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Invited Review

Tagged tumor cells reveal regulatory steps during earliest stages of tumor progression and micrometastasis

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Summary. Histochemical marker genes were used to "tag" mouse fibrosarcoma or human neuroblastoma cells, providing a better understanding of their subsequent progression and metastasis mechanisms in nude mice. Micrometastases in the lung were initiated from clusters of 2-6 cells rather than single cells in most cases; tumor cells were also visualized binding to the endothelium of small blood vessels to initiate these micrometastases. Shortterm, these mechanisms relied heavily on fluidity of cell surface proteins, rather than nuclear events. Micrometastases in some organs were transient and never became established. Angiogenesis was visualized in both primary tumor systems via "fixation" of the animal's circulation; very small microvessels were growing toward the primary tumor as soon as 48-72 hours post-injection. Marker genes were also valuable for quantitating genetic instability of specific tumor cell populations and potential gene regulatory mechanisms operating in specific organ sites. These latter studies have direct relevance to the significance of N-*myc* oncogene amplification in neuroblastoma during progression and CD44 gene plasticity of expression in fibrosarcoma during metastasis. Marker gene-tagged single tumor cells can now be analyzed for gene regulatory events in virtually any organ and in combination with laser capture microdissection and other high-resolution methodologies, providing insight into the very earliest gene-regulatory events during micrometastasis.

Key words: Histochemical marker gene, Tumor progression, Micrometastasis, Angiogenesis, Gene regulation

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Introduction and background

Primary tumors develop from the original malignantly-converted cell via activation of oncogene(s) and downregulation of specific tumor-suppressor gene(s). Progression of the primary tumor from a small collection of tumor cells into a large, palpable tumor, along with the generation of small subpopulations of these tumor cells competent to undergo metastatic spread to various target organs, presumably involve many other genes. The products of these genes are responsible for specific cellular physiological events associated with progression and metastasis. These multiple events are complex and difficult to analyze, even in experimental animal model systems.

In the mid-1970s, investigators began to analyze these progression and metastasis mechanisms using human and mouse melanoma cells that could be easily identified and quantitated in target organs based on their black pigment via expression of melanin genes (Fidler et al., 1978). In non-melanoma tumor systems, this ease of identification was impossible, although quantitation could be effected by transfection of a drug-resistance gene into the tumor population and enumerating drug-resistant colonies grown out in culture (Heppner and Miller, 1983; Miller et al., 1987). The frustration of "tracking" fibrosarcoma or neuroblastoma tumor cells during metastasis in our own studies (Radinsky et al., 1987; Culp and Barletta, 1990), combined with the novel use of histochemical marker genes transfected into embryo cells to track cell lineages (Sanes et al., 1986), induced us to try a similar approach in analyzing tumor progression/metastasis (Lin et al., 1990a,b). This has been a highly rewarding endeavor, allowing us in both fibrosarcoma and neuroblastoma tumor systems to analyze the earliest events in micrometastasis and progression at the single-cell level. Some of these studies will be reviewed here.

One novel finding involved characterization of

primary tumors containing two genetically different cell classes (Lin et al., 1993). Mouse 3T3 cells were transformed with the EJ-H-*ras* oncogene and subsequently transfected with the *E. coli lacZ* gene as a histochemical marker (referred to as LZEJ cells). In parallel, another population of 3T3 cells were transformed with the human *c-sis* oncogene and then transfected with the human placental alkaline phosphatase gene as an alternative histochemical marker (APSI cells). This provided two different color reactions to distinguish the two genetically different tumor classes (Lin et al., 1992). Both classes were highly tumorigenic but only the ras transformant was metastatic to lung and other organs (Lin et al., 1990a,b).

When the two cell classes were mixed prior to their injection into the subcutis of nude mice, palpable primary tumors were then analyzed to determine the spatial distribution of the two cell types (Lin et al., 1993). We expected the two cell classes to be completely intermixed. In fact, this was never the case for the many tumors analyzed. The tumors were comprised of regional concentrations of only one cell class while the neighboring region was comprised of the second cell class. Sectioning of these primary tumors also revealed the regional homogeneity of one of the cell types with rare intermixing of the two at the borders.

