Focal adhesion kinase and paxillin : mediators of breast cancer cell migration

Verkoeijen, S.

CHAPTER 3

Requirement for focal adhesion kinase in the early phase of mammary adenocarcinoma lung metastasis formation

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Running title: FAK is essential for metastasis formation

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ABSTRACT

An increased expression of focal adhesion kinase (FAK) in a variety of cancers is associated with a poor disease prognosis. To study the role of FAK in breast tumor growth and metastasis formation, we used conditional doxycycline-regulated expression of a dominant-negative-acting splice variant of FAK, FAK-related non-kinase (FRNK), in MTLn3 mammary adenocarcinoma cells in a syngeneic Fischer 344 rat tumor and metastasis model. In cell culture, doxycycline-mediated expression of FRNK inhibited MTLn3 cell spreading and migration in association with reduced formation of focal adhesions and phosphorylation of FAK on Tyr397, but FRNK did not cause apoptosis. Continuous expression of FRNK decreased the primary tumor growth in the mammary fat pad by 60%, which was not due to induction of apoptosis. Lung metastasis formation was almost completely prevented when FRNK was expressed from one day before tumor cell injection onwards, whereas expression of FRNK 11 days after injection did not affect lung metastasis formation. FRNK expression during the first 5 days was sufficient to block metastasis formation, excluding the possibility of FRNK-induced dormancy of tumor cells. Together, these data fit with a model wherein FAK is required for breast tumor cell invasion/migration processes that take place in the early phase of metastasis formation. Our findings suggest that FAK is a good candidate for therapeutic intervention of metastasis formation.

INTRODUCTION

Breast cancer is the most frequently occurring type of cancer in women. Primary breast tumors can be removed or irradiated relatively easily, but the distant metastases are hard to treat. Improved insights into the mechanisms involved in the tumor-metastasis process are necessary to define potential novel drug targets that can be used to combat metastasis formation. The formation of a metastasis involves different steps, including detachment, migration, invasion, extravasation, and proliferation of the cancer cells, which require the appropriate extracellular signals. Focal adhesions, the closest contacts between cells and the extracellular matrix (ECM), are important sites for these signaling events. Focal adhesion kinase (FAK) is a non–receptor protein tyrosine kinase that is important in cell-ECM-mediated signaling. Immunohistochemical staining of different cervical and breast carcinomas for FAK revealed that increased expression marks the malignant transformation of squamous cells of the uterine cervix and epithelial cells of breast ducts (1). Increased expression of FAK has been shown in various tumors, including tumors derived from the breast (1), head and neck (2), and ovary (3). Furthermore, the involvement of FAK in cancer is emphasized by the observation that amplification of chromosome 8q (the FAK-encoding region) is correlated to a poor disease prognosis (4). Moreover, FAK heterozygous mice develop less papillomas in a chemical skin carcinogenesis model (5).

FAK plays a central role in controlling cell migration, proliferation, and survival. Several domains of FAK are important for these functions. The NH_2-terminal region of FAK harbors a FERM domain that mediates binding of FAK to integrins and growth factor receptors and thereby FAK activation (6). The COOH-terminal focal adhesion targeting domain is required for focal adhesion localization through binding to paxillin and talin (7). FAK activity is controlled by autophosphorylation of Tyr397, providing a binding site for Src family kinases as well as phosphatidylinositol-3-kinase (8). Consequently, Src activity mediates phosphorylation of Tyr576/Tyr577 in the kinase domain and Tyr861 and Tyr925 in the COOH-terminal region (6). The FAK-Src complex mediates phosphorylation of several focal adhesion–associated adaptor proteins, including paxillin (9) and p130Cas (10). The focal adhesion targeting domain as well as a splice variant of FAK, the FAK-related non-kinase (FRNK), which lacks the entire NH_2 terminus

and the kinase domain, are often used to inhibit FAK localization at focal adhesions and thereby many of the cell biological functions of FAK (11).

Several studies suggest a direct role for FAK in tumor formation and progression. Increased expression of active CD2-FAK in Madin-Darby canine kidney cells enables s.c. tumor formation in nude mice (12). Similarly, overexpression of FAK in U-215MG human malignant astrocytoma cells results in increased tumor cell proliferation in a xenograft model (13). Expression of FRNK in human epidermoid carcinoma cells reduces cellular outgrowth when these cells are inoculated onto chorioallantoic membranes of chick embryos (14). Stable overexpression of FRNK in NIH-3T3 fibroblasts transformed with the dominant oncogene v-Src, inhibits cell invasion *in vitro* and reduces experimental metastasis formation in nude mice (15). Constitutive expression of FRNK in B16-F10 melanoma resulted in a 50% reduction in the number of lung metastases (16). Because FAK mediates survival signaling, it cannot be excluded that, in both v-Src NIH-3T3 cells and B16-F10 cells, selection occurred *in vitro* during stable transfection, favoring the selection of cells that were relatively resistant to FRNK-induced apoptosis. Such a selection may affect the characteristics and thereby the *in vivo* behavior of the tumor cells. Moreover, because FAK has been linked to both migration/invasion (17,18) and survival/proliferation (19, 20) processes, thus far, it remains unclear whether the *in vivo* effects of FRNK are related to early inhibition of migration/invasion processes and/or to inhibition of tumor cell survival/proliferation in the metastases. Alternatively, FRNK expression may cause dormancy of tumor cells in the distant target organs that eventually might grow out into full metastases when FAK is no longer inhibited. To study these important issues, conditional models to modulate the function of FAK are required.

Here, we examined the importance of FAK signaling during primary tumor growth as well as during the different steps in metastasis formation of breast tumor cells. For this purpose, we made use of the metastatic mammary adenocarcinoma cell line MTLn3 (21), which has been used both *in vitro* and *in vivo* to study the mechanisms of metastasis formation (22). We generated a cell line with a doxycycline-regulated expression of hemagglutinin (HA)–tagged FRNK: tetFRNK-MTLn3 cells. This cell line enabled us to study, to our knowledge for the first time, the importance of FAK at different phases in the process of metastasis formation. Conditional expression of HA-FRNK *in vitro* resulted in a reduced attachment, spreading, and migration of the tetFRNK-MTLn3 cells but did not

affect cell death. *In vivo*, HA-FRNK inhibited primary tumor growth, whereas HA-FRNK almost completely prevented experimental lung metastasis formation; expression of HA-FRNK during the first 5 days after tumor cell injection was sufficient for this effect. Expression of HA-FRNK starting 11 days after injection of the tumor cells did not significantly affect the number of lung metastases. Altogether, we conclude that FAK signaling is essential during the initial steps of metastasis formation, most likely by interfering with tumor cell invasion/migration processes.

