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# Experimental Metastasis of Oncogene-transformed NIH 3T3 Cells in Chick Embryo

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**By means of a highly sensitive and quantitative assay for specific detection of metastasized tumor cells in chick embryonic organs using the polymerase chain reaction (PCR), we have examined the experimental metastatic ability of individual clones of NIH 3T3 cells, transformed with oncogenes: v-Ki-*ras*, v-Ha-*ras*, v-*src*, v-*fos*, and v-*abl*. Such a transformed clone had different metastatic abilities in different embryonic organs. Among them, two clones of NIH 3T3 cells transformed with *ras*-oncogenes (v-Ki-*ras* or v-Ha-*ras*) metastasized to liver and lungs of chick embryo, and grew there more rapidly than the other clones. The parental NIH 3T3 cells were detected as slight bands of PCR products after iv injection, indicating some cells were trapped in chick embryonic organs, but did not grow. These findings indicate that the transformed cells are able to invade the organ tissues and grow in embryonic chick organs, but non-metastatic cells such as the untransformed-NIH 3T3 cells are not able to grow in the secondary sites. These experiments clearly demonstrate the usefulness of this assay system to study genes involved in malignant transformation.**

Key words: Chick embryo — Metastatic ability — Oncogene-transformed clone of NIH 3T3 — Polymerase chain reaction

Metastases are a major cause of morbidity and mortality for patients harboring malignant tumors. A metastatic colony is the end result of a complex series of steps through which tumor cells from the primary mass invade host tissue, enter the circulation, survive host defenses, and lodge and grow in distant organ sites.[1] Multiple genetic or epigenetic steps have been presumed to be required for completing the phenotypic changes necessary for cells to become metastatic. However, the genetic events in the acquisition of metastatic phenotypes are not well known.[2–4] To elucidate the role of oncogenes in the metastatic cascade, one approach is to introduce a cloned oncogene into a recipient non-metastatic cell line and to monitor the metastatic ability of the oncogene transfected-cells in appropriate hosts.[5–13] So, it is obviously essential to develop a simple and reliable quantitative assay method for investigating the molecular and genetic aspects of metastasis. The chick embryo as a naturally immunodeficient host has been used by many workers to examine tumorigenic and metastatic properties of cells.[14–23] The major disadvantage of using the chick embryo has been the time limitation of 7 to 10 days imposed by the development of the embryo and its immune system. Because most tumors cannot produce macroscopic metastases in this time, detection of metastatic cells in chick organs has been difficult, requiring techniques that do not permit the isolation of viable metastatic cells. Several quantitative assays have been reported to detect metastasized tumor cells in embryonic chicks using plasminogen activator or ouabain.[17,19] However, their applications for various tumor cells seem to be limited since tumor cells have different potential of producing plasminogen activator and different sensitivity to ouabain.

We have established a highly sensitive method for specific detection of metastasized human tumor cells in embryonic chicks using the polymerase chain reaction (PCR).[24] This method overcomes the time limitation that has previously restricted the use of this system. We have used this assay with specific primers and a probe for mouse $\beta$-globin gene here to assess the ability of NIH 3T3 cells transformed by oncogenes to form experimental metastases.

## MATERIALS AND METHODS

**Cells** We examined individual clones of NIH 3T3 cells, transformed with oncogenes such as v-Ki-*ras*, v-Ha-*ras*, v-*src*, v-*fos*, or v-*abl* for experimental metastatic ability in the naturally immunodeficient chick embryo. The v-Ki-*ras*-transformed cells were originally derived from a subclone of Kirstein murine sarcoma virus (Ki-MuSV)-transformed NIH 3T3 cells.[25] The v-Ha-*ras*-transformed cells were isolated from a transformed NIH 3T3 focus transfected with the genome of Harvey murine sarcoma virus (Ha-MuSV) cloned in pBR322.[6] The v-*src*-transformed cells were cloned from a transformed NIH 3T3 focus after injection with a murine retrovirus containing the v-*src* from Rous sarcoma virus.[7] The v-*fos*-transformed cells were derived from a transformed NIH

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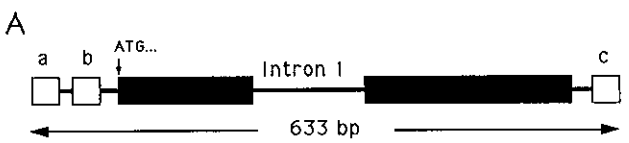
3T3 clone transfected with a cloned genemic DNA of FBJ murine sarcoma virus (FBJ-MuSV); they were kindly supplied by Dr. M. Noda of the Cancer Institute, Tokyo. The v-*abl*-transformed cells were obtained by transfection with a clone v-*abl* gene of Abelson murine leukemia virus (Ab-MuLV); they were kindly provided by Dr. K. Yanagihara of Hiroshima University, Hiroshima. The growth rates *in vitro* of these transformed NIH 3T3 cell lines were almost the same.

