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Cloning efficiency of human melanoma cells is modulated after invasion through a reconstituted basement membrane

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Summary

Three human malignant melanoma cell strains (C8146A, C8146C, and C83-2CY), three established human melanoma cell lines (A375P, A375M, and C8161), and one selected human melanoma subline (A375P-5) were studied to determine if invasion through a reconstituted basement membrane-coated filter (RBMF), which selects the most aggressively invasive cells, would also modulate the cloning efficiency of these cells in soft agar. With the use of the Membrane Invasion Culture System (MICS), all cell strains tested showed a significant increase in cloning efficiency (1.05—9.3-fold) following transit through the RBMF when compared to unmanipulated populations. The established cell lines (A375P, A375M, and C8161) and the A375P-5 subline showed either a decrease or unaltered status in cloning efficiency after invasion. However, all cells demonstrated a consistent decrease in clonogenicity following transit through an uncoated filter compared with RBMFs, thus suggesting the influence of the extracellular matrix on tumor cell clonogenic properties. In general, the established cell lines were more clonogenic before invasion of the RBMF compared with the cell strains, and no correlation was found between clonogenic potential and invasive or metastatic capability. These data may provide important insight into the underlying mechanisms of tumor cell invasion and the subsequent formation and dissemination of metastases in vivo.

Introduction

The most important clinical problem of cancer is metastasis — the migration of tumor cells through the vasculature and tissue parenchyma that gives rise to additional tumors in the body. Of the many types of cancers, malignant melanoma, considered one of the most severe and painful neoplastic diseases, is increasing in incidence across the globe. By the year 2000, it is estimated that one in 90 Americans will be a victim of melanoma [21]. Whatever the etiology of melanoma may be, alarmingly little is known about the fundamental biology of its aggressiveness in some individual and quiescence in others. In the absence of such specific knowledge, clinicians

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are forced to employ relatively nonspecific therapeutic regimes in the treatment of this malignant disease.

The acquisition of the capacity to invade or penetrate a basement membrane (BM) is of primary importance in both the development of malignant melanoma and the genesis of many kinds of advanced malignancy because the BM is the most ubiquitous biological barrier that a wandering tumor cell is likely to encounter during intravasation, extravasation and dissemination in the human body [13,15]. Within a primary tumor are subpopulations of cells [7] containing some cells capable of invasion, other cells capable of invasion and metastasis [3], and still other cells with unknown capabilities.

Since less than 2% of tumor cells from a primary tumor mass are capable of establishing secondary foci [19], it seems prudent to study the uniqueness of this small, but lethal, aggressive subpopulation which is more able to evade the body's immune system than other tumor cells.

In order to better understand and ultimately treat the pathogenesis of tumor cell dissemination, we have focused our efforts on the utilization of improved methods to predict the invasiveness and ultimately the metastatic aggressiveness of a patient's tumor. One of these methods measures anchorage-independent growth and the formation of tumor colonies in soft agar, which has been used extensively in clinical oncology and cancer biology as in vitro indicators of tumor proliferation [5]. In addition, the application of chemosensitivity testing in the soft agar assay offers the advantage of direct observation of the effects of various drugs on tumor cell growth in vitro, thus allowing the recommendation of specific agents to be used in vivo. Another in vitro model, which has recently been adapted for testing the effects of anticancer agents on human tumor cell invasion is the Membrane Invasion Culture System (MICS; [8,29]). This model, which mimics extravasation, allows the assessment of cell invasion through a reconstituted basement membrane-coated fil-

ter (RBMF) in culture over an extended period of time.

We combined the technology of the soft agar assay and the tumor cell invasion model to test the hypothesis that invasion of human melanoma cells through a RBMF modulates the cloning efficiency of the selected subpopulations. Three selected strains, three established cell lines, and one selected subpopulation of human malignant melanoma, which have all been found to be tumorigenic in athymic nude mice, were used in this study to investigate the complex interrelationships of clonogenicity, tumorigenicity, invasive potential and metastatic capabilities.

