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# Similar Self-Renewal Properties for Different Sizes of Human Primary Melanoma Colonies Replated in Agar<sup>1</sup>

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## ABSTRACT

Clonogenic assays currently define colonies as multicellular growth units above an arbitrarily designated cutoff size rather than by the biological function of different-sized growth units. To define the cutoff size between clusters and colonies in terms of the biological function of the cells within the growth units, we directly measured the self-renewal and proliferative capacity of cells from different-sized melanoma colonies. Primary colonies formed from cells of two patients were removed, pooled according to size, and replated, and the frequency and size distribution of the secondary colonies were analyzed. Cells from primary melanoma colonies that resulted from four to eight population doublings had similar extensive proliferative and self-renewal characteristics. The results demonstrated that self-renewal was not limited to cells in large colonies and suggested that the cutoff may be below 16 cells/growth unit. These data support the use of relatively small multicellular growth units to define colonies and measure highly proliferative human melanoma tumor cells. In addition, these methods may allow the determination of the cutoff size for other tumor types in terms of the biological function of cells rather than arbitrarily designating a cutoff size.

## INTRODUCTION

Human tumor clonogenic assays are in widespread use (1, 2, 14). They typically produce multicellular growth units, defined as clusters and colonies, with sizes ranging from 2 to greater than 100 cells/growth unit. Currently, the "cutoff" between clusters, small growth units, and colonies, larger growth units, is arbitrarily defined using growth units of a particular size. However, different sizes are used by different laboratories with 40 to 50 cells/growth unit as a commonly used cutoff. Furthermore, many laboratories (14, 15), including our own (10), use a growth unit diameter of 60  $\mu\text{m}$  as the cutoff. We have recently shown that, on the average, 60- $\mu\text{m}$ -diameter growth units contain only 8 to 10 cells (12). Thus, a colony defined as a 60- $\mu\text{m}$ -diameter growth unit contains far fewer cells than most investigators would consider necessary to define a colony.

However, because the definition of a colony has been arbitrarily set and evidence is lacking to define colonies in terms of their biological function, it is unclear what the cutoff should be. Thus, since no difference in biological function has been demonstrated for the cells within 16- versus 50-cell growth units, it is difficult to define one as a cluster and the other as a colony. The differences in growth unit size may reflect several properties, including different proliferative capacities of clonogenic cells and clonogenic cell kinetics.

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The stem cell model of human tumor growth and its implications for tumor cell clonogenic assays were recently proposed by Mackillop *et al.* (9). It may be a very useful guide in the determination of the cutoff between clusters and colonies, because it suggests that the range of growth unit sizes reflects a cell renewal hierarchy of the tumor, and it specifically predicts different biological functions associated with growth units defined as clusters versus colonies. The biological functions of clonogenic cells forming "clusters" would be consistent with those of a "transitional" cell of the renewal hierarchy. The limited proliferative potential of a transitional cell would allow only a few divisions in agar and give a small growth unit, a cluster. In contrast, the biological functions of clonogenic cells forming colonies would allow expression of extensive proliferative and self-renewal capacities and thus would be consistent with the properties of "stem cells" within the renewal hierarchy. Under appropriate culture conditions, these stem cells could form large growth units and secondary colonies after the renewal and replating of primary colonies.

In this study, to define the cutoff between melanoma clusters and colonies in terms of biological function, we replated growth units with 16 or more cells to determine their proliferative and self-renewal capacities. Using cells from 2 patients, we found very similar proliferative and self-renewal capacities for cells from growth units with 16 or more cells. These data suggest that for the melanoma clonogenic assay the cutoff between clusters and colonies may be below 16 cells/growth unit. This value is significantly lower than the traditional, arbitrarily designated value of 40 to 50 cells/growth unit.

## MATERIALS AND METHODS

**Preparation of Cells from Biopsies.** Tumor tissue was obtained under aseptic conditions by excisional biopsy of s.c. nodules from patients with metastatic malignant melanoma (protocol approved by the University of Arizona Committee on Human Subjects). Tumor tissue was processed to obtain single-cell suspensions as described previously (16). The cells were pooled, counted, tested for viability by exclusion of 0.4% trypan blue (Grand Island Biological Co.), and stored in liquid nitrogen in 10% dimethyl sulfoxide (spectrophotometric grade; Aldrich Chemical Co., Milwaukee, WI) in Ham's F-10 medium containing 10% heat-inactivated fetal calf serum. Cryopreserved cells were used in these experiments. Previous work using clonogenic human melanoma cells has shown that cryopreservation did not affect the replating of cells from primary colonies (16). The 2 patients studied were selected from a group of melanoma patients (40 of approximately 250 biopsied) whose biopsies gave excellent single-cell suspensions, confirmed by direct observation before and after plating, that formed primary colonies.

