Tumor Progression of Skin Carcinoma Cells *in Vivo* Promoted by Clonal Selection, Mutagenesis, and Autocrine Growth Regulation by Granulocyte Colony-Stimulating Factor and Granulocyte-Macrophage Colony-Stimulating Factor

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Tumor microenvironment is crucial for cancer growth and progression as evidenced by reports on the significance of tumor angiogenesis and stromal cells. Using the HaCaT/HaCaT-ras human skin carcinogenesis model, we studied tumor progression from benign tumors to highly malignant squamous cell carcinomas. Progression of tumorigenic HaCaT-ras clones to more aggressive and eventually metastatic phenotypes was reproducibly achieved by their *in vivo* growth as subcutaneous tumors in nude mice. Their enhanced malignant phenotype was stably maintained in recultured tumor cells that represented, identified by chromosomal analysis, a distinct subpopulation of the parental line. Additional mutagenic effects were apparent in genetic alterations involving chromosomes 11 and 2, and in amplification and overexpression of the *H-ras* oncogene. Importantly, *in vitro* clonal selection of benign and malignant cell lines never resulted in late-stage malignant clones, indicating the importance of the *in vivo* environment in promoting an enhanced malignant phenotype. Independently of their *H-ras* status, all *in vivo*-progressed tumor cell lines (five of five) exhibited a constitutive and stable expression of the hematopoietic growth factors granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor, which may function as autocrine/paracrine mediators of tumor progression *in vivo*. Thus, malignant progression favored by the *in vivo* microenvironment requires both clonal selec-

tion of subpopulations adapted to *in vivo* growth and mutational events leading to stable functional alterations. (Am J Pathol 2001, 159:1567–1579)

The development of cancer is a multistep process, in which cells accumulate genetic alterations thereby gradually progressing from a normal to a malignant phenotype. The stepwise evolution from premalignant lesions to malignant and finally metastasizing tumors is understood as the result of an increasing dysregulation of endogenous growth control mechanisms and independence of environmental growth regulatory factors.¹ One of the best studied models for tumor development and progression is that proposed by Fearon and Vogelstein² for colon carcinogenesis. Detailed genetic analysis of different stages in the process of tumor development revealed that an accumulation of multiple changes in tumor suppressor genes as well as oncogenes is necessary for the evolution of malignant tumors. To date it is not clear whether a specific number of genetic alterations or a defined sequence in which these alterations occur is prerequisite for a malignant tumor to develop. The understanding of the molecular mechanisms underlying tumor progression has been greatly improved by the development of *in vitro* model systems such as that for colon carcinoma.³ However, the specific role of the *in vivo* microenvironment in controlling or facilitating the transition to a more advanced stage of cell transformation is still poorly understood because of the complexity of the tissue influences.

We have previously developed an *in vitro* cell transformation system of human skin keratinocytes and characterized the genetic and phenotypic alterations associated with the subsequent stages of carcinogenesis to squa-

Supported by the Verein zur Foerderung der Krebsforschung e.V. and DFG SPP "Angiogenesis" (Fu 91-5).

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Accepted for publication July 11, 2001.

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mous cell carcinomas (SCCs).⁴ In parallel, we have studied the tissue-related changes occurring with tumorigenesis and malignant progression and have begun to characterize the accompanying alterations induced by transformed epithelia in the neighboring connective tissue, using a matrix-inserted surface transplantation assay.^{5,6} In these studies, we have demonstrated the critical role of the tissue microenvironment in controlling and enhancing distinct stages in skin carcinogenesis both by intraepithelial⁷ and epithelial-mesenchymal interactions.^{5,6,8} *In vitro* transformation of human cells requires, as a first step, the loss of cell senescence and acquisition of immortality, leading to permanently growing but nontumorigenic cell populations.^{9–11} This immortalization process is frequently induced by transfection or infection with oncogenic viruses such as simian virus 40 or human papilloma virus-16, -18, or -31.^{12–14} Tumorigenic conversion of these immortalized cells can then be induced by chemical and physical carcinogens or by specific oncogenes.^{15–18} In addition, long-term passage of some of these virally immortalized cells leads to spontaneous progression to a tumorigenic phenotype, suggesting a certain degree of genetic instability in these cells.^{19–24}

We have established a multistage skin carcinogenesis model system based on the spontaneously immortalized human keratinocyte line HaCaT.^{4,25} This cell line does not harbor any viral sequences that might add to genetic instability and thus has maintained its nontumorigenic phenotype for extended culture passages.²⁶ Moreover, HaCaT cells closely resemble normal human keratinocytes in their growth and differentiation potential^{25,27–30} and have thus become a frequently used model system to study keratinocyte biology and transformation. Immortalization of HaCaT cells was most likely a consequence of UV-type mutations in both alleles of the p53 tumor suppressor gene³¹ similar to those found in a high percentage of skin carcinomas and premalignant lesions.³² In addition, HaCaT cells show a loss of chromosomes 3p, 4p, and 9p, areas in which senescence genes have been identified or postulated.³³ Despite these genetic alterations, HaCaT cells retain a stable chromosome content and remain nontumorigenic throughout 320 passages.²⁶ Because of their genetic make-up and primarily maintained phenotypic normality, they are considered to represent a very early stage in skin tumorigenesis.⁴ Their tumorigenic conversion was achieved by transfection with the mutated *val-12 Harvey-ras* oncogene³⁴ but also by growth under elevated temperature (sunburn conditions)³⁵ or extremely stressful culture conditions such as repeated single cell cloning³⁶ or adaptation to autotrophy.³⁷ In epithelial skin tumors, an activated *ras* oncogene has been detected in squamous and basal cell carcinomas, suggesting its causal role at least in a subset of epithelial skin tumors.^{38,39} Tumorigenic conversion of the HaCaT cells resulted in clones growing as epidermoid cysts (benign tumors) after subcutaneous injection.³⁴ Additional loss of copies of chromosome 15 in the *ras*-transfected HaCaT cells was correlated with malignant tumor cells, forming well-differentiated SCCs in nude mice.^{33,40} Here we report that further progression of the malignant HaCaT-*ras* clones to even more aggressive

