Industasis, a Promotion of Tumor Formation by Nontumorigenic Stray Cells

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Abstract

A tumor cell is formed when a critical amount of endogenous and/or exogenous tumorigenic stimuli is exceeded. We have shown that the transient presence of nontumorigenic stray cells in tissues of experimental animals that contain cells with a subcritical set of genetic mutations can act as a tumor-promoting stimulus. To induce somatic mutations in all chicken tissues, we have used the MAV-2 retroviral insertion system that almost exclusively generates nephroblastomas. MAV-2 mutagenized animals i.v. inoculated with nonmalignant cells developed early clonal lung tumors before nephroblastomas. Importantly, the injected cells did not become a component of resultant tumors. Lung tumors displayed specific mutational signature characterized by an insertion of MAV-2 provirus into the *fyn-related kinase* (*frk*) promoter that results in the overexpression of the *frk* gene. In contrast, *plag1, foxP*, and *twist* genes were most often mutagenized in nephroblastomas. Based on such observations, we propose the mechanism termed industasis, a promotion of fully malignant phenotype of incipient tumor cell by stray cells, and hypothesize that it might be the underlying cause of human multiple primary tumors. [Cancer Res 2009;69(11):4605–12]

Introduction

Spontaneous tumors arise when multiple genetic and epigenetic changes amass in a single cell. Nongenetic factors, such as stromal microenvironment interactions (1) and the host immune response (2), have also been shown to play a role in oncogenesis. In addition, evidence has been published showing that tumor cells can remain dormant until a tumor-promoting stimulus triggers their uncontrolled proliferation (3).

Tumor cells of both metastatic and nonmetastasizing cancers are long known to circulate in the blood of patients (4, 5). Released potentially tumorigenic cells are able to persist in a second organ for an extended period of time (6, 7). In addition, normal cells can be liberated into the bloodstream as a result of an injury or surgical intervention (8). The seemingly nondeleterious ectopic presence of such stray (primary tumor or nontransformed) cells within secondary organs has been reported to induce changes within the affected local microenvironment. A fundamental link between the stromal microenvironment and behavior of transformed cells in terms of tumor development has recently been highlighted (9).

Over the last few decades, the incidence of multiple primary tumors within a single host has rapidly increased. The phenomenon is expected to become an even more serious threat in the future as a result of prolonged life span and, paradoxically, of improved healthcare. For example, individuals cured from a tumor exhibit an increased chance of developing second primary tumors in addition to the risk of metastases or a relapse of the first cured tumor (10). Two explanations have been put forward thus far: presence of an inherited genetic predisposition to tumor formation (11) and mutagenic effect of therapy for the first malignancy (12).

Retroviruses represent a potent tool for identifying cancer-related genes. Nonacute oncogenic retroviruses, such as the avian virus MAV-2, do not carry an oncogene; instead, they induce transformation through insertional mutagenesis when proviruses integrate into the host gene loci. Due to its high infectivity, the MAV-2 retroviral system ensures that essentially each host gene locus is affected through random integration in many cells of the target tissue. When a combination of mutations in a cell perturbs cellular functions critical for malignant transformation, the cell clone expands and forms a tumor. MAV-2 predominantly generates nephroblastomas after a 2- to 3-month latency period. Mutated gene loci are easily detectable as they are tagged by the proviral sequences. The genes *plag1, foxP1*, and *twist* have repeatedly been found hit by MAV-2 in nephroblastomas, thereby underlining their importance for malignant transformation of nephrogenic blastema (13, 14).

In this work, we have identified insertionally mutagenized and overexpressed *fyn-related kinase* (*frk*) gene as the salient feature of MAV-2–induced lung sarcomas. The *frk* gene encodes a nonreceptor tyrosine kinase synthesized predominantly in epithelial tissues (15, 16), which has been implicated in chondrogenesis and in the development of the islets of Langerhans (17). We have observed the substantial decrease of latency and increase in frequency of lung sarcomas when MAV-2–infected animals have been i.v. inoculated with nonmalignant cells. Activated *frk* represented the mutational signature in both late tumors induced by MAV-2 alone as well as in early tumors promoted by stray nontumorigenic cells.

Materials and Methods

Experimental Animals, Cells, and Viruses

Viruses. The MAV-2 was the MAV-2(N)–type virus isolated from the AMV-BAI-A complex stock by plaque purification as described (18).

Cells. Chick embryo fibroblasts (CEF) were prepared from CB or CC embryos, cultivated, and infected by MAV-2 virus stock as described (19).

A210 cells were prepared from kidney of 19-d-old CB White Leghorn embryo, infected at the 12th day of incubation by MAV-2 virus, and dispersed in trypsin-EDTA solution (Sigma) in PBS. The cells from each kidney were plated at concentrations of 5×10^6 to 7×10^6/100-mm Petri dish and cultivated as CEF. After 2 wk, the cells were plated in P60 tissue

