

## Review

# Tracking prostate carcinoma micrometastasis to multiple organs using histochemical marker genes and novel cell systems

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**Summary.** Studies of human prostate carcinoma (PCA) have been hampered by only a few cell systems from already-metastatic human disease. We have developed a novel cell system by using tissue cultured CWR22R cells from a xenograft of a primary tumor from a human patient. These cells were transfected with the bacterial *lacZ* gene to maximize their detection during progression and metastasis in nude mice. LZ-CWR22R cells are extremely stable for *lacZ* expression over 25 passages and metastasize to lung, liver, and bone from the subcutis - major sites of metastasis of the human disease. A matrigel vehicle facilitated development of primary tumors and micrometastases in all organs. While some micrometastases developed into overt metastases, others remained as micrometastases for long periods of time, possibly providing a model of latency of metastatic disease. An experimental metastasis model (tail vein injection) also generated micrometastases in lung, liver, and bone with differing kinetics of formation and stability. Serial sections of many individual lung micrometastases within one hour of injection indicated considerable heterogeneity in cellular composition (from 1 to 19 cells/site) while liver sites at later times were comprised of only 1 or 2 cells (the size of bone sites were comparable to those of liver). By combining use of these histochemically-tagged PCA cell systems with high resolution molecular analyses (laser-capture microdissection), it will now be possible to analyze gene expression patterns characteristic of micrometastases developing in several different organs.

**Key words:** Prostate carcinoma, histochemical marker gene, tumor progression, micrometastasis, gene regulation

## Introduction and Background

Animal models of human prostate carcinoma have principally used DU145, PC3, or LNCaP cell systems (Pretlow et al., 1994; Zhau et al., 1996; Lalani et al., 1997; Rubio et al., 1998; Yang et al., 1999). However, these cells are derived from metastases of human patients and therefore are already-selected metastatic variants. This obviates any analyses of how metastatic variants arise during formation of human primary tumors of prostate carcinoma (PCA).

New cell systems became available with development of several xenografts of primary tumors from human PCA patients passed in athymic nude mice (Pretlow et al., 1993; Wainstein et al., 1994; Cheng et al., 1996). One of these, CWR22R (Nagabhushan et al., 1996), was particularly notable because (a) it was metastatic to the lung in nude mice as a xenograft and (b) a tissue cultured cell line was developed from this xenograft that was also tumorigenic in nude mice (Sramkoski et al., 1999). The CWR22R cell line also shares many other properties characteristic of prostate cancer cells and the xenograft of origin (Sramkoski et al., 1999).

Our laboratory has had considerable success in analyzing the earliest events in progression and metastasis by transfecting a histochemical marker gene, such as bacterial *lacZ* or human placental alkaline phosphatase genes, into fibrosarcoma (Lin et al., 1990a,b, 1992) or neuroblastoma tumor cells (Kleinman et al., 1994) and tracking single tumor cells in any organ of nude mice via histochemical reactivity. These studies have been reviewed previously by us (Lin and Culp, 1992a,b; Culp et al., 1999) and others (Kruger et al., 1999).

We have now generated *lacZ* transfectants of the tissue cultured CWR22R PCA cell line to pursue progression and metastasis analyses in nude mice (Culp et al., 1998). Several transfectant clones of CWR22R demonstrated highly unstable or only moderately stable expression of the *lacZ* gene. However, clone H was



highly unusual (Culp et al., 1998). Its expression was extremely stable in cultured cell populations for >25 passages (>85% of the cells expressing it). This stability exceeded that of our previous analyses of many transfectant clones of fibrosarcoma and neuroblastoma (Lin and Culp, 1992a; Kleinman et al., 1994). Whether this is a novel property of this particular clone or is a general property of human prostate cancer cells derived from primary tumors will require analyses of many more xenografts and their complementary tissue cultured populations.

The tumorigenic properties of this LZ-CWR22R clone H cell line have been analyzed by Holleran et al. (2000). These and additional studies are reviewed below.