**Estimation of Live Tumor Cells.** Enumeration of live tumor cells has been described extensively elsewhere (16). Briefly, viability of fresh and cryopreserved cells derived from biopsies of human malignant melanoma was determined using 0.4% trypan blue dye exclusion. Cells that excluded the dye were considered as viable. Viable cells were divided into

2 groups based on size because morphological identification of the cells after cytocentrifugation and Papanicolaou staining showed that all the small nucleated cells were mature lymphocytes. Thus, the groups were defined as cells which were 4 to 7  $\mu\text{m}$  in diameter (corresponding to mature lymphocytes) and those greater than 7  $\mu\text{m}$  in diameter. The group of larger cells included both tumor cells and normal large cells such as macrophages. The morphology of cells was examined by light microscopy after Papanicolaou and Wright-Geimsa staining. Large host and malignant melanoma cells were clearly discriminated on the basis of the amount of cytoplasm, nuclear characteristics, and cellular staining pattern. It was difficult routinely to further distinguish the types of large viable cells as either melanocytes or macrophages because both cell types can contain pigment granules and both have the ability to phagocytize and adhere. Thus, in some cases in which normal large viable cells were present, the number of viable tumor cells may have been overestimated. This would result in an underestimate of the cloning efficiency. However, the estimate of large viable tumor cells excluded the dead cells and viable mature lymphocytes and thus was a better estimate of the cells that could form colonies than was the number of total nucleated cells, although it may not be perfectly accurate. Use of the number of large viable tumor cells to calculate cloning efficiency results in much higher cloning efficiencies than are generally cited (16).

**Bilayer Agar Microtiter Culture System.** Our clonogenic culture system was a modification of the agar system developed by Hamburger and Salmon (6) and described extensively elsewhere (16). We have simplified the culture conditions for clonogenic melanoma cells (10) and performed the assay in microtiter wells (16) to reduce the number of cells required. Colonies were enumerated after 16 to 18 days in culture by manual inverted microscopy. The diameters of 100 consecutive colonies/sample were measured with an inverted microscope equipped with a micrometer scale to obtain size distributions. We have previously shown by light and transmission electron microscopy (10, 13) and karyology (10, 18) that the cells within the colonies were human malignant melanocytes.

**Definition of a Colony and Cloning Efficiency.** We have used the term colony for multicellular groups of cells because designations of clusters and colonies have conventionally been chosen arbitrarily without reference to functional differences. Cloning efficiency was the number of multicellular growth units ( $\geq 2$  cells) formed divided by the number of viable large cells plated and was calculated from the slope of the curve obtained by plotting the number of growth units formed *versus* the number of live tumor cells plated.

**Replating and Culture of Cells.** Colonies were removed aseptically at room temperature using a micropipet connected to a micromanipulator as described previously (16). A portion of the cells were counted and used to determine trypan blue viability before replating the cells at various concentrations. Collection and transfer of small colonies (less than about 100 cells) results in greater than a 50% cell loss in the majority of cases (16). Therefore, colonies with similar diameters were collected, pooled, and replated as described previously (16). The actual number of cells plated was determined by counting the number of cells in each microwell after transfer.

**Direct Determination of Mean Diameter of Cells and Number of Cells in Colonies.** The number of cells cannot be determined while the colony is in agar, because the cells are stacked upon one another. Therefore, in a previous study (12), we removed colonies to count the number of cells directly. With these data, we developed a general equation and constructed a nomogram which allows calculation of the actual number of cells in a colony if the mean diameter of cells in colonies and the diameter of the colony are known (12). The general equation is

$$\text{No. of cells/colony} = \frac{2.40 (\text{Colony diameter})^{2.378}}{(\text{Cell diameter})^{2.804}}$$

To obtain the mean colony cell diameter, 20 individual cells within colonies were measured directly by visual observation using the inverted microscope at  $\times 200$ . The mean diameters of melanoma cells in the

growth units from Patients 1 and 2 were  $15.1 \pm 0.2$  (S.E.) and  $14.2 \pm 0.4$   $\mu\text{m}$ , respectively, and were used to calculate the number of cells within colonies.

**Analysis of Frequency Distribution of Colonies by Size (Diameter).** The frequency distribution of colony size was analyzed using nonparametric statistical procedures, including the 2-sample Wilcoxon test, Kolmogorov-Smirnov test, and a test to compare the 75th percentiles (7). We have shown recently (11) that growth unit size is markedly dependent on the number of cells plated, with smaller growth units forming when high concentrations of cells were plated. Thus, we used similar and low concentration of cells per well for comparisons of the size of colonies formed in primary and replated clonings.

## RESULTS

### Case 1

**Relationship of Number of Tumor Cells Plated and Number of Colonies Formed.** There was a linear relationship between the number of tumor cells plated and the number of primary colonies formed per well (Chart 1A). The linear regression fit the data well (correlation coefficient, 0.99) with an intercept close to zero, at 21 colonies, with a 95% confidence interval of 1 to 83. Colonies formed at the lowest number (28 cells) of tumor cells plated. The primary cloning efficiency, as determined by the slope, was 33.2% with a 95% confidence interval of 29.7 to 36.7%. The distribution of primary tumor colonies of different diameters is shown in Fig. 3A and discussed below.