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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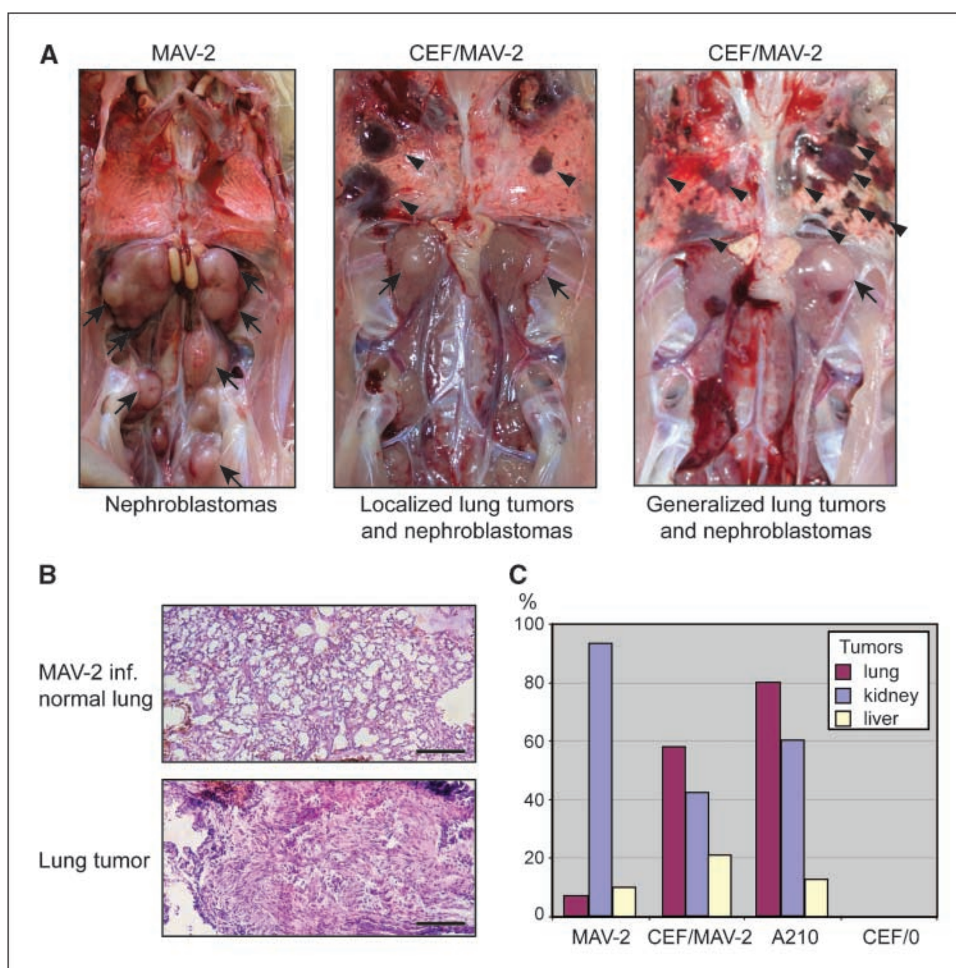

Figure 1. Injection of cells induces lung tumors. *A,* autopsy of animals injected with MAV-2 virus or CEF/MAV-2 cells. *Left, arrows,* MAV-2 alone induces nephroblastomas. Injected cells cause formation of lung tumors (*arrowheads*), either as distinct foci (*middle*) or generalized tumors (*right*). Nephroblastomas (*arrows*) also develop. *B,* histologic sections of nonmalignant (MAV-2 infected) lungs and of lung sarcoma (A210 induced). H&E staining. *Bar,* 100 μm. *C,* frequency of tumor induction in lungs, kidney, and liver by injection of MAV-2 virus, CEF/MAV-2, A210, and control CEF/0 cells. The reduction of nephroblastoma incidence in animals inoculated with the virus-producing cells is caused by earlier and frequently lethal onset of lung tumors. Groups of at least 20 animals were evaluated.

culture dishes at the density of 0.5×10^3 to 1×10^3 per one dish, and after another 2 wk, the largest foci of cells were isolated and further cultivated. Samples of cell suspension were counted in a cell counter CASY Model TTC (Schärfe Systems GmbH). The best proliferating culture, A210, turned out to be a cell clone. Cells for i.v. injections were prepared as follows: CEF, CEF/MAV-2, and A210 were grown to semiconfluency. If required, cells were treated with mitomycin C as described (19).

Animals. Chicks of inbred congenic CB and CC White Leghorns (20) were used. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the Academy of Sciences of the Czech Republic. Chicks were kept under standard laboratory conditions with free access to food and water. For cell inoculation, 1×10^5 to 2×10^5 cells detached by accutase were injected into chorioallantoic vein of 12-d-old embryo or into metatarsal vein of 1-d-old chick. MAV-2 infection was performed as described (14). Control animals were mock infected by an identical volume of PBS.

Sample Collection

The animals were sacrificed at the age between 20 and 120 d after hatching and tumor samples and control tissues were collected. The samples larger than ~4 mm in diameter were divided to thirds and processed immediately into DNA, RNA, and paraffin samples. Either genomic DNA or total RNA was isolated from the smallest tumor foci. DNA and RNA were isolated and quantitated by standard methods (14).

Southern Blot Analyses

Southern blot analyses were performed as described previously (14).

Histologic Investigations

Paraffin-embedded samples, microscopic preparations, and histologic procedures were made as described (21).

PCR, Reverse Transcription-PCR, and Quantitative PCR

cDNA was synthesized and long terminal repeat-rapid amplification of cDNA ends (LTR-RACE) was performed exactly as described previously (14). Real-time PCR was performed using DyNAmo HS SYBR Green qPCR kit (Finnzymes) on Chromo4 cycler (MJ Research/Bio-Rad) and analyzed using the included software. Semiquantitative PCR and integration site-specific PCR were performed using GoTaq polymerase system from Promega according to the manufacturer's instructions. Primers were present at 200 nmol/L, deoxynucleotide triphosphates at 0.2 mmol/L each, and Taq polymerase at 1 unit/50 μL. The standard cycling protocol was 25 cycles (95°C for 15 s, 60°C for 30 s, and 72°C for 30 s). The PCR products were resolved in agarose gels; and in cases when no product has been detected, five PCR cycles were added. Sequences of primers used in this study are in Supplementary Data.

