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Chapter 4

The Rac Activator Tiam1 and Ras-induced oncogenesis

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Abstract

The *Tiam1* gene encodes a guanine nucleotide exchange factor (GEF) that specifically activates the Rho-like GTPase Rac. *In vitro* studies indicate that Tiam1 localizes to adherens junctions and plays a role in the formation and maintenance of cadherin-based cell adhesions thereby regulating migration of epithelial cells. *In vivo* studies implicate Tiam1 in various aspects of tumorigenesis. In this chapter we discuss the use of the DMBA/TPA chemical carcinogenesis protocol in Tiam1-deficient mice to study the role of Tiam1 in Ras-induced skin tumors. This two-stage carcinogenesis protocol allows to study initiation, promotion and progression of tumors in a Tiam1-positive and -negative background. Moreover, we describe methods to study the role of Tiam1 in: susceptibility to apoptosis, cell growth, and Ras transformation by *in vivo* and *in vitro* experiments. The latter make use of tumor cells and primary embryonic fibroblasts and keratinocytes isolated from mice.

Introduction

Mouse genetic engineering technologies provide powerful means of elucidating the multistage process of tumorigenesis *in vivo*. Switch on/off systems for regulatable reversible gene expression include interferon, tetracycline and tamoxifen, possibly in combination with Cre-Lox or Flp-FRT recombinase conditional technologies (Jonkers and Berns, 2002;Hirst and Balmain, 2004). Mice with somatic expression of an oncogene or somatic inactivation of a tumor-suppressor gene mimic human sporadic tumor formation and are very useful to study the importance of particular genes in specific tumor types. Moreover, bioluminescence imaging allows quantification of tumor growth and metastasis in time. This method has been used initially in luciferase-expressing transplanted tumors (Edinger et al., 1999) but is now applicable for somatic tumors in Cre/loxP mouse models when crossed with a conditional reporter line for Cre-dependent luciferase expression (Lyons et al., 2003).

The Ras proto-oncogene is mutationally activated in many human cancers. Activated Ras induces multiple signaling pathways that are mediated by different effector proteins that bind to activated Ras. These effectors include Raf protein kinases, phosphoinositide-3 kinases, and guanine nucleotide exchange factors (GEFs) for the small GTPases Ral and Rac (Bar-Sagi and Hall, 2000;Repasky et al., 2004). The effector molecules act either in parallel or synergistically in oncogenic signaling downstream of active Ras. The Rac-specific GEF Tiam1 provides a direct link between Ras and Rac by activating Rac upon binding to activated Ras (Lambert et al., 2002). The *Tiam1* gene was initially identified in our laboratory by retroviral insertional mutagenesis in combination with *in vitro* selection for invasive T-lymphoma cells (Habets et al., 1994). Tiam1 binds to Ras via a conserved Ras-binding domain (RBD) (Fig. 1). The 1591 aa Tiam1 protein is further characterized by a C-terminal

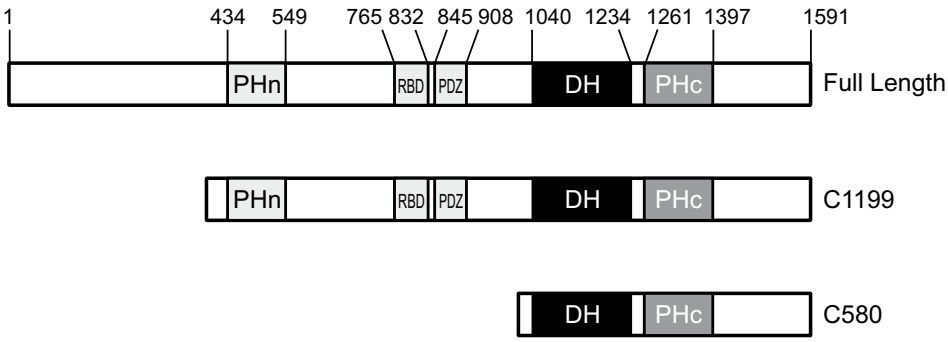

Figure 1. Schematic representation of the protein domains in Tiam1. Numbers indicate the amino acid positions. Catalytic Guanine nucleotide exchange (GEF)-activity resides in the Dbl-homology (DH) domain that is always flanked by a Pleckstrin homology (PH) domain. C1199 and C580 are amino-terminal truncated mutant versions of Tiam1. PHn and PHc: amino- and carboxy-terminal Pleckstrin Homology domain respectively; RBD: Ras-binding domain; PDZ: PSD-95 Discs large/ZO-1 homology domain.