Materials and methods

Preparation of cell cultures

Human melanoma cell strains C8146A, C8146C, and C83-2CY were previously developed in our laboratory. Low passage cells (from 3 to 7) were used in this study and were shown previously to be tumorigenic in athymic nude mice via subcutaneous administration [1]. The three established human melanoma cell lines used in this study were: (a) A375M, an intermediate metastatic variant (capable of producing a median of 146 tumors/mouse via tail vein administration), developed as a clone from the low metastatic parental line; (b) A375P (capable of producing a median of 3 tumors/mouse via tail vein administration); both of these lines have been previously characterized [11] and were a generous gift from Dr. I.J. Fidler, M.D. Anderson Cancer Center, Houston, TX; and (c) C8161, a highly metastatic line (capable of producing > 300 tumors/ mouse via tail vein administration) developed previously in our laboratory [1]. An additional subline was also used in this study — A375P-5, which was derived by allowing A375P cells to interact with a reconstituted basement membrane-coated filter (RBMF) in MegaMICS chambers for 24 h, then collecting the highly invasive subpopulation that invaded the RBMF, and subsequently reintroducing the subpopulation to additional RBMFs and

repeating the sequence a total of five times. Thus, the A375P-5 cells represent a highly invasive subpopulation or subline of the A375P line and has recently been shown to be metastatic in the nude mouse model (Dr. Danny R. Welch, Glaxo Research Company, Chapel Hill, NC, pers. commun.). The preparation of tumor cell suspensions for use as cell strains in the Hamburger-Salmon [5] soft agar assay has been described extensively [2,24]. Cells were maintained in Ham's F-10 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco; Grand Island, NY). Twenty-four hours prior to the invasion assays, the cells were transferred to Ham's F-10 supplemented with 10% (v/v) NuSerum (Collaborative Research; Bedford, MA) in preparation for assaying in the MICS chambers (see below).

For the determination of cloning efficiency of the human melanoma cells strains and lines in soft agar before invasion, each cell population was divided into three groups. These pre-invasion groups consisted of: (1) cells removed from plastic (after 48 h of growth) with EDTA and plated in 35-mm diameter culture dishes containing soft agar; (2) cells removed with dispase (Collaborative Research, Bedford, MA) from culture dishes coated with a reconstituted basement membrane material — matrigel (Collaborative Research) after 48 h, and then seeded in 35-mm diameter culture dishes containing soft agar; (3) cells removed with dispase from the upper surfaces of reconstituted basement membrane-coated filters (RBMF), which were harvested after a 48-h interval in the MegaMICS chambers (see Fig. 1) and then seeded in 35-mm diameter culture dishes containing soft agar. Determination of the cloning efficiency of cells that had invaded coated and uncoated filter barriers constituted the post-invasion groups: (1) cells collected with EDTA from the undersurfaces of uncoated, polycarbonate filters, which were harvested after a 48-h interval in the Mega-MICS chambers; (2) cells collected with EDTA from the undersurfaces of RBMFs following a 48-h interval in the MegaMICS chambers. All

post-invasive cells were centrifuged (15 min at 850 rev./min), resuspended in Ham's F-10 medium supplemented with heat-inactivated fetal bovine serum and plated immediately in 35-mm diameter culture dishes containing soft agar. For both groups of pre-invasive and post-invasive tumor cells, there were six replicates of the same concentration of cells (10^4) plated in soft agar in each experiment, and a minimum of two experiments were conducted per cell strain or line. All soft agar plates were incubated for 2 weeks at 37°C in a humidified incubator with 95% air and 5% CO_2. Colonies with 50 or more cells were scored for each experiment with an automated colony counter (FAS II; Bausch and Lomb, Rochester, NY).

