive proliferation to establish new neoplasms in distant sites. Not all tumor stem cells in our studies however give rise to metastatic tumors — only a proportion of the primary mammary tumors studied were spontaneously metastatic (30%) [43]. Of the 59 mice that developed tumors from implanted agarose colonies, only 22 had spontaneous lung metastases,

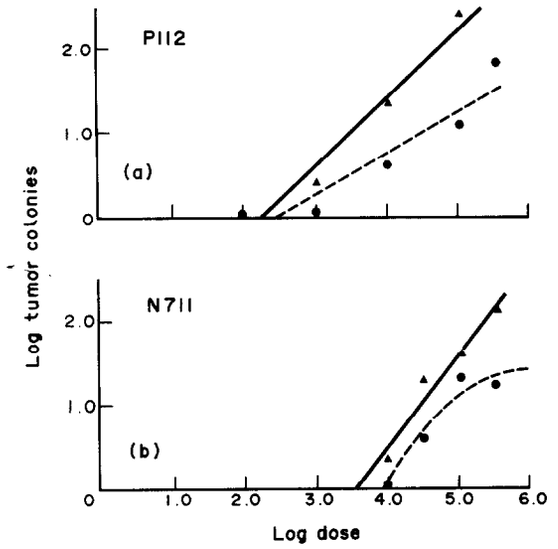

Fig. 1(*a*). *HCP tumour*: in vivo *and* in vitro *colony formation dose–response. Agarose colonies*: *correlation coefficient* = 0.96; *S.E.* = 0.26. *Lung deposits*: *correlation coefficient* = 0.89; *S.E.* = 0.29.
Fig. 1(*b*). *LCP tumor*: in vivo *and* in vitro *colony formation dose–response. Agarose colonies*: *correlation coefficient* = 0.96; *S.E.* = 0.18. *Lung deposits*: *correlation coefficient* = 0.86; *S.E.* = 0.27. —▲——, *agarose colonies*; ---●------, *lung deposits*.

which indicates that while clonogenicity is vital for distant tumor seeding and growth, it is not the only criterion of importance in the sequence of metastasis. In addition, it was noted that primary tumors which were spontaneously metastatic were not significantly more clonogenic than non-metastatic tumors [42].

It should be recalled that some tumors that are spontaneously metastatic are incapable or only weakly capable of colonising the lungs after intravenous inoculation and that others which are very potent in the lung colony assay are non-metastatic [43]. There is therefore no general correspondence between spontaneous metastatic capability and the results of the lung colony assay for a given tumor.

CONCLUDING REMARKS

By definition, tumors must contain at least some immortal stem cells which form heterogeneous clonal populations. Some of these cells may be able to migrate to other sites and set up new colonies with similar immortal clonogenic properties but microenvironmental and immunological influences exerted by the host can be superimposed and affect the outcome. The recent research discussed above has clarified the implications of some relevant properties attributed to malignant cells in experimental circumstances but it is clear from some of the apparent discrepancies seen in assays *in vivo* and *in vitro* that extrapolation from the experimental to the natural situation will need much caution and more investigation.

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