Figure 1. Pedigree of tumorigenic HaCaT clones with benign (A-5, HaCaTp), malignant (II-4, HaCaT40°) and, after *in vivo* passage, enhanced malignant (II-4RT, A-5RT1, HaCaTpT, and HaCaT40°RT) and metastatic (A-5RT3) HaCaT tumor cells.

and eventually metastatic phenotypes was not observed after extended passaging *in vitro* or after culture stress such as repeated single cell cloning. In contrast, their late passage cells exhibited reduced tumorigenicity when injected subcutaneously into nude mice, resulting in diminished tumor growth rates. On the other hand, cells recultured from established heterotransplants showed an increased malignant potential when reinjected into animals, indicating that the *in vivo* microenvironment exerted a selective pressure and favored malignant progression. We have studied this phenomenon in detail and observed a stepwise tumor progression to highly malignant and metastasizing tumor cell lines obtained through sequential *in vivo* passages. Characterization of the accompanying genetic and phenotypic changes strongly indicates both clonal selection and further genetic alterations as causal mechanisms for the development of advanced tumor stages. This tumor progression, shown to be stably maintained in recultured tumor cells, is associated with distinct genetic alterations and is phenotypically characterized by an altered growth regulation.⁴¹

Materials and Methods

Cell Lines

Cell lines used in this study are the benign HaCaT-*ras* clone A-5, the malignant HaCaT-*ras* clones II-3 and II-4,³⁴ as well as the benign tumorigenic HaCaT line HaCaTp³⁶ and the malignant line HaCaT40°³⁵ (Figure 1). The latter two cell lines had been converted to tumorigenicity by culture stress. The stress was exerted either by repeated single-cell cloning (HaCaTp) or by continued growth (>10 passages) at 40°C instead of 37°C (HaCaT40°). Under both stress conditions HaCaT cells were converted to tumorigenicity. The tumors formed were either noninvasive, slowly growing benign cysts HaCaTp³⁶ or locally invasive SCCs HaCaT40°.³⁵ The following cell lines were established after *in vivo* passage by recultivation of subcutaneously transplanted tumors: HaCaTpT, HaCaT40°RT, II-3RT, II-4RT, A-5RT1, and A-5RT3 (Figure 1).

Cell Isolation and Cell Culture

Cell lines were cultivated in 4× modified Eagle's medium with 5% fetal calf serum (FCS) following routine protocols.²⁵ Both benign and malignant HaCaT clones were serially passaged at a split ratio of 1:5 to 1:10 and tested for tumorigenicity at different passage levels. The cell lines A-5 and II-4 were cloned by ring isolation and clonal populations were again tested for tumorigenicity. For recultivation of tumor cells, subcutaneous tumors were surgically removed from the nude mouse, separated from stroma and necrotic areas, and proliferative tumor margins were minced into 1-mm³ pieces. These tumor fragments were transferred to 10-cm tissue culture plates containing 6×10^6 lethally irradiated (70 Gy) human fibroblast feeder cells in standard media. When islands of tumor cells had formed (after 5 to 10 days) the pieces of tumor tissue were removed by aspiration and the cells were further cultivated according to standard protocols.²⁵ Cells were routinely tested for mycoplasma contamination by standard procedures⁴² and always found to be negative. Their origin from transfected HaCaT-ras cells (containing the neo-resistance gene³⁴) as well as the absence of contaminating mouse cells were proved by the resistance of the recultivated cells to G418 (400 µg/ml) treatment. For HaCaT40° and HaCaTp cells elimination of mouse cell contamination was done as described.³⁵

Growth Curves and Colony-Forming Efficacy

Growth curves were recorded from cells seeded at a density of 4×10^3 cells/cm² in 4× modified Eagle's medium with 5% FCS by daily counting of the cells in three independent cultures with an electronic counter (Casy, Schaefer System, Reutlingen Germany). For determination of colony-forming efficacy, cells were plated at a density of 100 cells/6-cm dish in 4× modified Eagle's medium containing 1% FCS. After 24 hours, allowing for cell attachment, medium was shifted to 5% FCS or no FCS, respectively. After 10 days the medium was removed, cells were fixed in 3.7% formaldehyde, stained with Mayer's hematoxylin, and colonies were counted. Colony-forming efficacy was calculated as the ratio of colonies to the number of cells seeded. The colony-forming efficacy of A-5 cells was arbitrarily set to 100%.

Cytogenetic Analysis

Semiconfluent cultures were harvested by trypsinizing with 0.1% trypsin/ethylenediaminetetraacetic acid in phosphate-buffered saline without Ca²⁺ and Mg²⁺ containing 4 mg/ml colcemid, centrifuged, and the cell pellet resuspended in hypotonic solution of 70 mmol/L KCl. After incubation at 37°C for 15 to 25 minutes cells were fixed by three changes of methanol/acetic acid 3:1 and spread on glass slides. G-banding was performed after the slides had been aged for several days as described.⁴³ To analyze numerical chromosome alteration,

at least 50 metaphases were counted and 25 karyotypes were arranged and analyzed for structural changes.

Tumorigenicity and Metastasis Assays

Tumor formation was assayed after subcutaneous injection of 5×10^6 and 1×10^5 cells in 100 µl of culture medium into the interscapular region of 4- to 6-week-old athymic nude (Swiss nu/nu) mice and tumor size was measured in two axes throughout an observation period of 6 months. The tumor volume was calculated following published procedures.⁴⁰ The metastatic potential of tumor cells was assayed using two separate transplantation systems. Experimental metastasis was assayed by injecting 1×10^6 cells into the tail vein of athymic nude mice and lung colony formation was monitored for a period of 18 weeks.

Spontaneous metastasis from tumor cells grafted as skin-like surface transplants was determined after the transplantation of organotypic cultures as described previously.⁷ Briefly 7-day-old organotypic co-cultures of tumor cells were trimmed using a surgical punch (1.4 cm in diameter) and transplanted onto the back muscle fascia of nude mice. Animals were sacrificed at 9 weeks after transplantation, and grafts as well as regional lymph nodes were dissected and prepared for histology.