Preparation of the in vitro basement membrane model

For the collection of sufficient numbers of tumor cells that had invaded a coated or uncoated filter barrier, all human melanoma cells were removed from culture in log phase growth with 2 mM EDTA/Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS) and seeded at a concentration of 4 × 10^6 cells/well onto either matrigel-coated or uncoated, sterilized, polycarbonate filters (Nucleopore; Pleasanton, CA), which contained 10-μm diameter pores, as previously described [8]. At the time of seeding, cells were loaded in Ham's F-10 with 10% NuSerum in order to reduce the level of protease inhibitors present during the invasion assays. The coated and uncoated filters containing cells were arranged in specially designed, large Membrane Invasion Culture System (MICS) chambers, called MegaMICS ([7]; see Fig. 1, patent pending). Each invasion chamber contains two 30 × 50 mm diameter wells, located in the upper and lower plates, with a filter "sandwiched" in between these. (Matrigel is composed of laminin, type IV collagen, heparan sulfate proteoglycan and entactin, which is representative of most biological basement membranes found in vivo; [10]). The cells were allowed to interact with either the RBMF or the uncoated filter for 48 h at 37°C with 95% air and 5% CO_2. Those
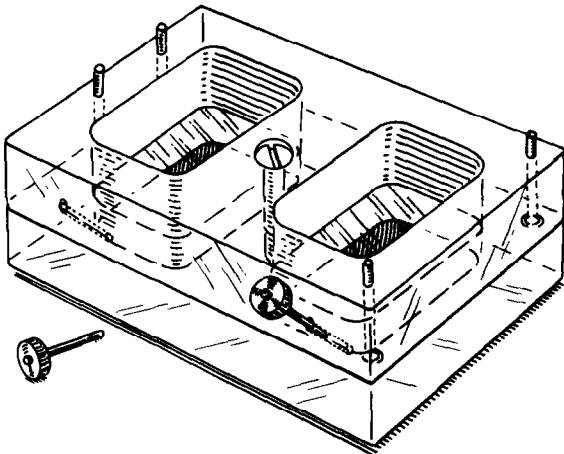
Fig. 1. The Mega Membrane Invasion Culture System (Mega MICS) was developed for the purpose of collecting large populations of invasive tumor cells after they have invaded a membraneous barrier. The chamber is composed of a top and bottom plate, containing upper and lower experimental wells, respectively, with side sampling ports. Briefly, the matrigel-coated filter is interposed between the top plate and bottom plate. Cells are then seeded into the upper wells, and invasive cells are subsequently collected in the lower wells via the side sampling ports.

cells that had invaded the membranes during that interval were collected from the lower experimental wells beneath the membrane via side-sampling ports (Fig. 1) and prepared for clonogenic studies, as described in the previous section.

The invasive potential of all the human melanoma cells was measured by using the analytical MICS chambers (shown in Fig. 2; patent pending), as previously described [8]. Briefly, a concentration of 10^5 cells was seeded per well onto matrigel-coated, sterilized polycarbonate filters within the MICS chambers. The cells were allowed to interact with the membrane for 48 h at 37 °C, and subsequently the invasive cells were collected from the lower experimental wells. The cells were stained and counted to determine percent invasion [8]. A minimum of six wells was used for each cell strain or line. Statistical analysis of all data was performed using the Student's t-test (SPSS/PC + program; IBM AT microcomputer).

Results

With the use of the analytical MICS chambers (Fig. 2), an evaluation of the number of tumor cells that had invaded the RBMFs after 48 h is reported as percent invasion (Fig. 3). These data were derived with the following equation:

$$\frac{\text{No. of Cells Collected Per Lower MICS Well}}{\text{No. of Cells Seeded Per Upper MICS Well}}$$

$$\times \ 100$$

The invasive potential of the tumor cells, ranging from the lowest to the highest percent, was: A375P, 0.05%; C8146C, 0.20%; A375M, 0.28%; C8146A, 0.30%; C83-2CY, 0.30%; C8161, 1.70%; and A375P-5, 2.90%. There is no significant difference among the invasion profiles of the cell strains
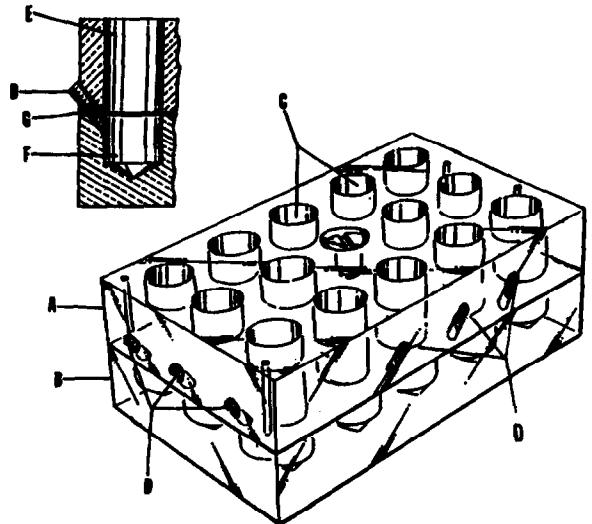

