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Advances in Brief

Conditional Expression of K-*ras* in an Epithelial Compartment that Includes the Stem Cells Is Sufficient to Promote Squamous Cell Carcinogenesis

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Abstract

***Ras* genes are the most frequently mutated oncogenes in human cancer. However, the contribution of *ras* to tumor initiation still is unclear because *ras* expression in primary cells can cause cell cycle arrest and even cell death by apoptosis. Furthermore, when expressed in the epidermis of mice, mutant *ras* promotes the formation of benign papillomas, only few of which will progress into carcinomas. However, in these cases, *ras*-transgene expression often is restricted to suprabasal or follicular epithelial cells that may lack self-renewal capacity. Thus, it still is conceivable that expression of active *ras* in other epithelial compartments may exert a distinct ability to promote malignant progression. To address this possibility, transgenic mice carrying the tetracycline-inducible system (*tet*-on receptor) targeted to the basal layer of stratified epithelium, which includes the epithelial stem cells, were engineered and crossed with mice expressing the K-*ras*^{G12D} oncogene under the control of *tet*-regulated responsive elements. On doxycycline administration, proliferative lesions ranging from hyperplasias, papillomas, and dysplasias to metastatic carcinomas developed in squamous epithelia of the skin, oral mucosa, salivary glands, tongue, esophagus, forestomach, and uterine cervix within just 10 to 20 days. The most noticeable lesions were invasive squamous carcinomas of the skin and oral mucosa. These findings suggest that the expression of oncogenes in an epithelial compartment that includes the stem cells may be sufficient to promote squamous carcinogenesis. They also provide a molecularly defined conditional animal model system in which the mechanisms responsible for cancer initiation, maintenance, and metastatic spread can be readily investigated.**

Introduction

Mutations in the three human *ras* genes, H-*ras*, K-*ras*, and N-*ras*, have been detected with high frequency in human cancers, including those from the lung, colon, urinary bladder, gall bladder, pancreas, breast, and ovary, with frequencies ranging from 10 to 90% (1). Activating mutations in K-*ras* and H-*ras* also have been reported in human squamous cell carcinomas (SCCs), primarily in those caused by exposure to carcinogens present in betel quid (2). Similarly, H-*ras* mutations frequently are found in experimental mouse models of chemical carcinogenesis (3). However, the role of *ras* in tumor initiation still is controversial because *ras* expression in primary cells can cause cell cycle arrest or even apoptosis (4, 5). The prevailing view is that human and experimental carcinogenesis involves the sequential accumulation of activating mutations in several oncogenes along with inactivation of a number of tumor suppressor genes (6). In line with this view, expression of mutant *ras* in the skin of mice, when caused by chemical carcinogens or by transgenic strategies, promotes benign papilloma formation with a variable rate of delayed carcinoma conversion (3). Because in these cases *ras* expression often is restricted to suprabasal or follicular cells that may lack self-renewal capacity, the possibility still exists that activation of *ras* in other epithelial compartment may display a distinct ability to promote tumor initiation and progression (3, 7). We show here that the conditional expression of activated K-*ras* in the basal layer of the epidermis, which includes the stem cell compartment (8), is sufficient to induce the rapid (10 to 30 days) formation of malignant SCC in the skin and other squamous epithelia.

Materials and Methods

Generation of Transgenic Mice. The K5-*rTA* transgene, referred herein as K5-*tet*-on, was constructed by adding a nuclear localization signal to the reverse tetracycline transactivator (rTA; ref. 9) and cloned into a unique *Nhe*I site in a pBSKII-derived vector, downstream from the bovine keratin 5 (K5) promoter and upstream from a polyadenylation signal (10). K5-*tet*-on mice were produced by injecting the 9.452 kb Asp718-excised fragment into FVB/N blastocytes. Founders were screened for transgene insertion by Southern blot analysis, and subsequent generations were screened by PCR using specific primers. *tet*-K-*ras* transgenic mice, expressing active K-*ras4b*^{G12D} oncogene, referred herein as K-*ras*^{G12D}, under the control of seven *tet*-responsive elements, have been described previously (11). Doxycycline (RPI) was administered via the drinking water at a concentration of 500 mg/L.

