Analysis of Clonal Evolution in a Tumor Consisting of pSV2neo-transfected Mouse Fibrosarcoma Clones

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The process of clonal evolution was analyzed in a line of methylcholanthrene-induced mouse fibrosarcomas. The tumor cells were transfected with pSV2neo gene and 22 clones were randomly isolated. Genetically tagged clones were mixed and inoculated into syngeneic mice. Southern blot analysis revealed that one of the clones, no. 11, dominated both in tumors *in situ* and in lung metastatic nodules. No. 11 clone and other clones were similar in growth rates *in vitro* and *in vivo*, in spontaneous and experimental metastatic abilities, in immunogenicity, and in the capacity of intercellular communication *in vitro*. Although no. 11 clone overgrew other clones *in vivo*, this was not the case when clones were mixed and maintained *in vitro*. We conclude that clonal interactions *in vivo* may be responsible for the dominance of no. 11 clone in the tumor. It is likely that the preferential metastasis of no. 11 clone to the lung may be a simple reflection of the proliferative advantage of the dominant clone in the tumor *in situ*.

Key words: Mouse sarcoma — pSV2neo — Clonal evolution — Metastasis

Despite major advances in general patient care, surgical techniques and adjuvant therapies, most deaths from malignant tumors are caused by the growth of metastases that are resistant to therapy. The resistance of tumors to therapy is generally thought to be due to the presence of variant cells arising in a tumor subpopulation.[1, 2] Malignant tumors are genetically unstable. The instability constantly generates subpopulations of tumors exhibiting a variety of biologic phenotypes. The heterogeneity of the tumor cells includes those of cell-surface properties, antigenicity, immunogenicity, growth rates, karyotypes, sensitivity to radiations and cytotoxic drugs, and metastatic ability.[3–5] On the other hand, the host environment selects a subpopulation of tumors which is best adapted to host conditions such as immunological attacks, presence of particular growth factors, tissue microenvironment, low oxygen supply, and nutrient supply.[6] Thus, the genetic instability and the selection pressure are two distinct forces operating on tumor cell populations. These forces determine the process of clonal evolution,[7, 8] one generating variant subpopulations and the other selecting a particular subpopulation. In order to understand the mechanism of tumor progression, a detailed analysis of clonal evolution is necessary. We developed genetic tagging of tumor cells for such analysis, and this method has also been used by others.[9, 10] With this method, we were able to follow the dynamics of clonal evolution and the process of metastasis.

MATERIALS AND METHODS

Animals Male and female (C57BL/Ka×C3H/He)F₁ mice (BCF₁ mice), bred in our laboratory, were used throughout the study.[11] Mice used were age- and sex-matched within a set of experiments. Mice were kept in metal cages, in an air-conditioned room. They were given commercial pellets and tap water *ad libitum*.

Cells The cells used in this experiment, 505-05-01, were a clonal line derived from an *in vitro*-adapted mouse fibrosarcoma, 505, which was induced originally by methylcholanthrene (MCA) in BCF₁ mice.[11] The cells were cultured at 37°C in 5% CO_2 and 95% air in alpha-modified minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin. The cells stably retained the properties of the parental tumor during the period of study. They are of fibrous morphology *in vitro* and produced a well-differentiated fibrosarcoma *in vivo* which spontaneously metastasized mainly to the lung, forming a few nodules.

DNA transfection DNA-mediated trnsfection was performed by the method of Graham and Van der Eb[12] with modifications according to Wigler *et al.*[13] Briefly, 5×10^5 cells were seeded onto 10 cm dishes 24 h prior to the transfection. Cells were transfected with 1 μg of pSV2neo plasmid together with 10 μg of calf thymus DNA. Plates were split 1:3 at 48 h after the transfection and maintained in a selective medium containing 400 μg/ml of G418 (Geneticin, GIBCO, N.Y.); resistant colonies were ring-cloned and propagated for further use.