Successive *in vivo* passages of tumor cells without intermittent *in vitro* growth were performed by subcutaneous transplantation of tumor fragments from the previous *in vivo* passage. Tumors were surgically removed, separated of stroma and necrotic areas, and 0.5 mm³ pieces of the proliferative tumor margins were subcutaneously transplanted via skin incision. Tumor growth was observed for up to 6 months and monitored by weekly measurements of two tumor diameters.

Southern Blot Analysis

Genomic DNA of cultured tumor cells was isolated by the sodium dodecyl sulfate lysis procedure,⁴⁴ and 10 mg of DNA were digested overnight at 37°C with the restriction endonucleases *Bam*HI and *Eco*RI, respectively. Hybridization conditions, electrophoresis, and autoradiographic procedures were as described previously.⁴⁵

Immunoprecipitation

Cellular proteins were extracted from 10⁷ trypsinized cells and the *ras* proteins immunoprecipitated as described previously.¹⁰ Samples were electrophoresed on 7.5 to 17.5% sodium dodecyl sulfate-polyacrylamide gels and electroblotted in buffers to an Immobilon P membrane (Millipore, Bedford, MA, USA) according to established procedures.⁴⁶ Antibody reactions and visualization of the immunoreactive proteins were performed as described previously.¹⁰ The antibody used was a pan-reactive *H-ras* antibody v-*H-ras* Ab-1-A clone Y13-259 (Oncogene Science, Boston, MA, USA).

Oligonucleotide Primers and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Sense and antisense primers for granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were synthesized according to the sequences extracted from GenBank and used as described previously.⁴¹ All oligonucleotide primer pairs spanned intron-exon splice sites ensuring that PCR products generated from any DNA present in the RNA preparations could be clearly distinguished from those generated in the RT-PCR reactions. RT-PCR was performed as described⁴¹ using the Perkin-Elmer (Branchburg, NJ) Geneamp RT-PCR kit. The identity of the PCR amplification products was confirmed by size and restriction digest with two restriction enzymes.

Results

After *H-ras* oncogene transfection into the immortal but nontumorigenic keratinocyte cell line HaCaT, two different classes of tumorigenic HaCaT-*ras* clones were isolated.^{10,34} Whereas one type of cells formed progressively enlarging tumors after subcutaneous injection into nude mice, which were histologically diagnosed as well-differentiated carcinomas, the other class of clones produced slowly enlarging and eventually stationary or regressing nodules composed of noninvasive keratinized cysts, diagnosed as benign tumors.³⁴ To elucidate whether these benign clones represented a mixture of premalignant and malignant cells or included a subpopulation sensitive to tumor progression *in vitro* or *in vivo*, we investigated their behavior in more detail after *in vivo* (heterotransplant) and *in vitro* passages and by single-cell cloning.

Tumor Progression of the Benign HaCaT-*ras* Clone A-5 by *In Vivo* Passaging

Early-passage (p16) benign HaCaT-*ras* A-5 cells were injected into eight nude mice. After forming an initial nodule, four out of eight tumors regressed within the first 50 days after injection or remained the same size (Figure 2a). Two tumors persisted as small stationary nodules, consisting of a rim of squamous epithelia surrounding a keratinized central part (Figure 3a), and two tumors progressively enlarged after a latency period of 40 and 60 days, respectively (Figure 2a). Vital tumor segments of one of these tumors (Figure 2a, arrowhead) were removed and processed *in vitro* to establish the recultivated tumor cell line A-5RT1. In addition, tumor fragments were subcutaneously transplanted into nude mice, resulting again in different tumor types (Figure 2b). Although two out of eight transplants had disappeared completely after 4 weeks, four tumors showed a faster growth than the original tumor, reaching a size of 0.8 to 1.2 cm³ after 30 to 40 days. The remaining two nodules grew slowly and finally remained stationary for several months. Vital segments of one of these latter tumors (Figure 2b, arrow),

Figure 2. a: Tumor growth curve of A-5 cells after subcutaneous injection of 5×10^6 cells. The arrowhead marks the tumor that was retransplanted and from which the A-5RT1 cell line was established. b: Tumor growth curve of the retransplanted tumor segments from animal 1 in a, the arrow marks the tumor whose segments were transplanted again into the nude mouse. c: Tumor growth curve of the retransplanted tumor segments from animal 2 in b, the arrowhead marks the tumor from which the cell line A-5RT3 was established. d: Tumor growth curve after subcutaneous injection of 5×10^6 cells of the established cell line A-5RT3 (passage 8).

which exhibited a late growth enhancement, were again transplanted subcutaneously. Despite its originally slow growth, retransplantation of this tumor resulted in a homogeneous series of very fast growing tumors reaching a tumor volume of 0.6 to 1.5 cm³ after 20 to 30 days (Figure 2c). One tumor of this third transplantation was again dissected and recultured to establish the recultivated tumor cell line A-5RT3 (Figure 2c, arrowhead).

Concomitantly with the enhanced growth behavior of the heterotransplants at each *in vivo* passage a step-wise shift in tumor histology was manifest indicating tumor progression from a benign to a highly malignant and aggressive phenotype. Whereas the original stationary HaCaT A-5 nodules consisted of a large cyst with a vital rim of stratified epithelia surrounding keratinized material (Figure 3a), the faster growing variants were still well-differentiated, although invasive, tumors (Figure 3b). After a further *in vivo* passage the resulting SCCs exhibited reduced differentiation accompanied by increased cell and nuclear atypia (Figure 3c). The rapidly growing tumors resulting from the third *in vivo* passage (see Figure 2c) were highly disorganized, infiltrating, and poorly differentiated SCCs with cellular atypia and large central necroses (Figure 3d).

