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In Vivo Gene Transfer Between Interacting Human Osteosarcoma Cell Lines Is Associated With Acquisition of Enhanced Metastatic Potential

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ABSTRACT

We report here in vivo gene transfer between cancer cells is associated with acquisition of high metastatic behavior. The 143B-GFP cell line with high metastatic potential and the MNNG/HOS-RFP cell line with low metastatic potential, both derived from the TE85 human osteosarcoma cell line, were either co-transplanted or transplanted alone in the tibia in nude mice. Upon mixed transplantation of the two differently labeled sublines, resulting metastatic colonies are single colored either red or green, thereby demonstrating their clonality and enabling facile color-coded quantification. When MNNG/HOS-RFP and 143B-GFP were co-transplanted in the tibia, the number of lung metastases of MNNG/HOS-RFP increased eight-fold compared to MNNG/HOS-RFP transplanted alone ($P < 0.01$). In contrast, no enhancement of MNNG/HOS-RFP metastases occurred when MNNG/HOS-RFP and 143B-GFP were transplanted separately in the right and left tibiae, respectively. This result suggests that the presence of 143B-GFP increased the metastatic potential of MNNG/HOS-RFP within the mixed tumor. We observed transfer of the Ki-*ras* gene from 143B-GFP to MNNG/HOS-RFP after they were co-implanted suggesting the Ki-*ras* played a role in increasing the metastatic potential of MNNG/HOS-RFP in the presence of 143B-GFP. These data suggest the possible role of in vivo gene transfer in enhancing the metastatic potential of cancer cells. The data also further demonstrated the power of color-coded imaging to visualize cancer-cell/cancer-cell interactions in vivo. J. Cell. Biochem. 108: 362–367, 2009.

KEY WORDS: GREEN FLUORESCENT PROTEIN; RED FLUORESCENT PROTEIN; REAL-TIME IMAGING; OSTEOSARCOMA; HIGH- AND LOW-METASTASIS SUBLINES; METASTASIS; INTERACTION; KI-*RAS*; LATERAL GENE TRANSFER

Tumors are heterogeneous and may contain cancer cells with very different metastatic potential [Nowell, 1976; Fidler and Kripke, 1977; Talmadge et al., 1982; Cotran et al., 1994; Tsuchiya et al., 1995]. The basis for the difference is both genetic and epigenetic. The interaction between heterogeneous cancer cells in a tumor is critically important, but poorly understood. The idea that cancer cells could exchange genes among one another has been intriguing for a long time. Horizontal transfer of genes has been reported in bacteria and fungi and plays an important role in the generation of resistance to antibiotics as well as adaptation to new environments [Mishra, 1985; Ochman et al., 2000]. A number of investigations have suggested that tumor aggressiveness and drug resistance are related to gene transfer between cancer cells [Holmgren et al., 1999; de la Taelle et al., 1999; Bergsmedh et al., 2001; Bergsmedh et al., 2002; Glinsky et al., 2006]. In this study, we have attempted to explore the possibility that in vivo transfer of genetic information from a high metastatic subline to a low metastatic subline of a human osteosarcoma clone may increase metastatic potential of the latter.

Abbreviations used: RPMI 1640, Roswell Park Memorial Institute 1640 medium; GFP, green fluorescent protein; RFP, red fluorescent protein.

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To demonstrate in vivo gene transfer between cancer cells which may cause an increase in malignancy, we used two different human osteosarcoma sublines, 143B and MNNG/HOS, which differ greatly in spontaneous metastatic potential. The two osteosarcoma cell lines are derivatives from the same osteosarcoma patient [McAllister et al., 1971; Rhim et al., 1977; Hensler et al., 1994]. The 143B cell line is a subline derived from the TE85 osteosarcoma line [McAllister et al., 1971] that has been further transformed with Ki-*ras* [Rhim et al., 1977], while the MNNG/HOS cell line is a *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)-treated subline derived from TE85.

When these cells are orthotopically transplanted into the proximal tibia of athymic nude mice, both 143B and MNNG/HOS cells were able to form tumors with high efficiency. Furthermore, the 143B-transplanted mice develop numerous pulmonary metastases, while the MNNG/HOS-transplanted mice only occasionally produced pulmonary metastases [Luu et al., 2005].