DNA extraction and Southern blotting High-molecular-weight genomic DNA was extracted as described previously.[14] Tissue-cultured cells were lysed in a buffer containing 1% sodium dodecyl sulfate (SDS), 0.1 M NaCl, 5 mM EDTA, 20 mM Tris-HCl pH 8.0 and 100 μg/ml RNase A. After incubation at 37°C for 1 h, proteinase K

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was added to 100 μg/ml and the lysate was further incubated. DNA was recovered by ethanol precipitation after phenol-chloroform extraction of the lysate. Solid tumors were quickly frozen by immersing them in liquid nitrogen and then ground to fine powder in a mortar. The tissues were then lysed in the buffer described above and DNA was extracted as before.

DNA (10 μg/sample) was digested with restriction enzymes, subjected to electrophoresis through a 0.75% agarose gel and transferred to a nitrocellulose filter according to the method of Southern.[15] Filters were baked for 2 h at 80°C. Prehybridization was done in 3×SSC (3 M NaCl, 0.3 M sodium citrate) at 65°C for 30 min, then in 3×SSC containing 10×Denhardt (50×Denhardt: 1% bovine serum albumin fraction V, 1% Ficoll 400, 1% polyvinylpyrrolidone) at 65°C for 1 h, and finally in 0.5×solution A (solution A: 100 mM Tris-HCl pH 7.8, 20 mM EDTA, 2 M NaCl, 20×Denhardt) containing 0.5% SDS at 65°C for 30 min. Filters were then hybridized in 0.5×solution A containing 50 μg/ml of denatured calf thymus DNA, 0.5% SDS and ^{32}P-labeled pSV2neo at 65°C for 16–24 h. The specific activity of the probes was around 1×10^9 cpm/μg. Filters were washed and exposed to X-ray film for autoradiography.

Inoculation of the mixture of clones The genetically tagged cells were inoculated subcutaneously into the back of BCF$_1$ mice. Usually, 1×10^5 cells of each clone were mixed and used for inoculation. The mice were killed 6 weeks after the inoculation and the lungs were examined for metastasis. In some cases such as those of

reconstruction experiments, mice were killed at 14 days or 28 days after inoculation.

Spontaneous metastasis and experimental metastasis BCF$_1$ mice were inoculated subcutaneously with 1×10^6 cells of each clone into the back. The size of the tumor was measured every 5 days. The mice were killed 42 days after inoculation and the lungs were examined for spontaneous metastasis of the cells.

BCF$_1$ mice were injected into the tail vein with 1×10^5 cells of each clone per mouse. The mice were killed 22 days after the injection and the lungs were examined for metastasis.

Immunization with tumor cells The cells were treated with mitomycin C (25 μg/ml) for 30 min at 37°C and 2×10^6 such cells were injected into mice as the immunogen. Injections were done subcutaneously into bilateral axillary and inguinal regions, and intraperitoneally into BCF$_1$ mice. Two weeks later, 1.0×10^6 live cells were inoculated subcutaneously into the back of the mice. The size of the tumors were measured two weeks later.

Scrape-loading dye-transfer method Gap junction-mediated intercellular communication *in vitro* was measured by the scrape-loading dye-transfer technique as described previously.[16]

RESULTS

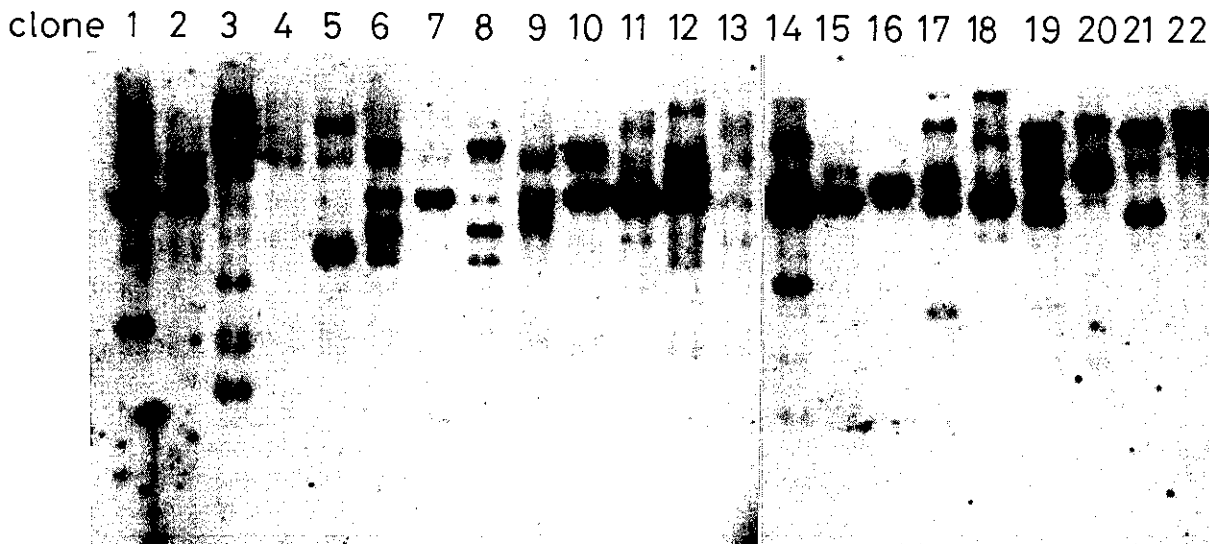**Effect of transfection on the growth rate *in vivo* and the spontaneous metastatic ability** We first examined