DNA Sequencing and Homology Searches

The sequencing reactions were performed using the BigDye Terminator Cycle Sequencing kit (PE Biosystems) and resolved on ABI PRISM 310 Sequencer. Sequence homology searches were conducted using the BLAT algorithm on the chicken genome assembly database.[3]

Results

The injection of virus-producing cells instead of virus changes the spectrum of tumors. When MAV-2 retroviruses are injected into either embryonic or newborn chicks, nephroblastomas (as a

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consequence of insertional mutagenesis) are almost exclusively induced (22) despite all tissues being similarly infected (see below). In ~5% of animals afflicted with nephroblastomas, late tumors of the lungs and liver can additionally be diagnosed. Analysis of these clonal tumors consistently revealed different proviral integration patterns when compared with nephroblastomas and as such were considered as second primary tumors. We investigated the possibility of changing the spectrum of MAV-2–induced neoplasms by injecting MAV-2–producing cells instead of virions. We used animals and cells of inbred congenic White Leghorn lines CB and CC, which differ only in the MHC(B) haplotypes (23). Differences in MHC locus could be used as molecular markers for unambiguous identification of injected cells. In preliminary experiments, a group of 20 CC 12-day-old embryos was injected with A210 cells. These are clonal virus-producing mesenchymal-type cells derived from a MAV-2–infected CB embryonic kidney. The use of A210 cells is favorable as they harbor six mapped MAV-2 provirus integration sites that can additionally aid in the cell identification following injection (Supplementary Fig. S1). Given that clonal A210 cells could potentially produce a mutant of MAV-2, a second group of embryos was injected with polyclonal unselected MAV-2–producing CB CEF cells (CEF/MAV-2). A third and fourth control group received MAV-2 virus collected from either A210 or CEF/MAV-2 cells, respectively. After injection, the birds were sacrificed and analyzed 20 to 90 days after hatching.

As expected, the majority of chicks injected directly with MAV-2 virions (third and fourth control groups) developed numerous focal nephroblastomas that were evident within 2 to 3 months. Analysis of older animals revealed additional rare tumor foci that developed on the lungs and liver. Surprisingly, chicks injected with A210 or CEF/MAV-2 cells were found to develop, in addition to nephroblastomas, early lung sarcomas in most animals. As early as 27 days after hatching, macroscopic tumor foci were apparent on the lungs of chicks and in ~30% of the aforementioned animal tumors accounted for the majority of lung tissue (Fig. 1*A*; Supplementary Fig. S2). The same frequency of lung tumor formation was recorded when CB CEF/MAV-2 cells were injected into CB animals, suggesting that the MHC haplotype plays no role. The histology of lung sarcomas (Fig. 1*B*) was independent of whether A210 or CEF/MAV-2 cells were injected.

No tumors were induced in animals injected with non–virus-producing cells alone. The combined results of several independent experiments are summarized in Fig. 1*C*. In addition to the lung tumors, we also observed, with less frequency, tumors of the liver and ovary.

The resultant lung tumors are clonal, host derived, and frequently invasive. To determine whether the resultant lung tumors originated from the injected A210 or CEF/MAV-2 cells, Southern blot analysis of tumor cell DNA was performed to detect the proviral integration sites. The Southern blots revealed that lung tumors as well as nephroblastomas and less common liver and ovary tumors were formed by single-cell clones. Various tumor types of a single animal represented unrelated clones, suggesting that these were independently originated tumors, not metastases