### Primary tumor formation

A critical issue was whether LZ-CWR22R cells formed tumors in the subcutis of nude mice as displayed by the original xenograft (Nagabhushan et al., 1996) and the original cell line (Sramkoski et al., 1999). This was the case under two different sets of conditions (Holleran et al., 2000). Animals injected with  $1-5 \times 10^6$  cells in a PBS vehicle yielded tumors 15-40% of the time over a lengthy period of time. In contrast, 100% of the animals developed tumors within 3-4 weeks when a matrigel vehicle was used for injection. This suggests that stabilization of these PCA tumor cells on a suitable extracellular matrix in the subcutis may be limiting for successful outgrowth. We had previously documented formation of the earliest primary tumors of fibrosarcoma (O'Connor and Culp, 1994) and neuroblastoma (Kleinman et al., 1994) tumor cells by tracking histochemically-stainable tumor cells in the subcutis at very high resolution within hours and days of injection. This type of analysis should be particularly revealing for LZ-CWR22R cells. By determining their fate in those first hours and days of residence in the subcutis, we can determine whether cells are cleared far more effectively from the subcutis when matrigel is not used or whether tumor cells persist in both cases but only with matrigel does outgrowth occur.

Another critical issue was the X-gal-stainability of tumors as they developed into large masses (Holleran et al., 2000). If tumors remained highly stainable throughout their development, then we could be confident of maximizing the discovery of micrometastases in any organ of the animal over lengthy periods. Indeed, this was the case (Holleran et al., 2000). Small or large tumors in the subcutis, whether cells were injected with PBS or matrigel vehicles, revealed excellent stainability with X-gal. Every primary tumor analyzed displayed excellent stainability. This was also true when tumors were cut into large segments and then these segments X-gal-stained, indicating that *lacZ*-nonexpressing cells were not clonally dominating at the interior regions of the tumor and that expressability persisted throughout the architecture of the large tumor mass.

### Spontaneous micrometastasis to multiple organs

While the original CWR22R xenograft was shown to metastasize to the lung but not to other organs (Nagabhushan et al., 1996), it was essential to determine whether the clonal population of LZ-CWR22R cells would also be metastatic to the lung and/or other organs. Our confidence in the sensitivity of this system was reinforced by the excellent and persistent X-gal staining of all primary tumors, as described above, but also by our previous analyses demonstrating our ability to detect single tumor cells in virtually any organ of the animal using histochemical reactivity (Lin and Culp, 1992a,b; Culp et al., 1998, 1999).

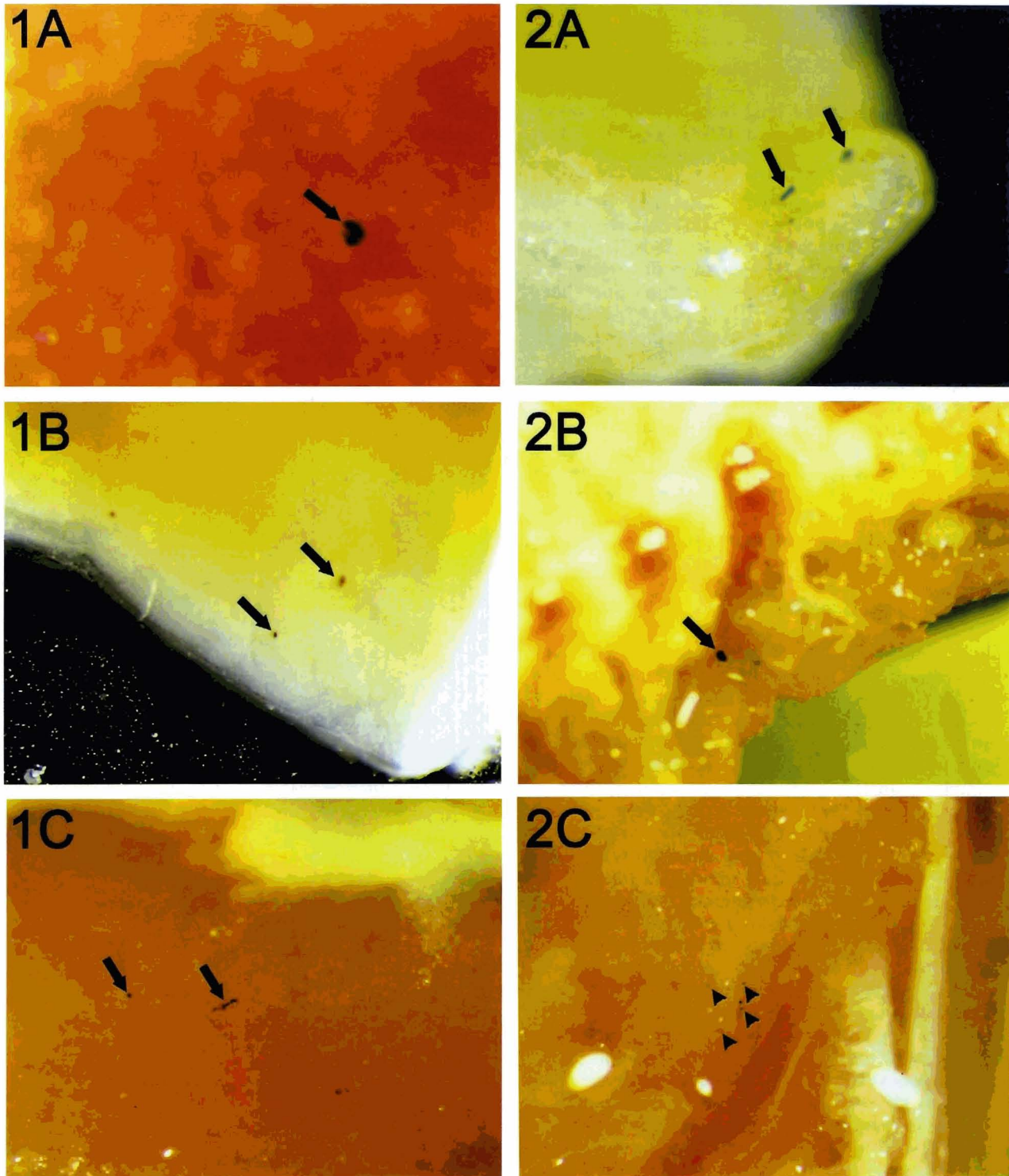
In animals injected s.c. with cells in a PBS vehicle and eventually developing primary tumors, spontaneous micrometastases were observed in the lungs of all these animals (Holleran et al., 2000). This was not the case for animals that did not yield sizable primary tumors, indicating that metastatic variants were indeed arising as subpopulations of the primary tumor. At lower frequencies, some animals also displayed micrometastases in liver, bone, kidney, and brain. These frequencies are enumerated as follows for PBS-injected cells: lung >>liver=bone (approx. 50%)>>kidney=brain (approx. 10%).