Fig. 1.　Southern blot analysis of the 22 clones. DNA was extracted from each clone. Genomic DNA (10 μg) was digested with *Bam*HI and separated by electrophoresis on 0.75% agarose gels. The numbers above the lanes correspond to those of the clones.
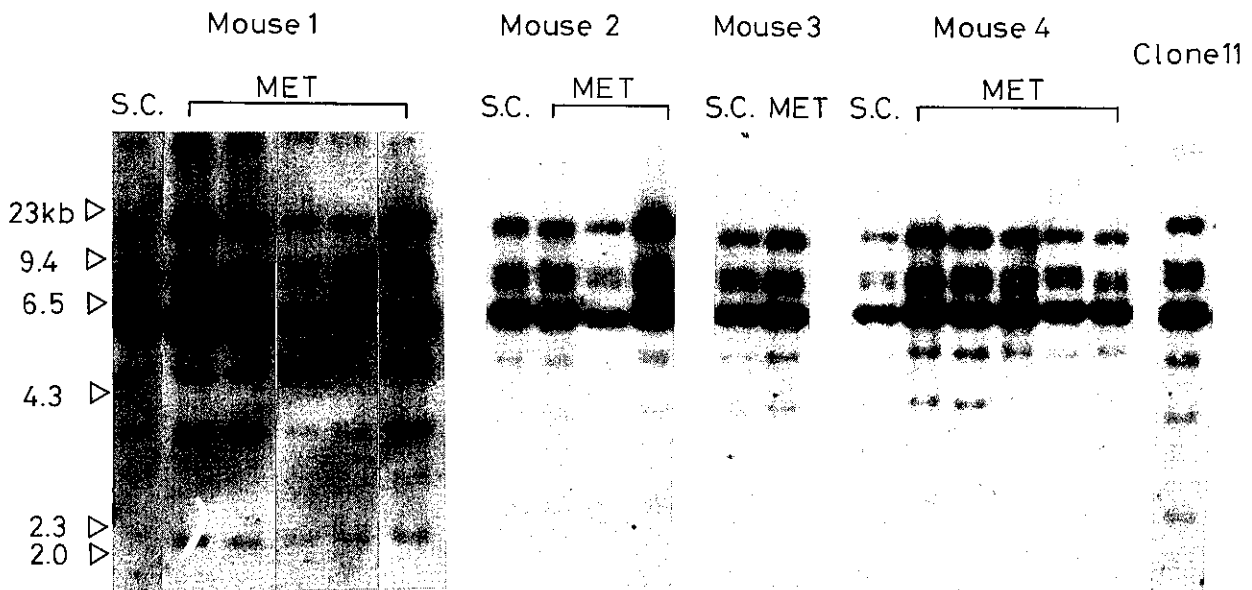
Fig. 2. Southern blot analysis of DNA from tumors *in situ* and lung metastatic nodules. Tumors *in situ* and lung metastatic nodules were surgically removed, minced with scissors and cultured for a short period of time in a medium containing G418 (400 μg/ml). DNA was purified from the cells in culture and digested with *Bam*HI. Four mice were inoculated with the mixture of 22 clones. S.C. represents the tumor *in situ* and MET represents metastatic nodules in the corresponding mice.

whether the growth rate *in vivo* and the spontaneous metastatic ability of 505-05-01 cells were altered by transfection with pSV2neo. The cells were collected from G418-selected dishes which contained more than 100 colonies, and were injected into mice. The growth of the tumors and the metastasis were examined. The growth rate *in vivo* and the number of metastatic nodules were not affected by transfection of 505-05-01 cells with pSV2neo (data not shown). Therefore, we randomly isolated 22 clones in G418-selected dishes and used them for further study.