Tissue Preparation, Histology, and Immunohistochemistry. Tissues were fixed in 4% paraformaldehyde overnight and transferred to 95% EtOH and embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and eosin or analyzed for expression of proliferating nuclear antigen (PCNA; Zymed, San Francisco, CA) and active phosphorylated forms of Akt and extracellular signal-regulated kinase (ERK) (Cell Signaling, Beverly, MA). Cryostat sections were obtained from snap-frozen, OCT-embedded tissues samples, fixed in 4% paraformaldehyde in 1× PBS, washed, and processed for β-galactosidase staining (10).

Results and Discussion

Transgenic expression of mutant *ras* genes in suprabasal epithelial cells or proliferating cells within the hair follicle causes hyperplasias and papillomas, <15% of which would slowly progress into carcinomas after several months (12, 13). Thus, expression of *ras* in differentiating or committed cells may not be sufficient to promote cancerous growth. Because available evidence suggests that squamous carcinomas may instead arise from genetic alterations in more primitive epithelial cells (3, 7), we decided to explore the consequences of expressing mutant *ras* from the K5 promoter, which targets transgene expression to a basal epithelial cell compartment that includes the stem cells (8). However, numerous attempts to express activated *ras* using this promoter failed to generate viable transgenic animals (data not shown), suggesting that *ras* expression may compromise mouse development and/or viability. Thus, as an alternative approach, we set out to express *ras* in the epithelium of adult animals using a tetracycline-inducible system. Mice were engineered to express the *rTA* (*tet*-on) transgene under the control of the K5 promoter and were first crossed with mice expressing the β-galactosidase gene under the

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control of *tet*-responsive elements to test the effectiveness of this *in vivo* gene expression system (Fig. 1). β-Galactosidase activity was readily detected in the epidermis of double transgenic animals but not in single transgenic mice and that was strictly dependent on the administration of doxycycline, a tetracycline analog, thus confirming the specificity of this system. β-Galactosidase activity was observed in follicular and interfollicular epithelium, as well as in other stratified squamous epithelium, such as in the tongue and forestomach, mirroring the pattern of expression of K5 (10).

We next crossed the K5-*tet*-on animals with mice expressing an activated allele of K-*ras*, K-*ras*^{G12D}, under the control of *tet*-respon-

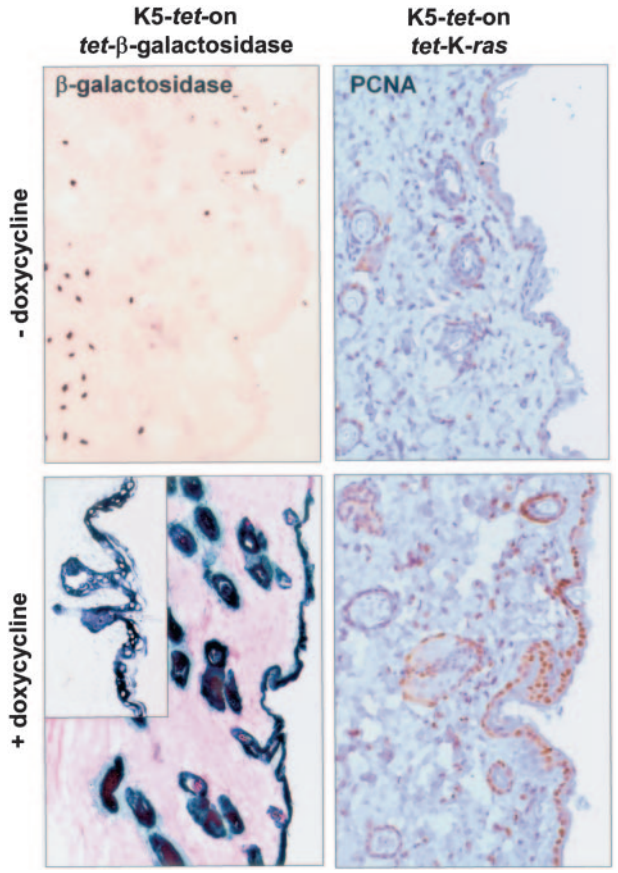

Fig. 1. K5-*tet*-on expression system. The indicated double transgenic mice were left untreated (−) or treated with (+) doxycycline. β-Galactosidase staining was strongly positive in the skin of treated animals (*top*, 40×). Most cells are positive for proliferating nuclear antigen (*PCNA*) within the epidermis of treated mice, whereas only a few immunoreactive cells are seen in untreated animals (*bottom*, 160×).