**Replating of Primary Colonies of Different Diameters (Sizes).** Determination of the secondary replating capacity of colonies containing different numbers of cells was assessed. Replating of colonies of 4 different sizes was accomplished; the data are shown in Chart 1, B to D, and summarized in Chart 1E. A linear relationship between the number of cells transferred and the number of secondary colonies formed was apparent. For replating of cells from colonies of each size class, the secondary cloning efficiencies were high (36.5 to 60.2%) and similar, with the 95% confidence intervals overlapping for all but the smallest size group (Table 1). The distribution of secondary colonies by diameter was also determined (Chart 3, B to E) and is discussed in detail below.

**Replating of Secondary Colonies of Different Sizes.** Secondary colonies 120 to 200  $\mu\text{m}$  in diameter generated from different size classes of primary colony cells were collected, transferred, and cultured. For cells from all size classes of primary colonies, the relationship between the number of secondary colony cells plated and the number of tertiary colonies formed was linear (Chart 2). Tertiary cloning efficiencies were high and similar, at 30.1 to 41.7% (Table 1). The distribution of tertiary clusters and colonies by diameter was also determined (Chart 4) and is discussed in detail below.

**Frequency Distribution of Primary and Secondary Colonies by Diameter.** Various sizes of colonies were formed in the agar, and the relative frequency distribution of the primary colonies by diameter is shown in Chart 3A. The relative frequency of the diameter of secondary colonies replated from cells of primary colonies of different diameters is present in Chart 3, B to D. In summary, the median diameter of secondary colonies generated from cells of primary colonies of different diameters is shown in Table 2. The median size of primary colonies (109.7  $\mu\text{m}$ ) was significantly greater than the 82.7- $\mu\text{m}$  median diameter of sec-

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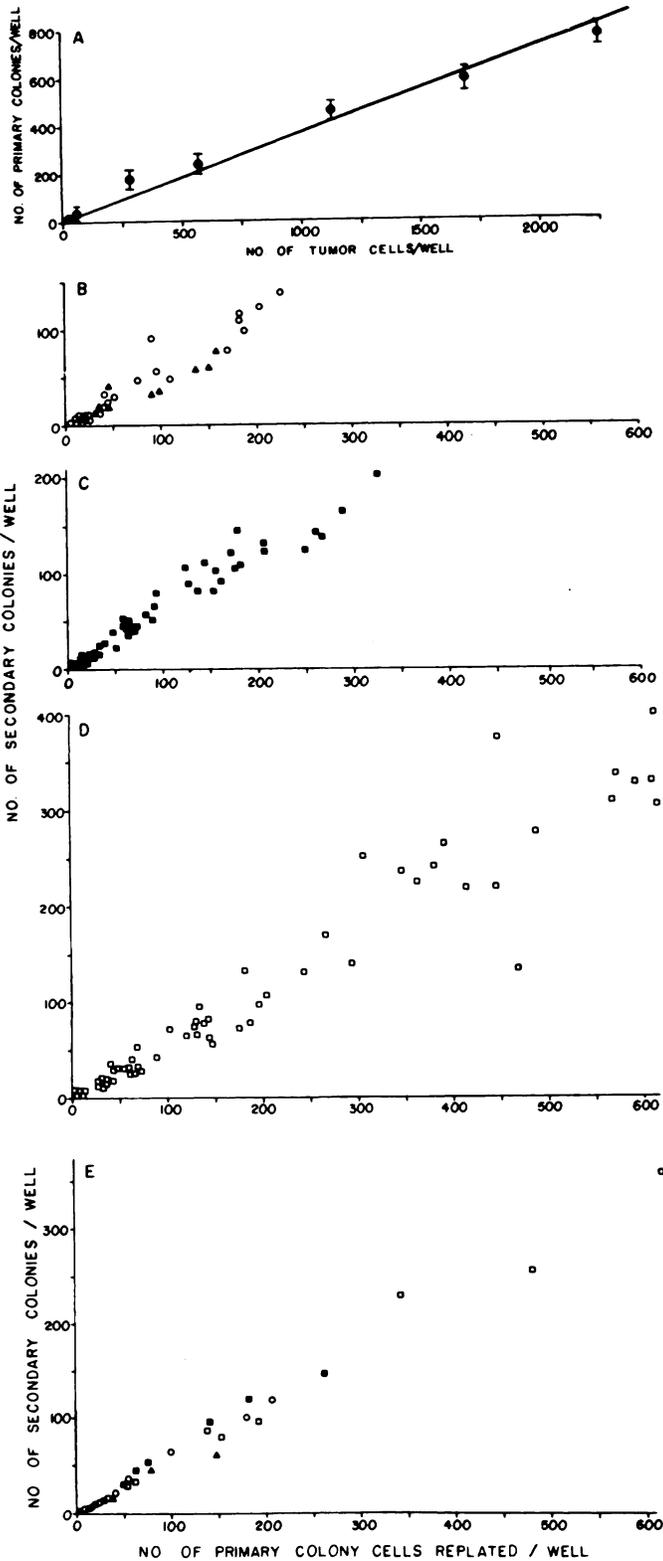