There are two possible mechanisms to explain this regional dominance of each tumor class. First, soon after injection, each tumor class could "sort itself out" by migrating into regions containing exclusively one cell class and then expand to give rise to one region of the tumor. Alternatively, the tumor could be displaying clonal dominance in a regional pattern where only one cell type is permitted to outgrow its neighbors in that region. This interesting and novel finding has yet to be explained mechanistically but may provide a model as to how metastatic variants become dominant in highly specific regions of a primary tumor.

Establishment and disestablishment of micrometastases

Earliest detection of fibrosarcoma micrometastases

The earliest events in metastasis for fibrosarcoma systems were also analyzed, along with more limited study of neuroblastoma metastasis. This involved the experimental metastasis model where tumor cells are injected directly into the tail veins of nude mice to decipher the later events in metastatic spread, particularly to the lung, as well as spontaneous metastasis from various ectopic and orthotopic injection sites (Lin et al., 1990a,b, 1993; Kleinman et al., 1994). With *lacZ*-tagged, *ras*-transformed 3T3 cells, micrometastases in the lung were evident within 5 minutes of injection into the tail vein (Lin et al., 1990b). At these earliest time points, fixation of lungs followed by sectioning revealed tumor cells bound to the endothelium of small blood vessels and presumably "in the act" of extravasation into the tissue site (Fig. 1) (Lin and Culp, 1992a). This was quite apparent from Fig. 1 when tumor cells bound to the endothelium were "captured" in sections while other tumor cells had already escaped into the tissue space. By 1 hour post-injection, tumor cells were only occasionally observed at the endothelium while many micrometastases were evident in the tissue space. As expected, greater than ninety percent of these micrometastases were transient and cleared within 24 hours while the remainder persisted and many grew out into overt metastases (Fig. 2) (Lin et al., 1990a,b, 1993). These high-resolution sections should provide greater insight in future studies as to the criteria by which some select micrometastases become established while the vast majority are