MATERIALS AND METHODS

Chemicals and antibodies

Alpha-modified MEM with ribonucleosides and deoxyribonucleosides (α-MEM), fetal bovine serum (FBS), PBS, trypsin, LipofectAMINE Plus, and geneticin (G418 sulfate) were from Life Technologies (Rockville, MD). Collagen (type I, rat tail) was from Upstate Biotechnology (Lake Placid, NY). Propidium iodide (PI), RNase A, and doxycycline were from Sigma (St. Louis, MO). Annexin V was from Boehringer Mannheim (Basel, Switzerland). Hoechst 33258 and the Alexa protein labeling kit were from Molecular Probes (Leiden, the Netherlands). Hygromycin was from Roche (Penzberg, Germany). All other chemicals were of analytic grade. Primary antibodies were anti-HA (clone 3F10, Roche, Indianapolis, IN) and anti-HA (clone 12CA5, Roche, Indianapolis, IN), anti-paxillin (clone 349; BD Biosciences, Rockville, MD), anti-FAK (Upstate Biotechnology), anti–active caspase-3 rabbit polyclonal (New England Biolabs, Beverly, MA), anti-paxillin-PY118, and anti-FAK-PY397 (Biosource Europe S.A., Nivelles, Belgium).

Cell culture and generation of stable cell lines

MTLn3 rat mammary adenocarcinoma cells (21) were cultured as described previously (23). For creating tetFRNK-MTLn3 cells, cells were first transfected with the pTet-on plasmid (Clontech, Mountain View, CA) using LipofectAMINE Plus. Colonies resistant to G418 were selected and tested for inducibility under the Tet-on system by transient transfection with a tetracycline response element promoter (pTRE) luciferase reporter. Next, proper clones were transfected with the pTRE-HA_3-FRNK construct (FRNK cDNA was kindly provided by Dr. T.J. Parsons (University of Virginia Health Science Center, Charlottesville, VA) and

inserted between two *Eco*RI sites in a pTRE-HA_3 vector) and a pTK-Hyg plasmid followed by selection with hygromycin. Hygromycin-resistant clones were evaluated for doxycycline (1 μg/ml)-induced expression of HA-FRNK. Selected tetFRNK-MTLn3 cells were cultured in complete medium supplemented with 50 μg/ml hygromycin and 100 μg/ml G418 and used for up to 10 passages during which more than 90% of the cells maintained doxycycline-induced expression of HA-FRNK.

Cell proliferation, apoptosis, and anoikis assays

tetFRNK cells were cultured for 24 hours in the absence or presence of doxycycline and subsequently trypsinized and replated in 24-well plates ($1x10^4$ cells per well). Cells were washed twice with PBS, 200 μl milliQ was added, and plates were frozen (−80°C). Lysed cells were incubated with Hoechst 33258 (2 μg/ml) and DNA concentration was measured in a fluorescence plate reader (HTS 7000 Bio assay reader, Perkin-Elmer, Norwalk, CT) with calf thymus DNA as a standard. Cell death was determined by staining the pooled attached and detached cells for Annexin V/PI or by cell cycle analysis as described previously (23). For anoikis experiments, cells were cultured in the absence or presence of doxycycline, serum was withdrawn for 1 hour, and cells were trypsinized. Cells were counted, resuspended to a final concentration of $1x10^6$ cells/ml, and incubated in a 15 ml tube on a roller bank in the absence of serum at 37°C, and cell death was determined by the Annexin V/PI method.

Immunoblotting

Frozen lung or tumor tissue was cryosectioned into 10 μm slices followed by lysis in TSE (10 mmol/l Tris-HCl, 250 mmol/l sucrose, 1 mmol/l EGTA (pH 7.4)) supplemented with inhibitors (1 mmol/l DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mmol/l sodium vanadate, 50 mmol/l NaF, 1 mmol/l phenylmethylsulfonyl fluoride). Cells were scraped in ice-cold TSE supplemented with the same inhibitors. After sonication of either tissue or cells, protein concentrations were determined by the Bio-Rad protein assay using IgG as a standard. Equal amounts of total cellular protein were separated by 7.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Blots were blocked with 5% (w/v) bovine serum albumin in TBST (0.5 mol/l NaCl, 20 mmol/l Tris-HCl, 0.05% (v/v) Tween 20 (pH 7.4)) and probed

with primary antibodies (overnight, 4°C) followed by incubation with secondary horseradish peroxidase–coupled antibodies and visualized with Enhanced Chemiluminescence Plus reagent (Amersham Biosciences, Uppsala, Sweden) on a multilabel Typhoon imager 9400 (Amersham Biosciences).

Immunofluorescence

Culturing and immunostaining of cells were performed essentially the same as described (23). For staining of tissue sections, paraffin-embedded tissue was cut into 4 μm sections. After deparaffinization, sections were blocked in 1.5% normal goat serum for 1 hour and incubated with primary antibodies (overnight, 4°C) followed by incubation with fluorescently-labeled secondary antibodies for 1 hour at room temperature. After washing, slides were incubated with 2 μg/ml Hoechst 33258 and mounted in Aqua PolyMount. Cells and tissue were visualized using a Bio-Rad Radiance 2100 MP confocal laser scanning system equipped with a Nikon Eclipse TE2000-U inverted fluorescence microscope and a 60× Nikon objective.

Cell attachment and wound healing assays

Cells incubated for 1 hour in the absence of FBS were trypsinized and resuspended to a final concentration of $4x10^5$ cells/ml in α-MEM (with or without doxycycline). Next, cells were replated into 6-wells plates (2.5 ml) coated with rat tail collagen type I. Cells were incubated for the indicated times at 37°C. Unattached cells were removed, phase-contrast pictures were taken, and attached cells were trypsinized and counted. For wound healing, confluent cells were serum-starved and subsequently wounded with a pipette tip. Photographs were taken at indicated times followed by measurement of wound width.