Characterization of pSV2neo-transfected clones Fig. 1 shows the banding pattern of the pSV2neo in each clone when DNA was digested with *Bam*HI. Each clone exhibited a unique pattern. When necessary, digestion with other restriction enzymes was carried out to facilitate identification of the clones. These banding patterns were stably conserved among clones throughout the study.

Clonal analysis of the tumor *in situ* and the lung metastatic nodules A mixture of the 22 clones (1×10^5 cells of each clone) was inoculated subcutaneously into the back of mice. Mice were killed six weeks after the inoculation. Tumors *in situ* and lung metastatic nodules were surgically removed, minced with scissors and cultured for a short period of time in a medium containing G418 (400 μg/ml), in order to remove contaminating

lymphocytes and fibroblasts. DNA was purified from the cells in culture. The clonal composition of the tumor *in situ* and the metastatic lung nodules was then assessed by Southern blot analysis of the DNA. As shown in Fig. 2, the banding patterns for pSV2neo in the tumor *in situ* and the lung metastatic nodules were indistinguishable from that of no. 11 clone in all of the four mice inoculated independently. This result indicated that among the 22 clones inoculated, no. 11 clone always overgrew other clones in the tumor. This dominant clone also metastasized to the lung of the mice.

In vitro and *in vivo* growth of pSV2neo-transfected clones We analyzed whether the apparent clonal dominance by no. 11 clone was due to a high growth rate of the clone. The no. 1, 3, 4, and 13 clones were chosen at random and compared with no. 11 in terms of the growth rate. The *in vivo* and *in vitro* growth rates were not different among these clones (Fig. 3 A and B). Therefore, the dominance of no. 11 clone cannot be explained simply by the growth rate.