**Injection of cells into chorioallantoic membrane (CAM) vein** Chicken eggs (Plymouth Rock×White Leghorn) were kept in an incubator at 37°C in a humidified atmosphere (relative humidity, about 70%). Eggs at 10 days after fertilization were used as recipients of transformed cells. Each egg was candled and a Y-shaped junction of blood vessels in the CAM was marked on the shell with pencil. The eggshell was cleaned with 70% ethanol. A portion of shell directly overlying a blood vessel was carefully removed, and the vein was made visible with a drop of paraffin oil applied to the shell membrane; injection of transformed cells was done with a 30G needle through the shell membrane. Embryos were returned to humidified, 37°C chick embryo incubators for 7 days (or for the stated times for growth kinetics experiments).

**Experimental metastasis assay in chick embryos** Embryonic livers and lungs were then dissected out and DNA was extracted using a rapid DNA preparation method.[26] Figure 1A shows the locations of the specific primers and probe. Figure 1B describes the sequences that we have designed for amplifying a 633-bp segment of the mouse $\beta$-globin gene. PCR was carried out in a Thermal Cycler (Perkin-Elmer/Cetus, Norwalk, CA) as described by Saiki *et al.*[27] with the precautions recommended by Kwok and Higuchi[28] in order to avoid false-positives. The PCR was done as follows. The reaction mixture contained 1 $\mu$g of genomic DNA, 1×amplification buffer [10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$], each primer at 1 $\mu M$, 200 $\mu M$ each of dNTP (dATP, dCTP, dGTP, TTP), and 2.5 units of *Taq* polymerase (Perkin-Elmer/Cetus) in a total volume of 100 $\mu$l. The samples were amplified by 25 cycles of PCR, each consisting of 2 min of denaturing at 94°C, 2 min of annealing at 50°C, and 2 min of extension at 72°C. After amplification, the DNA was concentrated by ethanol precipitation and was electrophoresed on a gel of 1.5% agarose in TAE buffer [40 m*M* Tris-base, 5 m*M* sodium acetate, 1 m*M* EDTA, pH 7.6]. Southern transfer to a nylon membrane was achieved by capillary blotting.[29] To verify the identity of the PCR products, the Southern-blotted membrane was hybridized with 5′-$^{32}$P-labeled probe specific for the amplified internal sequence within the PCR products at 43°C in a solution containing 6× SSC, 0.1% SDS, 5×Denhardt's solution,[30] denatured salmon sperm DNA (100 $\mu$g/ml), and 10% dextran sulfate. The membrane was then washed once in 2×SSC-0.1% SDS at room temperature, twice for 15 min at 43°C, and once in 0.1×SSC-0.1% SDS for 60 min at 43°C. The autoradiogram was exposed for 4–5 h with two intensifying screens at −80°C. Radioactivities in the PCR products hybridized with $^{32}$P-labeled probe was analyzed using a Fujix Bio-Image analyzer (Fuji Photo Film Co., Ltd., Tokyo).




B

(a) MGP-1: 5'-GGA TCA GTT GCT CCT ACA TT-3'

(b) MGP-2: 5'-GAC ATA GTT GTG TTG ACT CA-3'

(c) MGP-5: 5'-TAT CCG AAC TCT TGT CAA CA-3'

Fig. 1. Oligonucleotide primers and probe for PCR amplification and their locations in the mouse $\beta$-globin gene. (A) The locations of the specific primers and probe. These primers were designed for amplifing a 633-bp segment of the mouse $\beta$-globin gene. (a) The primer MGP-1 is complementary to the (−)-strand. (b) MGP-2 is complementary to the (−)-strand and was used as the probe to detect the amplified fragments. (c) The primer MGP-5 is complementary to the (+)-strand. The downward-pointing arrow indicates the $\beta$-globin initiation codon and filled boxes indicate the $\beta$-globin encoding regions. (B) The sequences of PCR primers and probe.