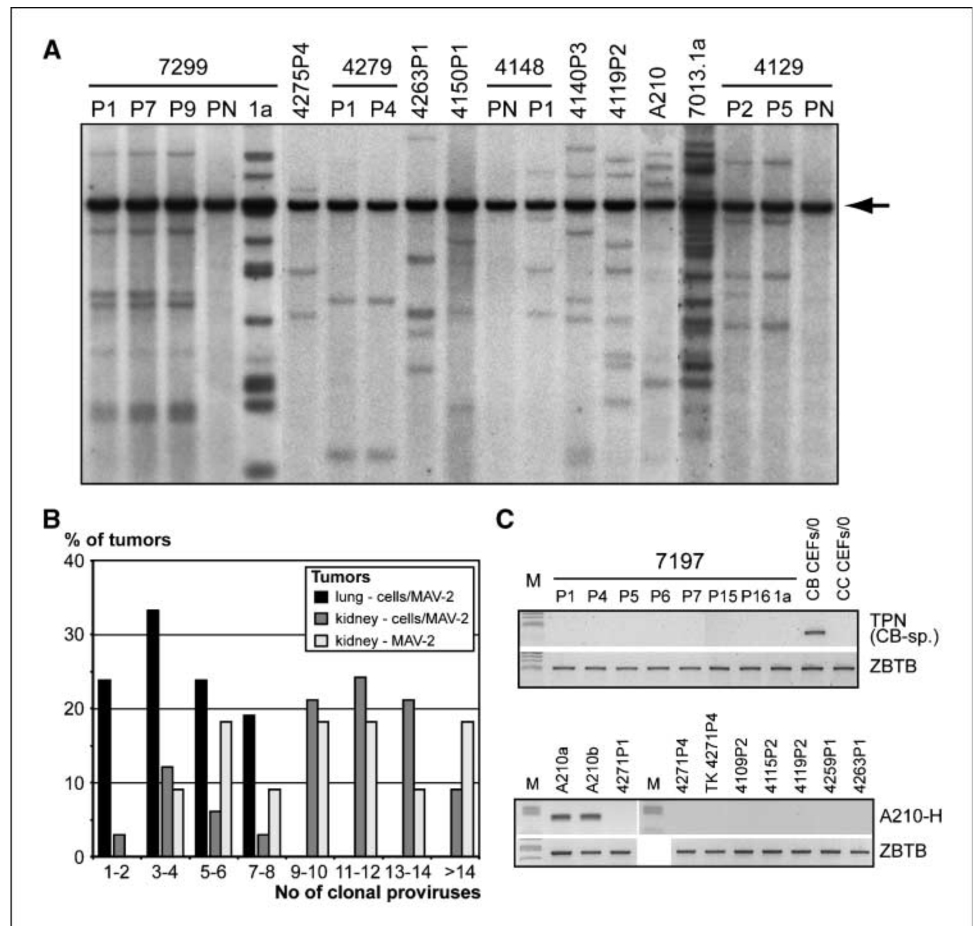

Figure 2. Lung tumors are clonal, host derived, and frequently invasive. *A,* the representative Southern blot analysis of 13 lung tumor foci (*P1–P5, P7*, and *P9*), 3 nonmalignant lung tissue (*PN*), 2 nephroblastomas (*1a*), and A210 cells. The identical integration pattern in various lung foci (*P1, P7*, and *P9* from the animal 7299 and *P2* and *P5* from the animal 4129) documents a frequent invasivity of the primary tumor clone. Normal lung tissue (*PN*) displays nonclonal pattern; nephroblastomas (7299.1a and 7013.1a) are different clones. *Arrow,* internal fragment of MAV-2 common to all integrated proviruses. *B,* distribution of clonally integrated MAV-2 proviruses (determined by Southern blot hybridization with the MAV-2–specific probe) in 20 lung tumors and in 33 and 11 nephroblastomas collected from animals injected with either CEF/MAV-2 or MAV-2, respectively. *C, top,* the representative result of PCR detection of CB cell–specific sequences (from *tpn* locus) in lung tumors (*P*) and nephroblastoma (*1a*) isolated from CC chick 7197 and in control fibroblasts (*CEF*) from both strains. The primer pair designed to check the amount and quality of DNA amplifies the fragment of the *zbtb* locus identical in both CB and CC strains. *Bottom,* representative result of detection of the A210-H integration site in A210 cells (*A210a*, 5th passage of stabilized cell culture; *A210b*, 20th passage) and in lung tumors from experimental animals (4271, 4109, 4115, 4119, 4259, and 4263). Cells from the tumor focus 4271P4 were grown in tissue culture for several passages (*TK 4271P4*) before analysis. *zbtb* control as in the top. The principle of the used PCR method is schematically depicted in Supplementary Fig. S3*A*.

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from a single primary tumor. Intriguingly, the lung tumor cells displayed a high invasive potential that frequently resulted in the dissemination of one clone into several foci in both lungs. In contrast, nephroblastomas or tumors of other tissues were non-invasive and adjacent foci were always formed from independent clones (Fig. 2*A*; ref. 14).

Close examination of various tumors isolated from animals injected with either A210 or CEF/MAV-2 cells revealed that none of the tumors was derived from the injected cells. This was first confirmed by PCR analysis of lung tumors from CC chicks injected with CB cells (A210 or CEF/MAV-2). Attempts to amplify genomic DNA isolated from lung tumors with a CB-specific primer pair consistently yielded negative results (Fig. 2*C, top*). Additional PCR analysis of tumor DNA with primer combinations that should identify the specific proviral integration sites unique to A210 cells was also negative as illustrated in Fig. 2*C* (*bottom*).

Further experiments were designed to rule out the possibility that tumors arose from a fusion of an A210 and host cell (when only a limited amount of tumorigenesis-related genetic material of A210 cells might remain in the resulting tumor cell). Southern blot analysis of DNAs isolated from A210-induced lung tumors of 20 different animals revealed that patterns of proviral integration were different from those in A210 cells and different in each animal. Notably, not even a single band on the blots (representing particular provirus insertion site) was shared between A210 and the tumors as well as between tumors (Fig. 2*A*).

To ensure that A210 cells do not contain a minute population of cells from which lung tumors could derive, we identified the genomic positions of viral integration sites in two lung tumors and designed PCR primers for their detection. We were unable to detect any shared proviral integration sites neither between the two tumors nor between tumors and A210 cells (Supplementary Fig. S3; Supplementary Table S1; data not shown). Taken together, the data conclusively show that the first malignant cell that eventually gives rise to a tumor originates from the host and exclude the possibility that the tumor cell arises from the fusion of an injected and host cell.

The tumor-promoting effect of injected cells is transient and independent of their abilities to divide and to infect surrounding cells. Figure 3*B* summarizes incidence of tumors in