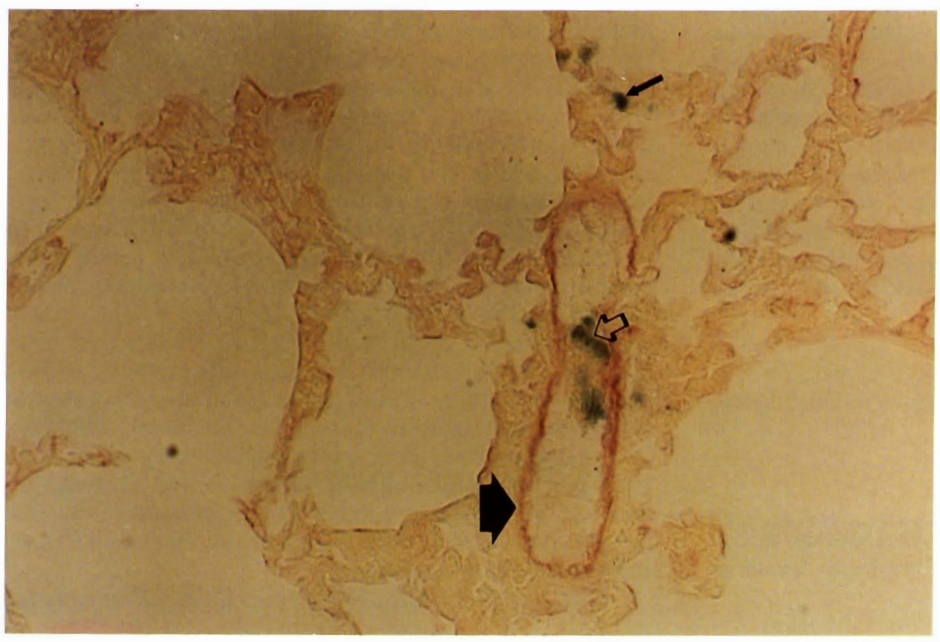

Fig. 1. Tumor cells adhering to the endothelium of a blood vessel. *Ras*-transformed, *lacZ*-tagged Balb/c 3T3 cells (LZEJ) (Lin et al., 1990a,b) were injected into the tail veins of athymic nude mice. At 30 minutes post-injection, animals were sacrificed; the lungs were then excised, fixed, and embedded in methacrylate at -20 °C and cut into 4μm sections for X-gal staining (Lin et al., 1990b). Blood vessels were detected with alkaline phosphatase staining (red-staining; broad solid arrow) while tumor cells stained blue with X-gal (small and open arrows). Note that there are tumor cells adhering to the endothelium within the blood vessel (open arrow), possibly in the process of extravasation, while other tumor cells have already escaped into the tissue space (small arrow). x 400. Taken from Lin and Culp, 1992a, with permission.