Spontaneous and experimental metastasis of the clones We examined the spontaneous and experimental metastatic ability of these clones (Tables I and II). The frequencies of spontaneous and experimental metastasis were similar for all the clones tested as well as for the parental 505-05-01 cells. These results indicated that no.
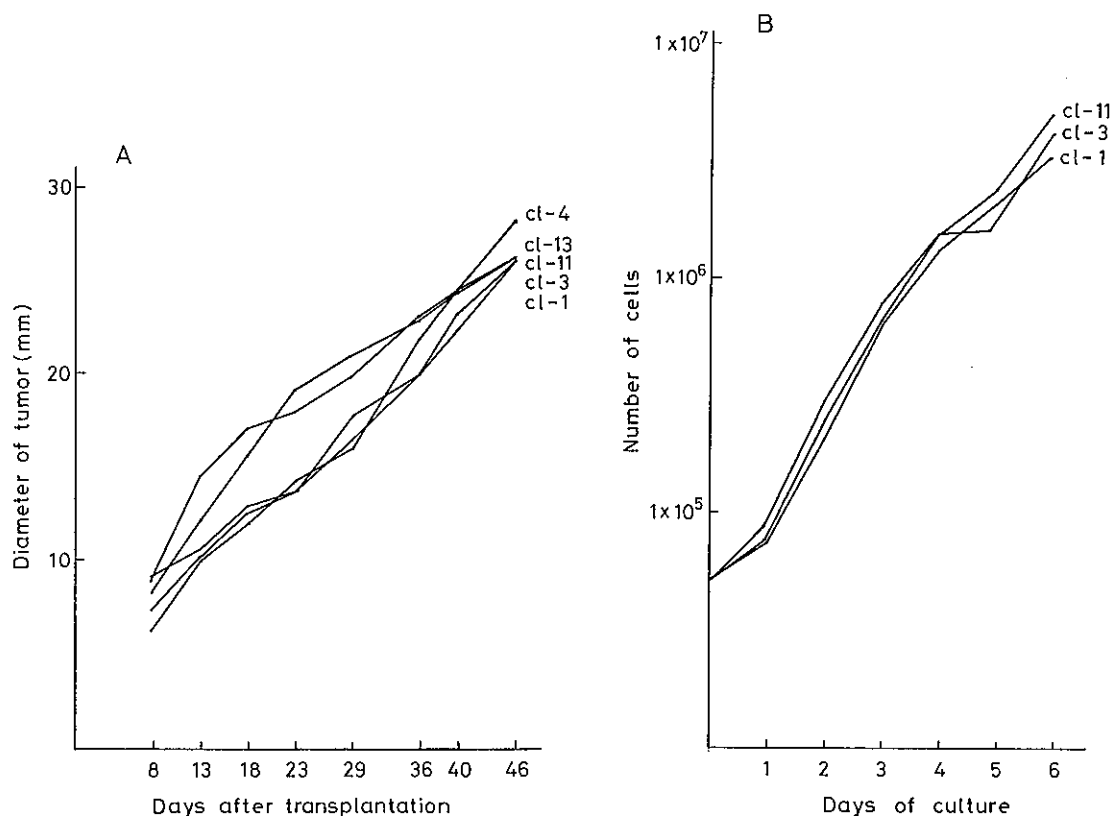
Fig. 3. The growth of clones *in vivo* and *in vitro*. A: *In vivo* growth of pSV2neo-transfected clones. Each clone (1×10^6 cells) was inoculated subcutaneously into BCF_1 mice. The size of the tumor was measured every 5 days. B: *In vitro* growth of pSV2neo-transfected clones. Each clone (1×10^5 cells) was seeded onto 10 cm dishes. The cells were cultured in a-MEM supplemented with 2.5% fetal bovine serum.

Table I. Incidence of Spontaneous Metastasis

Cells injected	Mice with metastasis	No. of metastatic nodules[a]/mouse
505-05-01	5/5	7.8 ± 2.8[b]
clone-1	5/5	8.2 ± 3.0
clone-3	5/5	2.4 ± 0.2
clone-11	3/5	6.2 ± 3.3

a) The cells (1×10^6) were injected sc and mice were killed 42 days after the injection.
b) Means ± SE.

Table II. Incidence of Experimental Metastasis

Cells injected	Mice with metastasis	No. of metastatic lung nodules[a]/mouse
505-05-01	4/4	74.0 ± 7.3[b]
clone-1	5/5	48.6 ± 14.2
clone-3	5/5	63.2 ± 13.0
clone-11	5/5	69.4 ± 13.2

a) The cells (1×10^5) were injected iv and mice were killed 22 days after the injection.
b) Means ± SE.

11 clone had similar growth rate and metastatic ability to the other clones. However, it nevertheless dominated the tumor *in situ* and metastasized to the lung when inoculated as a mixture with other clones.

Immunogenicity of the clones The possibility was examined that no. 11 was less immunogenic and so escaped suppression by the host animal. Mice were immunized with the mixture of 22 clones which had been treated with mitomycin C. All the clones used to challenge these immunized mice formed tumors. The results are shown in Table III. There was no difference in mean diameter of the tumors among clones tested. Therefore, the immuno-

Table III. Evaluation of Immunogenicity of the Clones

Clones	No. of tumor cells immunized[a]	No. of tumor cells challenged[b]	No. of mice with tumor/ No. challenged	Size of the tumor challenged[c]
505-05-01	2.0×10^6	1.0×10^6	5/5	9.7 ± 1.5[d]
clone-1	2.0×10^6	1.0×10^6	4/5	13.2 ± 0.6
clone-3	2.0×10^6	1.0×10^6	4/4	12.7 ± 1.2
clone-11	2.0×10^6	1.0×10^6	5/5	13.3 ± 1.3