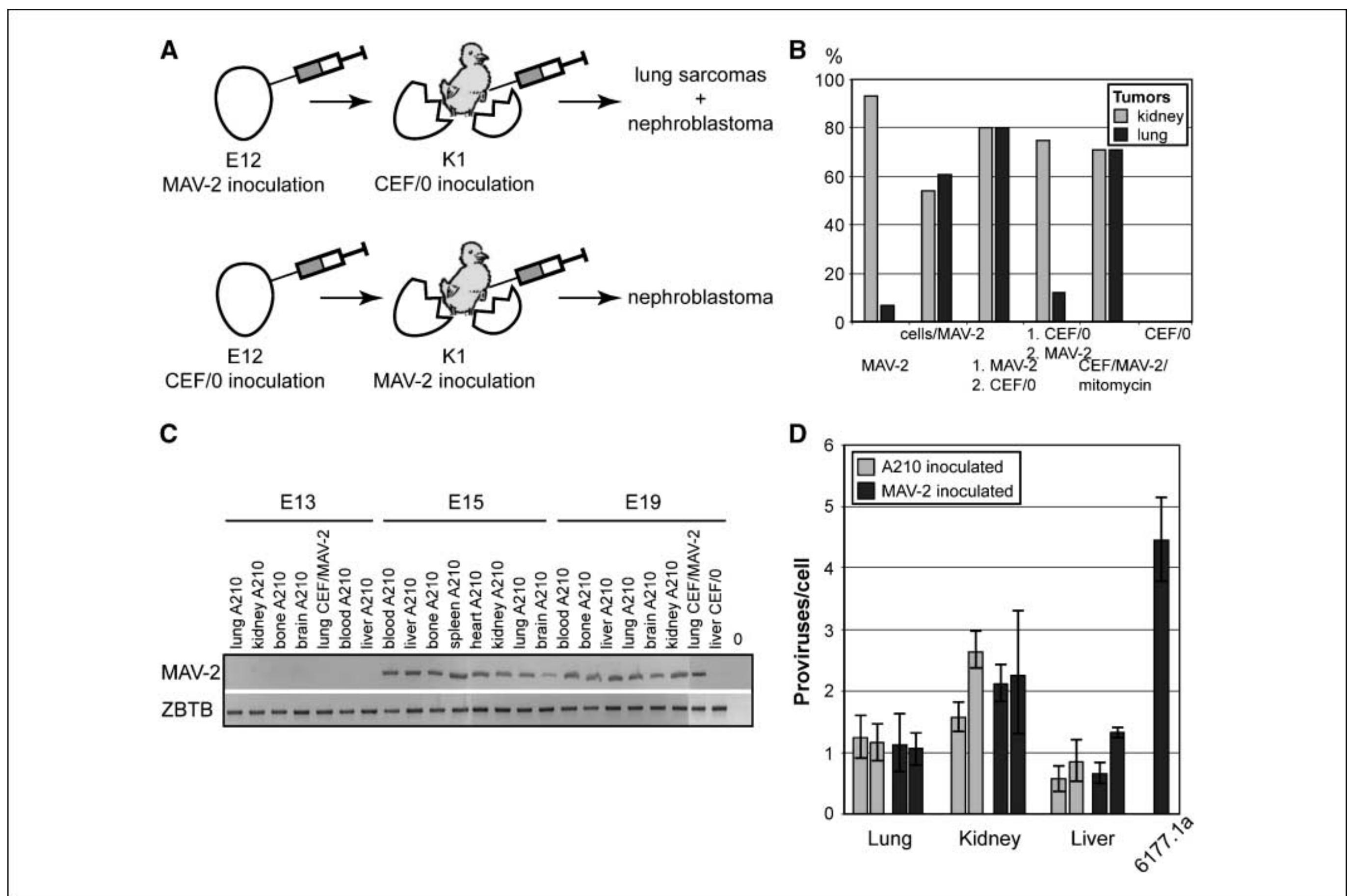

Figure 3. Contribution of the MAV-2 virus and cells to lung tumor induction and the extent of proviral integrations in different tissues. *A,* a schematic representation of successive injections of experimental chicks with virus (*MAV-2*) and cells (*CEF/0*) at E12 and at the first day after hatching (*K1*), and resulting tumors. *B,* incidence of tumors in chicks (groups of at least 10 animals) injected by MAV-2 virus at E12 (*MAV-2 columns*), A210 or CEF/MAV-2 cells at E12 (*cells/MAV-2 columns*), MAV-2 at E12 and CEF/0 cells at K1 (*1. MAV-2 2. CEF/0 columns*), CEF/0 at E12 and MAV-2 at K1 (*1. CEF/0 2. MAV-2 columns*), CEF/MAV-2 cells treated with mitomycin C at E12 (*CEF/MAV-2/mitomycin columns*), and noninfected CEF at E12 (*CEF/0 columns*). *C,* MAV-2 integration into various organs of chick embryos injected with MAV-2–producing cells. The virus-producing cells were injected at E12 into several embryos and DNAs from indicated tissues were analyzed by semiquantitative PCR using MAV-2–specific primers. Primers specific for the chicken *zbtb* locus (two copies per cell) were used as a control. *D,* quantification of MAV-2 proviruses integrated in nonmalignant tissues of 30-d-old experimental animals and in nephroblastoma 6177.1a. The marked tissues were isolated from two MAV-2–infected and two A210-infected chicks and subjected to quantitative real-time PCR with MAV-2–specific primers.

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chicks injected by MAV-2 virus or A210, CEF/MAV-2, or CEF/0 cells. To distinguish whether the dividing capability of injected cells is necessary for tumor formation, we injected MAV-2–infected CEFs that had been treated with mitomycin, a compound that permanently blocks cell division. After treatment, the cells were still able to promote tumor formation (Fig. 3*B*), indicating that the proliferative potential of an injected cell is dispensable. The result also further supports the notion of host origin of lung tumors.

To understand more precisely the role of stray cells and virus in lung tumor formation, a series of experiments were carried out in which virus-free CEFs and MAV-2 virus were injected stepwise in varying orders. The first agent was applied at embryonic day 12 (E12), whereas the second was injected after hatching (8–9 days after first injection, K1). The animals were sacrificed 40 to 60 days later and their lungs were examined. Interestingly, lung tumors were only evident in animals that received virus as the first treatment (Fig. 3*A* and *B*). This observation suggests that stray cells can promote tumor formation in tissues that have formerly been mutagenized and that the tumor-promoting potential of the stray cells persists not longer than several days in our model. The tumor-promoting capacity of virus-free cells injected into animals that have already been systemically infected also confirms that the effect of injected cells is independent of the dissemination of virus infection by injected cells.