***In vivo* tumor growth and metastasis formation**

Cells were injected into the mammary fat pad or into the lateral tail vein of Fischer 344 rats (7-8 weeks old, Charles River, Maastricht, the Netherlands). After the fat pad injection, the size of the primary tumors was determined by using calipers starting from day 20. Horizontal (h) and vertical (v) diameters were measured and tumor volume (V) was calculated: $V = 4/3\pi(1/2[\sqrt{(h \times v)}]^3)$. For the tail vein injection experiment, rats were pretreated with the NK-depleting antibody (mAb3.2.3) on days −3, −2, and −1 (150 μg in 0.5 ml PBS, i.p.) before the injection of the cells. tetFRNK cells (passage +1) were cultured in the absence or presence

of 1 μg/ml doxycycline for 24 hours and cells were trypsinized, counted, washed twice, and resuspended in PBS. Viable cells were injected into the lateral tail vein ($1x10^5$ cells in 0.2 ml PBS) or into the mammary fat pad ($1x10^6$ cells in 0.5 ml PBS). Where indicated, doxycycline (400 μg/ml in 2.5% (w/v) sucrose) was added to the drinking water; control animals received 1.5% (w/v) sucrose in their drinking water, which resulted in equal drinking volumes. At the indicated time points, animals were anesthetized with pentobarbital and the lungs were excised and rinsed in ice-cold PBS. The right lung was cut into three pieces and fixed in isopentane (used to prepare tissue homogenates for immunoblot analysis), 4% paraformaldehyde, or Carnoy's (10% (v/v) acetic acid, 30% (v/v) chloroform in 60% ethanol). The left lung was injected with ink solution, destained in water and fixed in Fekete's (4.3% (v/v) acetic acid, 0.35% (v/v) formaldehyde in 70% ethanol). Pictures of the stained lungs were taken and lung metastases (white dots) were counted.

Statistical analysis

Student's t test was used to determine significant differences between two means ($p < 0.05$). For the *in vivo* lung metastasis experiments, statistical significance was determined by the Mann-Whitney rank sum test.

RESULTS

Doxycycline-mediated expression of HA-FRNK in MTLn3 cells inhibits proliferation but does not affect cell survival.

To elucidate the role of FAK in the migratory, proliferative, and metastatic behavior of breast tumor cells *in vitro* and *in vivo*, we created a MTLn3 cell line that conditionally expresses the COOH-terminal splice variant of FAK, FRNK, that has an inhibitory effect on FAK. The obtained tetFRNK-MTLn3 clone had a tight concentration-dependent regulation of HA-FRNK expression and more than 90% of the cells stained positive for HA-FRNK after doxycycline treatment as indicated by antibody staining directed against the HA-tag followed by flow cytometric analysis and immunofluorescence (Supplemental fig. S1A and S1B). Immunostaining for active FAK (i.e., tyrosine phosphorylation on Tyr397) indicated that HA-FRNK expression strongly decreased pTyrY397-FAK levels at focal adhesions (Supplemental fig. S1C). Importantly, tetFRNK-MTLn3 cells kept

their potential for doxycycline-induced HA-FRNK expression for at least 10 passages (data not shown). These data show that we created a doxycycline-regulated HA-FRNK-expressing MTLn3 cell line in which HA-FRNK diminishes FAK phosphorylation at focal adhesions.

Several studies show that inhibition of FAK induces apoptosis. Transient transfection of MTLn3 cells with enhanced green fluorescent protein (GFP)-FRNK indeed induced apoptosis.[1] If HA-FRNK itself induces apoptosis in tetFRNK-MTLn3 cells, this could be mistaken for a reduction in tumor growth or a decrease in metastasis formation *in vivo*. HA-FRNK slightly reduced cell growth (Fig. 1A). Doxycycline exposure resulted in a 27% growth reduction after 6 days. Immunoblotting of the doxycycline-exposed cells revealed that the expression of HA-FRNK was not altered between day 2 (i.e., steady-state levels of HA-FRNK) and day 6 (Fig. 1B), indicating that HA-FRNK expression does not result in selection of HA-FRNK-negative cells during this time. The reduced cell growth was not caused by an increase in cell death, because no significant difference in the level of apoptosis between the control and the HA-FRNK-expressing cells could be detected in log-phase growing cells (Fig. 1C). When metastases are formed, tumor cells have to survive in the circulation. It is known that constitutively active FAK can rescue cells from anoikis (12). HA-FRNK did not increase anoikis in cells that were kept in suspension for 10 hours (Supplemental fig. S2). Altogether, these *in vitro* results show that in our inducible cell line HA-FRNK slightly inhibits proliferation but does not induce apoptosis by itself or promote the onset of anoikis when cells are kept in suspension.

HA-FRNK attenuates attachment and spreading and inhibits motility of cells.
Given the role of FAK in focal adhesion formation and turnover, we next investigated the effect of HA-FRNK expression on MTLn3 cell attachment, spreading, and migration. Four hours after replating the tetFRNK-MTLn3 cells, HA-FRNK-expressing cells were poorly attached to the collagen-coated support, whereas the control cells were almost completely spread (Fig. 2A). Quantification of the number of attached cells showed that after 8 hours more than 80% of the control cells were attached, whereas only 50% of the HA-FRNK-expressing cells were attached (Fig. 2B). At 4 hours after replating, hardly any Tyr397-phosphorylated FAK was present at focal adhesions when HA-FRNK was expressed (Fig. 2A).