Table 1 *Phenotype of doxycycline-treated* K5-*tet*-on/*tet-o*-K-*ras double transgenic mice*

Mouse (#)	Doxycycline treatment (days)	Skin	Oral mucosa and tongue	Esophagus and forestomach
1	7	Hyperplasia	Hyperplasia	Hyperplasia
2 *	7	Hyperplasia	Hyperplasia	Hyperplasia
3	7	Hyperplasia Papilloma	Hyperplasia	Hyperplasia
4	10	Hyperplasia SCC	Hyperplasia	Hyperplasia
5	11	Hyperplasia Papilloma	Hyperplasia Papilloma	Hyperplasia Papilloma
6	11	Hyperplasia Papilloma	Hyperplasia Papilloma	Hyperplasia Papilloma
7 †	15	Hyperplasia SCC	Hyperplasia	Hyperplasia
8	15	Hyperplasia	Hyperplasia	Hyperplasia
9	15	Hyperplasia Papilloma	Hyperplasia	Hyperplasia
10	15	Hyperplasia Papilloma SCC	Hyperplasia	Hyperplasia
11	16	Hyperplasia Papilloma SCC	ND	Hyperplasia
12	23	Hyperplasia	Hyperplasia	Hyperplasia
13	28	Hyperplasia	Hyperplasia	Hyperplasia
14	29	Hyperplasia	Hyperplasia	Hyperplasia
15	30	Hyperplasia	Hyperplasia Hyperplasia	Hyperplasia
16 ‡	32	Hyperplasia SCC Hyperplasia	Papilloma Microinvasive SCC §	Hyperplasia
17	33	Papilloma SCC	Hyperplasia	Hyperplasia

Abbreviations: SCC, squamous cell carcinoma; ND, not determined.
*A microinvasive carcinoma was observed in the uterine cervix.
†Metastasis was found in the cervical adipose tissue.
‡Metastasis was found in the cervical lymph node.
§Microinvasive SCC developed from a papilloma

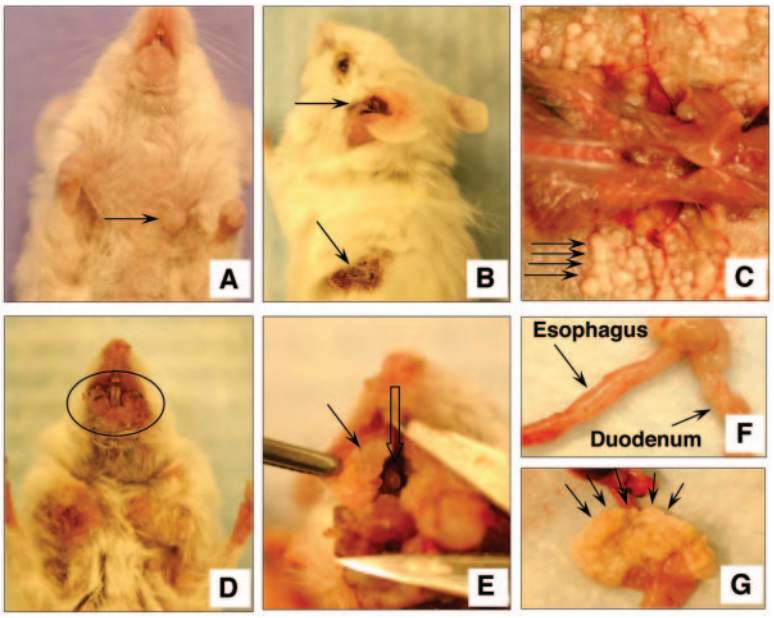

Fig. 2. Tumor development on induction of K-*ras*^{G12D} expression. *A,* skin papilloma (*arrow*). The lesion was histologically benign. *B,* Two ulcerative SCCs are shown in this animal (*arrows*), one of them originating in the left external duct of the ear. *C,* Numerous papillomatous and carcinomatous proliferations of the skin (white bumps) are seen protruding throughout the hypodermis (skin of the back). *D,* squamous hyperplasia compromising the lips (*circle*). *E,* This animal shows lip squamous hyperplasia (*arrow*) and oral papillomas (*empty arrow*). *F,* diffuse enlargement of the esophagus secondary to squamous hyperplasia. The organ has reached a diameter similar to that of the duodenum. *G,* forestomach, diffuse hyperplasia of the squamous epithelium. The organ seems to be covered by an irregular and thick white membrane.