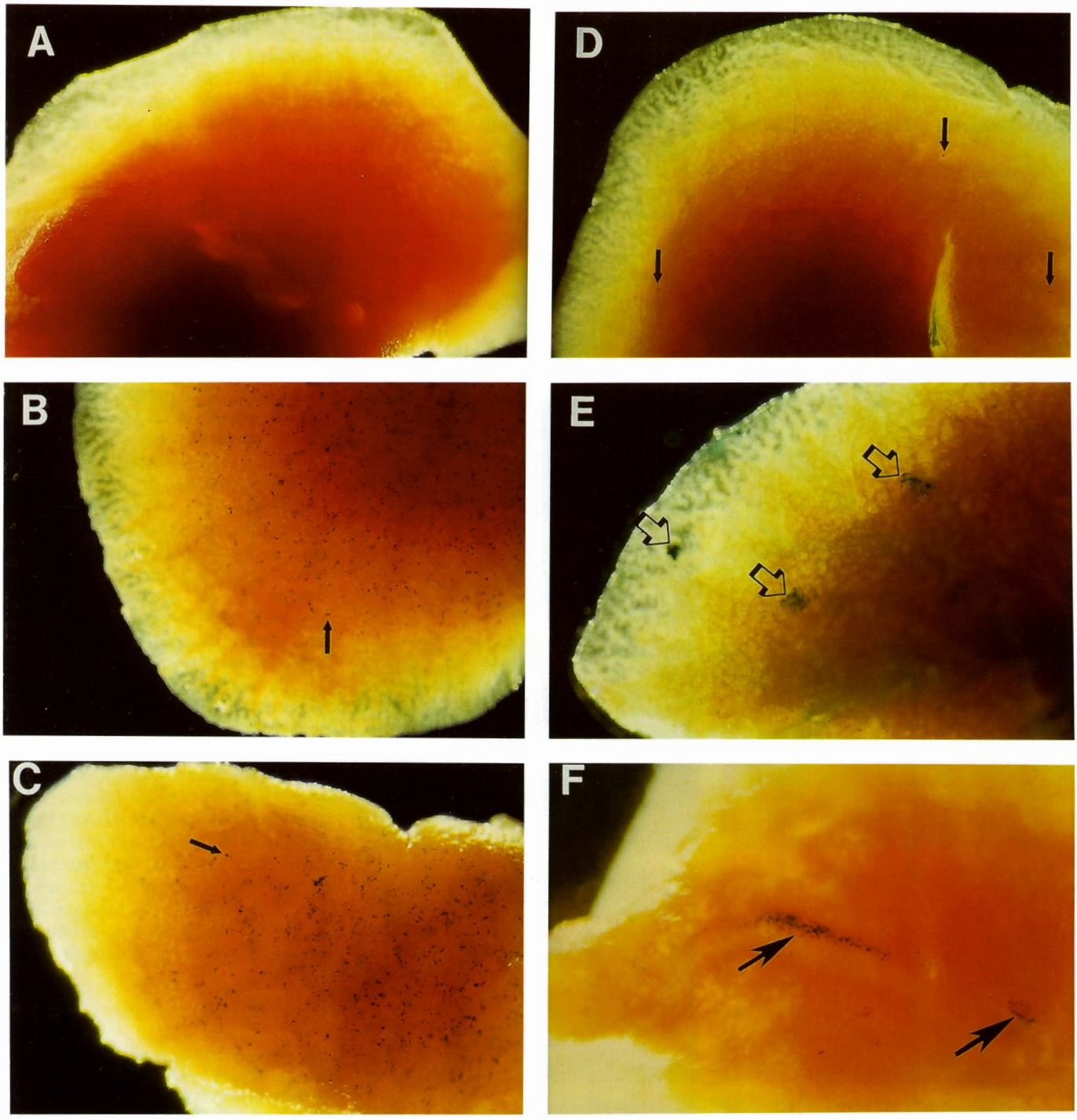

Fig. 2. Time course of establishment of experimental micrometastases in the lung. LZEJ cells ($1x10^5$; see legend to Fig. 1) were inoculated into the tail veins (or into the footpad as a control) of athymic nude mice. At various time points, mice were sacrificed; lungs were then excised, fixed, and X-gal-stained (Lin et al., 1990b). **A.** Lung from a mouse injected at the footpad, 5 hours after injection; note the complete absence of any staining since spontaneous metastasis had not yet occurred to this organ. x 15. **B.** Lung from mouse receiving cells via tail vein, 5 minutes earlier; many X-gal-staining foci are evident (e.g., black arrow) with a uniform distribution of tumor cell microfoci throughout the lung. x 15. **C.** Lung after tail vein injection, 1 hour earlier; many X-gal-staining microfoci persist at this time point (e.g., black arrow) and throughout the lung. x 15. **D.** Lung after tail vein injection, 24 hours earlier; most X-gal-staining microfoci have disappeared but a notable number (usually 1-3% of the original number) persist as stable micrometastases (e.g., black arrows). x 15. **E.** Lung after tail vein injection, 8 days earlier; three developing metastases (open arrows) are evident with expansion from the original micrometastases. x 22,5. **F.** Lung after tail vein injection, 14 days earlier; two expanding metastases (arrows) are evident and are following linear paths of development. x 22,5. Taken from Lin et al., 1990b, with permission.

disestablished from the tissue site (Fidler et al., 1978; Nowell, 1986; Nicolson, 1993). These discriminatory mechanisms continue to be an understudied area of tumor biology.

Micrometastases composed of multiple tumor cells

Serial sections were made of individual, established fibrosarcoma micrometastases in the lung of animals. These micrometastases were either spontaneously-generated or experimentally-injected (Lin and Culp, 1992b). Figure 3 provides an example of a micrometastasis composed of six tumor cells. Since this is well before tumor cell division could occur, this important finding implies that this micrometastasis became "established" by multiple tumor cells extravasating at that blood vessel site. This was the case for >85% of the micrometastases analyzed by this approach while only 10-15% contained a single tumor cell (Lin and Culp, 1992b). Multiple-cell foci could occur by two possible mechanisms: a) one or two tumor cells could penetrate the blood vessel, generating a channel for several other tumor cells to follow, or b) all six tumor cells could penetrate the blood vessel simultaneously after aggregating at the endothelium site. With better markers of endothelial cell and basal lamina integrity in these small blood vessels, it may soon be possible to resolve these two mechanisms.

Cell surface determinants are critical

These findings on the early events of micrometastasis to the lung led us to investigate what properties of the tumor cells are critical for their establishment (Lin and Culp, 1992b). In this paradigm, tumor cells were treated in culture with one of three cell-altering techniques: gentle fixation with a mild formaldehyde/glutaraldehyde mixture, irradiation with ^{60}Co, or treatment with mitomycin C. The first treatment reduces the mobility and some functions of cell surface proteins; the latter two treatments disrupt DNA replication, with limited disruption of transcription and translation and virtually no disruption of cell surface functions. In all experiments, the irradiation and mitomycin C treatments yielded cell populations that behaved the same as untreated cells: they formed multiple-cell foci in the lungs with clearance kinetics virtually unchanged. In contrast, the fixation protocol generated foci in the lung that