In animals injected with cells in a matrigel vehicle, the pattern of metastasis to multiple organs was qualitatively similar to those animals injected with PBS-borne cells. However, the quantitative relationships differed significantly (in addition to the excellent frequency of primary tumor formation reviewed above) [Holleran et al., 2000]. First, all animals developed micrometastases in their lungs - most animals with the number of sites comparable to PBS-injected cells but a few animals had a large number of lung micrometastases. Second, a higher frequency of bone micrometastasis was observed with matrigel-injected cells while the frequencies of liver and kidney sites were comparable. These frequencies are as follows: lung > bone (60%) > liver (20%) >> kidney or brain. This would indicate that the extracellular matrix provided by matrigel provides differing opportunities for selection of metastatic variants to bone and liver. This important finding should be addressed mechanistically with additional experiments.

Examples of spontaneous micrometastases are shown in Figs. 1, 2. A lung micrometastasis at fairly high magnification is shown in Fig. 1A while low magnification images of two liver micrometastases are illustrated in Fig. 1B (a third one is visible at the upper left in this panel). Kidney micrometastases (one with a highly unusual linear array of cells) are shown in Fig. 1C.

Particularly notable in our studies was the discovery that these cells could spontaneously metastasize to bone, a primary site of the human disease and a difficult target for spontaneous metastasis, except by using already-bone-metastatic PC3 cells (Lalani et al., 1997; Wu et al.,





**Fig. 1.** Micrometastasis to multiple organs with injection of LZ-CWR22R cells in a matrigel vehicle. Matrigel-suspended clone H cells were injected s.c. into nude mice. When the primary tumors had become large, animals were sacrificed and many organs were excised, fixed, and X-gal-stained to evaluate development of micrometastases. **A.** A lung micrometastasis (small arrow) at 44 days post-injection. x 102. **B.** Liver micrometastases (small arrows) at 72 days post-injection. x 34. **C.** Kidney micrometastases (small arrows) at 44 days post-injection. x 17. [Taken from Holleran et al., 2000, with permission.]

**Fig. 2.** Micrometastasis to bone. Clone H cells were injected s.c. in either PBS or matrigel vehicles. When primary tumors had become large, the animals were sacrificed for excision of many bones, followed by bone fixation and X-gal staining. **A.** Micrometastases (small arrows) in the long bone of the leg 17 days post-PBS-injection. x 42. **B.** Micrometastases (small arrow) along the spinal column 113 days post-PBS-injection. x 13. **C.** Micrometastases (small arrowheads) along the spinal column 44 days post-matrigel-injection. x 34. [Taken from Holleran et al., 2000, with permission.]