catalytic Dbl homology (DH) domain flanked by a Pleckstrin homology (PH) domain. This DH-PH unit is characteristic for Dbl-like guanine nucleotide exchange factors. Unique for Tiam1 is an additional NH_2-terminal PH domain, through which Tiam1 is localized to the membrane (Michiels et al., 1997). Further functional analysis of Tiam1 has shown that Tiam1-mediated activation of Rac promotes invasiveness of T-lymphoma cells *in vitro* and metastasis *in vivo* (Michiels et al., 1995). However, in epithelial cells Tiam1/Rac signaling prevents invasiveness by increasing the strength of E-cadherin-based cell-cell adhesions *in vitro* (Hordijk et al., 1997;Zondag et al., 2000). To elucidate Tiam1 functions *in vivo* we generated Tiam1 knockout mice (Malliri et al., 2002). In this chapter we describe the use of the Tiam1-deficient mice and cells derived from these mice to study the role of Tiam1 in Ras-mediated tumorigenesis in particular.

Tiam1-deficiency and mouse tumor models

Tiam1 knockout mice

To investigate the role of Tiam1 in Ras-mediated oncogenicity, mice deficient in Tiam1 ($Tiam1^{-/-}$) were used. A targeting vector was generated in which a promoterless GEO cassette (LacZ-Neo-polyA) was fused in frame with the translation initiation codon in the second exon of Tiam1. Insertion of a reporter gene such as β-galactosidase or green fluorescent protein (GFP) allows a rapid assessment of which cell types normally support the expression of that gene. $Tiam1^{-/-}$ mice are fertile and no major defects are observed in any of the organs analyzed. Based on LacZ activity found in the engineered mice, Tiam1 is widely expressed in most tissues but expression is most prominent in brain and testis. In the skin, Tiam1 is present in basal and suprabasal keratinocytes of the interfollicular epidermis and in hair follicles, where it is predominantly expressed in the infundibular portion. As Tiam1 is dispensable for development, tumors can be induced in various tissues in the Tiam1-deficient mice. In addition, Tiam1-deficient mice can be crossed with tumor-prone transgenic mice to study the involvement of Tiam1 in different oncogenic signaling pathways in specific cells and tissues.

Ras-induced skin carcinogenesis

Mutational activation of Ras is frequently found in skin cancers. Skin squamous tumors can be induced in mice by a well-established quantitative two-stage DMBA/TPA chemical carcinogenesis protocol (Quintanilla et al., 1986;Yuspa, 1994). This method is ideal to study the timing of qualitative and quantitative alterations which take place during the mechanistically distinct stages of chemical carcinogenesis, allowing analysis of the events that lead to the transitions from initiation to promotion and finally to malignant conversion and progression to carcinomas.

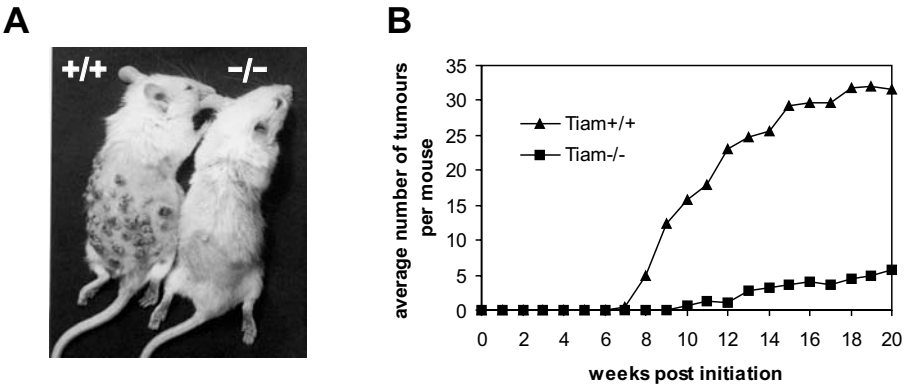

Figure 2. Analysis of Tiam1 in DMBA/TPA-mediated skin carcinogenesis. (A) Eighteen weeks after treatment, papillomas developed on the back of wild type mice (+/+) but not of Tiam1 knockout mice (-/-). (B) Tiam1$^{-/-}$ mice developed much less tumours than wild type mice.