a) The cells (2.0×10^6) were treated with MMC $(25 \mu g/ml)$ at 37°C for 30 min and injected into the bilateral axillary and inguinal region and ip.
b) Two weeks later the mice were challenged with 1×10^6 cells.
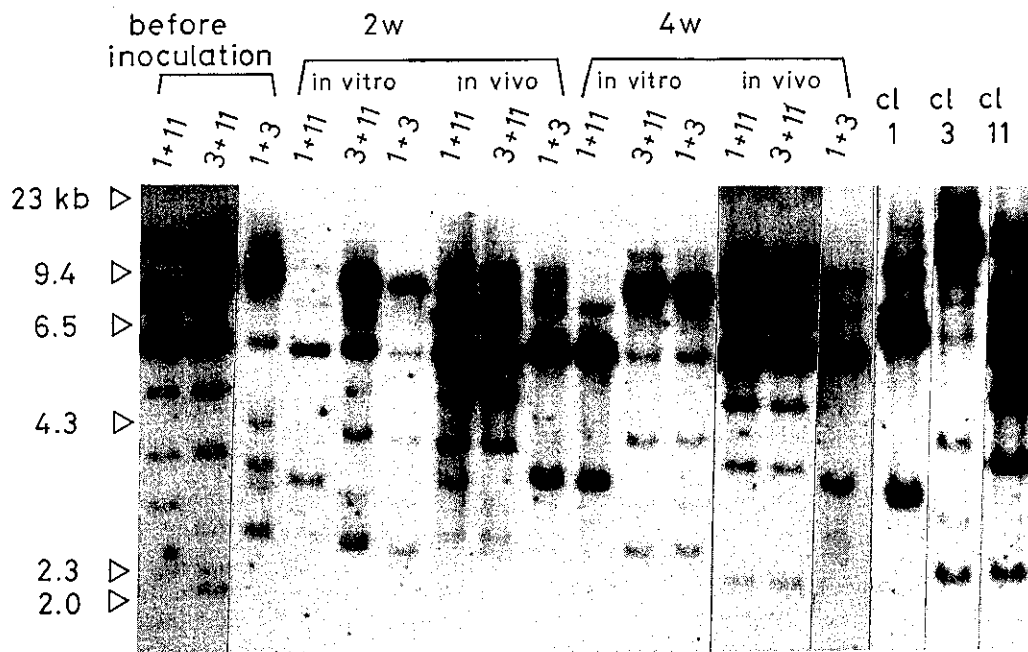c) Mean diameter (mm).
d) Means $\pm$ SE.

Fig. 4. Reconstruction experiments. Cells from two clones (each 1×10^5 cells) were mixed. The combinations were; no. 1 with no. 11, no. 3 with no. 11, and no. 1 with no. 3. The mixtures were passaged every 3 days *in vitro*. Another set of the mixtures was inoculated subcutaneously into groups of 2 mice. DNA was extracted from cells in tissue culture and tumors 2 and 4 weeks after inoculation.

genicity of these clones was similarly low and it cannot be the cause of the dominance of no. 11 clone.

Evaluation of the intercellular communication We used the scrape-loading dye-transfer technique to evaluate intercellular communication of the clones. Several studies have demonstrated that the loss of intercellular junctional communication correlates closely with high spontaneous metastatic ability.[17, 18] The intercellular communication capacity of the cells was estimated for each clone by observing the dye transfer from dye-entrapping cells to adjacent cells. The clones tested ex-

hibited a weak capacity for intercellular communication and the fluorescent dye remained in the primary loaded cells (data not shown).

In vivo and *in vitro* **reconstruction study** Although no. 11 clone was demonstrated to overgrow other clones, the degree of dominance among clones was difficult to analyze when all 22 clones were used in the experiments. Thus, we performed reconstruction experiments in which two clones were mixed and analyzed. Fig. 4 shows the results of Southern blot analysis of the reconstruction study. When DNA was extracted immediately after the

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clones were mixed, it showed a simple combination of banding patterns of each clones. However, when the mixture of no. 11 with no. 1 was passaged for 2 weeks *in vitro,* the bands corresponding to no. 11 clone disappeared. Similarly, the same clone disappeared from the mixture of no. 11 and no. 3. For the mixture of no. 1 and no. 3 clones, the latter dominated the former. These changes were more clearly seen when the mixture was passaged *in vitro* for 4 weeks. In this case. the bands of no. 11 were not observed. In the mixtures of no. 1 and no. 3, bands for both clones were still present.