## RESULTS AND DISCUSSION

Figure 2A shows the results of ethidium bromide staining of an agarose gel containing the PCR products from genomic DNA (0.1 $\mu$g) of untransformed NIH 3T3 and each transformed clone, and from serial dilutions of the NIH-3T3 DNA in chick embryonic liver DNA (total DNA amount, 1 $\mu$g). DNA from untransformed NIH 3T3 cells and transformed clone cells produced a single band of the expected size (633-bp) for the mouse $\beta$-globin gene. The relative amount of the PCR products did not vary among these DNAs (lanes 1–6). Also, in the serial dilutions of NIH 3T3 DNA the PCR products were accumulated in proportion to the amount of template and were clearly visible even at the concentration of $10^{-3}$ $\mu$g (lane 11). Figure 2B presents the result of Southern hybridization with the labeled oligonucleotide DNA (MGP-2) designed to recognize an internal sequence of the PCR primer. The bands observed in the autoradiograph were in complete concordance with those seen

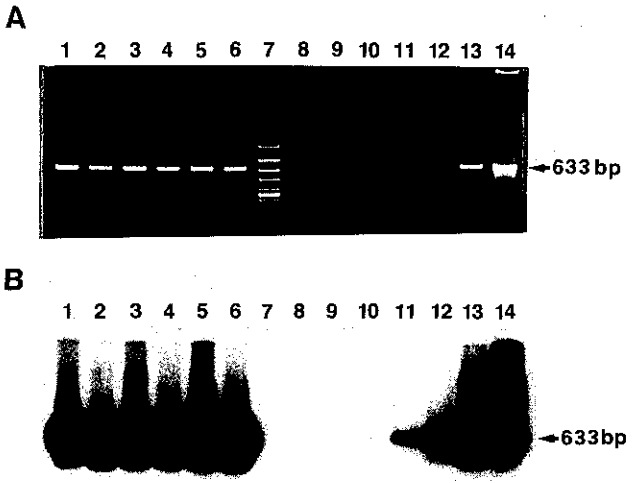



Fig. 2. PCR products resulting from amplification of genomic DNA (0.1 $\mu$g) of untransformed NIH 3T3 and each transformed clone, and serial dilutions ranging from 1 $\mu$g to 10 pg of the NIH 3T3 DNA in chick embryonic liver DNA (total DNA amount, 1 $\mu$g). The PCR was done essentially as described previously.[27] (A) Ethidium bromide-stained gel containing PCR products. (B) The same gel as in A following Southern blotting and hybridization to an internal oligomer probe, MGP-2. Lanes 1–6 contain 0.1 $\mu$g of each genomic DNA in chick embryonic liver DNA (total DNA amount, 1 $\mu$g), v-*fos*, v-*abl*, v-*src*, v-Ki-*ras*, v-Ha-*ras*, and parental NIH 3T3, respectively. Lane 7 contains *Hinc* II-digested $\phi$X174 DNA (0.5 $\mu$g) as size markers. Lane 8 contains chick embryonic liver DNA (1 $\mu$g). Lanes 9–13 contain various amounts of NIH 3T3 genomic DNA ($10^{-5}$ to $10^{-1}$ $\mu$g) in chick embryonic liver DNA (total DNA, 1 $\mu$g). Lane 14 contains 1 $\mu$g of NIH 3T3 genomic DNA.

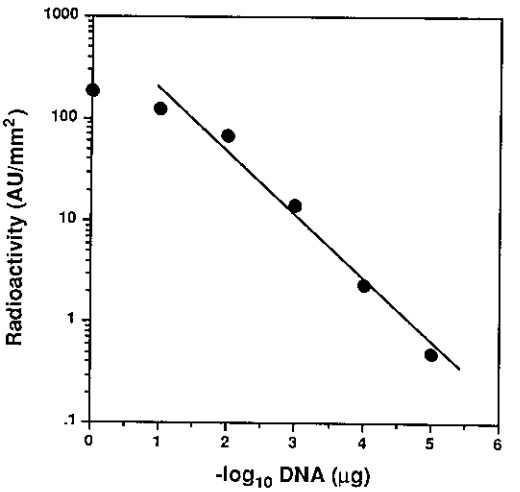



Fig. 3. Plot of the radioactivities of the PCR products from the dilutions of NIH 3T3 DNA against NIH 3T3 DNA concentration. Radioactivities in the PCR products hybridized with $^{32}$P-labeled probe as seen in Fig. 2B were analyzed using a Fujix Bio-Image analyzer. Radioactivity is given in arbitrary units (AU) per mm$^2$.