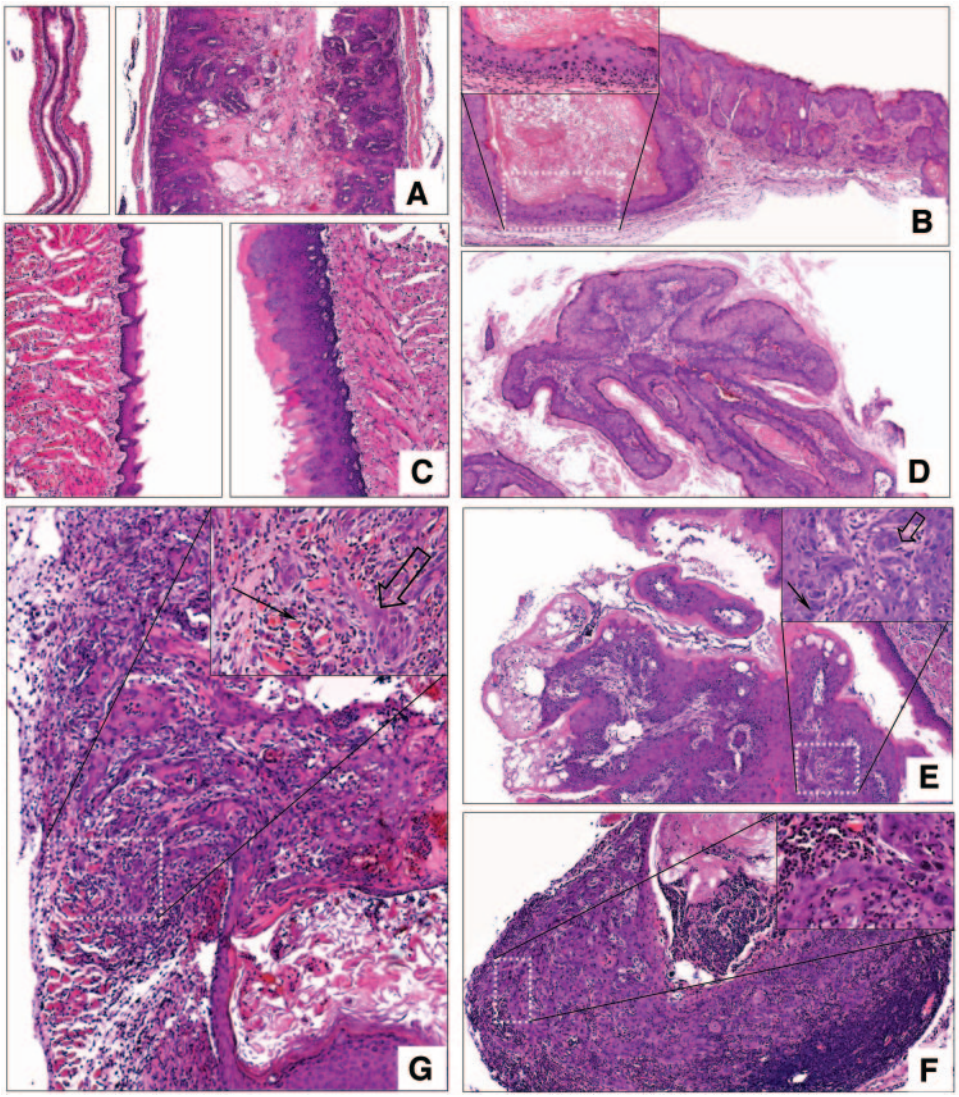

Fig. 3. Histopathology of epithelial lesions arising by expression of K-*ras*^{G12D}. *A,* papillary hyperplasia of the esophageal squamous epithelium with marked hyperkeratosis and parakeratosis. The lumen is completely obliterated, and the whole thickness of the organ is increased severalfold. Compare with a normal esophagus. *B,* skin hyperplasia in a doxycycline-treated animal. Dysplastic lesions are observed in some areas (*inset*). The cells show large, hyperchromatic nuclei with irregular shapes and coarsely granular chromatin. Nuclear/cytoplasmic ratios are markedly increased, and the nuclear membranes frequently show notches or indentations. *C,* Hyperplasia and hyperkeratosis of the tongue; focal parakeratosis also is evident. Compare with a normal tongue. *D,* papilloma of the skin. The hyperplastic squamous epithelium covers thin expansions of a fibroblastic stroma with mild chronic inflammatory infiltrates. *E,* This squamous cell papilloma of the oral mucosa shows hyperkeratosis and parakeratosis and vacuolization. At higher magnification (*insert*), mitotic figures are present in the basal layers (*arrow*), as well as areas of early stromal invasion (microinvasive carcinoma; *empty arrow*). *F,* metastasis of an SCC in a cervical lymph node. The neoplastic process has almost completely replaced the organ. The atypical features of the metastatic cells can readily been seen in the inset; the infiltrating atypical squamous cells display irregular shapes, with hyperchromatic nuclei and hypertrophic eosinophilic nucleoli (*arrow*). *G,* ulcerative SCC of the skin. The malignancy has infiltrated and destroyed the muscle layer (*arrow,* highly eosinophilic skeletal muscle cells; *empty arrow,* neoplastic cells). Images are from representative hematoxylin and eosin–stained sections, 64×; *insets,* 160×.

sive elements (*tet-o*-K-*ras*). Double transgenic animals were viable and healthy. Administration of doxycycline caused skin keratinocyte hyperproliferation, as judged by the immunodetection of proliferating nuclear antigen in nearly all of the basal epithelial cells, even after only 2 days of treatment (Fig. 1). Furthermore, these animals began to exhibit a wide range of visible lesions within 2 weeks of doxycycline administration, particularly in the skin and the mouth (Fig. 2). Animals sacrificed 3 to 4 weeks after initiating doxycycline treatment displayed overt lesions of the skin and squamous epithelium from other organs, such as