Injected cells have no effect on the spectrum of infected tissues or the level of infection. To examine whether the injected virus-producing cells might affect the spectrum of infected tissues, we measured steady-state levels of proviral sequences in the genomic DNA of several organs. This analysis revealed that all tissues, including the lungs, are fully infected 3 days after the cell injection (Fig. 3*C*) similarly as if animals were injected with MAV-2 virus (data not shown). The average number of proviruses in 2-month-old chicken tissues was the same independent of whether the animals were injected by virions or by virus-producing cells (Fig. 3*D*). To further investigate the possibility that the increased incidence of lung tumors resulted from a high local level of infection (and thus a high mutation load) caused by the residing virus-producing stray cells, we compared the number of integrated proviruses in lung tumors and nephroblastomas. The Southern blot analysis of 20 randomly selected lung tumor clones and 33 nephroblastomas from the same animals showed a significantly lower average number of integrations in lung tumors compared with nephroblastomas, contradicting the idea of high local level of infection in the lung inflicted by the A210 or CEF/MAV-2 cells. Importantly, the average number of integrations in nephroblastomas was independent of whether the animals were injected by virions or by virus-producing cells (Fig. 2*B*).

The injected cells preferentially reside in liver, kidney, and lung embryonic tissues. To elucidate the precise localization of injected cells in tissues, MAV-2–producing CB CEFs were metabolically labeled with [^{35}S]methionine before i.v. injection into embryonic chicks. After 1 or 3 days after injection, several embryonic tissues were collected and both protein lysates and paraffin sections were prepared. The radioactivity in lysates was measured and specific radioactivity was calculated. Radioactive label was found in all tissues analyzed, with markedly the highest levels in the liver, kidney, and lungs (Fig. 4*A*). Very low radioactivity was found in the blood already 20 hours after injection, indicating prompt passage of the cells into the target tissues. Examination of the paraffin-embedded sections by autoradiography revealed similar observations (Fig. 4*B*; Supplementary Fig. S4). These results

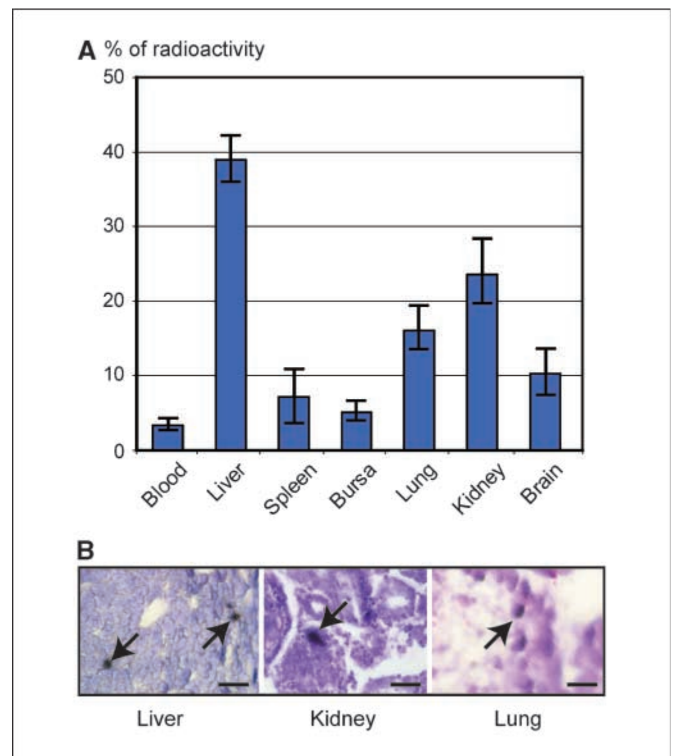

Figure 4. Homing and distribution of *in ovo*–injected MAV-2–producing cells in embryonic tissues. *A,* the distribution of injected [^{35}S]methionine-labeled cells was determined by calculating specific radioactivity in various embryonic tissues 20 h after injection. *B,* examples of radiolabeled cells (*arrows*) homed in liver, kidney, and lungs. For details, see Supplementary Fig. S4. *Bars,* 20 μm.

correlate well with the localization of the eventual tumors and show that the injected cells survive for at least 3 days in target tissues, sufficient time to influence the local microenvironment.

The recurrent target of MAV-2 insertional mutagenesis in lung tumors is the *frk* gene. The above observations are consistent with the hypothesis that a stray circulating cell can emit a tumor-promoting signal in a tissue with "silent tumor cells" that contain cancerous mutations, such as oncogene activation or tumor suppressor gene inactivation. If there are cancerous mutations typical for MAV-2–induced lung tumors, then common sites of MAV-2 integration should be found in tumor DNAs.

To identify loci hit by the provirus, we analyzed 17 chicks with cell promoted and 2 chicks with late MAV-2 lung tumors using the LTR-RACE method (14, 24). The study revealed recurrent integrations within the promoter/5′-untranslated region of the *frk* gene in 15 animals injected with cells and both animals injected only with MAV-2 (Supplementary Table S1). Proviruses were found to integrate in the same transcriptional orientation as the *frk* mRNA sequence within the narrow region of 1077 to 14 nucleotides upstream of the FRK initiation codon (Fig. 5*A*; Supplementary Table S2). Additional analysis by PCR, reverse transcription-PCR (RT-PCR), and Southern blotting confirmed the aforementioned results. As a consequence of proviral integration, the hybrid mRNA (starting in the proviral 3′-LTR and proceeding into the *frk* coding sequence) was highly expressed in all positive samples (Fig. 5*B* and *C*). The hybrid transcripts contained protein coding sequence identical with the proposed endogenous *frk* transcript. No *frk* expression was detectable neither in nontumor lung tissue nor in the two tumors with unaffected *frk* locus (Fig. 5*B* and *C*).