Figure 3. **a:** Histology of a benign A5 tumor after subcutaneous injection of 5×10^6 cells into the nude mouse. The tumor consists of a benign cyst with a rim of squamous epithelia surrounding a keratinized central part. **b:** Histology of one of the progressively enlarging A5 tumors exhibiting invasion of underlying connective tissue. **c:** Histology of a tumor originating after retransplantation of tumor segments. **Arrowheads** indicate the differentiated areas. **d:** Histology of a tumor from the third transplantation giving rise to the A-5RT3 cell line showing a highly invasive, poorly differentiated SCC. **e:** Histology of an A-5RT3 tumor after subcutaneous injection of 5×10^6 cells. The tumor has the same highly invasive and poor differentiation phenotype as the original tumor from which the cell line was established (Figure 2d). **Arrowheads** indicate invasion into muscle. **f:** Histology of a metastasis to an axillary lymph node after transplantation of A-5RT3 cells to the back muscle fascia. The **arrowhead** marks foci of tumor cells invading lymphatic tissue adjacent to the capsule of the lymph node while sheets of tumor cells are destroying node architecture (**arrow**). An enlarged axillary lymph node is seen in the insert next to a millimeter ruler. Scale bars, 50 μ m.

In a second set of experiments with a comparable passage of A-5 cells (p17) the tumorigenic progression by *in vivo* passage was shown to be highly reproducible resulting in a comparable yield of benign and malignant heterotransplants (data not shown).

The Recultivated Tumor Cells Maintain the Enhanced Malignant Phenotype

On subcutaneous reinjection of the newly established, recultivated tumor cell lines A-5RT1 and A-5RT3 we demonstrated that the *in vivo* growth potential and phenotype of the different steps of tumor progression were main-

tained in the tumor-derived cell lines *in vitro*. Thus, comparable to the results of the first heterotransplants, the recultured A-5RT1 cell line (p9) formed progressively growing, invasive SCCs at three out of eight injection sites, whereas the remaining five regressed after 2 to 8 weeks (data not shown). Subcutaneous injection of the cell line obtained after the third *in vivo* passage as tumor fragments (A-5RT3 passage 8) led to extremely rapidly growing tumors in all animals (Figure 2d). Thus, A-5RT1 and A-5RT3 cells show an *in vivo* growth behavior comparable to the original tumor cell populations from which the cell lines were established (compare Figure 2, c and d). Similarly, the histology of the resulting heterotrans-

plants exhibited the phenotype of moderately (A-5RT1) and poorly (A-5RT3) differentiating SCCs (data not shown for A-5RT1 but compare Figure 3c), the latter exhibiting rapid infiltration into the back muscle (Figure 3e).

Further confirmation of the enhanced malignancy of the A-5RT1 and A-5RT3 cells was obtained by the metastatic potential of these cells. Injection of A-5RT1 and A-5RT3 cells into the tail vein of athymic nude mice resulted in the establishment of lung colonies for A-5RT1 cells in two of four animals and for A-5RT3 cells in four of four animals after 10 weeks, respectively. In addition, A-5RT3 transplants of organotypic cultures grafted onto the dorsal fascia of nude mice⁷ resulted in spontaneous metastasis of these highly malignant tumor cells into axillary lymph nodes. Nine weeks after transplantation, all animals ($n = 4$) had large tumors at the site of transplantation, two animals showed two positive lymph nodes, and two animals had three positive nodes (Figure 3f, insert). Histological investigation of the enlarged lymph nodes showed the destruction of nodal architecture by infiltrating streaks of tumor cells that extended to the node capsule (Figure 3f, arrow). Infiltrating tumor cells were poorly differentiated with high nuclear atypia similar to A5-RT3 cells (Figure 3e).

Tumor Progression Is Associated with Specific Cytogenetic Changes

The reproducibility of this tumor progression under *in vivo* conditions and the stability of the malignant phenotype of the A-5RT1 and A-5RT3 cells after *in vitro* passage, raises the question whether the progressed tumor phenotypes were the result of selection of pre-existing tumor cell populations from the parental benign A-5 cell line and/or were the consequence of further genetic alterations induced by the *in vivo* microenvironment.

To address this question, we examined the chromosomal alterations that were associated with *in vivo* tumor progression of the A-5 cell population. To study the population dynamics, we first analyzed the median chromosome content of the three cell lines. In the benign A-5 cells, 70% of the metaphases contained 75 to 80 chromosomes with a mean of 76 chromosomes (Figure 4a). Both the mean and the variation in chromosome content were clearly reduced in A-5RT1 cells, ranging from 72 to 76 chromosomes in 73% of cells with a mean of 74 chromosomes. The reduction in chromosome number indicates a selection of a population with fewer chromosomes. This tendency was most pronounced in the A-5RT3 cells, where 72% of metaphases only exhibited 69 to 72 chromosomes with a mean of 70 (Figure 4b). Thus, with each *in vivo* passage and resulting tumor progression, the mean chromosome number was decreased, indicating a continuous reduction in heterogeneity of the parental A-5 cell population by selection of subpopulations.

To perform a more detailed analysis of the genetic characteristics of populations of A-5RT1 and A-5RT3 tumor cells, G-banded karyograms of these cells were

Figure 4. Chromosome numbers per metaphase in benign A-5 cells (a) and derived malignant A-5RT1 and A-5RT3 cells (b).

examined and compared with those of A-5 cells (Table 1). These data unequivocally demonstrated: 1) the descent of the advanced tumor lines A-5RT1 and A-5RT3 from the A-5 cell line as proved by the unaltered maintenance of the HaCaT and A-5 marker chromosomes M1, M4, M5, M7, M9, and M10; 2) that the two recultivated tumor cell lines represent subpopulations of the A-5 cell line because the modified marker chromosome M1*, present in only 16% of A-5 metaphases, was found in all cells of both recultivated tumor lines and, because M2, present in two copies in 93% of A-5 cells, was only detected as a single copy in the recultivated tumor cell lines; 3) that the recultivated tumor lines had unique markers (M1RT and M2RT) not discovered in A-5 cells; and finally 4) that the second recultivated tumor marker M2RT had duplicated during the progression from A-5RT1 to A-5RT3 (Table 1).

Thus, our data clearly demonstrate that the *in vivo* tumor progression from the benign A-5 to the malignant A-5RT1 and more aggressive A-5RT3 is driven by a selection of genetic subpopulations and is associated with the development of new genetic aberrations.