We have used green fluorescent protein (GFP) and red fluorescent protein (RFP) to color-code these human osteosarcoma sublines. Upon mixed transplantation of the two differently labeled sublines, resulting metastatic colonies are either red or green, thereby demonstrating their clonality. Using color-coded in vivo imaging, our results suggest that the high metastatic subline 143B-GFP greatly enhanced metastasis of the low metastatic MNNG/HOS-RFP subline due to horizontal gene transfer between the two sublines.

MATERIALS AND METHODS

PRODUCTION OF GFP AND RFP RETROVIRUS

The pLEIN retroviral vector (Clonetech Laboratories, Inc., Palo Alto, CA), expressing GFP and the neomycin resistance gene on the same bicistronic message, was used as a GFP vector. PT67, an NIH3T3-derived packaging cell line, expressing the 10 Al viral envelope, was purchased from Clontech Laboratories, Inc. PT67 cells were cultured in DMEM (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-products, Calabasas, CA). For vector production, packaging cells (PT67), at 70% confluence were incubated with a precipitated mixture of DOTAP reagent (Boehringer Mannheim, Indianapolis, IN) and saturating amounts of pLEIN plasmid for 18 h. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy 48 h after transduction. For selection, the cells were cultured in the presence of 500–2,000 μg/ml G418 (Life Technologies, Inc., Grand Island, NY) for 7 days to select for a clone producing high amounts of a GFP retroviral vector (PT67-GFP).

For RFP retrovirus production, the *Hin*dIII/*Not*I fragment from pDsRed2 (Clontech), containing the full-length RFP cDNA, was inserted into the *Hin*dIII/*Not*I site of pLNCX2 (Clontech) that has the neomycin resistance gene, to establish the pLNCX2-DsRed2 plasmid. The PT67 cells, at 70% confluence, were incubated with a precipitated mixture of Lipofectamine reagent (Life Technologies, Inc.) and saturating amounts of pLNCX2-DsRed2 plasmid, as described above for GFP vector production. For selection of a clone producing high amounts of a RFP retroviral vector (PT67-DsRed2), the cells were cultured in the presence of 200–1,000 μg/ml G418 for 7 days.

CELL CULTURES AND CHEMICALS

Human osteosarcoma cell lines 143B and MNNG/HOS were purchased from ATCC (Manassas, VA), and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin and streptomycin at 37°C in 5% CO_2.

GFP AND RFP GENE TRANSDUCTION OF 143B AND MNNG/HOS HUMAN OSTEOSARCOMA CELL LINES, RESPECTIVELY

For GFP gene transduction, 70% confluent 143B human osteosarcoma cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67-GFP cells and RPMI 1640 containing 10% fetal bovine serum for 72 h. Fresh medium was replenished at this time. Cells were harvested with trypsin/EDTA 72 h after transduction and subcultured at a ratio 1:15 into selective medium, which contained 200 μg/ml G418. The level of G418 was increased stepwise up to 800 μg/ml. Clones of 143B expressing high level of GFP (143B-GFP) were isolated with cloning cylinders using trypsin/EDTA and amplified by conventional culture methods.

For RFP gene transduction, 70% confluent MNNG/HOS human osteosarcoma cells were incubated with a 1:1 mixture of retroviral supernatants of PT67-DsRed2 cells and RPMI 1640 containing 10% fetal bovine serum for 72 h. Fresh medium was replenished at this time. Cells were harvested with trypsin/EDTA 72 h after transduction and subcultured at a ratio 1:15 into selective medium, which contained 200 μg/ml G418. The level of G418 was increased stepwise up to 800 μg/ml. Clones of MNNG/HOS expressing high level of RFP (MNNG/HOS-RFP) were isolated with cloning cylinders using trypsin/EDTA and amplified by conventional culture methods.