1998; Boyce et al., 1999; Koeneman et al., 1999; Lange and Vessella, 1999; Yang et al., 1999). Fig. 2A illustrates two micrometastases in the long bone of the leg under conditions where background staining by X-gal is minimized. Spinal column micrometastases are shown in Fig. 2B,C. There is some evidence (e.g., Jacob et al., 1999) that bone-recognizing adhesion protein, osteonectin, may be playing an important role in establishment of tumor sites in bone. It will now be important to screen for the participation of osteonectin and a variety of other adhesion ligands and their receptors to evaluate any critical participation in establishing prostate carcinoma micrometastases in various bone sites of the animal.

A highly remarkable aspect of these studies is the consistent metastasis of LZ-CWR22R cells to lung, liver, and bone. These three sites are the most common targets of the human disease process and offer the greatest difficulty in clinically treating metastatic disease in patients. Therefore, this cell model offers an excellent example for dissecting the gene- and cell-regulatory events that make prostate carcinoma such a devastating disease.

#### Outgrowth into overt metastases

These analyses also addressed whether micrometastases could grow out into overt metastases in these various organs. Micrometastases were unstable in the brain and kidney and eventually disappeared by unknown mechanisms. In contrast, micrometastases in the lung persisted over several months of animal observation as small foci. Rarely were overt metastases developing in the lungs of animals, indicating that this environment was not suitable for aggressive cell division of these populations while it was conducive for cells persisting at these sites for long periods of time (Tarin and Price, 1981). Several experimental approaches can now be used to determine how outgrowth of lung metastases could be facilitated by environmental cues.

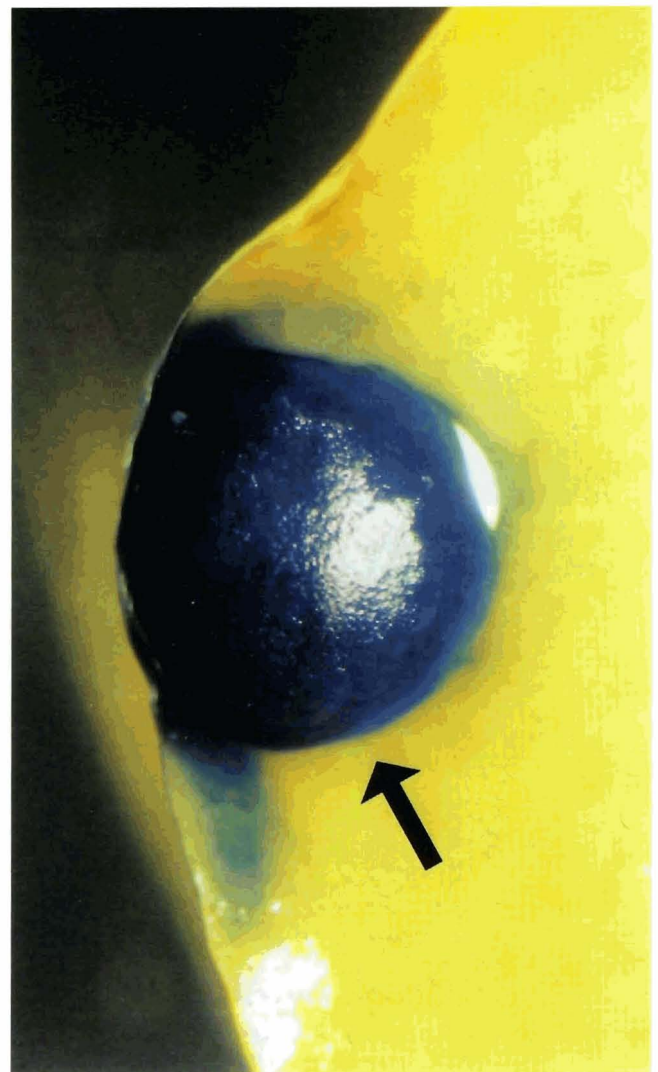
In contrast, overt metastases were observed in the liver. This is particularly notable because the lung was populated with a large number of micrometastases while the liver was populated with very few. Therefore, the "specific activity" of outgrowth of liver-selected tumor populations was very high. An example of a large liver metastasis is shown in Fig. 3. This tumor displayed excellent X-gal stainability after a lengthy period of outgrowth (possibly as long as 65 days). This again indicates the persistence of expression of the *lacZ* gene even when selection pressures are very high.