Table 1. Comparison of Marker Chromosomes Present in A5, A-5RT1, and A-5RT3 Cells

	Marker chromosome	A5 benign	A-5RT1 malignant	A-5RT3 malignant/ metastasizing
M1	der(3;4)t(3;4)(q10;q10)	1* (100%) [†]	1 (100%)	1 (95%)
M1*	der(3;4)t(3;4)(q10;q10)add(4)(q35)	1 (16%)	1 (100%)	1 (95%)
M2	i(9)(q10)	2 (93%)	1 (98%)	1 (95%)
M4	t(4;18)(p10;q10)	2 (97%)	2 (95%)	2 (95%)
M5	t(15;22)(q10;q10)	1 (93%)	1 (100%)	1 (90%)
M7	add(6)(p25)	1 (93%)	1 (95%)	1 (98%)
M9	der(17)dup(17)(q21q25)del(17)(q25)	1 (97%)	1 (100%)	1 (90%)
M10	del(1)(p22)	1 (90%)	1 (100%)	1 (98%)
M1RT	add(11)(q23)		1 (96%)	1 (100%)
M2RT	del2(p23)		1 (100%)	2 (95%)

*Mean N° per metaphase.

[†]In percent of metaphases.

H-ras Oncogene Amplification and Overexpression during Tumor Progression

Transfection of the activated *H-ras* gene into immortal HaCaT cells originally resulted in their tumorigenic conversion, demonstrating a functional role of the *H-ras* oncogene in the transformation process. We examined the *H-ras* status in the benign A-5 and malignant A-5RT1 and A-5RT3 cells to determine the potential role of this oncogene during tumor progression as well.

In Southern blot hybridization of *Bam*HI- (not shown) and *Eco*RI-digested genomic DNA with a *H-ras*-specific probe, the endogenous *H-ras* proto-oncogene is visible as a 23-kb band (Figure 5a). The exogenous *H-ras* oncogene gave rise to an additional 13-kb band for A-5 cells and showed a significant (eightfold) increase in intensity for both A-5RT1 and A-5RT3 cells (Figure 5a). The alterations in band intensity at 13 kb and the additional 6.6-kb band might be the result of an amplification and novel integration of the oncogene during tumor progression. Alternatively, this pattern in the recultivated tumor cell lines may have been caused by selection of a pre-existing but minor population of A-5 cells during the *in vivo* progression.

The amplification of the *H-ras* oncogene in A-5RT1 and A-5RT3 cells is clearly reflected at the expression level as shown by protein analysis of the allele-specific expression of mutant and wild-type *H-ras* in the benign A-5 cells and the derived A-5RT1 and A-5RT3. All *ras*-type-specific antibodies tested showed a strong cross-reactivity with more than one of the *ras*-oncogenes (data not shown). Thus, discrimination of wild-type (*Gly*-12) and mutant (*Val*-12) *H-ras* was only possible through the higher mobility of the wild-type protein, as seen in HaCaT cells *versus* the mutant protein expressed by EJ-bladder carcinoma cells.²⁵ Nevertheless, immunoprecipitation of the *H-ras* proteins clearly showed an increase in the expression of the mutant *H-ras* protein during tumor progression in both A-5-derived tumor cell lines A-5RT1 and A-5RT3 (Figure 5b). This increase in *H-ras* oncogene expression was similarly demonstrated at the RNA level using RT-PCR with specific primers discriminating wild-type and Val-12-mutated *H-ras* (data not shown). Thus, tumorigenic progression of benign A-5 cells to the high-grade malignant phenotype seems associated with an amplifi-

Figure 5. a: Southern blot of *Eco*RI-digested genomic DNA from HaCaT cells; EJ bladder carcinoma cells; and the HaCaT-*ras* cell lines A-5, A-5RT1, and A-5RT3 with a *H-ras*-specific probe. The 23-kb band represents the endogenous *H-ras* gene, whereas integration of the transfected *H-ras* gene results in a band of 13 kb in size. In the malignant A-5RT1 and A-5RT3 an additional band of 6.6 kb is visible. **b:** Immunoprecipitation of protein extracts from HaCaT, EJ bladder carcinoma cells, and the benign HaCaT-*ras* A-5 cells, as well as the malignant A-5RT1 and A-5RT3 cells with a pan-reactive anti-*H-ras* antibody. HaCaT cells show the signal for the internal wild-type *H-ras* gene, EJ bladder carcinoma cells serve as control for the mutated *H-ras*. Both mutated and wild-type *H-ras* are visible in A-5 and A-5RT cells. The expression of the mutated gene is considerably increased in the highly malignant A-5RT cells.

Figure 6. Tumor growth after subcutaneous injection of 5×10^6 cells of II-3, II-3RT, II-4, II-4RT, and HaCaTp, HaCaTpT, as well as HaCaT40° and HaCaT40°RT, determined after 4 weeks of observation time.

cation and a relatively strong increase in the mRNA and protein expression of the integrated (Val-12) *H-ras* oncogene, suggesting a role of the oncogene during tumor progression.

Tumor Progression through *in Vivo* Microenvironment Is Not Restricted to *ras* Transfectants

Acquisition of a more aggressive growth behavior after *in vivo* passage of premalignant tumor cells was not restricted to the *ras*-transfected HaCaT A-5 cell line. Similarly, two other *ras*-transfected HaCaT clones, manifesting a malignant behavior, exhibited enhanced malignant tumor growth as subcutaneous heterotransplants after recultivation from excised tumors. Thus, the HaCaT-*ras* lines II-3 and II-4, which generate well-differentiated SSCs after subcutaneous injection in nude mice,³⁴ displayed significantly faster tumor growth rates than their parental cell lines when recultivated from subcutaneous tumors (cell lines II-3RT and II-4RT) and reinjected into nude mice (Figure 6).