ANIMALS

Female nude mice (BALB/c-nu/nu) aged 4–6 weeks were used. They were purchased from Sankyo Laboratory, Inc. (Toyama, Japan). Mice were kept in a barrier facility under HEPA filtration. They were bred under germ-free and specific pathogen-free conditions. All animal studies were conducted in accordance with the principles and procedures outlined in the Kanazawa University's guidelines for the care and use of laboratory animals and national laws on the care and use of laboratory animals.

ORTHOTOPIC TRANSPLANTATION OF 143B AND MNNG/HOS IN THE TIBIA

For intratibial transplantation, a cell suspension in 20 μl RPMI 1640 containing 10 μg Matrigel™ (Becton Dickinson, Bedford, MA) prevents the suspension from leaking out of the tibia. A suspension of 2×10^5 MNNG/HOS-RFP cells or 2×10^5 143B-GFP cells or a mixture of 1.6×10^5 MNNG/HOS-RFP cells and 0.4×10^5 143B-GFP cells, or 1.6×10^5 MNNG/HOS-RFP cells were implanted. The mice were anesthetized with pentobarbital sodium (40 mg/kg). The knee of the nude mouse was fixed beyond 90° and an incision was made to expose the tibia. A pin hole was made in the medial tibia and 20 μl of the cell suspension was transplanted in the tibia through the hole using a 25 gauge needle. Five mice were transplanted for each group.

As another control, a suspension of 0.4×10^5 143B-GFP cells in 20 μl RPMI 1640 containing 10 μg Matrigel™ was

transplanted into the right tibia and a suspension of 1.6×10^5 MNNG/HOS-RFP cells in 20 μl RPMI 1640 containing 10 μg Matrigel™ was transplanted into the left tibia in five mice as described above. Six weeks after transplantation, all mice were sacrificed and lung metastases were evaluated under fluorescence microscopy.

TUMOR GROWTH AT THE PRIMARY SITE

The tumors were allowed to form and the dimensions of the leg were measured every week. The volume of the leg and tumor was calculated by using the following equation: volume = 4π (A/2)(B/2)(C/2)/3. The value assigned as the width (A) was an average of the distance in the medial–lateral plane. The value assigned as the length (B) was an average of the distance in the proximal–distal plane. The value assigned as the width (C) was an average of the distance in the anterior–posterior plane.

LUNG METASTASIS ANALYSIS

Both lungs were harvested from each mouse at necropsy and were placed on a 65 cm^2 dish with covered glass. Images were captured directly with a Hamamatsu C7780 3CCD camera (Hamamatsu Photonics, Japan). For macroimaging, a Leica fluorescence stereo microscope model MZFL3 was used. This microscope was equipped with a GFP filter set and a xenon lamp with a 100-W power supply. High-resolution images were captured directly on a PC (Fujitsu, Japan). Images were processed for contrast and brightness and analyzed with the use of Adobe Photoshop v9.0 software. The total number of metastases per lung was counted and averaged among the mice.

RT-PCR ANALYSIS

For confirmation of gene transfer and expression of the Ki-*ras* genes, RNA was isolated from cultured 143B-GFP and MNNG/HOS-RFP cells, nude mouse lung and red fluorescent metastatic lung colonies resulting from co-transplantation of 143-GFP and MNNG/HOS-RFP cells, using an RNeasy kit (NucleoSpin RNA II, MACHEREY-NAGEL, Germany). The reverse transcription reaction was performed using ReverTra Ace (TOYOBO, Japan) and oligo (dT). Reverse transcription reaction products derived from the above specimens were amplified and isolated using the following set of primers: GATATTCACCATTATAGAGAACAATTA (F); CAGGAGTC-TTTTCTTCTTTGC (R). The PCR protocol for Ki-*ras* was performed using GoTag DNA polymerase (Promega) following thermal cycling parameters: 94°C (5 min); 30 cycles: 94°C (30 s); 51.5°C (30 s); 72°C (30 s); hold: 72°C (5 min). For confirmation of the presence of the human β-actin gene in red fluorescent lung colonies resulting from co-transplantation of the two cells types, reverse transcription reaction products were amplified and isolated using the following set of primers: CAAGAGATGGCCACGGCTGCT (F); TCCTTCTT-GCSTCCTGTCGGCA (R). The PCR protocol for human β-actin was performed using the following thermal cycling parameters: 94°C (5 min); 30 cycles: 94°C (30 s); 59°C (30 sec); 72°C (30 s); hold: 72°C (5 min). For confirmation of the presence or absence of 143B-GFP cells in the red fluorescent lung colonies resulting from co-transplantation of the two cell types reverse transcription reaction products for the GFP gene were amplified and isolated using the following set of primers: AAACGGCCACAAGTTCAGCGT (F); GTCCTTGAAGAAGATGGTGCG (R). The PCR protocol for GFP was performed using the following thermal cycling parameters: 94°C (5 min); 25 cycles: 94°C (30 s); 56°C (30 s); 72°C (30 s); hold: 72°C (5 min). Aliquots of the reaction were electrophoresed on 2% agarose gels in 40 mM Tris acetate (pH 8.0)/1 mM EDTA buffer. The gels were stained with ethidium bromide, viewed under UV light, and photographed.