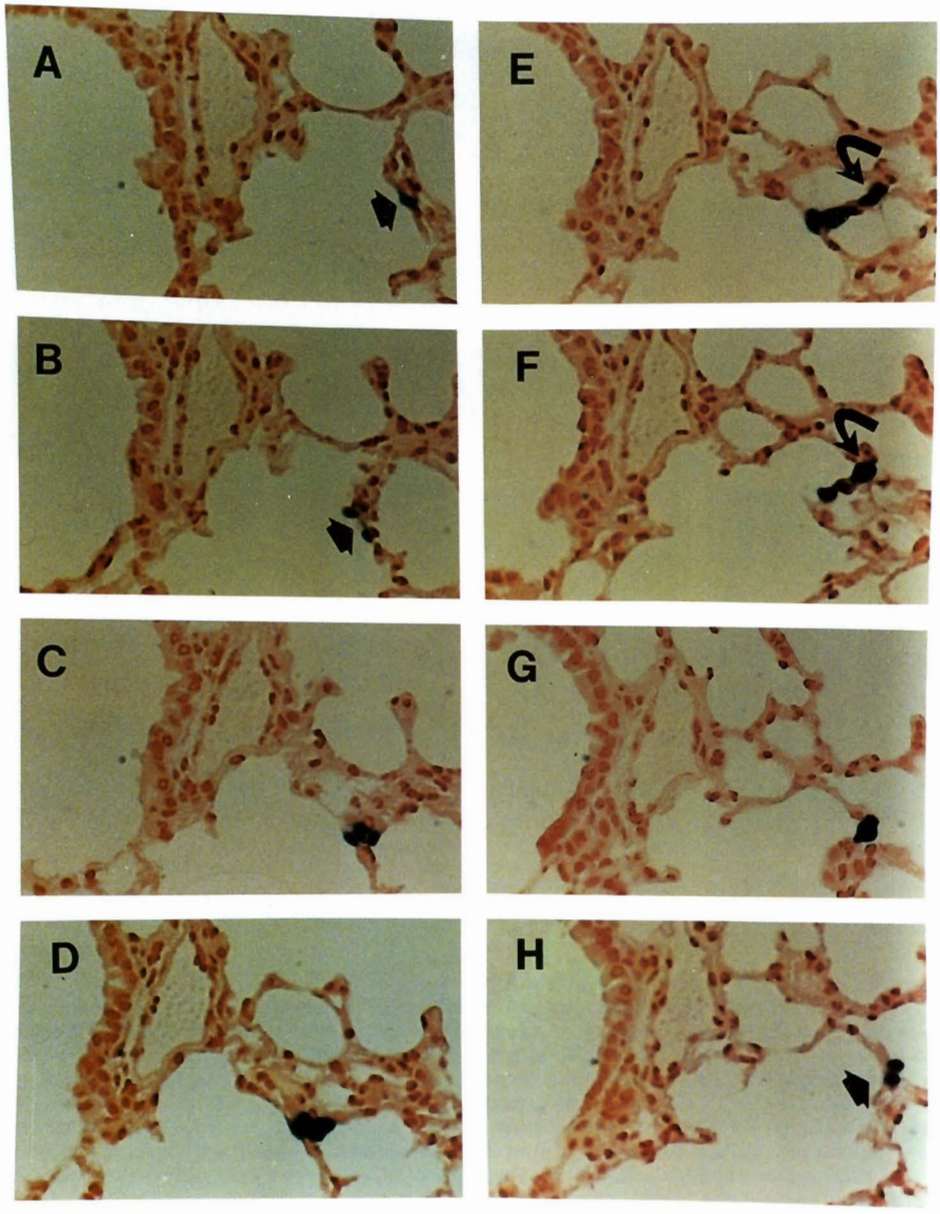

Fig. 3. Serial sections of a single micrometastasis. LZEJ tumor cells were injected into the tail vein of an athymic nude mouse. At 1 hour postinjection, the mouse was sacrificed; the lungs were excised, fixed, and X-gal-stained. One micrometastasis was carefully cut out of the tissue which was serially sectioned to give the sections [A]-[H] shown here. Note that this micrometastasis is comprised of 6 or 7 cells (the broad solid and curved arrows). The curved arrows also indicate a tangential turning point of this micrometastasis along a lung air sac structure. x 360. Taken from Lin and Culp, 1992b, with permission.

contained only single tumor cells (not multiple-cell foci) and their clearance was slowed down but was complete within one week, indicating that these cells were incapacitated for forming stable micrometastases (Lin and Culp, 1992b). First, these results illustrate that cell surface functions are critical for generating multiple-cell foci, probably because tumor cell:cell interactions become essential at these sites in blood vessels. Second, they establish that cell surface functions are also critical for stabilization of micrometastases in the lung, once extravasation from the blood vessel has occurred.