in Fig. 2A. The amplified products could be detected at the concentration of $10^{-5}$ $\mu$g of NIH 3T3 DNA (lane 9) but DNA from chick embryonic liver did not produce any detectable band under our standard conditions. The radioactivities of the PCR products from the dilutions of NIH 3T3 DNA were plotted against concentration of NIH 3T3 DNA used as a template (Fig. 3). The plot of radioactivity versus template concentration was linear in the range of $10^{-5}$–$10^{-1}$ $\mu$g. This calibration curve was used to measure metastasized cells in chick embryo. These data indicate that the PCR with these primers is capable of selectively amplifying and measuring this region of the mouse $\beta$-globin gene.

DNA samples from embryonic organs 7 days after injection of $2\times10^6$ cells of each transformed clone into the CAM vein produced a visible band of the PCR products, which was detected by ethidium bromide staining after electrophoresis, but DNA samples of embryonic organs injected with parental NIH 3T3 cells did not (data not shown). The results of Southern blot analysis with the mouse $\beta$-globin specific probe are presented in Fig. 4. The PCR products were detected in DNA samples from embryonic liver and lung which were injected with each transformed clone. The data indicate that transformation by these oncogenes has induced a metastatic potential in the chick embryonic organs. In accordance with the result of PCR analysis, metastases of these transformed cells to the embryonic liver were confirmed histologically 7 days after cell inoculation (Fig. 5). The existence of a small subpopulation of metastatic cells in the parental NIH 3T3 cells can not be ruled out,[5] but the slight signals in embryonic liver samples injected with untransformed NIH 3T3 cells were considered to reflect the presence of trapped cells.

We examined the kinetics of growth of each clone in embryonic liver after injection into the CAM vein. Six groups of 10-day embryos received individual clones; v-Ki-*ras*, v-Ha-*ras*, v-*src*, v-*fos*, v-*abl*-transformed, or untransformed NIH 3T3 cells ($10^6$ cells). Embryos were incubated for 3, 5, and 7 days after cell inoculation and their livers were dissected out and weighed. Then the DNA of the combined liver tissues from 4 embryos at each of the stated times was extracted. One $\mu$g of DNA was tested by PCR. The amplified fragments from the liver DNA of embryos injected with cells transformed by v-Ha-*ras* and v-Ki-*ras* increased gradually from 3 to 7 days after cell inoculation and their amounts were more than that of the other transformed clones (Fig. 6). The