STATISTICAL ANALYSIS

Statistical analysis of the volume of the primary tumors and the number of pulmonary micrometastases were made using a two-tailed paired Student's *t*-test to evaluate the statistical significance of the difference between the means.

RESULTS AND DISCUSSION

LABELED GFP- AND RFP-EXPRESSING CLONES

The selected fluorescent-protein transduced 143B cells and MNNG/HOS cells have a strikingly bright GFP and RFP fluorescence, respectively, in vitro (Fig. 1).

TUMOR GROWTH AT THE PRIMARY SITE

After orthotopic transplantation in the tibia, the primary tumors were measured every week. The 143B-GFP and MNNG/HOS-RFP formed tumors that were detectable by 3 weeks whether transplanted in the tibia of nude mice alone or co-transplanted (Fig. 2). At 6 weeks, the average tumor volumes resulting from 143B-GFP and the mixture of 143B-GFP and MNNG/HOS-RFP were significantly greater compared to MNNG/HOS-RFP transplanted alone ($P < 0.05$ and $P < 0.01$, respectively).

DEVELOPMENT OF SPONTANEOUS OSTEOSARCOMA PULMONARY METASTASES

At 6 weeks after transplantation, the lungs from the 143B-GFP-transplanted mice exhibited numerous surface metastases. Micrometastasis containing as few as single or several cells could be visualized under fluorescence microscopy (Fig. 3A). The MNNG/HOS-RFP-transplanted mice produced much fewer lung metastases at 6 weeks (Fig. 3B). The lungs from the mice transplanted with the cell mixture had numerous red or green colonies indicating their clonality [Yamamoto et al., 2003] (Fig. 3C). Green and red colonies were found approximately at the same ratio on the lung surface. As shown in Figure 4, an inoculum of 1.6×10^5 MNNG/HOS-RFP cells in the tibia of the mice produced an average of 46 red fluorescent micrometastases. In contrast, the mixture of 0.4×10^5 143B-GFP cells and 1.6×10^5 MNNG/HOS-RFP cells, when co-innoculated in the tibia, produced an average of 393 red fluorescent micrometastases. The mixed suspension resulted in an eight-fold increase in red-fluorescent micrometastases resulting from MNNG/HOS-RFP cells compared to when this cell line was implanted alone ($P < 0.01$). No enhancement of MNNG/HOS-RFP metastases occurred in mice in which each cell line was simultaneously, but separately transplanted, in the left tibia and in the right tibia, respectively. 143B-GFP and MNNG/HOS-RFP sublines produced an average of 673 green fluorescent and 8 red fluorescent