#### Androgen-relatedness for LZ-CWR22R cells

These initial studies of a reporter gene-tagged PCA cell system led to some additional and important experiments. Those of Holleran et al. (2000) were performed in young male nude mice without androgen supplementation. These animals have very low levels of

androgen in their circulation so that these initial experiments fail to address any androgen-relatedness of tumor progression and metastasis in this model. Clearly, androgen does regulate to some extent the fate of primary tumor development in humans and the evolution of metastatic variants (Pretlow et al., 1994; Zhou et al., 1996; Lalani et al., 1997; Boyce et al., 1999; Koeneman et al., 1999; Lange and Vessella, 1999).

To further test the significance/insignificance of androgen in this system, female mice or male mice in which testosterone pellets were implanted s.c. were evaluated after s.c. injection of tumor cells (J.L. Holleran & L.A. Culp, unpublished data). In both cases, matrigel was used as a vehicle to maximize efficiency of primary



**Fig. 3.** Overt metastasis in liver. LZ-CWR22R clone H cells were injected s.c. in PBS. When primary tumors had become large (in this case, 65 days post-injection), animals were sacrificed for excision of many internal organs, followed by their fixation and X-gal staining. A large liver metastasis (large arrow) was developing, staining throughout for *lacZ* expression. x 13. [Taken from Holleran et al., 2000, with permission.]



tumor formation and animals were observed for as long as six months.

For female mice, primary tumor formation occurred at a lower frequency (20% of animals never developed them) and with a longer latency. Micrometastases were observed in all lungs of tumor-bearing animals but at a lower frequency than the experiments described above. Only 2/15 animals displayed bone micrometastases and no micrometastases were observed in any other organs, particularly liver.

In contrast, male mice supplemented with testosterone pellets displayed a latency of primary tumor formation comparable to unsupplemented animals. All animals developed large primary tumors and they displayed an elevated frequency of micrometastases in liver and bone, as well as a much greater number of sites in the lungs. These experiments taken together indicate that testosterone is not absolutely critical to demonstrate micrometastasis to some organs but that it does elevate the efficiency of the selection process to other organs as well as increase the efficiency of primary tumor formation (J.L. Holleran & L.A. Culp, unpublished data).

#### Experimental metastasis model using LZ-CWR22R cells

The ability of LZ-CWR22R cells to spontaneously metastasize to lung, liver, and bone during one cycle of primary tumor outgrowth led us to question the origin of these metastatic variants. Were they present in the tissue cultured cell population already or were they only selected after lengthy outgrowth of the primary tumor in the subcutis? One approach for addressing these issues is the experimental metastasis model in which cells are injected into the tail veins of nude mice to test their ability to colonize one or more target organs (Fisher and Fisher, 1967; Fidler et al., 1978; Fidler and Hart, 1982; Kawaguchi and Nakamura, 1986; Fornabaio et al., 1988; Fidler and Ellis, 1994). This route obviates any participation of selection in the primary tumor and determines whether subsets of cells in the cultured population are competent for invading and colonizing specific organs. This approach was used successfully by our laboratory to demonstrate the importance of cell surface CD44s in fibrosarcoma colonization of the lung (Kogerman et al., 1997).

These experiments become quantitative when individual tumor cells can be tracked in target organs minutes, hours, and days after injection - e.g., radiolabeled cells (Fisher and Fisher, 1967; Fidler, 1970) or melanin-producing melanoma cells (Fidler and Hart, 1982). In our case, expression of *lacZ* has been ideal for these purposes (Lin et al., 1990b; Lin and Culp, 1992a,b; Culp et al., 1998, 1999); in both fibrosarcoma and neuroblastoma (Kleinman et al., 1994) systems, we demonstrated that cultured cells injected into tail veins could only populate the lung and no other organs.