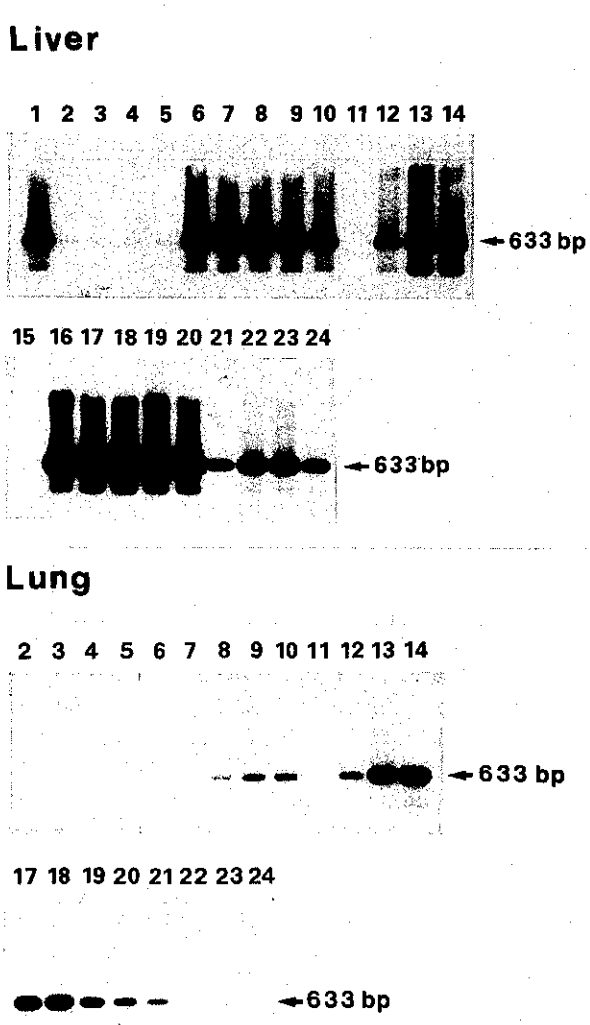



Fig. 4. Detection of metastasis in embryonic liver and lung following i.v. injection of untransformed NIH 3T3 cells and transformed cells. Individual clones of NIH 3T3 cells, transformed with the oncogenes v-Ki-*ras*, v-Ha-*ras*, v-*src*, v-*fos*, and v-*abl* were examined for experimental metastatic ability in the chick embryo. Cells were injected into the CAM vein; from 2 to 4 embryos received each clone at the inoculum size of $2\times10^6$ cells. Embryos were incubated for 7 days after cell inoculation, their livers were dissected out and weighed, and the DNA was extracted. DNA samples were amplified by 25 cycles of PCR and hybridized to the probe MGP-2 as described in Fig. 1B. Lanes are as follows. Lanes 1 and 16: nude mouse liver DNA as positive control; lane 15: chick embryonic liver DNA as negative control; lanes 2–5: DNA from embryo inoculated with parental NIH 3T3 cells; lanes 6 and 7: DNA from embryo inoculated with v-*abl*-transformed cells; lanes 8–10: DNA from embryo inoculated with v-*src*-transformed cells; lanes 11–14: DNA from embryo inoculated with v-Ki-*ras*-transformed cells; lanes 17–20: DNA from embryo inoculated with v-Ha-*ras*-transformed cells; lanes 21–24: DNA from embryo inoculated with v-*fos*-transformed cells.

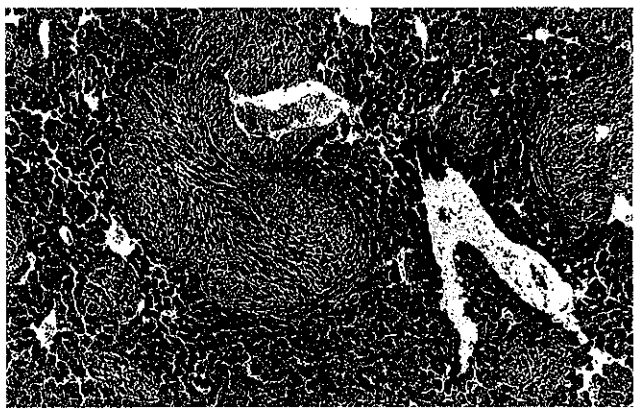


Fig. 5. Histological section of 17-day chick embryonic liver 7 days after CAM vein injection of the v-Ki-*ras*-transformed cells ($10^6$ cells/embryo). ×40. Hematoxylin and eosin stain.

radioactivities in the PCR products hybridized with $^{32}$P-labeled probe were analyzed by using a Fujix Bio-Image analyzer. The cell numbers per liver estimated from the amount of template, which was obtained from the calibration curve, were plotted as shown in Fig. 7. The v-Ha-*ras* and v-Ki-*ras*-transformed cells showed marked growth ability in the chick embryonic liver. The cell numbers per embryonic liver tissue were $2.8\times10^5$ on day 3, $5.8\times10^6$ on day 5 and $1.5\times10^7$ on day 7 for the v-Ha-*ras*-transformed cells, and $5.6\times10^5$ on day 3, $5.7\times10^6$ on day 5 and $6.0\times10^6$ on day 7 for the v-Ki-*ras*-transformed cells. On the other hand, the cell numbers of untransformed NIH 3T3 cells in liver were not detectable on day 3, $4.8\times10^3$ on day 5, and $1.6\times10^3$ on day 7. Three clones of NIH 3T3 cells transformed with v-*src*, v-*fos*, and v-*abl* showed metastatic potential, but the metastasized cell numbers were constant from day 3 to day 7. The v-*src*-transformed cells had a lower signal on day 7 than the signals which were detected from the embryonic liver at the inoculum size of $2\times10^6$ cells, as shown in Fig. 4. This lower growth rate of the v-*src*-transformed clone at the inoculum size of $10^6$ cells may result from lower density of the transformed cells in liver. It may be necessary to have more cells trapped in the liver for the v-*src*-transformed cells to metastasize and proliferate.