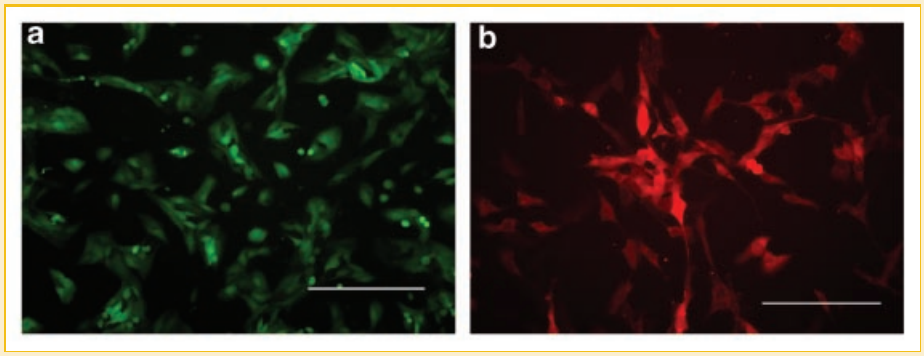

Fig. 1. Stable high GFP-expressing human osteosarcoma cells (143B) and RFP-expressing human osteosarcoma cells (MNNG/HOS) in vitro. Human osteosarcoma cells (143B) were transduced with GFP and the neomycin-resistance gene in a retrovirus vector. Human osteosarcoma cells (MNNG/HOS) were transduced with RFP and the neomycin-resistance gene in a retrovirus vector. See Materials and Methods Section for details. A: 143B-GFP. B: MNNG/HOS-RFP. Bars, 200 μm.

micrometastases, respectively, when separately transplanted in the right and left tibua of nude mice (Fig. 5).

IN VIVO TRANSFER OF THE KI-*RAS* GENE FROM 143-GFP TO MNNG/HOS-RFP CELLS

We then demonstrated the occurrence of in vivo transfer of the Ki-*ras* gene from 143-GFP cells to MNNG/HOS-RFP cells when they were co-inoculated in the tibia. RT-PCR was performed on RNAs extracted from both the parental cell lines, from the nude mouse lung, as well as from red fluorescent micrometastases formed from transplantation of the mixture of the two sublines in the tibia. As shown in Figure 6, the Ki-*ras* gene was not amplified from the cDNA of MNNG/HOS-RFP or normal nude mouse lung. However, the Ki-*ras* gene was consistently amplified from cDNA obtained from 143B-GFP and red fluorescent micrometastases resulting from transplantation of the mixture of MNNG/HOS-RFP and 143B-GFP in the tibia. As a positive control, the human β-actin gene was amplified from cDNA from red fluorescent micrometastases resulting from the transplantation of the mixture of MNNG/HOS-RFP and 143B-GFP, the MNNG/HOS-RFP cell line alone, and the 143B-GFP cell line alone. The GFP gene was not amplified from cDNA of red fluorescent micrometastases resulting from the transplantation of the cell mixtures (Fig. 6).

We have therefore observed that the low metastatic MNNG/HOS-RFP subline had an elevated frequency of metastasis due to mixed transplantation in the tibia with the high metastatic 143B-GFP

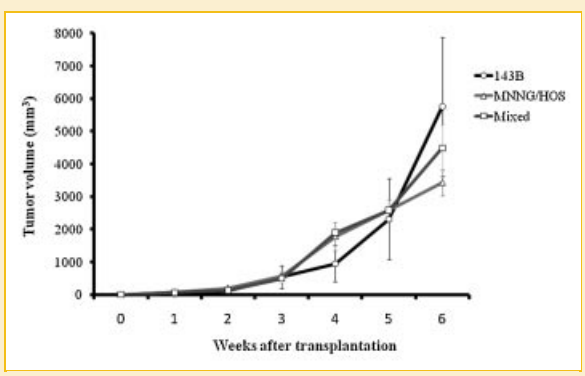

Fig. 2. Orthotopic tumor formation in the tibia. Tumors were measured every week and the volumes calculated. After 6 weeks, the average tumor volumes of 143B-GFP and the mixture of 143B-GFP and MNNG/HOS-RFP were significantly increased compared to MNNG/HOS-RFP transplanted alone ($P < 0.05$ and $P < 0.01$, respectively). *P*-values were derived using the two-tailed Student's *t*-test.