Treatment of the skin with DMBA (7,12-dimethylbenz(a)anthracene) almost invariably introduces oncogenic mutations of the Ha-Ras gene in epidermal keratinocytes. A to T transversions in the Ras codon 61 are the most frequent mutations found in this protocol (Finch et al., 1996). Subsequent repeated TPA (12-*O*-tetradecanoylphorbol-13-acetate) treatment for twenty weeks lead to tumor promotion that causes the selective clonal outgrowth of the initiated cells targeted by Ras mutation to produce benign lesions (papillomas). Approximately 20% of the produced papillomas undergo malignant progression into squamous cell carcinomas, which can undergo epithelial to mesenchymal transition (EMT) to spindle cell carcinomas. The incidence of carcinomas can be substantially enhanced by treating papilloma-bearing mice with mutagens such as urethane, nitroquinoline-N-oxide or cisplatinum suggesting that distinct additional genetic events are responsible for malignant conversion.

To dissect the different stages of the carcinogenesis process, variations on the two stage carcinogenesis applications can be performed. Increasing concentrations of DMBA and/or TPA during the treatments result in an increasing number of lesions per mouse. When the repeated TPA treatments are stopped, existing papillomas may regress or reduce in size. Mice can also be repeatedly treated with DMBA alone. This complete carcinogenesis protocol leads directly to the formation of predominantly squamous cell carcinomas. To study effects on growth and survival of epidermal keratinocytes, skin hyperplasia can be induced by TPA treatment with or without previous DMBA treatment. The genetic background of treated mice can influence the carcinogenesis process, i.e. the number of tumors produced or the progression of these tumors. Although DMBA/TPA-induced tumors have been described in Black 6 mice, we have better experience when using this protocol on FVB mice.

We found that Tiam1$^{-/-}$ mice are resistant to the development of skin tumors induced by DMBA/TPA treatment (Malliri et al., 2002) (Fig. 2). Moreover, the few tumors produced in Tiam1$^{-/-}$ mice grew much slower than did tumors in wild type mice. Tiam1-deficiency was associated with increased apoptosis in the basal layer of the epidermis during initiation and with impeded proliferation during promotion. Although the number of tumors in Tiam1$^{-/-}$ was small, a greater proportion progressed to malignancy, suggesting that Tiam1-deficiency promotes malignant conversion. Tiam1 is required for the proper formation and maintenance of E-cadherin-mediated cell-cell adhesion, of which the loss is associated with tumor progression (Zondag et al., 2000;Malliri et al., 2004). Therefore, Tiam1-deficiency might favour malignant progression by reduced strength of E-cadherin-mediated adhesion *in vivo*.

Genetic ablation of other potential Ras effectors has also been shown to reduce tumor formation in the DMBA/TPA skin carcinogenesis model. As we found for Tiam1, deficiency of both RalGDS and Phospholipase Cε (PLCε) leads to reduced initiation and growth of skin tumors (Gonzalez-Garcia et al., 2005;Bai et al., 2004). However, while papillomas in Tiam1$^{-/-}$ mice progressed more frequently to malignant carcinomas than in wild type mice, RalGDS and PLCε deficient mice show a lower percentage of malignant tumors as compared to control tumors in wild type mice. Apparently, different Ras effectors cooperate with Ras in the oncogenic process by regulating different aspects of tumorigenicity.