It was clear from these findings that metastasized cells could be quantitatively detected using the PCR detection technique, and that the NIH 3T3 cells transformed with oncogenes, v-Ha-*ras*, v-Ki-*ras*, v-*src*, v-*fos*, or v-*abl*, were capable of forming metastases in the chick embryonic organs. Transfection with activated *ras* or v-*src* oncogenes resulted in induction or enhancement of metastatic potential as assessed by other methods.[5–12] Our results

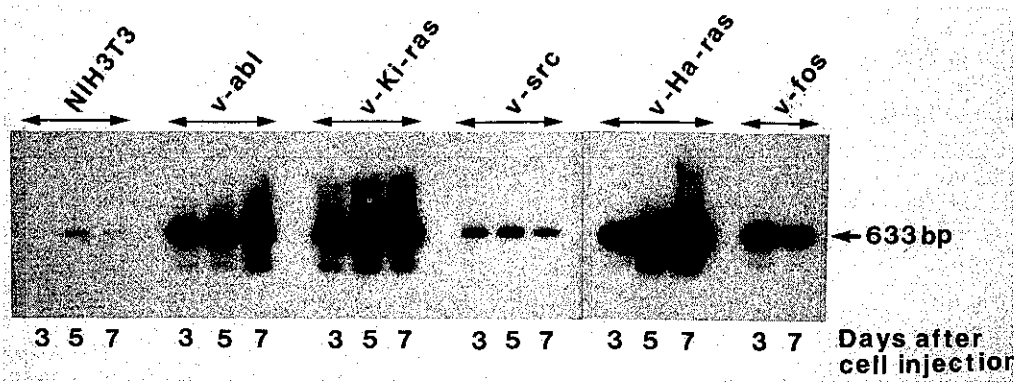



Fig. 6. Kinetics of growth of each clone in embryonic liver after injection into the CAM vein. Six groups of 10-day embryos received one of the clones, v-Ki-*ras*, v-Ha-*ras*, v-*src*, v-*fos*, v-*abl*-transformed, or untransformed NIH 3T3 cells ($10^6$ cells). Four embryos from each group were killed at 3, 5, and 7 days after cell inoculation. Embryo livers were dissected out and weighed, and the DNA was extracted. DNA from each embryo liver was mixed and 1 $\mu$g of DNA was tested by PCR and Southern blot analysis as described in the legend to Fig. 2.

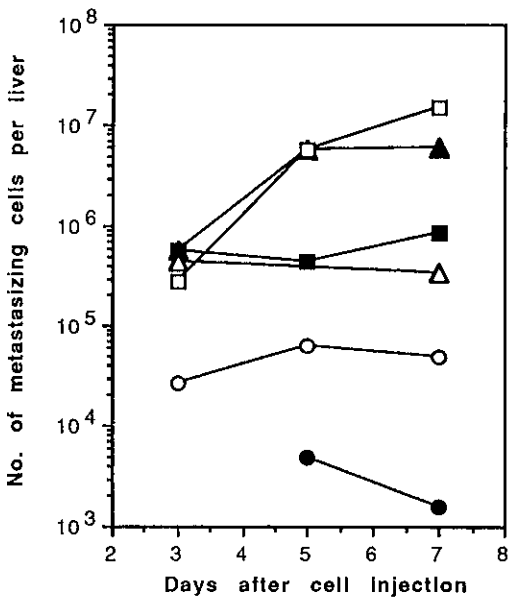



Fig. 7. Growth curve of metastasizing cells in chick embryonic liver after cell inoculation. The same transfer membrane as shown in Fig. 6 was used to measure the radioactivities of the PCR products hybridized with $^{32}$P-labeled probe, using a Fujix Bio-Image analyzer. The cell numbers per liver tissue were estimated from the amount of template which was obtained from the calibration curve shown in Fig. 3. Results are represented as □=v-Ha-*ras*, ▲=v-Ki-*ras*, ■=v-*abl*, △=v-*fos*, ○=v-*src* or ●=NIH 3T3.

are consistent with those findings. The v-Ha-*ras*- or v-Ki-*ras*-transformed cells were detected in embryonic livers and lungs after i.v. injection and their metastatic abilities were higher than those of other transformed clones since the DNA samples from embryos injected with these clones produced more PCR-amplified fragments. The v-*src*-transformed cells also metastasized in embryonic organs. On the other hand, it was not clear whether single oncogene transfection with v-*abl* or v-*fos* was sufficient to induce the metastatic phenotype, whereas v-*fos* oncogene transfer to rat 3Y1 cell line which was transformed with v-*src* oncogene was reported to enhance metastatic potential.[13] Our results showed that individual v-*abl*- or v-*fos*-transformed lines acquired malignant phenotypes that were necessary for metastasis in chick embryo after i.v. injection.