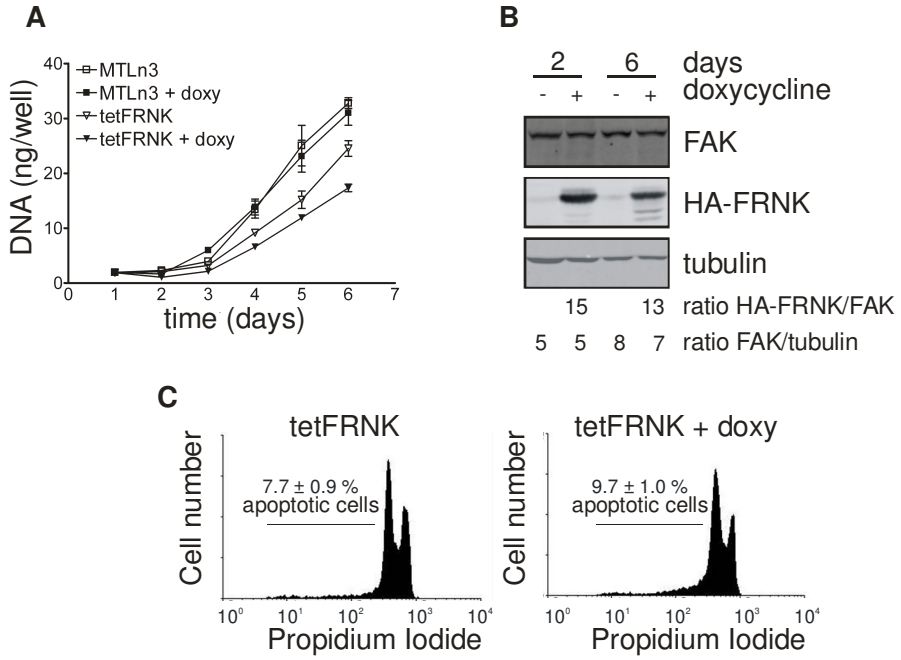

Figure 1. HA-FRNK inhibits proliferation without affecting apoptosis. MTLn3 and tetFRNK-MTLn3 cells were seeded on day 0. Cells were grown in the absence or presence of doxycycline (*doxy*) and medium was replaced daily. (A) The total amount of DNA was quantified daily as described in Materials and Methods. (B) Expression of FAK, HA-FRNK, and tubulin was determined by immunoblotting using anti-COOH-terminal FAK and anti-tubulin antibodies. (C) After 3 days, attached and floating cells were pooled and the percentage of apoptotic cells was measured by flow cytometric cell cycle analysis. Representative of three independent experiments.

Western blot analysis of the tetFRNK-MTLn3 cells displayed a ~50% reduction in overall pTyr397-FAK upon HA-FRNK expression (data not shown). To determine the effect of HA-FRNK on cell motility, we studied the migration of tetFRNK-MTLn3 cells using a wound healing assay. To prevent growth of the cells, which could be mistaken for migration, we performed the experiments in the absence of serum. HA-FRNK-expressing cells were not able to initiate wound closure within 8 hours. In contrast, in the control cells, 30% of the wounds were closed within 8 hours (Fig. 2C and D). Because MTLn3 cells die in the absence of serum, we were not able to study migration after 24 hours under these conditions. However, in the

presence of low concentrations of serum, both control and HA-FRNK-expressing cells closed the wound in 24 hours (data not shown).

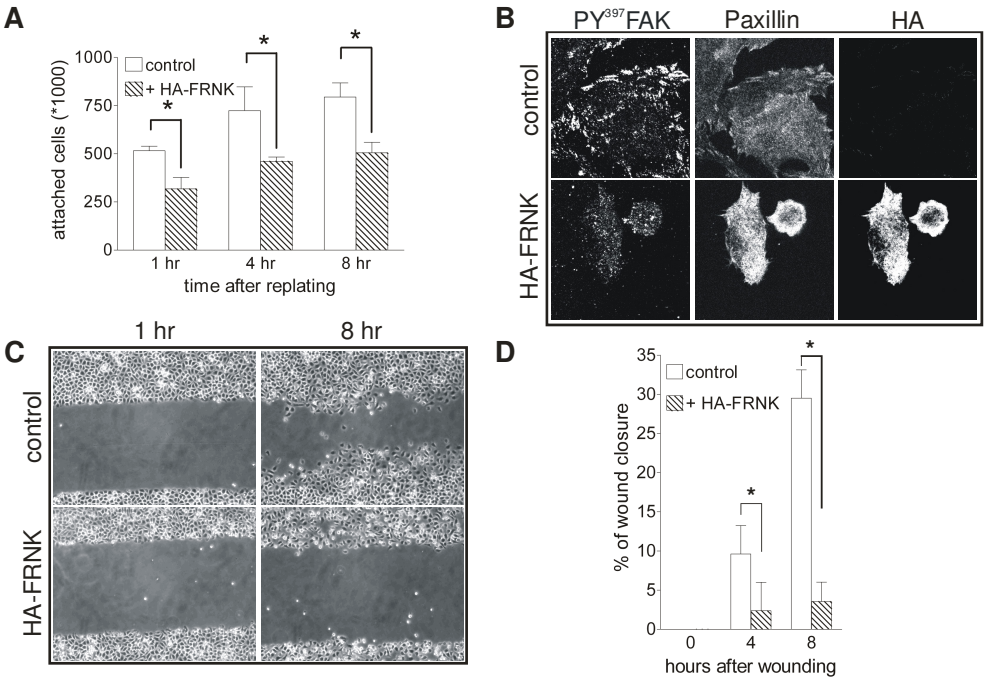

Figure 2. HA-FRNK inhibits attachment, spreading, and migration of MTLn3 cells. tetFRNK-MTLn3 cells (incubated for 24 hours in the absence or presence of doxycycline) were trypsinized and kept in suspension for 1 hour. Thereafter, cells were replated on collagen-coated plastic culture dishes. (A) At the indicated time points after replating, the number of attached cells was counted. (B) Immunofluorescence analysis of the attached tetFRNK-MTLn3 cells 4 hours after replating by confocal laser scanning microscope (CLSM) with anti-pTyr397-FAK, anti-paxillin, and anti-HA antibodies. (C) Confluent tetFRNK-MTLn3 cell layers (with or without doxycycline) were wounded as described in Materials and Methods. At the indicated time points, phase-contrast pictures of the wounds were taken. (D) Cell motility was quantified by measuring wound width at three different locations in the wound. Columns: mean of three independent experiments. Bars: SE.

HA-FRNK reduces primary tumor growth.

Next, the effect of HA-FRNK on the *in vivo* behavior of MTLn3 cells was studied. We first tested the effect of inhibition of FAK function on primary tumor formation. For this purpose, tetFRNK-MTLn3 cells were injected into the fat pad of female Fischer 344 rats and doxycycline was added to the drinking water of the experimental animals. The tumors of the doxycycline-exposed animals showed a significant reduction in volume compared to tumors of animals that did not receive doxycycline. After 30 days, this reduction in volume corresponded to a reduction in tumor weight (Fig. 3A). The expression of HA-FRNK in the tumors of the doxycycline-exposed animals was confirmed by Western blot analysis; control animals did not express HA-FRNK (Fig. 3B). Doxycycline itself did not affect tumor formation or the size of the formed MTLn3 tumors (Supplemental fig. S3A). To check the effect of HA-FRNK on cell survival *in vivo*, tumor sections were stained with antibodies directed against active caspase-3. Only a few caspase-3-positive cells were observed and the percentage of active caspase-3-positive cells in the HA-FRNK-expressing tumors was comparable with that in the control tumors (Fig. 3C). This suggests that the HA-FRNK-induced tumor reduction is most likely due to effects on proliferation rather than apoptosis.