This progression to enhanced malignant tumor cells through *in vivo* passaging, however, was not restricted to the *ras*-transfected clones, but was reproducibly obtained with benign and malignant HaCaT cells converted into tumorigenicity by culture stress. The stress was exerted either by repeated single-cell cloning (HaCaTp)³⁶ or by growth at 40°C instead of 37°C (HaCaT40°).³⁵ Under both stress conditions HaCaT cells were con-

Figure 7. Expression of G-CSF and GM-CSF mRNA in fibroblasts, HaCaT, and HaCaT-*ras* cells determined by RT-PCR (DNA size standard, ϕ X174/*Hae*III digest). Fibroblasts and enhanced malignant HaCaT cells, HaCaTpT and HaCaT40°RT, as well as HaCaT-*ras* cells A-5RT1, A-5RT3, and II-4RT expressed G-CSF and GM-CSF mRNA, whereas benign A-5, HaCaTp, and HaCaT40°, and malignant II-4 cells did not.

verted to tumorigenicity. HaCaTp cells formed noninvasive, slowly growing benign cysts,³⁶ whereas HaCaT40° cells gave rise to well-differentiated SCCs. Cell lines established from these heterotransplants (HaCaTpT and HaCaT40°RT, respectively) gave rise to faster growing tumors, which show an earlier onset of tumor growth and enhanced malignant growth behavior (Figure 6). This *in vivo* progression of HaCaT clones, which had been converted into tumorigenicity without the influence of the *H-ras* oncogene to an enhanced malignant tumor phenotype, has been reproducibly observed.^{35,36}

Altered Growth Regulation through Microenvironment-Induced Tumor Progression

The most prominent feature of the heterotransplant-derived cell lines was their enhanced growth potential *in vivo* as manifested by the accelerated enlargement of tumors. This was indicative of an improved adaptation of cells to growth in the subcutaneous environment and was particularly obvious with the metastatic A-5RT3 cells, which exhibited a comparable graft-take and tumor growth rate when the number of injected cells varied from 5×10^6 to 1×10^5 cells. Tumor growth in 100% of animals was indeed still observed after subcutaneous injection of as little as 1×10^5 cells (data not shown).

This growth potential indicated an improved autocrine growth regulation and independence of environmental growth control mechanisms. Investigation of the growth-factor expression profile of the highly malignant tumor cell lines established after *in vivo* passage revealed a common characteristic. Both HaCaT tumor cell lines HaCaTpT and HaCaT40°RT, as well as the HaCaT-*ras* tumor cell lines II-4RT, A-5RT1, and A-5RT3, exhibited a *de novo* expression of the hematopoietic growth factors G-CSF and GM-CSF (Figure 7) (protein expression data as determined by enzyme-linked immunosorbent assay not shown).⁴¹ Because all HaCaT cell variants expressed the cognate receptors for G-CSF and GM-CSF,⁴¹ the novel expression of both factors in these highly malignant tumor cells represents a shift from microenvironmentally determined paracrine to an autocrine growth regulation. Indeed, we have quite recently demonstrated that GM-CSF is a major paracrine growth factor, expressed in fibroblasts and regulating keratinocyte growth and differ-

entiation.⁴⁷ An autocrine mechanism of tumor cell growth stimulation and enhanced migration by G-CSF and GM-CSF has been demonstrated *in vitro*.⁴¹ Furthermore, there is evidence that both factors modulate the tumor micro-environment by enhancing stromal activation and inducing angiogenesis in heterotransplants (MM Mueller and NE Fusenig, manuscript in preparation). Thus, expression of G-CSF and GM-CSF is a common characteristic associated with the *in vivo* progression of HaCaT cells to a highly malignant phenotype, independently of the *H-ras* status. By their autocrine and paracrine functions both newly expressed growth factors may well contribute to tumor progression.

A-5RT1 and A-5RT3 Show a Reduced Growth Potential *in Vitro*

In marked contrast to their drastically increased growth potential in heterotransplants, the tumor-recultivated cell lines did not exhibit improved growth rates in culture. This was evident on the initial adaptation of the outgrowing tumor cells from dissected tumor specimen, where growth initiation in culture required the use of fibroblast feeder cells. Whereas the benign parental A-5 cells showed clonal growth potential in media with low or even no serum content (data not shown), the more malignant tumor cells did not possess a growth advantage under *in vitro* conditions, and were even less adapted to growing in culture than their parental cells. This was still evident after several passages of culture adaptation, when the metastatic A-5RT3 cells maintained a clearly reduced growth rate (ie, increased population doubling time) *in vitro* (31 hours) compared to the malignant A-5RT1 (23.6 hours) and benign A-5 cells (20 hours).

Furthermore, when plated at clonal cell densities in media containing 5% FCS, A-5RT1 and A-5RT3 exhibited a reduced colony-forming efficacy compared to A-5 cells, whereas the size of the colonies formed was similar (data not shown). At a density of 100 cells per 6-cm dish colony-forming efficacy of A5 cells was set to 100%, whereas A-5RT1 and A-5RT3 cells only showed a colony-forming efficacy of 57% and 63%, respectively. Under serum-free culture conditions A-5 cells were still able to form 5% colonies, whereas A-RT1 and A-5RT3 cells did not grow at all (data not shown). This reduced growth potential *in vitro* was maintained throughout many (up to 25) passages in culture.

In Vitro Cloning Does Not Select for a Malignant Phenotype

The chromosomal data indicated that selection of an A-5 subpopulation may be involved in tumor progression to the recultivated tumor lines. Thus, it might be possible to select for such a subpopulation by single-cell cloning *in vitro*. Although continued passaging of the A-5 cell population did not result in a gain of the malignant phenotype and the *in vitro* propagation of the malignant HaCaT-ras clone II-4 had not increased its malignant potential (data

Figure 8. A: Tumor growth of A-5 subclones after subcutaneous injection into the nude mouse. Median tumor growth values of eight animals for each A-5 subclone are shown. The late onset of enlarged tumor growth for A-5/a is seen in three of eight animals; A-5/c cells caused tumor growth in one of eight animals, and A-5/g cells formed tumors in one of eight animals. **B:** Tumor formation of cells of different passages of A-5 and A-5RT3 cells after subcutaneous injection of 5×10^6 cells into the nude mouse.

not shown), we tried to select and isolate a malignant subpopulation of the A-5 cells. A-5 cells were cloned with ring isolation and 15 clones were obtained and further propagated. However, none of these clones formed malignant tumors after subcutaneous injection (Figure 8A). On the contrary, most of the resulting clones were non-tumorigenic and only three gave rise to persistent benign tumors. Interestingly, two of the subclones A-5/c and A-5/a formed progressively enlarging tumors in one of eight and two of eight injection sites, respectively. However, this occurred only after a long lag period of more than 3 months. Because these A-5 subclones are already the result of single-cell isolations (ie, of clonal origin), this late tumor progression *in vivo* suggests a development of new mutations under the influence of the *in vivo* micro-environment. The failure to clone a progressively malignant subpopulation *in vitro* was also confirmed by two sets of experiments attempting to isolate malignant subclones from the benign HaCaT-ras cell line I-7 and the low-grade malignant line II-4, respectively (P Tomakidi and NE Fusenig, unpublished observations). Single cell clones of both cell lines showed no malignant invasive growth in

the short-term tumorigenicity assay of surface transplants during an observation period of 4 weeks.