When assessing metastatic ability in mice, it is difficult to decide whether failure to metastasize represents an intrinsic metastatic inability of the cells or an active rejection of otherwise metastatic cells by the host.[31–37] Therefore the use of a variety of *in vivo* hosts with a range of immunocompetencies and deficiencies is advocated, to clarify the nature of phenotypic changes contributing to both intrinsic metastatic ability and immune escape. The chick embryo assay system may be adequate to study the basic role in metastasis of many oncogenes. Because the chick embryos are naturally immunodeficient, there is no influence of host-derived immune cells such as NK cells. Moreover, this assay with high reproducibility is less costly and more rapid than assays using nude mice.

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REFERENCES


1) Fidler, I. J., Gersten, D. M. and Hart, I. The biology of cancer invasion and metastasis. *Adv. Cancer Res.*, **28**, 149–250 (1978).
2) Hart, I. R., Goode, N. T. and Wilson, R. E. Molecular aspects of the metastatic cascade. *Biochim. Biophys. Acta*, **989**, 65–84 (1989).
3) Greenberg, A. H., Eagan, S. E. and Wright, J. A. Oncogenes and metastatic progression. *Invasion Metastasis*, **9**, 360–378 (1989).
4) Sanchez, J., Baker, V. and Miller, D. M. Basic mechanisms of metastasis. *Am. J. Med. Sci.*, **292**, 376–385 (1986).
5) Thorgeirsson, U. P., Turpeenniemi-Hujanen, T., Williams, J. E., Westin, E. H., Heilman, C. A., Talmadge, J. E. and Liotta, L. A. NIH/3T3 cells transfected with human tumor DNA containing activated *ras* oncogenes express the metastatic phenotype in nude mice. *Mol. Cell. Biol.*, **5**, 259–262 (1985).
6) Muschel, R. J., Williams, J. E., Lowy, D. R. and Liotta, L. A. Harvey *ras* induction of metastatic potential depends upon oncogene activation and the type of recipient cell. *Am. J. Pathol.*, **121**, 1–8 (1985).
7) Anderson, S. M. and Scolnick, E. M. Construction and isolation of a transforming murine retrovirus containing the *src* gene of Rous sarcoma virus. *J. Virol.*, **46**, 594–605 (1983).
8) Bradley, M. O., Kraynak, A. R., Storer, R. D. and Gibbs, J. B. Experimental metastasis in nude mice of NIH 3T3 cells containing various *ras* genes. *Proc. Natl. Acad. Sci. USA*, **83**, 5277–5281 (1986).
9) Greig, R. G., Koestler, T. P., Trainer, D. L., Corwin, S. P., Miles, L., Kline, T., Sweet, R., Yokoyama, S. and Poste, G. Tumorigenic and metastatic properties of "normal" and *ras*-transfected NIH/3T3 cells. *Proc. Natl. Acad. Sci. USA*, **82**, 3698–3701 (1985).
10) Vousden, K. H., Eccles, S. A., Purvies, H. and Marchall, C. J. Enhanced spontaneous metastasis of mouse carcinoma cells transfected with an activated c-Ha-*ras*-1 gene. *Int. J. Cancer*, **37**, 425–433 (1986).
11) Egan, S. E., McClarty, G. A., Jarolin, L., Wright, J. A., Spiro, I., Hager, G. and Greenberg, A. H. Expression of H-*ras* correlates with metastatic potential: evidence for direct regulation of the metastatic phenotype in 10T1/2 and NIH3T3 cells. *Mol. Cell. Biol.*, **7**, 830–837 (1987).
12) Egan, S. E., Wright, J. A., Jarolim, L., Yanagihara, K., Bassin, R. H. and Greenberg, A. H. Transformation by oncogenes encoding protein kinases induces the metastatic phenotype. *Science*, **238**, 202–205 (1987).
13) Taniguchi, S., Kawano, T., Mitsudomi, T., Kimura, G. and Baba, T. *fos* oncogene transfer to a transformed rat fibroblast cell line enhances spontaneous lung metastasis in rat. *Jpn. J. Cancer Res.*, **77**, 1193–1197 (1986).
14) Murphy, J. B. Transplantability of malignant tumors to embryos of foreign species. *J. Am. Med. Assoc.*, **59**, 874–875 (1912).
15) Karnofsky, D. A., Ridgway, L. P. and Patterson, P. A. Tumor transplantation to the chick embryo. *Ann. N.Y. Acad. Sci.*, **55**, 313–329 (1952).
16) Leighton, J. Invasion and metastasis of heterologous tumors in the chick embryo. *Prog. Exp. Tumor Res.*, **4**, 98–125 (1964).
17) Ossowski, L. and Reich, E. Experimental model for quantitative study of metastasis. *Cancer Res.*, **40**, 2300–2309 (1980).
18) Sherbet, G. V., Lakshmi, M. S. and Guy, D. A possibility of predicting metastatic ability of tumours by implantation into chick embryos. Epigenetic grading of metastasizing and non-metastasizing forms of a hamster lymphosarcoma. *Eur. J. Cancer*, **6**, 561–568 (1980).
19) Chambers, A. F., Shafir, R. and Ling, V. A model system for studying metastasis using the embryonic chick. *Cancer Res.*, **42**, 4018–4025 (1982).
20) Chambers, A. F. and Wilson, S. Cells transfected with a *ts* viral *src* mutant are temperature sensitive for *in vivo* growth. *Mol. Cell. Biol.*, **5**, 728–733 (1985).
21) Chambers, A. F., Denhardt, G. H. and Wilson, S. M. *Ras*-transfected NIH3T3 cell lines, selected for metastatic ability in chick embryos, have increased proportions of p21-expressing cells and are metastatic in nude mice. *Invasion Metastasis*, **10**, 225–240 (1990).
22) Nishikawa, K., Sasaki, T., Tanaka, M., Uchida, H., Endo, Y., Fukuma, H., Chuman, H., Beppu, Y., Matsumoto, K. and Nitta, K. Experimental model for predicting metastatic ability of tumors using chick embryo. *Jpn. J. Clin. Oncol.*, **17**, 319–325 (1987).
23) Uchida, H., Sasaki, T., Tanaka, M., Endo, Y., Nitta, K., Nishikawa, K., Chuman, H., Fukuma, H. and Matsumoto, K. Response to antitumor agents of murine transplantable tumors implanted onto chorioallantoic membrane of chick embryo. *Jpn. J. Cancer Res.*, **78**, 729–736 (1987).
24) Endo, Y., Sasaki, T., Harada, F. and Noguchi, M. Specific detection of metastasized human tumor cells in embryonic chicks by the polymerase chain reaction. *Jpn. J. Cancer Res.*, **81**, 723–726 (1990).
25) Noda, M., Selinger, Z., Scolnick, E. M. and Bassin, R. H. Flat revertants isolated from Kirsten sarcoma virus-transformed cells resistant to the action of specific oncogenes. *Proc. Natl. Acad. Sci. USA*, **80**, 5602–5606 (1983).
26) Davis, L. G., Dibner, M. D. and Battey, J. F. "Basic Methods in Molecular Biology," pp. 4–43 (1986). Elsevier Science Publishing Co., Inc., New York.
27) Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239**, 487–491 (1988).
28) Kwok, S. and Higuchi, R. Avoiding false positive with