HA-FRNK inhibits experimental lung metastasis formation.

Next, we investigated the effect of HA-FRNK on metastasis formation. tetFRNK-MTLn3 cells were injected into the tail vein of rats to induce experimental lung metastases. To improve the efficiency of *in vivo* experimental metastasis formation, we depleted NK cells from the Fischer 344 rats, thereby preventing NK cell–mediated killing of circulating MTLn3 cells.[1] An advantage of the HA-FRNK-inducible cell line is that HA-FRNK can be expressed after the tumor cells have been injected. This allowed us to discriminate between the effect of inhibition of FAK on attachment, invasion, and secondary tumor formation by exposing three groups of rats to doxycycline at different time points (i.e., days 0, 1, and 11) after injection of the tetFRNK-MTLn3 cells. The control group did not receive doxycycline. Lungs of animals injected with doxycycline-exposed cells that were continuously exposed to doxycycline (the day 0-group), contained 14% of surface tumors compared to the control animals (Fig. 4A). Induction of the expression of HA-FRNK starting *in vivo* with no doxycycline pretreatment *in vitro* (the day 1-group) resulted in a lung tumor metastasis burden of ~41% relative to control.

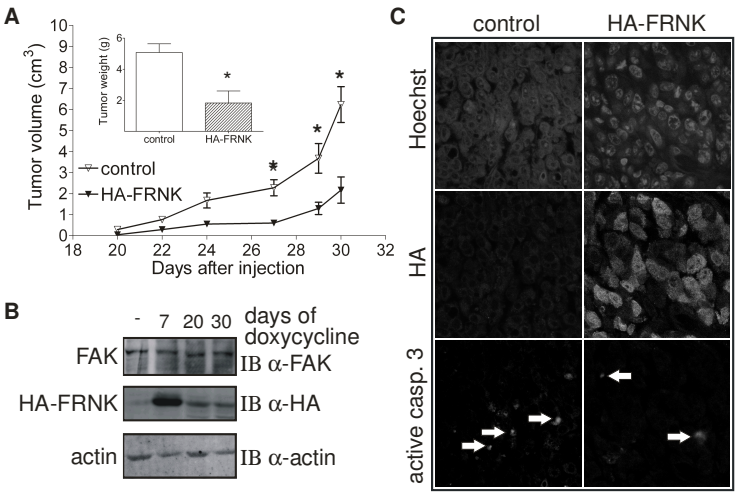

Figure 3. HA-FRNK decreases primary breast tumor growth without affecting apoptosis. Female Fischer 344 rats were injected with $1x10^6$ tetFRNK-MTLn3 cells (either left untreated or pretreated *in vitro* with doxycycline (1 μg/ml)) into the mammary fat pad and tumor growth was assessed by measuring tumor size as described in Materials and Methods. (A) At day 30, tumors were isolated and tumor weight was determined (inset). Points/columns: mean of five animals. Bars: SE. (B) Cryosections of primary tumor material of animals that were exposed to doxycycline for 7, 20, or 30 days or left untreated were analyzed for FAK and HA-FRNK expression by immunoblotting with anti-HA and anti-FAK antibodies. (C) Immunofluorescence analysis of Carnoy's fixed breast tumor sections of control and doxycycline-exposed animals with antibodies directed against HA (green) and active caspase-3 (red). Hoechst 33258 (blue) was used for nuclear staining. White arrows: apoptotic cells. Note that no increase in apoptosis in HA-positive cells is seen.

When doxycycline was added to the drinking water from day 11, a non-significant decrease in the number of lung metastases was seen: 69% of the control (Fig. 4A). H&E staining of lung tissue showed that the effect of HA-FRNK on the number of surface metastases was a reflection of the metastasis burden inside the lungs (Fig. 4B). In a control experiment, no statistically significant difference in lung tumor metastasis burden of the founder cell line Tet-on-MTLn3 clone 25 was found between animals that were treated or left untreated with doxycycline (Supplemental fig. S3B). This indicates that the effect observed in tetFRNK-MTLn3 cells is

mediated by HA-FRNK. Next, we verified the expression of HA-FRNK in the metastases. Cryosections of the lungs of the two animals with the highest number of surface metastases out of the six that received doxycycline both *in vitro* and *in vivo*, (for median and range, see Table 1), were analyzed by Western blotting and clearly showed HA-FRNK expression (Fig. 4C). In addition, the analyzed lungs of animals that were exposed to doxycycline at later time points also expressed HA-FRNK (Fig. 4C).

HA-FRNK reduces cell growth both *in vitro* (Fig. 1A) and *in vivo* in the primary tumor model (Fig. 3A). The average size of the HA-FRNK-positive lung tumor metastases was significantly decreased when the animals were directly exposed to doxycycline regardless of doxycycline pretreatment *in vitro*. This was not seen when HA-FRNK was expressed 11 days after injection of the cells (Table 1). This further supports the *in vitro* and primary tumor *in vivo* observations. Immunofluorescence analysis of the lungs revealed that in all animals exposed to doxycycline, HA-FRNK was expressed in the lung metastases (Fig. 4D). Fifty to seventy percent of the lung metastases contained HA-FRNK-positive cells when doxycycline was provided *in vitro* and *in vivo* from day 0 (Table 1). For doxycycline treatment that started only *in vivo* from day 1, 70 to 90% of the metastases had HA-FRNK-positive cells. This number was 80 to 90% when animals were treated from day 11 with doxycycline (Table 1). No evident changes in the structure of the tumors were observed between all the different groups.