Alternative to the DMBA/TPA protocol, Ras activation in mouse models can be achieved using transgenetic approaches. In the case of oncogenic Ras, constitutive expression cannot be used to study tumorigenesis as these animals show early developmental defects and malformations. Even in conditional systems, a minor leakage of oncogenic Ras expression often results in severe phenotypes. Examples of models with conditional mutant Ras expression are v-Ha-ras expression targeted to the mammary gland by MMTV promoter for mammary tumorigenesis (Sinn et al., 1987), expression of mutant Ras from different keratin promoters directing expression to specific cells in the skin (Bailleul et al., 1990;Brown et al., 1998), and Cre-mediated somatic induction of Ras in the lungs, a model for non-small cell lung cancer (Meuwissen et al., 2001). A tetracycline-inducible system for K-Ras expression in epidermal stem cells has been described to study the promotion of squamous cell carcinomas (Vitale-Cross et al., 2004). In a 4-hydroxytamoxifen (4OHT)-regulated system expressing Ras under control of the keratin 14 promoter, Ras reversibly induced massive cutaneous hyperplasia and suppressed differentiation (Tarutani et al., 2003;Perez-Mancera and Tuveson, 2006)

Protocols for *in vivo* studies

Protocol for two-stage DMBA/TPA carcinogenesis

The backs of 8-week-old mice are shaved using a hair clipper. The next day initiation is carried out by a single application of 25 μg DMBA (Sigma) in 200 μl acetone topically on the shaved area of the dorsal skin. Control mice receive acetone only. A week after DMBA initiation, mice are skin painted with 200 μl of a 10^{-4}M solution TPA (Sigma) in acetone twice weekly at the site of DMBA application for 20 weeks. For complete carcinogenesis mice are treated biweekly with 5 μg DMBA alone in 200 μl acetone for 20 weeks. Mice are visually examined twice weekly for tumor formation and number and size of tumors is determined. Mice are killed when moribund, if any individual tumor reaches a diameter of 1 cm, or at the termination of the experiment at 30 weeks. Tumors and organs can be isolated and fixed for histochemical examination. Cells from tumors can be isolated as described below (II-1-1) for *in vitro* studies.

Proliferation and Apoptosis assays *in vivo*

In proliferating cells, exogenous 5-bromo-2-deoxyuridine (BrdU) is incorporated into genomic DNA during DNA replication in the S-phase of the cell cycle. Therefore, BrdU incorporation can be used to detect cycling cells. To measure the number of proliferating cells in tumors and organs, mice are injected intraperitoneal with BrdU (Sigma) at 50 mg/kg in 200 μl PBS, usually 2-4 hrs before sacrificing the mice. Alternatively, BrdU can be applied in the drinking water of the mice at 0.8 mg/ml. BrdU incorporation in proliferating cells is detected on paraffin sections using an anti-BrdU antibody (1/50; DAKO) according to the immunohistochemistry protocol as described below. Alternatively to the BrdU protocol, sections are stained for the proliferation marker PCNA by immunohistochemistry (CS-56 antibody 1/500; Santa Cruz).

Apoptosis, or programmed cell death, is associated with changes in several cellular processes. For example, it alters plasma membrane asymmetry, cleaves cellular DNA into histone-associated DNA fragments, and activates ICE-like proteases. In the TUNEL (terminal deoxynucleotidyl transferase(TdT)-mediated dUTP nick end labeling) assay, apoptotic cells are labeled *in situ* by a TdT reaction tailing labeled nucleotides into DNA strand breaks that occur during early apoptosis. For detection of apoptotic cells in paraffin-embedded tissue sections we use the *in situ* cell death detection kit, POD (Boehringer). For pre-treatment of the sections we refer to the immunohistochemical protocol as described below with the exception that antigen retrieval is performed by a proteinase K (20 μg/ml) treatment for 15 min at room temperature (RT). Using the reagents of the kit, the TdT-mediated incorporation of fluorescein-dUTP is performed for 1 hour at 37°C. TUNEL POD solution, containing an anti-fluorescein antibody conjugated with peroxidase (POD), is

applied for 30 min at 37°C. After washing in PBS, DAB detection is performed as described below in the protocol for immunohistochemistry.

Histology and Immunohistochemistry

Tumors and organs are dissected and embedded in Tissue-Tek® OCT™ compound (Sakura) and frozen or fixed in 10% buffered formalin for 24-48 hours at room temperature as required. Paraffin-embedded tissues sections (3 μm) are prepared using a semi-automatic microtome (Leica, 2255) and captured on Superfrost Plus object glasses in a water bath. Sections of the tumors and tissues are stained with haematoxylin and eosin for histological classification.