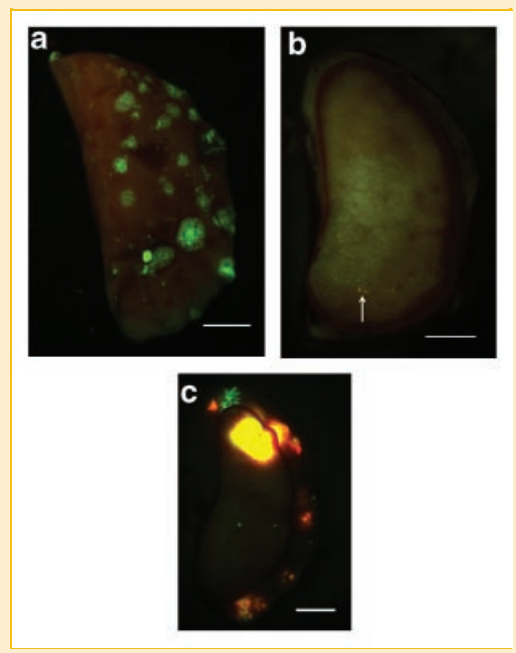

Fig. 3. Visualization of spontaneous lung micrometastasis 6 weeks after transplantation in the tibia. A: Visualization of lung metastases 6 weeks after 143B-GFP cells were transplanted. Bar, 2 mm. B: Visualization of lung metastases 6 weeks after MNNG/HOS-RFP cells were transplanted. Bar, 2 mm. C: Visualization of lung metastases 6 weeks after the mixture of 143B-GFP and MNNG/HOS-RFP cells was co-transplanted. Bar, 2 mm.

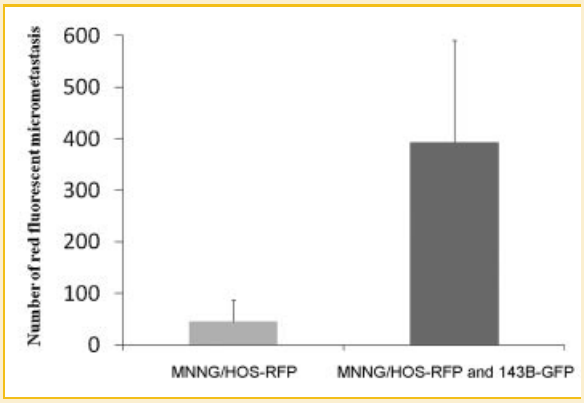

Fig. 4. Quantitation of MNNG/HOS-RFP lung metastasis 6 weeks after transplantation. 1.6×10^5 MNNG/HOS-RFP cells transplanted alone produced an average of 46 red fluorescent micrometastases. In contrast, the co-transplantation of the mixture of 0.4×10^5 143B-GFP cells and 1.6×10^5 MNNG/HOS-RFP cells produced an average of 393 red fluorescent micrometastases. The mixed transplantation produced an eight-fold increase in red fluorescent micrometastases compared to that of 1.6×10^5 MNNG/HOS-RFP cells transplanted alone ($P < 0.01$). *P*-values were derived using the two-tailed Student's *t*-test.

subline. However, when the MNNG/HOS-RFP and 143B-GFP sublines were transplanted separately in the left and right tibia, respectively, the MNNG/HOS-RFP subline did not have an elevated metastasis frequency. This suggests that the 143B-GFP cell line influenced the metastatic potential of MNNG/HOS-RFP during tumor formation when they were co-transplanted in the tibia. Our data suggest that the increased metastatic potential of MNNG/HOS-RFP after co-transplantation with 143-B-GFP was due at least in

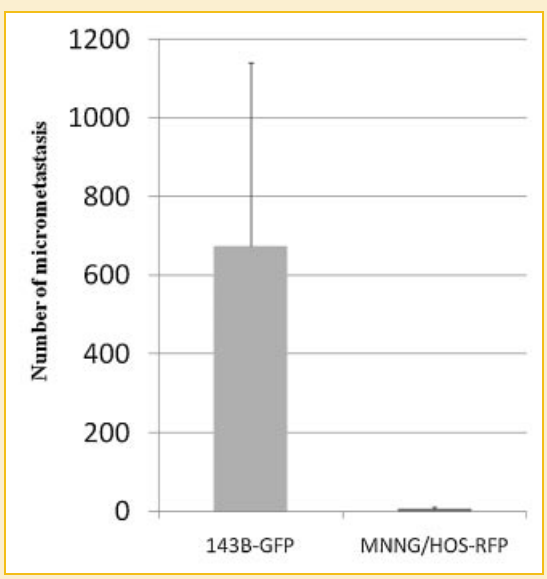

Fig. 5. Quantitation of lung metastasis 6 weeks after separate transplantation. When separately transplanted in the left and right tibia of the same mouse, respectively, the 143B-GFP subline produced an average of 673 green fluorescent micrometastases and the MNNG/HOS-RFP subline produced an average of eight red fluorescent micrometastases.