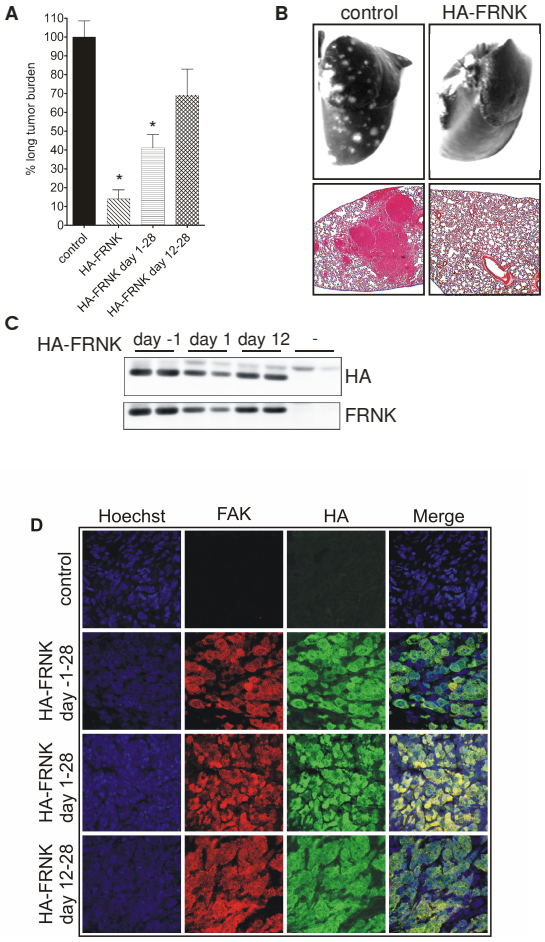

Figure 4. Inhibition of FAK strongly reduces the number of experimental lung metastases. tetFRNK-MTLn3 cells ($1x10^5$), either pretreated *in vitro* overnight with doxycycline (1 µg/ml) or left untreated, were injected into the tail vein of female Fischer 344 rats. When indicated, drinking water of the animals was supplemented with doxycycline to induce the expression of HA-FRNK. (A) After 28 days, lungs were injected with ink and the number of surface tumors was counted and is displayed as a percentage of the lung tumor burden in the control animals (no doxycycline exposure). (B) Representative images of lungs of control animals and animals that were continuously exposed to doxycycline. Top: surface lung metastases. Bottom: lung sections stained with H&E. (C) Immunoblotting of lung tissue cryosections for HA-FRNK expression using antibodies directed against HA and COOH-terminal FAK. For each treatment group, different animals were chosen that possessed lung tumor metastases. (D) HA-FRNK expression in lung metastases was verified by CLSM after immunofluorescence staining for FRNK and HA; nuclear counter-staining was with Hoechst 33258. Columns: mean. Bars, SE.

Table 1. Effect of HA-FRNK expression on tetFRNK-MTLn3 experimental metastasis formation

Group	*Incidence**	*Median # mets (range)*	*Significance †*	*Metastasis size (size class) ‡*	*Significance §*	*% range HA-FRNK positive mets ‖*
A no doxy	**11/11**	**130 (57-180)**		**2.7±0.3**		**0**
B doxy in vitro	**5/6**	**17 (0-42)**	**p<0.0005**	**0.4±0.2**	**p<0.001**	**50-70**
C doxy d0	**13/13**	**63 (3-128)**	**p<0.05**	**1.5±0.2**	**p<0.01**	**70-90**
D doxy d11	**12/13**	**100 (0-220)**	**NSD**	**2.5±0.2**	**NSD**	**80-90**

* Incidence, number of animals with visible lung surface metastases at day 28/ total number of animals in the group.

† Statistical significance was determined by the Mann-Whitney Rank Sum test; NSD, no statistical difference for group D lung tumor burden compared to the lung burden in the control group A. p indicates the confidence level for the statistical difference between group C or B and control group A.

‡ Metastasis size is determined by classification of the size of the metastases in H&E stained lung sections in four groups: ranging from 0 (very small) to 3 (very large).

§ Statistical significance of the difference in metastasis size was determined by Student's t-test; NSD, no statistical difference in metastasis size between group D and A. p indicates the confidence level for the statistical difference between group C or B and control group A.

‖ Range of the percentage of HA-FRNK-positive cells per metastasis.

Inhibition of lung metastasis formation by HA-FRNK is not caused by tumor cell dormancy.

Inhibition of FAK is known to induce dormancy, which can be overcome by activating the downstream extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway (14). To determine whether HA-FRNK expression resulted in dormant MTLn3 cells that were still able to grow out into metastases when FAK was no longer inhibited, we did another experimental metastasis formation experiment. Again, tetFRNK-MTLn3 cells (either preincubated with doxycycline *in vitro* or left untreated) were injected into the tail vein of female Fischer 344 rats. One group was exposed to doxycycline for the duration of the experiment, the other group was kept on doxycycline-containing drinking water only for the first 5 days after tumor cell injection. The animals that were continuously exposed to doxycycline had only 5% of the tumor burden compared to the control animals (Fig. 5A). When HA-FRNK was only expressed for the first 5 days of the experiment, the lung tumor burden was 13% of the control. Immunohistochemical analysis of the lungs showed HA-FRNK-positive tumor cells in animals continuously exposed to doxycycline, whereas no HA-FRNK could be detected in either control animals or animals that were exposed to doxycycline during the first 5 days of the experiment (Fig. 5B).

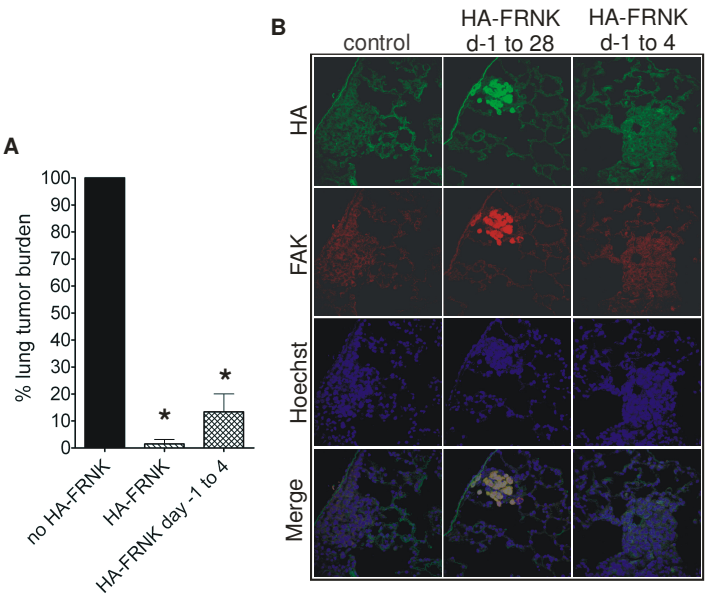

Figure 5. Inhibition of tumor metastasis formation by HA-FRNK is not related to MTLn3 cell dormancy. tetFRNK cells were pretreated with doxycycline *in vitro* or left untreated, followed by injection into the tail vein. Rats were either continuously treated with doxycycline in the drinking water or only for the first 5 days. Control animals did not receive doxycycline. (A) The number of surface tumors was counted after 28 days and is displayed as a percentage of the lung tumors in the control animals (no doxycycline). Columns: mean (n = 8). Bars: SE. (B) To verify HA-FRNK expression, tissue sections were immunostained with anti-COOH-terminal FAK and anti-HA followed by CLSM. Representative images of the different treatment groups. Note that the metastases in the animals that were exposed to doxycycline from days −1 to 4 no longer express HA-FRNK.