PCR. *Nature,* **339,** 237–238 (1989).
29) Southern, E. M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.,* **98,** 503–517 (1975).
30) Denhardt, D. T. A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.,* **23,** 641–646 (1966).
31) Poste, G. and Fidler, I. J. The pathogenesis of cancer metastasis. *Nature,* **283,** 139–146 (1980).
32) Feldman, M. and Eisenbach, L. What makes a tumor cell metastatic? *Sci. Am.,* **259,** 40–47 (1988).
33) Johnson, P. W., Baubock, C. and Roder, J. C. Transfection of a rat cell line with the v-Ki-*ras* oncogene is associated with enhanced susceptibility to natural killer cell lysis. *J. Exp. Med.,* **162,** 1732–1737 (1985).
34) Johnson, P. W., Trimble, W. S., Hozumi, N. and Roder, J. C. Enhanced lytic susceptibility of Ha-*ras* transformants after oncogene inducing is specific to activated NK cells. *J. Immunol.,* **138,** 3996–4003 (1987).
35) Hanna, N. Expression of metastatic potential of tumor cells in young nude mice is correlated with low levels of natural killer cell-mediated cytotoxicity. *Int. J. Cancer,* **26,** 675–680 (1980).
36) Hanna, N. and Fidler, I. J. Role of natural killer cells in the destruction of circulating tumor emboli. *J. Natl. Cancer Inst.,* **55,** 801–809 (1980).
37) Hanna, N. and Fidler, I. J. Relationship between metastatic potential and resistance to natural killer cell-mediated cytotoxicity in three murine tumor systems. *J. Natl. Cancer Inst.,* **66,** 1183–1190 (1981).