Transient micrometastasis to some organs

In addition to the rapid clearance mechanisms of some micrometastases from the lung described above, a second mechanism appears to operate in other organs. An example is provided in the fibrosarcoma system where micrometastasis to the brain of the animal was evident both in experimental models and spontaneously (Fig. 4) (Lin et al., 1990a). In these cases, micrometastases were observed over a period of several days to several weeks. They never expanded into overt metastases, possibly because they were missing some critical growth factor in that particular organ (Nicolson, 1993). Invariably, they were cleared from this organ. The same was true of the kidney, again indicating that some longer-term mechanism was not operating to provide a nutritionally suitable environment for these tumor cells.

Neuroblastoma versus fibrosarcoma comparisons

Extrapolating from our findings in the fibrosarcoma tumor system, we then challenged human neuroblastoma cells, *lacZ*-tagged, in similar paradigms (Kleinman et al., 1994). After tail vein injection, experimental micrometastases became established with the same kinetics as those from fibrosarcoma cells. They also were comprised of multiple cells and only rarely from a single cell, indicating that this phenomenon was not tumor type-specific. Also, primary tumor development was monitored as a function of time at two ectopic sites, the subcutis and the dermis. In both sites, clearance of some peripheral tumor cells, condensation of tumor cells into a focal mass, and expansion into a palpable tumor occurred with very similar kinetics. Injection of *lacZ*-tagged neuroblastoma cells into the adrenal gland (an orthotopic site for neuroblastoma) yielded excellent primary tumors and, in contrast to ectopic injections, spontaneous metastases to several organs (Flickinger et al., 1994; Judware et al., 1995).

Earliest stages of angiogenesis in primary tumors

Primary tumor development becomes limiting when interior tumor cells can no longer get sufficient nutrition (Blood and Zetter, 1990; Fidler and Ellis, 1994; Folkman, 1995). This led us to investigate whether microvessel development could be visualized at the primary tumor site by taking advantage of the color contrast between blue-staining tumor cells and the red staining of hemoglobin in blood vessels. To do this, fixative was perfused into the left ventricle of the heart after maximal anesthesia had been achieved; this left the blood virtually "fixed" in all tissue sites of the animal and provided an excellent contrast between X-gal-stained tumor cells and blood vessels (Kleinman et al., 1994).

Fig. 4. Spontaneous micrometastases developing in the brain. LZEJ fibrosarcoma tumor cells were injected subcutaneously into athymic nude mice. At 14 days post-injection when a medium-sized primary tumor (approx. 4-5mm diameter) had developed, mice were sacrificed and various organs excised for fixation and X-gal staining. Shown here is the brain with several micrometastases evident at this time point (open arrows). By 28 days, no micrometastases were evident in the brain, indicating that a clearance mechanism had been efficient at targeting these cells and/or the tumor cells lacked sufficient nutritional support to persist very long in the brain.

A surprising finding in this study was that very small microvessels were beginning to grow toward the primary tumor site within 48-72 hours after injection into the subcutis or the dermis (Kleinman et al., 1994). Within several days, these microvessels had developed into sizable vessels that were penetrating the tumor "space". By 2-3 weeks, very large vessels were serving the tumor effectively. These experiments (Kleinman et al., 1994) raise the intriguing question as to what soluble factors emitted by the injected tumor cells induce these very early microvessels to grow chemotactically toward the tumor cell population. Some candidate gene products can now be evaluated for this activity (Folkman, 1995).

Marker Genes: Tools for analyzing genetic instability

Histochemical marker genes integrate randomly into the tumor cell genome, using the eucaryotic expression plasmids described by Lin et al (1990a,b, 1992). Therefore, we might expect that these nonessential genes would be exposed to the same genetic instability mechanisms that operate in tumor cells for endogenously-expressed genes (Lin and Culp, 1992a). Furthermore, their ease of detection with histochemical reactions provides an ideal opportunity to quantitate and evaluate various mechanisms of this instability.