DISCUSSION

In this study, we inhibited FAK function in breast tumor cells by conditional expression of a dominant-negative-acting splice variant of FAK, FRNK. Using a syngeneic rat MTLn3 adenocarcinoma tumor/metastasis model, we obtained results that indicate that (*a*) FAK is important in primary breast tumor growth, (*b*) FAK is essential in breast tumor lung metastasis formation, (*c*) FAK-mediated control of metastasis occurs in the initial phase of the lung metastasis process, and (*d*) inhibition of FAK does not cause dormancy of tumor cells. These effects of FAK

are not dependent on the control of cell survival; FAK rather interferes with MTLn3 breast tumor cell migration and invasion. Our observations suggest that FAK is a potential target for therapeutic intervention in the attachment and invasion steps of metastasis formation.

We found that FAK is essential in the early phase of metastasis formation. This phase involves both invasion and migration processes, and leads to colonization of the lungs by MTLn3 breast tumor cells: inhibition of FAK during the first 5 days of metastasis formation dramatically reduced the number of experimental lung metastases (Fig. 4 and 5). The importance of FAK in the control of adhesion and migration of MTLn3 cells was supported by our *in vitro* observations: expression of HA-FRNK reduced attachment, spreading, and migration of the tumor cells. These observations agree with the effect of FRNK in various cell types *in vitro* (11,24–26). Localization of FRNK at focal adhesions seems crucial in the inhibition of *in vivo* metastasis formation. Wild-type FRNK reduced invasion and metastasis formation of v-Src-transformed 3T3 cells through a mechanism that is independent of effects on cell motility. The FRNK-L1034S mutant, which is impaired in focal adhesion localization and therefore cannot compete with FAK at focal adhesions, did not affect the metastatic phenotype of v-Src 3T3 cells (15). In the MTLn3 cells, HA-FRNK inhibits FAK localization at focal adhesions; prevention of this localization likely impairs the turnover of focal adhesions. In contrast to the v-Src 3T3 cells, in which FRNK does not reduce motility, HA-FRNK-expressing MTLn3 cells showed a reduced cell spreading and attenuated focal adhesion formation as well as a reduced cell migration. This distinct effect of FRNK on motility in the v-Src 3T3 versus MTLn3 cells is probably due to the v-Src transformation; v-Src can phosphorylate other targets (i.e., cdc42, p190Rho-GAP, Vav1/2, and ezrin) that (in)directly regulate actin cytoskeletal organization and migration (27). The role of FRNK in impairment, but not complete prevention, of FAK-mediated focal adhesion formation/turnover in our cells explains why MTLn3 experimental metastasis formation was not fully inhibited. We found that despite HA-FRNK expression at the time of the tail vein injection of the tumor cells, after 28 days still some HA-FRNK-positive tetFRNK-MTLn3 colonies could be found in a subset of metastases-bearing animals (Table 1). This indicates that also under *in vivo* conditions HA-FRNK inhibits but does not completely block focal adhesion turnover, a requirement for cell migration. Alternatively, given the fact that the levels of HA-FRNK expression after

doxycycline exposure vary within the population of tetFRNK-MTLn3 cells (Supplemental fig. S1B), it is possible that the formed HA-FRNK-positive metastases were derived from cells that initially expressed low levels of HA-FRNK and, as a consequence, had a higher change of invading the lungs. Based on our data, we think that FAK is essential in the metastasis process. Because we used an experimental metastasis model, we can only be certain that FAK is important in the later steps of the entire metastasic process: extravasation and tissue invasion. Because HA-FRNK inhibited tumor growth in the mammary fat pad, we were unable to study a role for FAK in the early steps of the metastasis formation process: detachment of the primary tumor and intravasation into the bloodstream.

Although our combined *in vitro* and *in vivo* data support a role for FAK in the migration and invasion processes involved in metastasis formation, the exact downstream substrates that mediate these effects remain to be identified. FAK interacts with c-Src to form a FAK/Src tyrosine kinase complex to phosphorylate downstream signaling (adaptor) proteins, including paxillin and p130Cas. Indeed, HA-FRNK expression inhibited the early tyrosine phosphorylation of paxillin (data not shown). Alternatively, FAK can function as a docking protein to target other kinases (or kinase-linkers) to the focal adhesions. In v-Src-transformed 3T3 cells, FRNK inhibited c-Jun NH_2-terminal kinase (JNK) activation in association with a reduced expression of matrix metalloproteinase-2; pharmacologic inhibition of JNK with SP600125 decreased the invasive phenotype of v-Src 3T3 cells (15). In tetFRNK-MTLn3 cells, no overall difference in the phosphorylation of JNK could be detected in the presence or absence of HA-FRNK (data not shown). However, because FAK binds to, for instance, MAPK kinase 1 and can thereby target other kinases, including JNK, to focal adhesions, loss of FAK at focal adhesions may indirectly affect the phosphorylation of proteins that are crucial in focal adhesion turnover. In this respect, we should note that paxillin can be phosphorylated at Ser178 by JNK; this is required for the turnover of focal adhesions (28). MTLn3 cells that stably overexpress a paxillinS178A mutant show impaired migration *in vitro*.[2] Possibly, the decreased cell motility caused by inhibition of FAK localization at focal adhesions by HA-FRNK is related to a decreased FAK-mediated targeting of JNK to the focal adhesions with reduced paxillin Ser178 phosphorylation and focal adhesion turnover as a consequence. By using genome-wide gene expression analysis of HA-FRNK-MTLn3 cells, we are currently also

trying to identify downstream targets that are involved in the HA-FRNK-induced inhibition of experimental metastasis formation.