Standard ABC (Avidin-Biotin Complex) techniques are used for antigen-specific immunohistochemical detection (Malliri et al., 2002). The principle is based on the irreversible high affinity binding of avidin to biotin (Hsu et al., 1981). Avidin has four binding sites for biotin and is used to complex biotinylated horseradish peroxidase (HRP) to biotin-conjugated secondary antibody used for immunohistochemistry. De-paraffinized slides are cooked for 20 min in 0.1M citrate buffer pH 6.0 for antigen retrieval. After cooling down, endogenous peroxidase is blocked in 3% H_2O_2 in methanol at RT for 10 min. Slides are pre-incubated for 30 min at RT in 5% normal goat serum in PBS with 1% BSA. Primary antibodies are applied overnight at 4°C. Subsequent slides are incubated for 1 hour at RT with the appropriate anti-mouse or anti-rabbit biotin-conjugated secondary antibodies (DAKO). After washing, slides are incubated for 30 min at RT with preformed Avidin and Biotinylated HRP Complex (ABC; DAKO) in PBS/BSA. For detection we use the HRP substrate DAB (3,3-diaminobenzidine tetrahydrochloride) which produces an alcohol-insoluble brown precipitate in the sections. Slides are rinsed in 0.05M Tris/HCl pH 7.6 and treated for 5 min at RT with substrate buffer (0.05% DAB (Sigma) 0.01% H_2O_2 in 0.05M Tris/HCl-0.1M imidazole pH 7.6). After counterstaining with Haematoxylin slides are rinsed with tap water for 10 min, subsequently rinsed in raising concentrations alcohol and xylene and embedded in DePeX mounting medium (Gurr; BDH laboratory supplies). For Tiam1 staining we use different dilutions (1/500-1/1.500) of C16 (Santa Cruz) and a house-made anti-DH polyclonal antibody (Habets et al., 1994).

Protocol for β-Gal detection

Freeze coupes made from dissected mouse organs or whole embryos are fixed for 5 min at RT in 2% paraformaldehyde; 0.2% glutaraldehyde. Tissues are washed in PBS and stained overnight at 37°C in X-Gal solution (0.1M sodium phosphate pH 7.3, 2 mM $MgCl_2$, 5mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/ml X-Gal (dissolved in DMSO; Invitrogen)). After washing in PBS, tissues are counterstained using Nuclear Fast Red, dehydrated and mounted in non-aqueous DePeX mounting medium (Gurr; BDH laboratory supplies).

IN VITRO ANALYSIS OF TIAM1/RAC SIGNALING

Overexpression or downregulation of gene of interest in cells cultured *in vitro* are ideal tools to study the function of the respective gene. Although we found that Tiam1 is expressed at low levels in most murine tissues, some established human and rodent cell lines do not show detectable expression of Tiam1 at the RNA (Habets et al., 1995) or protein level (Sander et al., 1999). Ectopic expression of Tiam1 in cells has been used extensively for functional studies. Besides full-length Tiam1 expression constructs, we have used more stable N-terminally truncated versions of Tiam1 (Fig. 1). C1199 Tiam1, referring to the C-terminal 1199 amino acids, resembles an activated mutant of Tiam1 that is encoded by a truncated Tiam1 transcript as found after proviral insertion (Habets et al., 1994). The C580 Tiam1 mutant contains the minimal DH-PHc catalytic GEF domains only. By overexpression studies we have investigated the role of Tiam1-mediated Rac activation in invasiveness of lymphoid cells (Habets et al., 1994;Michiels et al., 1995), in lamellar spreading and neurite formation in neuronal cells (Leeuwen et al., 1997), in inhibition of invasion by upregulation of the E-cadherin/catenin-complex in epithelial cells (Hordijk et al., 1997;Sander et al., 1998), and in epithelial to mesenchymal transition (EMT) (Sander et al., 1999;Zondag et al., 2000).