This approach has now been adapted to our LZ-

CWR22R cell system (J.L. Holleran, C.J. Miller, N.L. Edgehouse, T.P. Pretlow and L.A. Culp, 2001, submitted for publication). Minutes after tail vein injection, LZ-CWR22R cells were colonizing the lung. While measures were taken to verify that a single-cell suspension was being injected (verified by plating them into tissue culture dishes at the same time), it appeared that the sizes of lung sites were quite heterogeneous. The number of these experimental micrometastases maximized during 1-2 hours after injection but were virtually all cleared from the lungs within 24 hours. This contrasts with *lacZ*-tagged fibrosarcoma cells whereby 1-2% of the sites persisted in the lung for many days and weeks after injection (Lin et al., 1990b; Lin and Culp, 1992a,b).

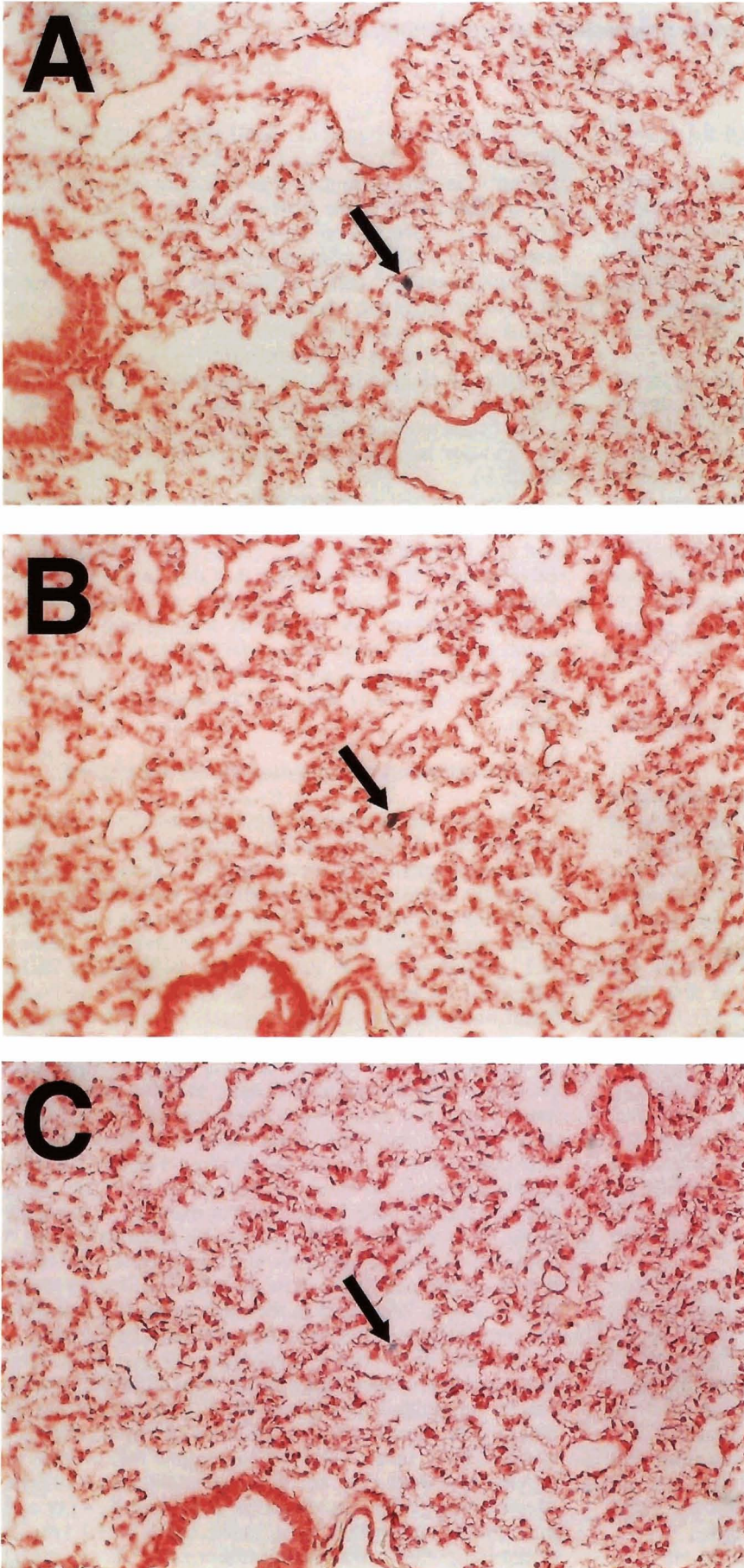
Experimental micrometastases were also observed in the liver and bones of these animal at a lower frequency and at a later time point (J.L. Holleran, C.J. Miller, N.L. Edgehouse, T.P. Pretlow and L.A. Culp, 2001, submitted for publication). However, these sites were much smaller and of a uniform size indicating homogeneity in their initial formation. These sites persisted for as long as one week before being cleared from the organ. At a lower frequency some sites were also observed in kidney and brain.