Chart 1. Relationship of number of tumor cells plated and number of primary or secondary colonies formed (Patient 1). A, primary colony formation. Means of 6 replicates. Bars, S.E. B to D, secondary colonies formed from primary colonies of different diameters (in  $\mu\text{m}$ ) at different cell concentrations.  $\blacktriangle$ , 50 to 60;  $\circ$ , 60 to 70;  $\blacksquare$ , 70 to 120;  $\square$ , 120 to 200. E, mean of 6 replicates. Symbols as for B to D.

Table 1

Cloning efficiencies of replated cells from colonies of different sizes

Patient <sup>a</sup>	Size of primary colonies		% of cloning efficiencies <sup>b</sup>	
	Diameter ( $\mu\text{m}$ )	No. of cells <sup>c</sup>	Secondary	Tertiary
1	50-60	13-20	36 (21-52) <sup>d</sup>	35 (33-38)
	60-70	20-29	59 (54-64)	30 (26-34)
	70-120	29-104	60 (57-63)	34 (31-36)
	120-200	104-351	57 (52-61)	42 (38-45)
2	50-70	16-35	13 <sup>e</sup>	
	70-110	35-102	10	
	110-200	102-422	9	

<sup>a</sup> The primary cloning efficiencies of Patients 1 and 2 were, respectively, 33 (95% confidence interval, 29 to 36) and 9%.

<sup>b</sup> Slope of the curve which relates number of large viable cells plated and colonies formed.

<sup>c</sup> We have presented in detail elsewhere (13) the relationship between cell and colony diameter and the number of cells per colony, derived from the equation:

$$\text{No. of cells/colony} = \frac{(2.40) (\text{colony diameter})^{2.378}}{(\text{cell diameter})^{2.804}}$$

<sup>d</sup> Numbers in parentheses, 95% confidence interval.

<sup>e</sup> Confidence interval not calculated because curve was nonlinear at low cell number plated.

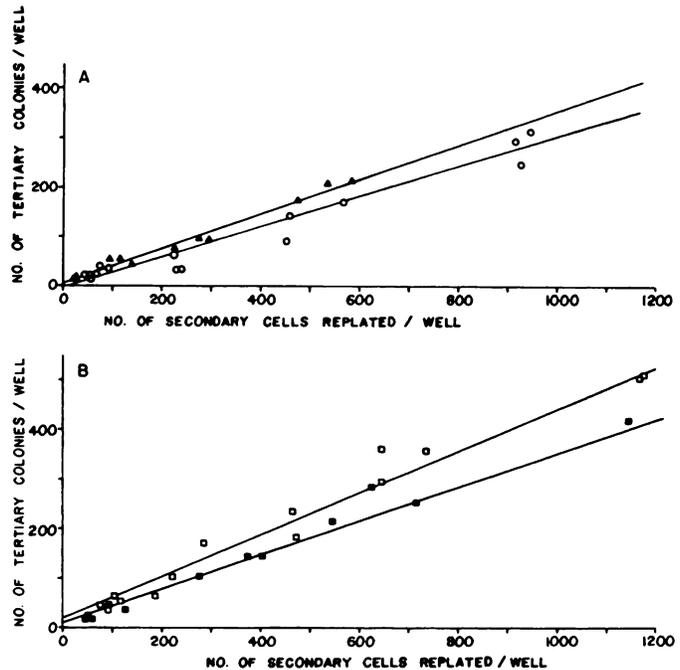


Chart 2. Relationship between the number of cells from secondary colonies plated and tertiary colonies formed. Colonies 120 to 200  $\mu\text{m}$  in diameter generated from different size classes ( $\mu\text{m}$ ) of primary colonies were collected, transferred, and cultured. A:  $\blacktriangle$ , 50 to 60;  $\circ$ , 60 to 70; B:  $\blacksquare$ , 70 to 120;  $\square$ , 120 to 200.

second-generation colonies derived from 50- to 60- $\mu\text{m}$  primary generation colonies ( $p = 0.001$ ). However, distribution of second-generation colonies derived from 60- to 70-, 70- to 100-, and 120- to 200- $\mu\text{m}$  primary colonies were not significantly different ( $p = 0.20$ ); these secondary colonies as a group did have a slightly greater median (120.7  $\mu\text{m}$ ) than did the primary colonies ( $p = 0.02$ ).