LZEJ fibrosarcoma cells, being the first transfectant analyzed with *lacZ*, were shown to be relatively stable (Lin and Culp, 1992a). After 15 passages in culture, >70% of the cells still stained with X-gal. In contrast, APSI fibrosarcoma cells were unstable (Lin et al., 1992). After 5 passages in culture, <40% of the cells retained stainability.

The same differences were evident in the neuroblastoma tumor system (Kleinman et al., 1994). LZPt-1 clone, when injected into nude mice, generated nonstaining primary tumors or primary tumors where only a small segment retained stainability. In contrast, LZPt-2 and LZPt-3 clones (independent transfectants with *lacZ*) yielded very uniformly-staining tumors, comparable to those generated with LZEJ cells. The rate of loss of expression of the histochemical marker gene can be quantitated by performing colony-growth assays and enumerating the percentage of colonies that stain as a function of continued growth in vitro or in vivo.

There are several mechanisms that could operate to turn a histochemical marker gene, that was originally highly-expressing in a transfectant, into a non-expressing state. The first would involve some genetic rearrangement of the *lacZ* gene at its chromosomal integration site such that it has lost promoter activity. Alternatively, the gene could be interrupted with integration of irrelevant DNA such that it has lost its integrity. A second general mechanism would involve transcriptional down-regulation. In this case, hypermethylation of the LTR or CMV promoter of the *lacZ* gene could result in considerable, if not total, loss of activity. There are many examples of this epigenetic regulation in tumor cells (Kogerman et al., 1997a,b, 1998; Lengauer et al., 1997; Smith, 1998). If this latter mechanism applies, then expression should be revertable upon treatment of the tumor cells with the methylation inhibitor, 5-aza-2'-deoxycytidine (Gorzowski et al., 1995; Merlo et al., 1995; Kogerman et al., 1997a). There may also be other mechanisms of transcriptional down-regulation, e.g., mutation of cis-acting sites in the promoter or loss of a critical trans-acting factor in the tumor cell population.

Gene activities in select tumor cells at the earliest stages of metastasis

Future studies in the fibrosarcoma and neuroblastoma systems

In our own studies, histochemical marker genes can be used in novel ways. Two such systems will be described here. First, amplification and overexpression of the N-*myc* oncogene in progressing human neuroblastoma lead to more highly aggressive and metastatic tumors (Judware et al., 1995). Transfection of an episome-harbored N-*myc* oncogene and selection for its amplification in neuroblastoma tumor cells containing only 1 or 2 copies of this gene (i.e., SKNSH) led to much more aggressive tumors (Flickinger et al., 1994) and downregulation of the expression of the ß1 integrin subunit gene (Judware and Culp, 1995). Closer scrutiny of the regulation of integrins in this system revealed two different mechanisms of downregulation of their expression; α2 and α3 integrin subunit genes were downregulated transcriptionally while the ß1 subunit was post-translationally regulated (Judware and Culp, 1997a). The same regulatory changes in integrin expression were found when the episomal N-*myc* was overexpressed in human Saos-2 osteosarcoma cells, indicating that these regulatory mechanisms are not necessarily cell-type or tumor-type specific (Judware and Culp, 1997b). It will be highly informative to transfect histochemical marker genes into these various N-*myc* transfectants and track the progression and metastasis of these cells as described in the paragraphs above.