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Two conclusions can be drawn from the presence of an activated *frk* gene in ∼90% of the MAV-2–induced lung tumors, both cell promoted and MAV-2-only induced. First, it indicates that this dominant mutation is critically important for the tumor formation. Second, it suggests that injected cells promote tumor formation from the same pool of dormant mutagenized cells that also give rise to the rare long latency lung tumors in animals injected with MAV-2 only.

Discussion

Common presence of cancer-related mutations in cells of normal tissue has been well documented. The mutant cells are subject to microenvironmental and systemic regulation that enables them to suppress any potential malignant characteristics of the cells. This homeostasis can only be maintained as long as a critical amount of tumorigenic stimuli is not exceeded. As proven by cancer patients that relapse years after a successful therapy, a potential cancer cell can be maintained and suppressed for a long period of time. The experimental systems typically used to study the mechanisms that push potential cancer cells beyond the control of tissue homeostasis have been based on the use of tumor promoters such as phorbol esters that, although not mutagenic in themselves, induce tumors in animals previously mutagenized by chemicals (25).

We have used an experimental model that takes advantage of the ability of MAV-2 to randomly integrate its provirus into the host genome to create somatic mutations. In chicks, the process of infection and proviral integration is essentially completed within a few days after infection. Additional insertions are strongly limited due to a viral interference mechanism (26). However, before interference develops, permissive cells can acquire multiple proviruses. This model mimics the slow accumulation of naturally occurring somatic mutations. An advantage of the retroviral system is that mutated loci contain retroviral sequences so that they can readily be identified in tumor clones (27). Furthermore, the specific integration patterns allow independent tumor clones to be distinguished one from another.

In chickens, it is well established that the predominant tumors caused by MAV-2 infection are clonal nephroblastomas (22). Occasionally, we have also observed the formation of other tumor types predominantly of lung and liver origin that arose independently of