#### Serial sectioning of individual micrometastases

To date no laboratory has addressed the cellular complexity of individual micrometastases soon after they have formed. One reason may have been the lack of a suitably sensitive marker to identify individual cells in 4-5  $\mu\text{m}$  thick sections. Such an analysis was undertaken in our model with lung sites (J.L. Holleran, C.J. Miller, N.L. Edgehouse, T.P. Pretlow and L.A. Culp, 2001, submitted for publication). At the 15 minute time point, serial sections were made of X-gal-stained/neutral-red-counterstained lungs and 22 individual micrometastases enumerated for the number of cells per site. Most sites contained 1 to as many as 5 cells per site. However, a small proportion of sites contained more cells - as many as 19 cells in one case. Since these cells have not had sufficient time to divide, this survey verifies the size heterogeneity of sites in the lung directly as a consequence of their formation. An example is provided in Fig. 4 in which a 2-cell site can be observed across 3 adjacent sections; sections adjoining these failed to reveal any stained tumor cells. In a few rare cases, tumor cells could be visualized at this time point still bound to the endothelium of the small blood vessels of the lung.

These data raise question as to how this heterogeneity in lung sites arises. Two different models can be suggested to explain these findings, either of which will require more sophisticated experiments to address. First, tumor cells may aggregate into clumps of varying size once they are in the animal's blood stream. Alternatively, cells may circulate in the blood singly and then "home" to specific sites in the small blood vessels of the lung where "pioneering tumor cells" have opened





**Fig. 4.** Serial sections of a single micrometastasis. Clone H tumor cells were injected into the tail vein of an athymic nude mouse. At 30 minutes post-injection, the mouse was sacrificed; the lungs were excised, fixed, and X-gal-stained. Pieces of lung were cut out of the tissue, one of which was serially sectioned to give the sections (A-C) shown here harboring only one micrometastasis. Sections were 5  $\mu\text{m}$  thick. Note that this micrometastasis is comprised of 2 cells (arrows). Several sections on either side of these three sections did not reveal any tumor cells. x 65



a route into lung tissue architecture.

A similar analysis was made of liver micrometastases (J.L. Holleran, C.J. Miller, N.L. Edgehouse, T.P. Pretlow and L.A. Culp, 2001, submitted for publication). However, there are far fewer of these sites and only 8 individual sites could be dissected by this approach. In all cases, only 1 or 2 cells could be identified in these 8 sites. This is consistent with the relative homogeneity of liver sites and/or their smaller sizes compared to many of the lung sites. Although bone micrometastases have not yet been serially sectioned, their sizes are also quite small and similar to those of liver, suggesting that only 1 or 2 cells comprise these sites.

### Major findings from these studies and future directions

Several conclusions can be drawn from this experimental metastasis analysis of LZ-CWR22R, particularly when compared to the spontaneous metastasis studies deciphered by Holleran et al. (2000). First, the tissue cultured population contains subsets of cells that are competent for establishing transient micrometastases in lung, liver, and bone without going through any selection process in a primary tumor. Whether these cell types are identical to those that form spontaneous micrometastases from the subcutis-borne primary tumor remains to be tested. This may be unlikely given the instability of the experimental micrometastases versus the relative stability of the micrometastases in these same organs based on selection from a subcutis-localized primary tumor.

It will now be possible to do gene-array analysis of both spontaneous and experimental micrometastases (individual ones at that) to test their gene expression patterns. Laser-capture microdissection (Emmert-Buck et al., 1996; Schutze and Lahr, 1998; Simone et al., 1998) can be used to capture these very small micrometastases from X-gal-stained lung, liver, and bone for several comparisons: (a) among sites from the three target organs to test for organ specificity in gene expression; (b) between spontaneous and experimental micrometastases in one organ to determine if the same cell subpopulations are being selected; (c) between micrometastases forming in liver or bone from primary tumors developing in the subcutis (ectopic injection) versus the prostate gland (orthotopic injection) to determine if routing selects for different cell types; and (d) between micrometastases forming in lung after injection of LZ-CWR22R cells versus *lacZ*-tagged fibrosarcoma cells to determine any common gene activities shared by these two different tumor classes.