**Frequency Distribution of Tertiary Colonies Generated from Cells of Secondary Colonies.** A study of the size distribution of tertiary colonies generated from cells in secondary colonies can be approached in one of 2 ways. First, secondary colonies

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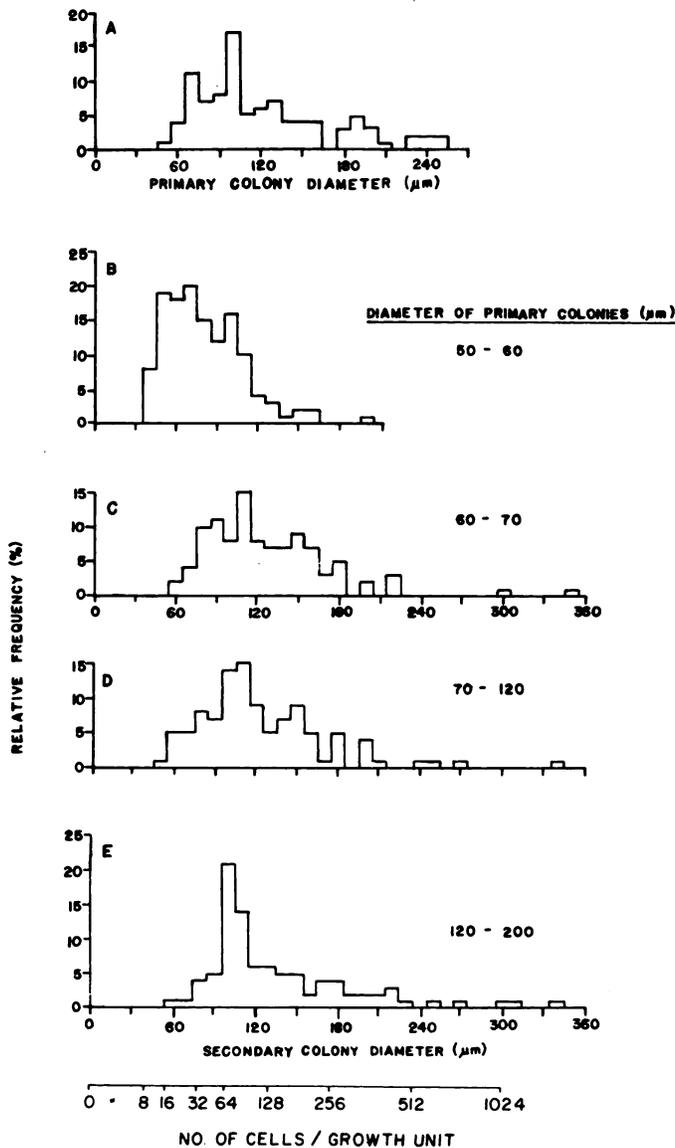


Chart 3. Relative frequency distribution of clusters and colonies by diameter (in  $\mu\text{m}$ ). A, primary colonies; B to E, secondary colonies grown from cells plated from primary colonies of indicated diameters. Data based on sizing of 100 growth units/histogram.

Table 2

Median diameter of secondary colonies generated from cells of primary colonies of different sizes

Patient	Primary colonies		Median diameter ( $\mu\text{m}$ ) <sup>a</sup>	
	Diameter ( $\mu\text{m}$ )	No. of cells	Secondary colonies	Tertiary colonies
1	50-60	13-20	70	79
	60-70	20-29	120	95
	70-120	29-104	110	95
	120-200	104-351	120	102
2	50-70	16-35	85	
	70-110	35-102	103	
	110-200	102-422	123	

<sup>a</sup> The median diameters of secondary and tertiary colonies generated from primary colonies of different sizes were compared using a number of statistical tests as discussed in "Materials and Methods." The results are presented in the text.