For functional studies we have downregulated Tiam1 to circumvent Tiam1-independent side effects in artificial overexpression. Efficient dominant-negative versions of Tiam1 are not available but recently we have made use of the siRNA technique (Brummelkamp et al., 2002) to downregulate endogenous Tiam1. We cloned different Tiam1 RNA targeting oligonucleotides containing a 9-bp hairpin loop in pSuper and pRetrosuper for transfection and retroviral infection experiments respectively. Infection of MDCK cells with canine Tiam1 siRNA results in a knock-down of Tiam1 protein levels of at least 50%, which results in a transition to a mesenchymal morphology resembling EMT and in dramatic effects on the migratory behaviour of the cells (Malliri et al., 2004). The siRNA technique is highly specific and can be used for transient and stable gene silencing. However, the knock-down efficiency for a given gene may vary considerably and different constructs have to be tested for each gene. Silencing by siRNA also creates highly variable cell populations with different knock-down levels and complete downregulation of the protein of interest is never achieved.

To ensure complete knock-out of Tiam1 in homogeneous cell populations we have isolated cells from Tiam1$^{-/-}$ mice in which the *Tiam1* gene has been ablated by homologous recombination. Mouse embryonic fibroblasts (MEFs) can be easily isolated (see II-1-2) and can be cultured for prolonged times after immortalization. Wild type (WT) MEFs grow in tightly packed colonies with few scattered cells at the periphery while Tiam1$^{-/-}$ MEFs grow in irregular colonies that frequently show dispersed cells at the edges. Tiam1$^{-/-}$ MEFs show reduced levels of active Rac when compared to WT MEFs (Malliri et al., 2004;van Leeu-

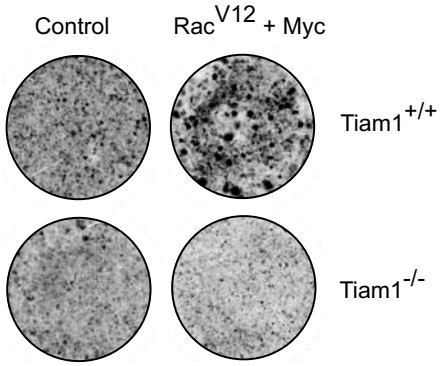

Figure 3. Focus formation assay in primary embryonic fibroblasts. Focus formation induced by co-expression of Ras^{V12} and Myc in primary embryonic fibroblasts (MEFs) derived from wild type and $Tiam1^{-/-}$ mice. The number of foci is strongly reduced in $Tiam1^{-/-}$ MEFs when compared to wild type MEFs.

wen et al., 2003) and fail to undergo E1A-induced mesenchymal to epithelial transition (MET), a process dependent on Tiam1 (Malliri et al., 2004). Primary WT and $Tiam1^{-/-}$ MEFs have also been used in focus formation assays (see II-1-4). Co-expression of dominant active Ras^{V12} and Myc in MEFs induces much more foci in WT than in $Tiam1^{-/-}$ cells (Fig. 3), illustrating the importance of Tiam1 in Ras-mediated transformation of cells (Malliri et al., 2002;Lambert et al., 2002). Besides MEFs many other primary cell types can be isolated from mice such as neuronal cells, endothelial cells, lymphoid cells, and epithelial cells from skin, lung or intestine. In addition, tumors induced in recombinant mice can be source of cells (see II-1-1). Keratinocytes, derived from the epidermis of newborn $Tiam1^{-/-}$ mouse, provide an excellent tool for *in vitro* studies on epithelial cells. Primary keratinocytes have to be immortalized (e.g. by SV40 large T antigen) to prevent apoptosis or terminal differentiation (see II-1-3). We are using $Tiam1^{-/-}$ keratinocytes to study the role of Tiam1 in Rac-mediated cell-cell and cell-matrix interactions as well as in cell polarization, cell migration and apoptosis susceptibility.

Protocols for *in vitro* studies

Isolation of skin tumor cells

We isolated skin tumor cells from lesions that are formed in mice treated with the DMBA/TPA protocol as described above. Tumors are excised and washed in PBS. The tissue is chopped using a scalpel and subsequently incubated for 1 hour at RT in a digestion solution (3 mg/ml collagenase (Sigma) and 1.5 mg/ml trypsin (Difco) in DMEM) to dissociate cell-cell and cell–matrix adhesions. The cell suspension is filtered through a 70 µm nylon cell strainer, centrifuged and resuspended in DMEM supplemented with 10% FCS, 100 U/

ml penicillin and 100 µg/ml streptomycin (P/S) before plating in standard tissue culture dishes.