Adhesion of cells to the ECM is an important cell survival signaling route, which is mediated in part by the activity of FAK. Various studies showed that inhibition of FAK, by expression of either FRNK or focal adhesion targeting domain or by down-regulation of FAK levels using small interfering RNA, resulted in an increase in apoptosis of both attached and suspended cells, including breast tumor cells (29–32). Transient transfection of MTLn3 cells with GFP-FRNK resulted in an increase in the percentage of apoptotic cells.[1] These apoptosis-inducing effects are probably the result of high levels of FRNK: increasing expression levels of GFP-FRNK resulted in increasing percentages of apoptotic cells, whereas cells with relatively low levels of GFP-FRNK were not susceptible to apoptosis. In tetFRNK-MTLn3 cells, the expression level of HA-FRNK is lower than the expression level of transiently-transfected GFP-FRNK; therefore, HA-FRNK was not able to induce apoptosis. This allowed us to study more subtle biological effects that are rather related to focal adhesion organization and turnover than to interference with cell survival signaling. Indeed, no effects of HA-FRNK expression were observed on the levels of apoptosis *in vitro* or on the primary tumor and lung metastasis *in vivo*. *In vivo*, neither prolonged expression of HA-FRNK (Fig. 3B) nor expression of HA-FRNK for only 3 days resulted in an increase in apoptosis of HA-FRNK-positive cells (data not shown). Because low levels of HA-FRNK affect migration, whereas high levels affect cell survival, a divergence in the signaling mechanism involved in both processes is likely. Therefore, although not all breast tumor cell lines are sensitive toward FRNK-mediated apoptosis (29), inhibition of FAK-mediated focal adhesion turnover may be a general strategy to prevent the attachment and invasion steps involved in the formation of metastases irrespective of apoptosis susceptibility.

In conclusion, the data indicate that proper FAK signaling at the focal adhesions is essential in the early steps of metastasis formation. Therefore, FAK should be recognized as an important therapeutic target for the development of anticancer drugs. Such a pharmacological modulation of FAK function would be sufficient at the level of the focal adhesions (i.e., by preventing FAK localization to the focal adhesions). Preferably, this would be mediated by small molecules that prevent FAK from binding to its partners, such as paxillin and talin. A selective inhibition of FAK localization would likely result in a reduced number of

unwanted side effects compared with the alternative approach, inhibition of overall FAK activity by blocking kinase activity.

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FOOTNOTES

[1] van Nimwegen et al., in preparation.

[2] Verkoeijen and Van de Water, in preparation.

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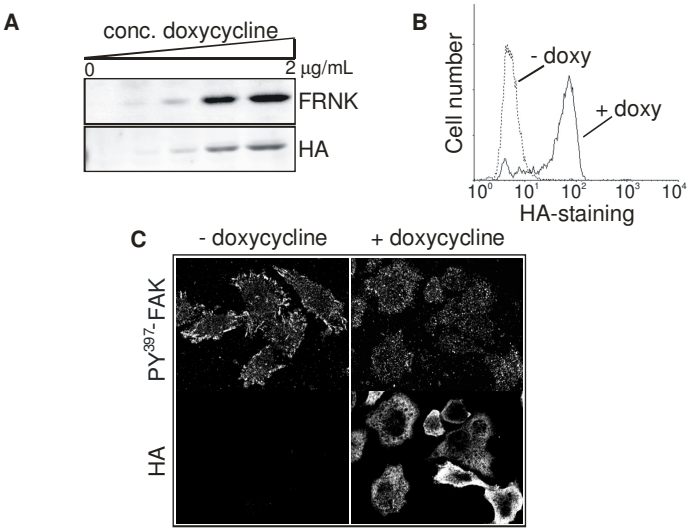
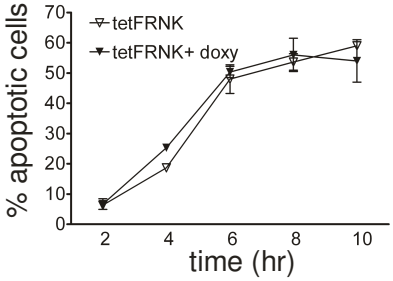
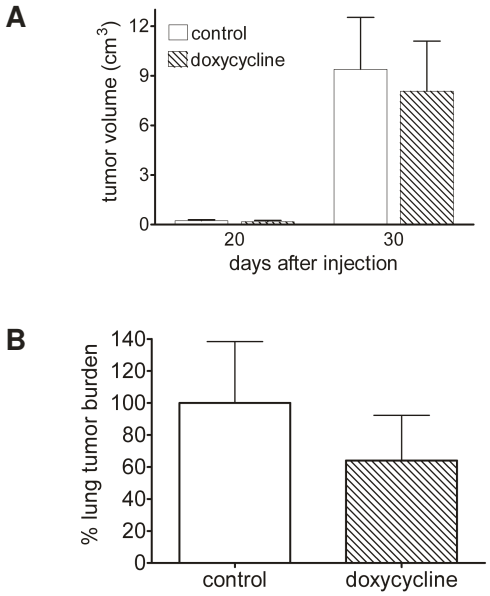
SUPPLEMENTAL DATA



Supplemental figure 1. Characterization of tetFRNK-MTLn3 cells. (A) TetFRNK-MTLn3 cells were exposed to increasing concentrations doxycycline (0, $1x10^{-2}$, $1x10^{-1}$, 1 and 2 µg/ml) for 24 hours and cell lysates were immunoblotted for C-terminal FAK and HA. (B) TetFRNK-MTLn3 cells (control (dotted) or exposed to doxycycline (bold)) were fixed and stained for intracellular HA expression and subsequently analyzed by flow cytometry as described in Materials and Methods. (C) Immunofluorescence analysis with antibodies directed against FAK-PY397 and HA of tetFRNK-MTLn3 cells exposed to 1 µg/ml doxycycline for 24 hours.



Supplemental figure 2. HA-FRNK expression does not affect anoikis. TetFRNK-MTLn3 cells were kept in tubes and incubated on a rollerbank for the indicated periods of time. The amount of anoikis was determined by annexin V/PI staining and flow cytometric analysis. Data represent three independent experiments ± SEM.



Supplemental figure 3. Doxycycline does not affect primary tumor growth and experimental lung metastasis formation. (A) Tet-on cells were injected into the fat pad of female Fischer 344 rats. One group of rats was continuously exposed to doxycycline. At day 20 and day 30 tumor diameter was determined and tumor volume was calculated. (B) Tet-on cells were injected into the tail vein of female Fischer 344 rats. One group of rats was continuously exposed to doxycycline. At day 28 the lungs were isolated and the number of surface tumors was determined. The number of lung metastases in the control group was set to 100%. Data represent mean ± SEM (n=8).