Fig. 2. Membrane Invasion Culture System (MICS) chamber: (A) top plate, (B) bottom plate, (C) experimental wells, (D) sampling ports, (E) upper well, (F) lower well, (G) matrigel-coated nucleopore filter is interposed between the top and bottom plate. Tumor cells are then seeded into the upper wells, attached to the coated filter, and are subsequently assayed at 48 h in the bottom wells via the side sampling ports.
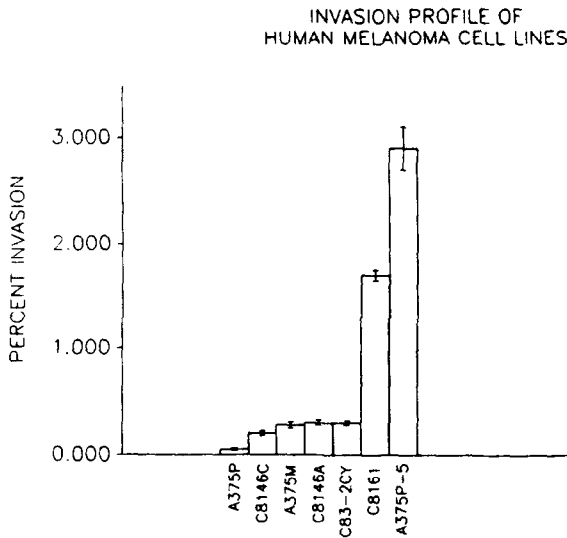
INVASION PROFILE OF
HUMAN MELANOMA CELL LINES

Fig. 3. Bar graph representing percent invasion of human melanoma cell strains and cell lines A375P, C8146C, A375M, C8146A, C83-2CY, C8161, and A375P-5 cells/well through matrigel-coated polycarbonate filters at 48 h (n = 6 for each cell; bar = standard error of 0.01% for A375P with 0.05% invasion, 0.02% and 0.20% invasion for C8146C, 0.03% and 0.28% invasion for A375M, 0.03% and 0.30% invasion for C8146A, 0.02% and 0.30% invasion for C83-2CY, 0.05% and 1.70% invasion for C8161, 0.02% and 2.90% invasion for A375P-5.

(C8146C, C8146A, and C83-2CY) and the A375M established cell line after 48 h in the MICS. However, a highly significant difference exists between this group and the A375P, C8161, and A375P-5 cells, such that $P <$ 0.001.

The analysis of percent cloning efficiency of the human melanoma cells tested in soft agar before (pre-invasion) and after (post-invasion) transit through RBMFs is presented in Table 1. The categories of the pre-invasion group represent: (a) cells grown on plastic for 48 h prior to seeding in soft agar, (b) cells grown on matrigel (RBM)-coated culture dishes for 48 h prior to seeding in soft agar, and (c) cells collected from the uppersurface of the RBMFs in the MegaMICS after 48 h (these cells did not

invade the filters during that time interval, whereas subpopulations of these cells did invade and were collected for cloning). The post-invasion group consists of: (d) cells collected from the undersurface of uncoated filters in the MegaMICS after 48 h and seeded immediately in soft agar, and (e) cells collected from the undersurface of RBMFs in the MegaMICS after 48 h and then seeded immediately in soft agar. The data show that all three cell strains (C8146A, C8146C, and C83-2CY) demonstrated a significant increase in cloning efficiency following transit through the RBMF compared to unmanipulated populations. C8146A cells collected from the undersurface of RBMFs showed a 1.5% increase in cloning efficiency compared with pre-invasive cells grown on plastic, a 0.9% increase compared with pre-invasive cells grown on RBM-coated culture dishes, and a 7.8% increase compared with pre-invasive cells attached to the uppersurface of the RBMFs during the 48 h MegaMICS assay. The C8146C cells collected from the undersurface of RBMFs showed a dramatic 9.3% increase in cloning efficiency compared with their pre-invasive counterparts grown on plastic, a 12.6% increase compared with cells grown on RBM-coated culture dishes, and a 23.1% increase compared with the cells attached to the uppersurface of the RBMFs. C83-2CY cells collected from the undersurface of RBMFs showed a 2.2% increase in cloning efficiency compared with pre-invasive cells grown on plastic, a 1.8% increase compared with the cells grown on RBM-coated culture dishes, and a 75.9% increase compared with cells attached to the uppersurface of RBMFs. Cells from all strains showed a significant decrease in cloning efficiency of post-invasive cells collected from the undersurface of uncoated filters.

In general, percent cloning efficiency for the established human melanoma cell lines (A375P, A375M, and C8161) and subline (A375P-5) was either unaltered or significantly reduced after cells invaded RBMFs. However, these cells were all more clonogenic before

Table 1. Percent cloning efficiency (mean ± S.E.) of human melanoma cells in soft agar: per-invasion vs. post-invasion.