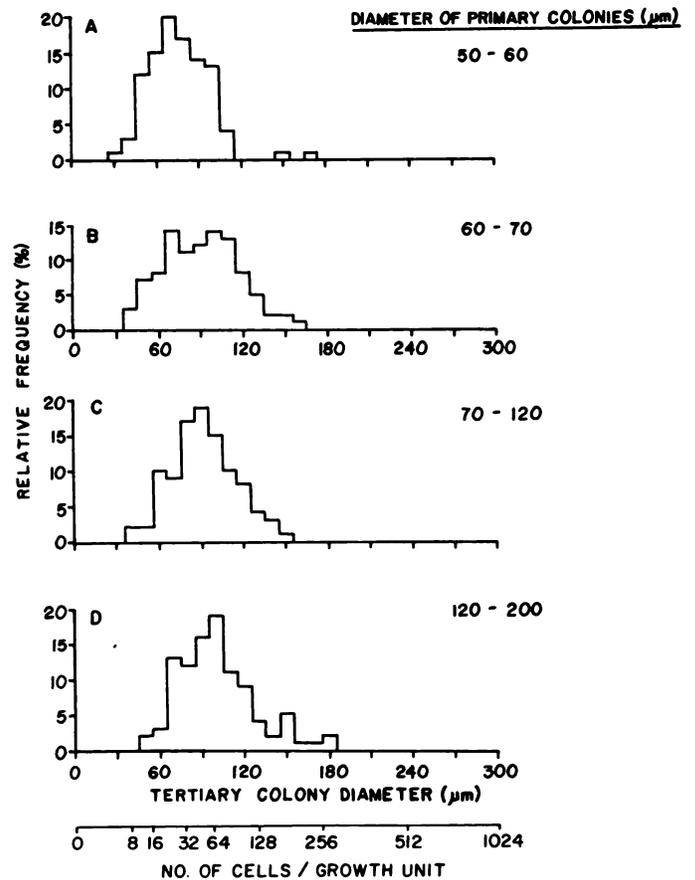


Chart 4. Frequency distribution of tertiary colonies generated from cells of secondary colonies. Cells from secondary colonies 120 to 200  $\mu\text{m}$  in diameter generated from cells of primary colonies of indicated diameters were plated. Data are based on sizing of 100 growth units/histogram.

generated from cells of each of the size classes of primary colonies could be subdivided by diameter of colonies and cells from each size class replated. Alternatively, if secondary colonies were formed from cells with a limited proliferative capacity, then after proliferation to large colonies the cells may be close to their limit when plated the third time. Therefore, we replated cells from large secondary colonies 120 to 200  $\mu\text{m}$  in diameter (128 to 512 cells), which were generated from cells of primary colonies of the different diameters. The frequency distribution of tertiary colonies by diameter is shown in Chart 4. In summary, the median diameter of tertiary clusters and colonies generated from cells of secondary colonies with similar (120- to 200- $\mu\text{m}$ ) diameters is shown in Table 2. Third-generation colonies derived from 50- to 60- $\mu\text{m}$ -diameter primary-generation colonies yielded a smaller medium colony diameter (79.8  $\mu\text{m}$ ) compared to the other tertiary colonies ( $p = 0.001$ ). Third-generation colonies derived from the larger primary colonies (60- to 70-, 70- to 120-, 120- to 200- $\mu\text{m}$  diameters) had similar size distributions. However, the tertiary colonies derived from the smaller 2 groups had a median colony size significantly less than the largest colonies ( $p = 0.007$ ).

Case 2

**Relationship between Number of Tumor Cells Plated and Number of Colonies Formed.** Chart 5A demonstrates the relationship between the number of tumor cells plated and the

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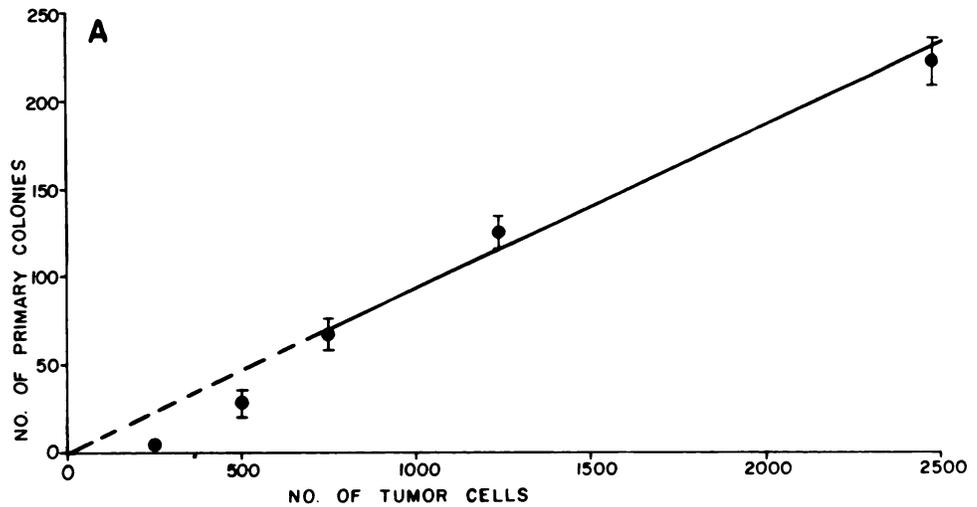
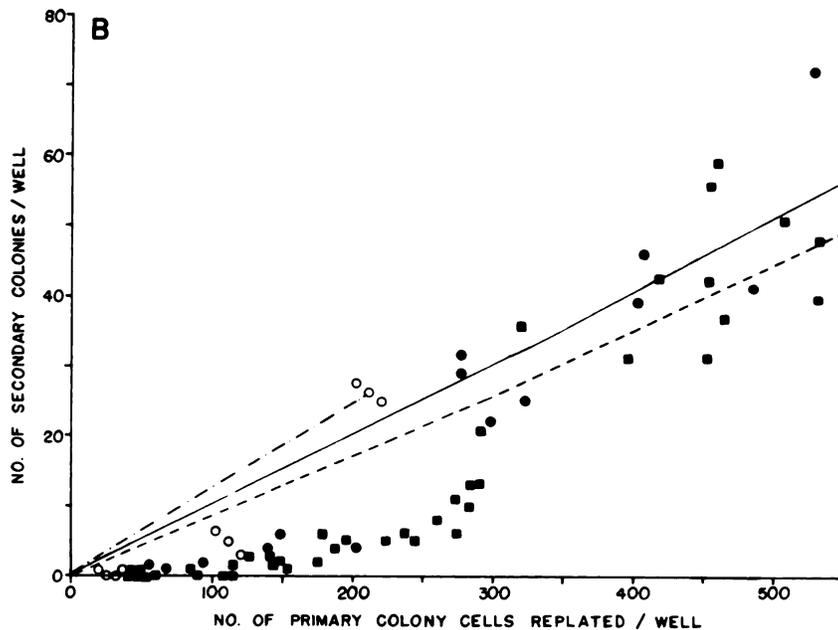