A second system has been developed with fibrosarcoma tumor cells in which the plasticity of expression of the CD44 gene correlated remarkably with the metastatic competence of cells and inversely with their outgrowth as aggressive tumors. In this system, *sis*-transformed 3T3 cells (tumorigenic but not metastatic) were transfected with an overexpressing cDNA to the human CD44s isoform (Kogerman et al., 1997a). Transfectant cells became highly metastatic from the subcutis and all micrometastases express high levels of the human CD44s. In contrast, both large primary tumors and overt metastases in various organs have lost expression of CD44s (Kogerman et al., 1997a). Therefore, overexpressed hCD44s conveys metastatic competence to these normally-nonmetastatic tumor cells and is required for metastatic spread to the lung and some other organs. Conversely, its overexpression is selected against during primary tumor or overt

metastasis outgrowth for unknown reasons. Similarly, transfection of this gene into 3T3 cells makes them tumorigenic and metastatic (Kogerman et al., 1998). Experimental metastasis assays (i.e., tail vein injections) shed some insight into the mechanism of this metastatic competence conveyed by hCD44s (Kogerman et al., 1997b). Overexpression of this cell surface molecule increased the efficiency of colonization of the lung severalfold by transfectant cells and increased the stability of micrometastases once established. Unfortunately, the interactions between tumor cells and the vascular endothelium of lungs and other organs are not well understood. Cells transfected with histochemical marker genes should prove useful in resolving the mechanistic details of these interactions.

Laser-capture microdissection and histochemical tagging

The recent development of laser-capture microdissection as a means to evaluate gene expression in single tumor cells in virtually any tissue (Emmert-Buck et al., 1996) provides a powerful strategy that complements our own use of histochemical marker genes. Single tumor cells tagged with these marker genes can now be visualized in any organ and at any location within organs or within developing primary tumors or metastases. After histochemical staining, sections can be subsequently analyzed by laser-capture microdissection with the tumor cells easily visualized. Then many specific questions regarding expression of individual genes can be answered at the single-cell level. Two examples relate to our neuroblastoma and fibrosarcoma gene regulation studies described above. We can address questions as to when and where integrin subunits are downregulated during N-*myc* amplification and during progression of the neuroblastoma tumor. Similarly, when and where hCD44s becomes downregulated during aggressive fibrosarcoma outgrowth can be addressed, as well as the significance of the hCD44s cell surface protein as CD44s interacts with the target ligand on the endothelium of blood vessels in the lung (Kogerman et al., 1997b).

Further resolution of genetic instability in tumor populations

Having three different histochemical marker genes which stain different colors provides a powereful tool for examining genetic instability mechanisms (Lin et al., 1992). For example, a clonal population of tumor cells which is highly unstable in terms of *lacZ* expression and/or another gene product (e.g., CD44s) can be mixed with a second population tagged with human placental alkaline phosphatase or with *Drosophila* alcohol dehydrogenase and that is highly stable in its expression. The mixture can be injected into animals at ectopic or orthotopic sites to quantitate the ratios of the two cell types with time, as well as to measure the metastatic competence of the two cell classes. Furthermore, it is relatively easy now to determine if the unstable class conveys this instability to the "stable-cell" population or, conversely, if stability is conveyed to the "unstable-cell" population (Lin et al., 1993). Not only can these studies be executed with homologous tumor cells (i.e., both cell classes would be fibrosarcoma) but also with heterologous tumor populations (e.g., mixing genetically-unstable neuroblastoma with genetically-stable fibrosarcoma) to determine if there are tumor-specific mechanisms that apply to complementation mechanisms.

Chimeric genes to dissect functions during progression and metastasis

The gene encoding green-fluorescent protein (GFP) has been fused to other genes to generate chimeric gene products that can be tracked subcellularly in the fluorescence microscope. Unfortunately, fluorescence microscopy obviates the ability to visualize complex tissue architecture in tumor systems, leading us originally to use histochemical marker genes rather than fluorescing gene products such as GFP or luciferase. However, the functional domain of bacterial ß-galactosidase has been refined to a protein sequence of approximately 12Kd such that this minigene of *lacZ* could be fused to the C-terminus of a cell surface receptor gene and histochemical staining used to evaluate localization and intensity of receptor expression during progression and metastasis. As far as we are aware, this important experiment has not been performed in any tumor system. Such an experiment could clarify the roles of specific proteins which may exhibit highly plastic expression levels (e.g., CD44s[Kogerman et al., 1997a,b, 1998]). For example, this approach may be particularly useful in deciphering the rules of angiogenesis induction during tumor expansion. Many other cases for utility of such experiments will rely on specific questions being addressed in each experimental model system.

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