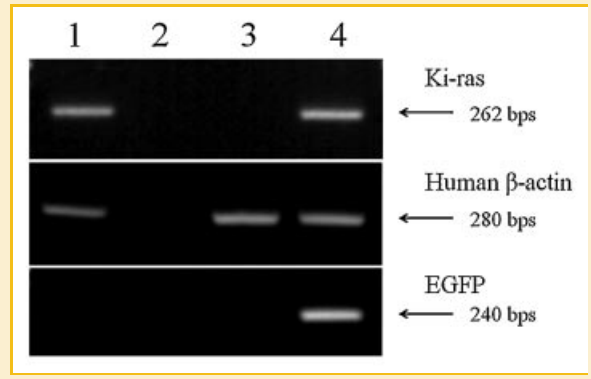

Fig. 6. PCR amplification of Ki-ras. RT-PCR amplification of Ki-ras, human β-actin, and EGFP from RNAs extracted from MNNG/HOS-RFP lung micrometastasis formed after co-transplantation of MNNG/HOS-RFP and 143B-GFP (Lane 1); nude mouse lung (Lane 2); MNNG/HOS-RFP cells (Lane 3); 143B-GFP cells (Lane 4).

part to transfer of the Ki-*ras* gene from 143B-GFP to MNNG/HOS-RFP. Other genes may have been transferred as well. This will be investigated in future studies.

Glinsky et al. [2006] isolated yellow-fluorescent highly metastatic cancer cells from the circulation of mice orthotopically implanted with mixtures of RFP- and GFP-expressing human prostate carcinoma cells, suggesting that gene transfer occurred within the primary tumor resulting in elevated metastatic potential. In the present study, we observed that the Ki-*ras* gene derived from 143B-GFP subline was transferred to the MNNG/HOS-RFP subline during tumor formation in the tibia. It is notable that the metastatic clones were either red or green, not a mixture of yellow, suggesting the clonality of the metastases, a phenomenon we observed previously using color-coded imaging [Yamamoto et al., 2003]. The transferred Ki-*ras* gene may have enabled, by itself or in concert with other not-as-identified transferred genes, the MNNG/HOS-RFP cells to form lung metastases at high frequency, which they cannot do when transplanted alone. These results are consistent with the idea that spontaneous genetic exchange between tumor cells in vivo contributes to increased metastatic capability. The Ki-*ras* protein is associated with many cellular processes such as cell-cell and cell-matrix adhesion which could affect metastasis [Yan et al., 1997a,b].

In our study, the use of color-coded fluorescence imaging [Hoffman, 2005] enabled us to observe the increased metastatic capability of MNNG/HOS-RFP cells when co-transplanted with 143-GFP cells in the tibia. Thus, with color-coded fluorescence imaging, the origin of each micrometastasis could be readily determined either from the MNNG/HOS-RFP cell line or from the 143B-GFP cell line. The clonality of the metastasis enabled us to make this observation. Additional experiments will demonstrate whether other genes were transferred in vivo and if they affected metastasis.

We propose that the gene transfer between cancer cells may be one mechanism by which genetic diversity is generated within tumors. The color-coded model described in this study should be a very valuable tool to investigate the role of tumor heterogeneity, cancer cell-interaction and gene transfer in metastasis and to the identification of genes influencing metastatic potential. Studies in

many types of organisms have demonstrated the phenomenon of lateral gene transfer [Bushman, 2002], including plant grafts, which used different colored fluorescent proteins to identify lateral gene transfer [Stegemann and Bock, 2009]. The current study and our previous study [Glinsky et al., 2006] indicate lateral gene transfer may play a key role in metastasis.

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