Cell line	Pre-invasion			Post-invasion	
	Plastic	RBM-Coated culture dish	Uppersurface of RBM-coated filter/Mega MICS	Undersurface of uncoated filter/Mega MICS	Undersurface of RBM-coated filter/Mega MICS
C8146A	1.28 ± 0.12	2.08 ± 0.20	0.25 ± 0.02	1.14 ± 0.14	1.95 ± 0.06
C8146C	0.60 ± 0.03	0.44 ± 0.02	0.24 ± 0.09	0.81 ± 0.03	5.55 ± 0.20
C83-2CY	2.46 ± 0.10	3.00 ± 0.29	0.07 ± 0.00	2.45 ± 0.05	5.31 ± 0.08
A375P	5.90 ± 0.27	10.80 ± 0.53	9.54 ± 0.99	1.77 ± 0.08	3.22 ± 0.12
A375P-5	5.10 ± 0.27	6.12 ± 0.30	8.76 ± 1.38	0.27 ± 0.04	4.35 ± 0.16
A375M	26.98 ± 0.47	31.55 ± 0.45	29.5 ± 0.28	21.66 ± 0.67	28.98 ± 1.11
C8161	23.45 ± 0.06	26.93 ± 0.41	26.43 ± 0.50	14.78 ± 0.42	19.48 ± 0.37

Percent cloning efficiency (mean ± S.E.) of human melanoma strains C8146A, C8146C, and C83-2CY, cell lines A375P, A375M, C8161, and A375P-5 in soft agar before and after invasion through RBM-coated membranes are shown as the average of two to four experiments per cell strain or line. As a control the effect of cell-attachment-to-RBM vs. plastic on subsequent tumor cloning efficiency in soft agar and the effect of transit through an uncoated filter are shown. A significant increase in cloning efficiency is found in the post-invasive cells (RBM-coated filter) of the cell strains, but not in the post-invasive populations of the cell lines $(P < 0.01)$. For all cells except C8146C, there is a significant decrease in cloning efficiency of post-invasive cells that have traversed the uncoated-filter $(P < 0.01)$.

invasion of RBMFs compared with the cell strains. Collectively, a significant decrease in cloning efficiency was seen in the post-invasive A375P, C8161, and A375P-5 cells collected from the undersurface of the RBMFs in the MegaMICS compared with their pre-invasive counterparts on plastic, RBM-coated culture dishes, and on the uppersurface of RBMFs. The A375M cells showed a 1.1% increase in cloning efficiency in the post-invasive subpopulation collected from the undersurface of the RBMFs only when compared with their pre-invasive counterparts grown on plastic; however, compared with pre-invasive A375M cells on RBM-coated culture dishes and on the uppersurface of RBMFs, a decrease in cloning efficiency was observed. The post-invasive cells from the established lines and the subline, collected from the undersurface of uncoated filters, consistently showed a significant decrease in cloning efficiency compared with the RBM-coated filters.

A typical colony, containing 50 or more cells, in soft agar appeared to have the same morphology whether it consisted of pre-invasive or post-invasive cells from established lines or strains or the subline.

Discussion

From these experiments, we have observed a dichotomy in the clonogenic potentials of pre-invasive and post-invasive cell populations from cell strains and established cell lines of human melanoma. With respect to the data generated from the cell strains, a significant increase in cloning efficiency occurred (1.5—9.3-fold) between the post-invasive cells (collected from the undersurface of the RBMFs) and the unmanipulated, pre-invasive cells grown on plastic. In addition, an even greater increase in cloning efficiency (7.8—75.9-fold) was seen between the same group of post-invasive cells and the pre-invasive cells

removed from the uppersurface of the RBMFs after 48 h. These data suggest that those cells that invaded the RBMFs constitute not only a more invasive population, but also represent a selected subpopulation with more clonogenic potential compared with the pre-invasive cells attached to the uppersurface of the RBMFs. This idea of a selection process occurring during invasion can be further substantiated when considering the clonogenic data of the C8146A cell strain. This strain was unique among the three tested, in one set of controls, since no increase in cloning efficiency was seen in post-invasive cells (collected from the undersurface of the RBMFs) compared with the unmanipulated, pre-invasive cells grown on RBM-coated dishes. We suggest that the cells on the coated dishes consist of two potential subpopulations: (a) the aggressive invaders that could transit the RBMF within 48 h and possess a higher cloning efficiency, and (b) the non-invaders that will remain attached to the uppersurface of the RBMF during the 48 h time interval and possess a lower cloning efficiency potential.