Another important contrast in these studies is the marked homogeneity of micrometastases in liver and bone with only single or doublet cells in each site and the heterogeneity in size of lung sites. Since the lung is the first organ encountered after tail vein injection, this may indicate that any aggregation in the blood leads to

“filtration” of aggregates once in the lung circulation. Subsequent circulation to the liver and skeletal sites would only include single cells. However, this does not exclude the possibility that lung sites for tumor cell colonization are intrinsically different from sites in liver and bone by permitting many cells to “collect” at specialized lung microvasculature sites for whatever reasons (Blood and Zetter, 1990; Folkman, 1995). Perhaps the best way to address these questions is to inject single-cell suspensions of tumor cells into other blood vessels of the animal and monitor the size and cellular composition of experimental micrometastases at the first organs encountered versus those at distant organs. Other experimental approaches may also be useful - e.g., injecting various dilutions of tumor cells to determine if cell concentration plays any role, injecting two different cell types tagged with different histochemical marker genes to determine cellular complexity (Lin et al., 1993), and performing sequential injections to determine if there are a limited number of lung sites that are “saturable”.

Comparisons of our previous studies with *lacZ*-tagged fibrosarcoma cells (Lin et al., 1990b; Lin and Culp, 1992a,b) and these LZ-CWR22R PCA cells (Holleran et al., 2000) lead to several other hypotheses. Why are LZ-CWR22R cells cleared so completely from the lungs of animals after tail vein injection while a sizable subset of fibrosarcoma cells persist? These differences may reflect differential attack by natural killer cells or relative efficiencies of two different immuno-modulatory mechanisms operating in the lung as observed in other tumor systems in animal models (Glaves, 1980; Barlozzi et al., 1983; Kawaguchi and Nakamura, 1986; Ramani and Balkwill, 1988; Aslakson et al., 1991). One approach for addressing these differences is co-injection of LZ-CWR22R cells with fibrosarcoma cells tagged with a different histochemical marker gene (e.g., human placental alkaline phosphatase). This approach has been used by us to test two different oncogene-transformants of fibroblasts to demonstrate synergy between two genetically-different subpopulations (Lin et al., 1993).

Overall, this LZ-CWR22R cell system is an ideal model of the human disease processes of progression and metastasis for several reasons. First, this cell type was derived from a primary human tumor and, therefore, is not an already-metastatically-selected cell subpopulation such as DU145, PC3 or LNCaP. Second, LZ-CWR22R cells metastasize to the three principal organs involved in the human disease beyond the prostate gland in both spontaneous and experimental metastasis model systems - i.e., lung, liver, and bone. Third, the efficiency of selection of metastatic variants targeting the bone as spontaneous events offers promise for the first time of evaluating the gene regulatory events required to populate this unusual organ; the environment of bone must be very foreign to these transformed prostate epithelial cells. We are currently undertaking such studies (J.L. Holleran, J. Dennis and L.A. Culp,



unpublished data). Finally, the efficiency of outgrowth of liver micrometastases into overt metastases contrasts with the inefficiency of these events in lung and bone; therefore, these overt liver metastases should be evaluated with cell and molecular biological approaches to determine the basis for this efficiency. Reporter gene-tagging of these PCA cell systems offers us the highest degree of sensitivity for identifying the earliest events in micrometastasis formation and combining this biological system with ultra-sensitive gene evaluation techniques should identify organ-specific gene expression patterns.

**Acknowledgements.** The authors acknowledge partial support for some of these studies from the Comprehensive Cancer Center of the Ireland Cancer Center at Case Western Reserve University (NCI-supported via P30-CA43703) and the support of research grant DAMD 17-98-1-8587 from the U.S. Army. Athymic nude mouse experiments were conducted in the Athymic Animal Facility (AAALAC-I-approved) of the Case Western Reserve University/Ireland Cancer Center, assisted by Pamela Steele, and approved by the Animal Care and Use Committee of this University. The authors thank Drs. Thomas and Theresa Pretlow of the Department of Pathology for assistance with tumor and organ tissue sectioning and immunohistochemistry protocols, pathology consultation, consultation of PCA xenograft biology, and use of testosterone pellets implanted into nude mice. The assistance of Joseph Giaconia in the Pretlow lab is also acknowledged for implantation of testosterone pellets. The CWR22Rv1 cell line was kindly donated by Dr. James Jacobberger of the Cancer Center.

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Accepted March 25, 2001