the forestomach and esophagus (Table 1). Histologically, a wide variety of epithelial alterations were observed, ranging from benign hyperplasia to dysplasia and SCCs. The latter were detected in ~40% of the animals (6 of 17 mice), some of which had already metastasized to lymph nodes (Fig. 3), indicating that expression of activated alleles of K-*ras* in this particular epithelial compartment promotes malignant transformation. Furthermore, tumor progression correlated with the persistent activation of *Ras*-initiated biochemical pathways, such as the activation of extracellular signal-regulated kinase and Akt, as judged by the immunodetection of the active, phosphorylated form of these signal-transducing molecules (data not shown).

Whereas *ras* readily transforms cell lines in culture, this oncogene does not stimulate cell proliferation but instead causes cell cycle arrest or even apoptosis when expressed in primary rodent and human fibroblasts (4, 5). In our animal model, however, conditional expression of K-*ras* potently stimulated cell proliferation, an observation in line with recent reports that activated *ras* promotes cell growth when conditionally expressed in other epithelial tissues, such as the lung, pancreas, and colon (14, 15). Collectively, the available data support the emerging concept that *ras* can display a potent proproliferative activity *in vivo*, which is distinct from its biological effects in primary cells *in vitro*. However, the observation that the sole expression of *ras* can result in the rapid malignant conversion of squamous epithelium is intriguing because mutations in the *ras* oncogene usually are considered insufficient to directly promote carcinogenesis (16). Because mice have long telomeres, unlike humans, one explanation could be that murine cells may be more prone to cell transformation because they do not need to reactivate telomerase activity to initiate tumorigenesis (17). We also have observed that *ras*-transformed cells display abnormal nuclear morphology consistent with changes in ploidy, whose nature is under investigation. Thus, chromosomal instability secondary to *ras* expression may promote the rapid genetic and epigenetic inactivation of tumor suppressor genes. Alternatively, functional inactivation of key tumor suppressor proteins may be achieved by the ability of *Ras* to stimulate signal-transducing path-

ways and cell cycle–regulating molecules. These, as well as additional possibilities, are under current investigation.