On the contrary, we found that prolonged passage *in vitro* reduced the tumorigenic potential not only of the A-5 but also of the A-5RT1 and A-5RT3 cells. A-5 cells showed a reduction in tumorigenic potential with increasing passage number resulting in smaller tumors that formed during the observation period (Figure 8b). Although the enhanced malignancy of the A-5RT cell lines was in principle maintained throughout 45 *in vitro* passages, including their altered cytology, the *in vivo* growth rate of malignant tumors was delayed with increasing number of *in vitro* passages (Figure 8B, inset).

Discussion

Transformation of human cells is a multistep process resulting from the accumulation of genetic alterations disturbing the stable balance between proliferation and differentiation required for normal tissue homeostasis. These alterations are accompanied by an increasing independence from the complex regulatory network that cells are subjected to in their tissue environment. For SCC of the skin, the progression from premalignant lesions to malignant tumors is histopathologically well documented.⁴⁸ However, little is known about the molecular mechanisms underlying this progression. To elucidate these mechanisms we established a progression model from benign to high-grade malignant SCCs based on the HaCaT/HaCaT-ras-keratinocyte tumor model.^{4,25,41} *In vivo* passage of tumorigenic cells resulted in tumor progression to more aggressively growing SCCs with reduced differentiation potential. Moreover, after two additional retransplantations, very rapidly growing, poorly differentiating, highly invasive, and metastatic SCC tumors had developed. The cell lines A-5RT1 and A-5RT3 established from the first and third retransplantation, respectively, exhibited the same *in vivo* growth and differentiation potential as the tumor that was dissected for the recultivation of these tumor cells and maintained this phenotype stably *in vitro*. Thus, these cell lines confirmed a stable tumor progression from benign to highly malignant and metastatic cells in the *in vivo* environment. The selection of a more malignant phenotype *in vivo* has also been reported for other tumor systems such as prostate carcinomas,⁴⁹ colon carcinomas,⁵⁰ transitional cell carcinomas of the bladder,⁵¹ and pancreatic carcinomas.^{52,53} In these studies, *in vivo* passage of tumor cells resulted in increasingly malignant tumor cell populations with a higher metastatic potential than exhibited by the parental cell lines. Thus, the *in vivo* environment generally seems to exert a characteristic selection pressure in favor of increasingly malignant tumor phenotypes.

In contrast, the *in vitro* environment did not support tumor progression to a more malignant phenotype. *In vitro* passage of benign HaCaT-ras clone A-5 and malignant HaCaT-ras clone II-4, did not lead to progressive tumor cell populations (P Tomakidi and NE Fusenig, unpublished observations). Additionally, the benign tumorigenic phenotype of A-5 cells was stably maintained after

prolonged passage *in vitro* up to 72 passages, however, despite its phenotypic stability, we observed a reduction in tumor growth rate after prolonged passage. A similar decrease in tumorigenicity after prolonged *in vitro* passage was observed with all tumorigenic cell lines tested as well as in a cell line derived from a human skin SCC.⁵⁴ This difference in selective pressure for tumor progression between the *in vitro* and the *in vivo* environment is also reflected in the phenotype of *in vivo* progressed populations, which exhibit a decreased *in vitro* growth capacity, as was seen when A-5RT cells were compared to the parental A-5 cells. Although A-5RT cells, to survive *in vitro*, originally required growth support by feeder fibroblasts that mimic a mesenchymal compartment⁵⁵ they became independent of these feeder cells during prolonged passages in tissue culture. However, they retained a reduced *in vitro* growth potential compared to A-5 cells, as is evident from population doubling times and cloning experiments. This discrepancy between *in vitro* and *in vivo* growth capacity in our system is not surprising, because even tumor-derived cells of fast growing tumors such as basal cell carcinomas were difficult to propagate in culture.^{56,57} There are a few examples in the literature describing an *in vitro* progression to a more malignant phenotype.^{19,24} However, this *in vitro* progression either occurred in simian virus-40 immortalized cell lines,¹⁹ that are characterized by an increased genetic instability, or required the action of an additional mutagen such as radiation.²⁴ We have found a decrease in tumor progression *in vitro* for the HaCaT-ras model system and these findings are in agreement with reports demonstrating an *in vitro* selection against tumorigenic subpopulations in breast adenocarcinomas,⁵⁸ SCCs arising in Bowen's disease,⁵⁹ and prostate carcinomas.⁶⁰

Tumor progression *in vivo* is thought to be associated with specific genetic alterations and/or the selection of genetic subpopulations supposedly induced by adverse environmental conditions, eg, hypoxia.^{61,62} Genetic analyses of our A-5/A-5RT cell progression model supports both mechanisms. The selection of A-5RT cells form a pre-existing subpopulation of the benign A-5 cells was clearly confirmed by the conservation of all A-5 marker chromosomes as well as by the presence of one copy of M1* and M2 in the malignant tumor clones. On the other hand, the detection of novel aberrations, with progression to the malignant phenotype in A-5RT cells, such as the M1RT (add(11)(q23)) and M2RT (del2(p23)) marker chromosomes, points to the acquisition of new genetic rearrangements as a second mechanism active during *in vivo* tumor progression. The role of chromosomal alterations, and, more specifically, the loss of chromosomal material in tumor progression, is well established.⁶³ Interestingly, the involvement of chromosomes 11 and 2p affects two locations known to harbor a number of genes involved in tumor development, such as the Wilms' tumor suppressor genes⁶⁴ and the cyclin D1 gene⁶⁵ on chromosome 11, or the MSH2 gene⁶⁶ and the fra-2 gene on 2p,⁶⁷ respectively. However, further studies are needed to identify the function of these chromosomal aberrations and the role such respective candidate genes may play during tumor progression in our skin carcinoma model system.