Isolation of mouse embryonic fibroblasts (MEFs)

To isolate MEFs female mice are sacrificed at day 12.5 of pregnancy. The peritoneum is opened and the uterus is removed and cut along the upper side. The embryos are taken out and placed in separate wells. Embryos are decapitated and soft tissue is removed. Embryo carcasses are minced and transferred to cold PBS. After centrifugation, cells derived form each embryo are resuspended in 5 ml PBS containing 50 µg/ml trypsin, 50 µM EDTA and P/S, and incubated overnight at 4°C. Trypsin is blocked by addition of complete culture medium (DMEM, 10% FCS, 0.1 mM β-mercaptoethanol, P/S). The suspension of cells and tissue debris is allowed to settle down for 2 min and supernatant is transferred to a culture flask. Primary MEFs can be efficiently immortalized by transfection with large T antigen. Alternatively, continuous passaging of the cells will eventually lead to spontaneous immortalization.

Isolation of Keratinocytes

To isolate primary keratinocytes newborn (1-3 days old) mice are decapitated and washed in water and 70% ethanol. Limbs and tail are amputated with scissors under sterile conditions. The skin of the mice is cut on the dorsal side all along the length of the body and carefully separated from the rest of the carcase. The skin is washed in PBS (supplemented with P/S) and remaining fat tissue and blood vessels are removed. The isolated skin is stretched with the dermal side faced down on a sterile Whatmann paper that is soaked with trypsin (2.5 mg/ml, EDTA free) and incubated overnight at 4°C. The next day the dermis is separated from the epidermis, and both are minced with tweezers and scissors. Suspensions of epidermis and dermis are incubated separately for 1 hour at 4°C in DMEM (supplemented with 10% FBS and P/S) under gentle stirring. Subsequently, cell suspensions are filtered through a 70 µm cell strainer, centrifuged at 900 rpm for 5 min. and resuspended in DMEM containing 10% FBS, P/S. Dermal and epidermal cells are plated separately on 6 well plates coated with collagen I. The following day culture medium is replaced by serum-free keratinocyte medium supplemented with growth factors (Cascade Biologics) and $CaCl_2$ (0.02 mM). Keratinocytes can be immortalized by introduction of SV40 large T antigen.

Focus formation assay

Fibroblasts (NIH3T3) or MEFs, grown to a density of 40-50%, are infected with retroviruses carrying the desired oncogenes (e.g. *Ras*^{V12} and/or c-*Myc*). After 24-48 hours when cells reach confluency, the medium is refreshed with DMEM containing 2% FBS (for MEFs) or 5% NCS (for NIH-3T3). Cells are cultured for 14 days with medium refreshments every

three days. At the end of the experiment cells are fixed with methanol and stained with 1% crystal violet.

Apoptosis assay on *in vitro* cultured cells

Apoptosis can be induced in MEFs or keratinocytes by several means including growth factor deprivation, TNF-alpha and TRAF treatment, UV and gamma-irradiation, surface detachment (anoikis), hyperosmotic conditions or heat shock (43-45°C). Cells that undergo apoptosis expose phosphatidylserine on the external side of the cell membrane. Annexin-V is a protein that specifically binds phosphatidylserine. Therefore, apoptotic cells can be specifically stained with annexin-V protein conjugated to a fluorochrome like APC. In the case of growth factor starvation-induced apoptosis in keratinocytes, normal growth medium is replaced for growth factor-free medium and the degree of apoptosis is analyzed after 24 hours. For this, the cells are trypsinized, washed twice with cold PBS and are resuspended in annexin-V-binding buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). 2-4 µl APC-labeled annexin-V (Becton Dickinson) is added to each sample containing $1x10^5$ to $1x10^6$ cells and incubated on ice for 15 min protected from light. Subsequently, 400 µl of annexin-binding buffer containing 1.25 µg/ml propidium iodide is added to the samples and cells are analyzed by flow cytometry. Unstained and single stained samples are used for proper calibration of the Flow cytometer.

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