When the mixtures were inoculated *in vivo,* no. 11 clone began to dominate the other clones within 2 weeks. This is consistent with the previous results. In the tumors formed by inoculation of the mixture of no. 1 and no. 3, banding patterns corresponding to both of the clones were observed at 2 weeks. In the tumors recovered 4 weeks after the inoculation, no. 11 clone completely dominated other clones. In the mixture of no. 1 and no. 3, the banding pattern of no. 1 was only distinguishable. This indicated that no. 1 is dominant over no. 3 in the absence of no. 11 clone. The dominance of no. 11 over other clones was observed only in the tumors *in vivo.* In contrast, no. 11 clone was suppressed *in vitro* by no. 1 clone as well as by no. 3 clone.

DISCUSSION

In the present study, we have analyzed the clonality of the tumor *in situ* and the lung metastatic nodules by genetically tagging the mouse fibrosarcoma cells with the neomycin resistance marker. This marker was stably retained during the course of the experiments. It was reported that transfection with pSV2neo affected the metastatic ability of the mouse mammary adenocarcinoma cells.[19] We could not observe any alteration in the tumorigenicity or metastasis of the 505-05-01 cells, a fibrosarcoma line, after introduction of the plasmid. This may be due to the difference in tumor lines used for the experiment. The results of Southern blot analysis revealed that no. 11 clone dominated other clones in the tumor *in situ* as well as in all the lung metastatic nodules.

The emergence of a dominant clone in a tumor was previously reported by Kerbel *et al.*[9, 10, 20] They also exploited the method of genetic tagging of tumor cells and analyzed the clonality of the primary and the lung metastatic nodules of mouse mammary adenocarcinomas. They tagged the cells either by transfecting them with pSV2neo or by infecting them with a retrovirus vector. These authors utilized a tumor cell population that consisted of a mixture of 360 clones which were not characterized individually. Their data indicated the dominance of one or two clones in a tumor cell population. We exploited a defined tumor population for the analysis

in which the clones used were characterized individually. Our data demonstrate that 505-05-01 cells and their transfectants were highly tumorigenic and grew at a similar rate *in vivo* and *in vitro* when tested individually. Also, no difference was found in spontaneous or experimental metastatic ability between parental and transfected clones. Moreover, we could not find any difference in the immunogenicity or intercellular communication of these clones. Therefore, no significant difference was found in the behavior of the clones. Nevertheless, no. 11 clone and other clones seem to differ in their growth in the tumor when mixed together.

From the reconstruction studies, we found that the dominance of no. 11 clone is observed only *in vivo.* The no. 11 clone was suppressed *in vitro* by other clones. The no. 1 and no. 3 clones grew equally in mixed culture when grown *in vitro* as well as *in vivo.* Thus, the behavior of the cells *in vitro* did not accurately reflect that of the cells in tumors. We think that the dominance of no. 11 clone might have been brought about through the interaction of clones in the tumor.[21-23]

Many studies have reported the existence of clonal interactions *in vivo* and *in vitro.* A variety of mechanisms are involved in the clonal interactions. Miller *et al.* have demonstrated that the presence of certain mouse mammary adenocarcinoma subpopulations can alter the tumor take rate, latency period, or actual growth kinetics of other subpopulations *in vivo.* Besides, immunogenicity, sensitivity to drugs, and metastatic ability were altered by the clonal interaction.[21] The mechanism by which the no. 11 clone dominates other clones *in vivo* is not clear yet. It may be due to autocrine/paracrine growth regulatory factors[23] or intercellular communication *in vivo.*[24] In either case, host factors seem to be involved.

The reconstruction experiments showed that the dominance of no. 11 clone *in vivo* was evident as early as 2 weeks after the inoculation. The no. 11 and other clones exhibited a similar capacity to metastasize to the lung when inoculated individually. However, we found that no. 11 clone preferentially metastasizes to the lung when inoculated as a mixture with other clones. Thus, we think that the dominance of no. 11 clone in the lung nodules is simply a reflection of overgrowth of the clone in the tumor *in situ,* which in turn offers a better chance for the clone to metastasize.

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