Chart 5. Relationship of number of tumor cells plated and number of primary or secondary colonies formed. A, primary colony formation (Patient 2); B, secondary colonies formed from primary colonies of different diameters (in  $\mu\text{m}$ ):  $\circ$ , 50 to 70;  $\bullet$ , 70 to 110;  $\blacksquare$ , 110 to 200. Means of 6 replicates.



number of primary colonies formed per well. There was a linear relationship between the number of cells plated and primary colonies until less than 500 cells/well were plated. A linear regression line extrapolated through zero allowed calculation of a cloning efficiency from the slope; this was 9%. The distribution of colonies by diameter is shown in Chart 6A and discussed below.

**Replating of Primary Colonies of Different Sizes.** Determination of the secondary replating capacity of colonies containing different numbers of cells was assessed. Replating of colonies with the 3 selected diameters was performed (Table 1); the data are summarized in Chart 5B. Similar proportions of secondary colonies were formed for replating of cells from colonies of each size class. For replating of cells from colonies of each size class, the secondary cloning efficiencies were high (9 to 13%) and similar. The distribution of secondary clusters and colonies by diameter was also determined (Chart 6, B to D) and is discussed below.

**Frequency Distribution of Primary and Secondary Colonies by Diameter.** Various sizes of colonies were formed in the agar, and the relative frequency distribution of the primary colonies by diameter is shown in Chart 6A.

The relative frequency of the diameter of secondary colonies replated from cells of primary colonies of different diameters is presented in Chart 6, B to D. The median diameter of secondary colonies generated from cells of primary colonies of different diameters is shown in Table 2. Secondary colonies derived from the cells of smallest colonies (50 to 70 and 70 to 110  $\mu\text{m}$ , respectively) as contrasted to the median colony size with a primary distribution (133.0  $\mu\text{m}$ , both  $p$  values = 0.004). However, second-generation colonies derived from primary colonies with diameters of 110 to 200  $\mu\text{m}$  generated similar median-sized colonies (133.0 versus 123.0  $\mu\text{m}$ ;  $p$  = 0.94). Secondary colonies derived from the largest primary colonies did generate a significantly greater number of large (>162  $\mu\text{m}$ ) as compared to the primary colonies ( $p$  = 0.04).

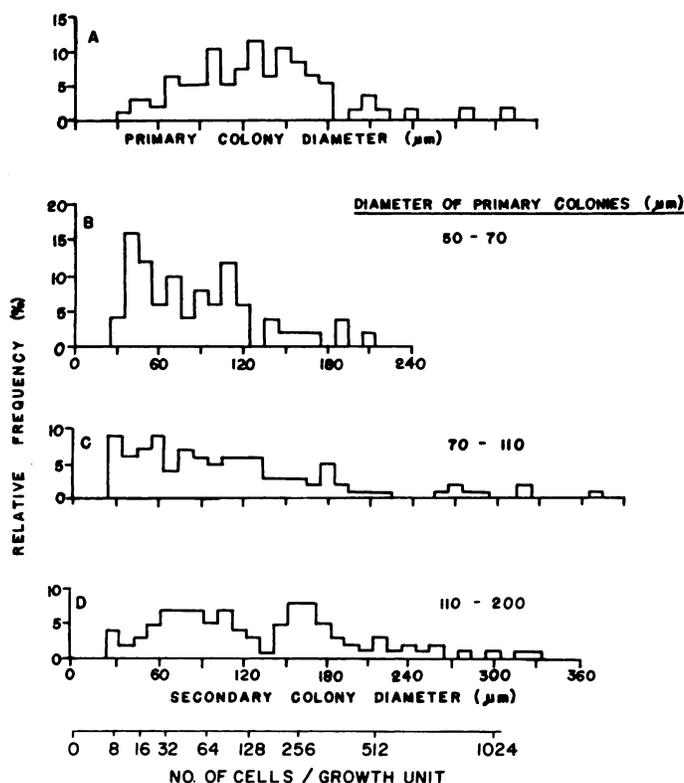


Chart 6. Frequency distribution of clusters and colonies by diameter (in  $\mu\text{m}$ ). A, primary colonies; B to E, secondary colonies grown from cells plated from primary colonies of indicated diameters. Data are based on sizing of 100 growth units/histogram.