In this regard, it is tempting to speculate that the remarkable effects caused by *ras* in our animal model could be because of the expression of this oncogene in a particularly susceptible cell population, such as the epidermal stem cells, which might exhibit distinct requirements for malignant transformation. In contrast to the fast onset of malignant conversion observed here, when *ras* is expressed in other epithelial cell compartments, SCCs arise only after an extended period in which additional stochastic genetic lesions are likely to occur (3). Classical chemical carcinogenesis studies further support this explanation because activation of endogenous *ras* by carcinogens appears to occur primarily in cells that have left the stem cell niche (3, 7), thus also causing benign tumors, few of which can progress into frank malignancies after prolonged treatment with tumor promoters. The primitive stem cells, few in number and buried deep into the epidermis, may be protected from mutagens because they are less accessible to environmental carcinogens than are the more superficial epithelial transit-amplifying cells (3, 7). Stem cells also divide infrequently; therefore, they have fewer chances to incorporate mutations or can repair them more effectively (18) and are susceptible to apoptosis secondary to DNA damage, often choosing to self-eliminate rather than undergo extensive error-prone DNA repair (18). Conversely, recently developed mathematical models suggest that exponential growth during development may result in the accumulation of mutations in a small population of stem cells, which, although not tumorigenic in nature, may nonetheless predispose to certain late-life cancers (19). Thus, it is possible that in our animal model the seemingly explosive growth of cancerous cells may result from the transformation of this limited pool of already predisposed cells that do not require the accumulation of additional mutations subsequent to *ras* activation for malignant conversion. This exciting possibility may be relevant to human cancer. Individuals may harbor few already predisposed epithelial stem cells or cells that have regained self-renewal capacity, which may accumulate additional mutations over the years by the exposure to carcinogens, but without presenting any clinically identifiable lesion. In this scenario, aberrant activation of proliferative pathways (*e.g.,* by mutations in *ras* or other oncogenes) may promote the rapid cancerous growth of this particular population of susceptible cells. Additional work in this novel experimental animal model may help address this possible stem cell origin of *ras*-induced tumors, as well as the nature of the preexisting or subsequent genetic and epigenetic events that enable tumor progression. We envision that the future use of this molecularly defined conditional animal model system may help in current efforts to unravel the mechanisms responsible of cancer initiation, maintenance, and metastatic spread, thus aiding in the search for novel chemopreventive strategies and molecular-targeted human cancer therapies.

References

1. Bos JL. ras oncogenes in human cancer: a review. Cancer Res 1989;49:4682–9.
2. Das N, Majumder J, DasGupta UB. ras gene mutations in oral cancer in eastern India. Oral Oncol 2000;36:76–80.
3. Perez-Losada J, Balmain A. Stem-cell hierarchy in skin cancer. Nat Rev Cancer 2003;3:434–43.
4. Serrano M, Lin AW, McCurrach ME, et al. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997;88:593–602.
5. Joneson T, Bar-Sagi D. Suppression of Ras-induced apoptosis by the Rac GTPase. Mol Cell Biol 1999;19:5892–901.
6. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
7. Owens DM, Watt FM. Contribution of stem cells and differentiated cells to epidermal tumours. Nat Rev Cancer 2003;3:444–51.
8. Tumbar T, Guasch G, Greco V, et al. Defining the epithelial stem cell niche in skin. Science 2004;303:359–63.
9. Gossen M, Freundlieb S, Bender G, et al. Transcriptional activation by tetracyclines in mammalian cells. Science 1995;268:1766–9.
10. Ramirez A, Bravo A, Jorcano JL, Vidal M. Sequences 5′ of the bovine keratin 5 gene direct tissue- and cell-type-specific expression of a lacZ gene in the adult and during development. Differentiation 1994;58:53–64.
11. Fisher GH, Wellen SL, Klimstra D, et al. Induction and apoptotic regression of lung adenocarcinomas by regulation of a K-Ras transgene in the presence and absence of tumor suppressor genes. Genes Dev 2001;15:3249–62.
12. Brown K, Strathdee D, Bryson S, et al. The malignant capacity of skin tumours induced by expression of a mutant H-ras transgene depends on the cell type targeted. Curr Biol 1998;8:516–24.
13. Bailleul B, Surani MA, White S, et al. Skin hyperkeratosis and papilloma formation in transgenic mice expressing a ras oncogene from a suprabasal keratin promoter. Cell 1990;62:697–708.
14. Tuveson DA, Shaw AT, Willis NA, et al. Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. Cancer Cell 2004;5:375–87.
15. Hingorani SR, Petricoin EF, Maitra A, et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 2003;4:437–50.
16. Land H, Parada LF, Weinberg RA. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 1983;304:596–602.
17. Blasco MA, Hahn WC. Evolving views of telomerase and cancer. Trends Cell Biol 2003;13:289–94.
18. Cairns J. Somatic stem cells and the kinetics of mutagenesis and carcinogenesis. Proc Natl Acad Sci USA 2002;99:10567–70.
19. Frank SA, Nowak MA. Cell biology: developmental predisposition to cancer. Nature 2003;422:494.

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