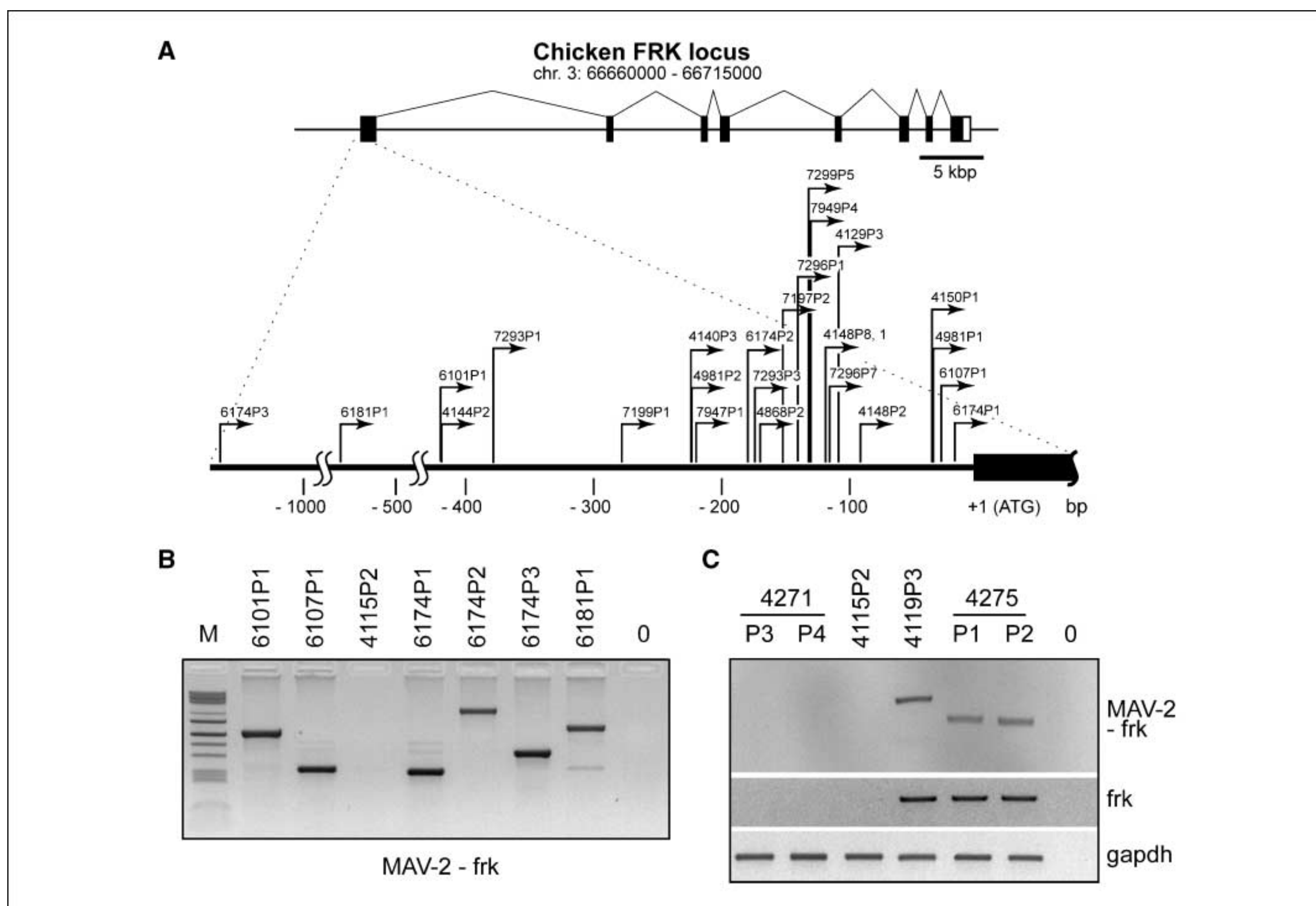

Figure 5. Common site of proviral integration in the *frk* locus. *A,* the exon-intron structure of chicken *frk* genomic locus and sites of proviral integrations upstream from ATG initiation codon. Arrows indicating integration sites and orientation of transcription are marked by the code number of lung tumors in which they were found. *B,* an example of detection of hybrid MAV-2-frk mRNAs synthesized as a result of MAV-2 integration, by integration site-specific RT-PCR (described in Supplementary Fig. S3*A*). The variation in lengths of PCR products reflects the distance of individual integration sites from the *frk*-derived primer. The tumor 4115P2 has no integration in *frk* gene. *0,* reaction without cDNA. *C,* RT-PCR analyses of *frk* expression using MAV-2 and frk (*top*) or only frk primers (*middle*). The majority of *frk* mRNA is produced from the proviral promoter. The tumors without integration in the *frk* locus do not express the *frk* gene. *Bottom,* control *gapdh* expression.

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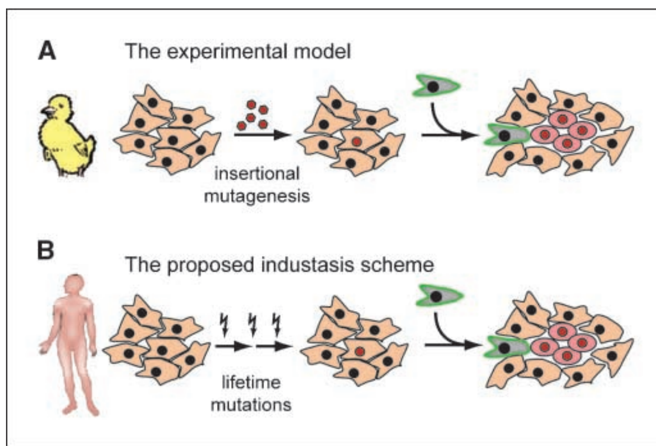

Figure 6. Industasis concept. *A,* in the chicken model (*top scheme*), the insertional mutagenesis by MAV-2 (*red hexagons*) results in the formation of a precancerous initiated cell (*red nucleus*) and is immediately followed by a tumor-promoting effect of an injected stray nontumorigenic cell (*gray*), resulting in the formation of a tumor clone (*red oval cells*). *B,* in the proposed general model (*bottom scheme*), mutations accumulate throughout the life span of an individual forming the genetically transformed initiated cell. A stray nontumorigenic cell liberated for instance from a primary tumor affects the microenvironment of an initiated cell promoting its malignant progression.

the nephroblastomas. As such, these tumors induced by MAV-2 insertional mutagenesis represent multiple primary tumors. Compared with nephroblastomas, these second primary tumors displayed a different mutation signature. In nephroblastomas, the *twist, plag1*, and *foxP1* loci represent dominant common sites of proviral integration (14). In contrast, lung tumors had very frequent insertions in the promoter region of the *frk* gene, never found in nephroblastoma. This observation for the first time documents oncogenic capability of *frk* overexpression, previously being only suspected (28). This result supports the concept that distinct mutational signatures can exist in cancers derived from different tissues (29).

The injection of cells into the blood circulation of MAV-2 mutagenized animals resulted in the appearance of numerous early lung tumors with the same mutation signature as the rare late lung tumors in animals injected with the MAV-2 alone (i.e., proviral integration in the *frk* gene). Thus, the presence of stray cells changed the formerly rare and late second tumors to frequent and early primary neoplasias. We suggest that the preferential promotion of lung tumors by stray cells is due to the hemodynamics, specific characteristics of the walls of fine pulmonary capillaries, and tissue-specific activity of the retroviral promoter/enhancer.

Based on the above observations, we propose the concept of industasis (Fig. 6). This mechanism of cancer promotion may take place in single preneoplastic cells or cell compartments (cancerized fields) that have accumulated cancerous mutations yet remain under the control of tissue homeostasis. Stray cells may interfere with the regulation of the local microenvironment that maintains tissue homeostasis through cell-cell adhesion/communication and cell-extracellular matrix interactions. By locally weakening homeostasis, stray cells may reduce the amount of tumorigenic alterations required for predisposed cells to express their malignant character. Eventually, presence (or death) of stray cells may elicit signals stimulating proliferation normally associated with wound healing. Once the tumor-predisposed cells start to proliferate, they lose contact with suppressive microenvironment and may become self-sustaining by creating supportive microenvironment for themselves, including recruitment of supportive stromal cells (30). The proposed mechanism could be the final step in genesis of several human multiple primary tumors (Fig. 6*B*). Human cells accumulate numerous somatic mutations during their lengthy life span, and it is well documented that the suppression of genetically transformed cells by the surrounding microenvironment represents an important defense against tumor outburst (3). The organism hosting one primary tumor is often flooded by stray cells liberated from the tumor (31). Their role is currently supposed to constitute a substantial risk of forming metastases (32). However, these stray cells could disturb the suppressive defense mechanisms, allowing pretransformed cells to grow.

Our hypothesis suggests that a substantial portion of human second primary tumors might be provoked by industasis. Such cases might be frequent as there is evidence that several presumed metastases could be in fact misdiagnosed second primary tumors (33).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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