## DISCUSSION

A simple stem cell model of human tumor growth has been proposed by Mackillop *et al.* (9). Its implications for human tumor cell cloning specifically predict that clusters may form from cells with limited proliferative potential, consistent with the "transitional" cells of a renewal hierarchy. In contrast, colonies may form from cells with extensive proliferative and self-renewal capacities, consistent with the stem cell of the renewal hierarchy. Thus, the cutoff size between clusters and colonies theoretically could be determined by testing the proliferative and self-renewal capability of different-sized growth units rather than just arbitrary designating a cutoff size. The present study tested this theory and found similar proliferative and self-renewal capacities for cells from colonies that resulted from 4 to 8 population doublings. Thus, based on the extensive data from 2 patients, the cutoff size for melanoma colonies would be less than 16 cells/growth unit. Whether clonogenic melanoma cells which form colonies with less than 16 cells represent clusters (transitional cells) or colonies (stem cells) cannot be determined from the present study because primary colonies with less than 16 cells were not replated. However, in one case, the secondary cloning efficiency of the smallest colonies was slightly lower, which suggests that colonies with less than 16 cells may lose some self-renewal capacity. Another point is that, although we found relatively high cloning efficiencies for primary and replating colony formation, the majority of cells failed to proliferate in agar. This suggests that a cell renewal hierarchy may be operating *in vivo* to produce these nonproliferating "end cells," although it may also just reflect

suboptimal culture conditions.

The kinetics of clonogenic cellular proliferation provides an alternative explanation of these results. We have recently shown that smaller melanoma colonies can result from a variable delay in the onset of clonogenic cell proliferation, with the smallest growth units forming from clonogenic cells with the most delay before they started growing in agar (17). These small colonies were formed because they started growing later, not because there was a limitation on growth imposed by the clonogenic cells being transitional cells of a cell renewal hierarchy. Furthermore, our culture conditions may tend to select cells with extensive proliferative capacities, leaving the less proliferative transitional cells as single cells in the agar. Indeed, in these 2 and other cases of melanoma, there are few colonies with 2 to 4 cells. In contrast, more small colonies have been seen for ovarian clonogenic growth by Buick (3), which may reflect a difference in the size of the transitional cell compartment for melanoma *versus* ovarian tumors or culture conditions which allow more ovarian transitional cell growth.

These results must be cautiously interpreted since these experiments were performed with pooled colonies and our studies assume that the frequency of secondary colony formation was not observed by a few colonies containing cells with an unusually high (or low) replating ability within a size class of primary colonies. Studies with one patient suggest that this explanation is unlikely (16). However, the possibility cannot be completely ruled out except by extensive studies of replating of cells from single primary colonies, a very difficult proposition for reasons outlined in "Materials and Methods" and as discussed elsewhere (16).

Other studies of self-renewal of human tumor clonogenic cells are limited to acute myelogenous leukemia (5) and ovarian cancer (4). Buick *et al.* have demonstrated that a subpopulation of primary colony cells grown from these 2 cancers can replate in agar. Although the effect of the size of primary colonies on secondary replating was not performed, transfer of cells from individual colonies showed that self-renewal was not limited to a few colonies and that it varied widely (4). These studies and fractionation experiments with ovarian cancer cells (8) suggested that human tumor cell growth may simulate a classical stem cell model with stem, transitional, and end cells (9).

The primary and secondary plating efficiencies were high for the present 2 and previously studied melanoma tumors (16), suggesting an extensive stem cell compartment. Furthermore, our data show that small multicellular growth units have high self-renewal and proliferative capacity. This suggests that, at least for clonogenic human melanoma cells, the probability of self-renewal,  $P_{SR}$ , may be high, and the clonal expansion number,  $n$  may be small, if not zero (9). The clonal expansion number ( $n$ ), the maximal number of generations of cell divisions that occur in the process of clonal expansion, may be different for different tumors (9). Indeed, the high primary cloning efficiencies, self-renewal, and proliferative capacities of melanoma compared to acute myelogenous leukemia (5) and ovarian cancer (4) suggest that  $n$  is much lower for melanoma tumors. This parallels the range for normal tissues, 10 to 17 for hematopoietic and 3 to 6 for epithelial tissues (9). Thus, it would not be unexpected that different tumors have stem cell compartments which are fundamentally different, paralleling this range of  $n$  for normal tissues.

In summary, our techniques allowed the direct assessment of

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the proliferative and self-renewal capacity of different sizes of melanoma colony-forming cells in order to define the cutoff size between melanoma clusters and colonies. Our results suggest that a high proportion of clonogenic melanoma cells have extensive proliferative self-renewal capacities as measured by replating in agar and may be representative of the stem cell compartment. These investigations provide further support for the use of this bioassay to explore biological responses and experimental therapeutics of human melanoma tumor cells *in vitro*.

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# Cancer Research



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