In addition to these chromosomal alterations that may result from mutational events exerted by the *in vivo* environment, progression of the A-5 cells was associated with an apparent gene amplification and with an increased expression of the *H-ras* oncogene. However, it remains unclear whether this is the result of the selection of a pre-existing subpopulation already carrying this amplification or the consequence of genetic rearrangements induced by the *in vivo* environment. The oncogenic activation of *ras* has been observed in ~15% of SCCs of the skin^{38,39} and shown to contribute to the tumorigenic conversion of HaCaT skin keratinocytes.^{10,34} Additionally, there are other reports associating the dose of *ras* protein with malignant conversion or tumor progression.^{68,69} On the other hand, several authors have questioned the dominant role of *ras* in the process of malignant transformation and tumor progression.⁷⁰⁻⁷² In the original benign and malignant HaCaT-*ras* clones the degree of malignancy did not correlate with the level of expression of the mutated *H-ras* oncogene.³⁴ Additionally, *in vivo* tumor progression in the HaCaT system can also occur independently of an *H-ras* oncogene activation or overexpression, as demonstrated for the progression of the HaCaT40° and HaCaTp cells to the more aggressive HaCaT40°RT and HaCaTpT cells. We therefore consider the amplification of the *H-ras* oncogene in the A-5RT cells, to be a marker for the mutagenic influence of the tumor microenvironment that might result in a gene amplification of the *H-ras* oncogene similar to those seen under stressful growth conditions for the *c-myc* oncogene.⁷³

A clear indication for additional genetic alterations causing the phenotypic changes occurring during malignant progression is the novel and constitutive expression of the hematopoietic growth factors G-CSF and GM-CSF that is shared by all highly malignant HaCaT tumor cells arising as a consequence of tumor progression *in vivo*. Interestingly, despite the possible role that *H-ras* oncogene activation may play in developing a tumorigenic phenotype in HaCaT cells, this expression of G-CSF and GM-CSF in all highly malignant tumor cells is independent of a *H-ras* activation. Thus, expression of G-CSF and GM-CSF is a common characteristic associated with the *in vivo* progression of HaCaT cells to a highly malignant phenotype, independently of the initial *H-ras* status. In normal skin, G-CSF and GM-CSF are expressed by stromal fibroblasts.⁷⁴ They can be induced by interleukin-1⁴⁷ and stimulate keratinocyte growth and differentiation. Their novel constitutive expression in the highly malignant HaCaT cells leads to a shift from a paracrine to an autocrine growth regulatory loop without the need for interleukin-1 mediated G-CSF and GM-CSF induction, resulting in the stimulation of tumor cell proliferation and migration, and facilitating tumor invasion and metastasis.⁴¹ Comparably, *de novo* expression of both growth factors has been observed in high-grade malignant gliomas⁷⁵ and head and neck tumors (MM Mueller, C Herold-Mende, S Ninde, C Reisses, NE Fusenig, manuscript in preparation). Additionally, tumor cell-expressed G-CSF and GM-CSF might act in a paracrine manner on endo-

thelial cells contributing to tumor-induced angiogenesis.⁴¹ Therefore, both factors may contribute to the increasingly malignant growth of these cells *in vivo*.

Thus, the increase in malignancy of the *in vivo* passaged cells results from their better adaptation to the *in vivo* growth environment as indicated by their increased capacity to induce stromal activation and tumor angiogenesis. A-5RT cells induce an earlier and stronger activation of the tumor stroma and an enhanced angiogenic response, partly resulting from strong vascular endothelial growth factor expression (S Vosseler, M Skobe, and NE Fusenig, unpublished observations). *In vivo* selection of more malignant tumor cell populations with a higher metastatic potential has also been reported for prostate carcinomas,⁴⁹ colon carcinomas,⁵⁰ transitional cell carcinomas of the bladder,⁵¹ and pancreatic carcinomas.^{52,53} Tumor progression in these systems is also associated with the increased expression of angiogenic growth factors such as vascular endothelial growth factor and basic fibroblast growth factor and other cytokines such as interleukin-8.^{51,52}

Thus, the *in vivo* growth environment generally seems to exert a characteristic selective pressure resulting in the development of an ever more malignant tumor phenotype, which is frequently associated with alterations in the expression profile of growth factors and cytokines. Recent evidence shows that this tumor progression is not only the result of a malignant conversion of the tumor cells themselves but rather is dependent on a critical contribution of the tumor stroma.^{76,77} Activation of the stroma by constitutive expression of PDGF in nontumorigenic HaCaT cells promotes their growth as benign tumors.⁷⁸ Olumi and colleagues⁷⁹ documented the crucial role of fibroblast from the tumor stroma in determining the malignant phenotype of premalignant prostate cells, however the mechanisms by which the tumor stroma contributes to malignant progression remain primarily unclear. Besides a selective pressure of the stroma that might further the survival of more malignant tumor cells, Yuan and colleagues^{61,62} discuss fluctuation in hypoxia, low pH, and nutrient deprivation as potential mutational influences of the microenvironment, resulting in secondary modifications of critical proteins involved in replication, repair or cell cycle regulation. In this context the hypoxia-induced expression of interleukin-1⁸⁰ may initially contribute to an activation of G-CSF and GM-CSF expression in the tumor cells that eventually becomes constitutive through the mutational influences that the hypoxic, low pH tumor microenvironment would exert on the tumor cells. However, much work is needed to better understand the mechanisms of tumor progression by selection and mutation via the microenvironment. We conclude that the *in vivo* progression model of SCCs established in our group, representing distinct and consecutive steps in tumor development and progression, provides an excellent basis to investigate and better understand the complex mechanisms controlling the interaction of tumor cells with their microenvironment and contributing to tumor progression *in vivo*.

Acknowledgments

We thank Dr. Christopher Bauser and Mrs. Susanne Wieber for expert technical assistance, and Brigitte Nagel-Plagens for carefully reading this manuscript.

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