In contrast, the established cell lines exhibited relatively unchanged (in the case of A375M cells) or slightly reduced cloning efficiency of the post-invasive subpopulations of the A375P, C8161, and A375P-5 cells. However, it is interesting to note that the established cell lines and subline were more clonogenic before invasion of the RBMF compared with the pre-invasive groups of the cell strains. These data might suggest the following intriguing possibilities: (a) the ability to select tumor cells with higher cloning efficiencies among subpopulations that have invaded RBMFs might only be possible during early culturing of cell strains; these cells have not yet lost many of the properties of the original tumor; and (b) cell strains are often more heterogeneous in composition compared with established lines that have been maintained ad infinitum in culture; therefore, selection of subpopulations of tumor cells possessing a more aggressive phenotype and demonstrating a higher cloning efficiency

could stimulate the metastatic behavior of these cells in vivo.

One of the most interesting observations from this study was the significant increase in cloning efficiency seen in the pre-invasive cells grown on RBM-coated dishes vs. plastic, regardless of the cell origin (exception: C8146C cell strain). Furthermore, all cells demonstrated a highly significant reduction in cloning efficiency among the post-invasive subpopulations collected from the undersurface of uncoated filters in the MegaMICS compared with the coated filters. These data strongly support previous reports showing the enhanced effects of matrigel on cell growth [9,12].

Since it has been shown that tumor cell invasion is a critical step in the complicated cascade of dissemination and metastases formation [13], we tried to simulate a portion of this dynamic process by combining two well-known assays — the soft agar assay and the MICS assay. Cloning capability and invasive potential of other human cell types, including the well-studied HeLa cell line, have been reported previously [22]. In that study, colony forming ability was correlated with invasion in a muscle organ culture assay system. In our study, the data generated by the post-invasive cells from the established lines and subline, would support the notion that there is no correlation between invasive ability and cloning efficiency. However, the data generated by the cell strains supports the opposite conclusion.

From experiments performed with the B16 murine melanoma model, it it known that a tumor can consist of a variety of subpopulations that differ in clonogenicity, tumorigenicity, in vitro invasive potential as well as metastatic potential in vivo [3,4,6,15]. The relationship among these four properties is complex and extremely important if a tumor is going to become invasive and metastasize.

The relationship between clonogenicity in vitro and tumorigenicity has been quite controversial. Several studies have emphasized the heterogeneous composition of clonal populations and warn that microenvironmental and

immunological influences exerted by the host can affect the data generated by such studies [18,20,28]. In particular, the biology of melanoma metastasis has been shown to be a selective process, which is influenced by the interactions of tumor cells with their host environment [19]. Most recently, studies by Nicolson and co-workers [16] have shown no correlation between the spontaneous metastatic potential of in vivo or in vitro grown tumor cells and their in vitro cloning efficiency in soft agar. Our study also demonstrated no correlation between the invasive and metastatic ability and clonogenic potential of human melanoma cell lines. However, a direct correlation does exist between the metastatic potential of a few lines tested — A375P, A375M, and C8161 and their invasive profiles generated in the MICS [29]. It is interesting to point out that the A375P-5 cells, which were highly selected for their increased invasive capability in the MICS showed a similar low cloning potential to the A375P (parental cells), both in the pre-invasive and post-invasive populations, thus demonstrating the lineage of that particular parental trait.

In order to effectively assimilate the information propagated by various in vitro models, the best approach in terms of creating effective treatment modalities would be to correlate data from multi-model systems. We have presented the initial experiments toward the establishment of such an approach. For instance, the soft agar assay has been applied to the study of chemotherapeutic agents on fresh tumor biopsies [14,23,25—27], and the MICS assay, which simulates extravasation, has recently been adapted for the same purpose [29]. By combining these two assays, it might be possible to better determine in vitro which chemotherapeutic agents would be effective against the most invasive cells in a primary tumor, for it is this highly aggressive subpopulation which we envision as establishing micrometastases. Such an approach might conceivably lead to improved clinical prognoses for patients with malignant melanoma as well as other highly aggressive neoplasms.

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