Am J Physiol Lung Cell Mol Physiol 299: L393–L400, 2010.
First published June 25, 2010; doi:10.1152/ajplung.00437.2009.

Effect of doxycycline on proliferation, MMP production, and adhesion in LAM-related cells

William Y. C. Chang, Debbie Clements, and Simon R. Johnson

Division of Therapeutics and Molecular Medicine and Respiratory Biomedical Research Unit, University of Nottingham, Queen's Medical Centre, Nottingham, United Kingdom

Submitted 29 December 2009; accepted in final form 25 June 2010

Chang WY, Clements D, Johnson SR. Effect of doxycycline on proliferation, MMP production, and adhesion in LAM-related cells. *Am J Physiol Lung Cell Mol Physiol* 299: L393–L400, 2010. First published June 25, 2010; doi:10.1152/ajplung.00437.2009.—Matrix metalloproteinases (MMPs) have been implicated in lung cyst formation in lymphangioleiomyomatosis (LAM). As doxycycline inhibits MMP activity in vivo, some patients take doxycycline, as one report has suggested a possible benefit in LAM. However, there have been no randomized controlled clinical trials of doxycycline for LAM, and any mechanism of action is unclear. Here, we examine previously proposed mechanisms of actions. Cell proliferation and adhesion were examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and Cytomatrix cell adhesion kits. Apoptosis was examined by TdT-mediated dUTP nick end labeling (TUNEL) assay. MMP-2 expression was examined by quantitative real-time PCR and zymography in doxycycline-treated ELT3 cells and tumor growth using angiomyolipoma-derived tumor xenografts in nude mice. In ELT3 cells, ≥25 μg/ml doxycycline decreased proliferation, increased apoptosis, and caused a change in cell morphology associated with redistribution of actin stress filaments. Reduction in proliferation was also seen in human angiomyolipoma-derived cells. Cell adhesion to ECM proteins was decreased by doxycycline at 50 μg/ml and prevented detachment of already adherent cells. There was no effect of doxycycline on MMP-2 expression or activity in vitro. In the xenograft model, doxycycline (30 $mg \cdot kg^{-1} \cdot day^{-1}$) had no effect on tumor growth, final tumor weight, or tumor lysate MMP levels. Doxycycline at doses ≥ 25 μg/ml inhibited cell proliferation and adhesion, possibly by a toxic effect. Doxycycline had no effect on MMP-2 expression or activity or tumor growth in the xenograft model. Any possible in vivo effect is unlikely to be mediated by MMP-2 or reduced cell proliferation.

lymphangioleiomyomatosis; matrix metalloproteinases

LYMPHANGIOLEIOMYOMATOSIS (LAM) is a rare disease of the lungs and lymphatics occurring almost exclusively in women. LAM occurs sporadically or in association with the autosomal dominant condition tuberous sclerosis complex (TSC). Both sporadic and TSC LAM are associated with mutations in the tuberous sclerosis genes (2). LAM affects women of childbearing age and nearly always presents before menopause (21, 35). It is characterized by the abnormal growth of atypical smooth muscle-like LAM cells in the lungs and axial lymphatics, leading to progressive cystic destruction of the lung parenchyma, obstruction of lymphatics and airways, and in most cases progressive respiratory failure (22). Currently, there are no proven treatments for LAM.

Address for reprint requests and other correspondence: W. Y. C. Chang, Division of Therapeutics and Molecular Medicine, Univ. of Nottingham, D Floor, South Block, Queen's Medical Centre, Nottingham, NG7 2UH UK (e-mail: william.chang@nottingham.ac.uk).

Recent interest in the possible role of matrix metalloproteinases (MMPs) in the pathogenesis of cystic lung destruction in LAM offers a potential therapeutic target (26). MMPs are a family of enzymes for which the main function is to degrade components of the ECM, which are involved in the turnover of molecules such as collagens, elastin, proteoglycans, and glycoproteins (29, 41). MMPs are present in normal tissues and are secreted in a latent form as inactive zymogens, which require activation for proteolytic activity. They are inhibited by tissue inhibitors of metalloproteinases (TIMPs), which play an important part in their regulation. Imbalance between MMPs and their inhibitors has been implicated in the pathogenesis of other lung disorders, including asthma and COPD (40), idiopathic pulmonary fibrosis, diffuse alveolar damage, and Langerhans cell histiocytosis (LCH) (15). Protease imbalance has been described in LAM lesions: overexpression of membrane type 1 MMP, a membrane-associated protein that activates MMP-2, has been demonstrated in spindle-shaped LAM cells (26). A rapamycin-insensitive increase in MMP-2 expression has been described in a TSC2-null immortalized angiomyolipoma (AML)-derived cell line from a LAM patient (24). Increased expression of MMP-1, -2, and -9 has also been demonstrated in LAM tissues (18), whereas TIMP-3 is repressed in LAM cells through the aberrant induction of serum response factor, a transcription factor normally expressed in immature smooth muscle cells (18, 42). As MMP-related alterations of the ECM in malignant tumors contribute to cell migration, invasion, and metastasis (4), a similar mechanism may facilitate LAM cell migration and entry into the circulation (17).

Doxycycline, a tetracycline antibiotic, has been shown to inhibit some MMPs independent of its antimicrobial activity (14, 34). In some cell types, doxycycline can directly inhibit the catalytic activity of MMPs (11) or pro-MMP activation, resulting in diminished activation, accelerated enzyme degradation, and loss of enzymatic activity (36). Tetracyclines are also known to downregulate gene expression (39) and scavenge the action of oxygen species that play a role in the activation of procollagenases and progelatinases in vitro (31). Doxycycline also modulates other cellular functions including proliferation and matrix remodeling (7). Doxycycline has already been trialed with some success in the treatment of pulmonary capillary hemangiomatosis (8), where angiogenesis is known to be dependent on MMP activity. A case report suggested that doxycycline treatment in LAM was associated with improvement in forced expiratory volume in 1 s (FEV_1) and reduction in urine MMP levels (27). An increase in shuttle walk distance was also seen in a preliminary study of 10 patients presented at the 2006 LAM Foundation International Research Conference in Cincinnati, Ohio. However, there is

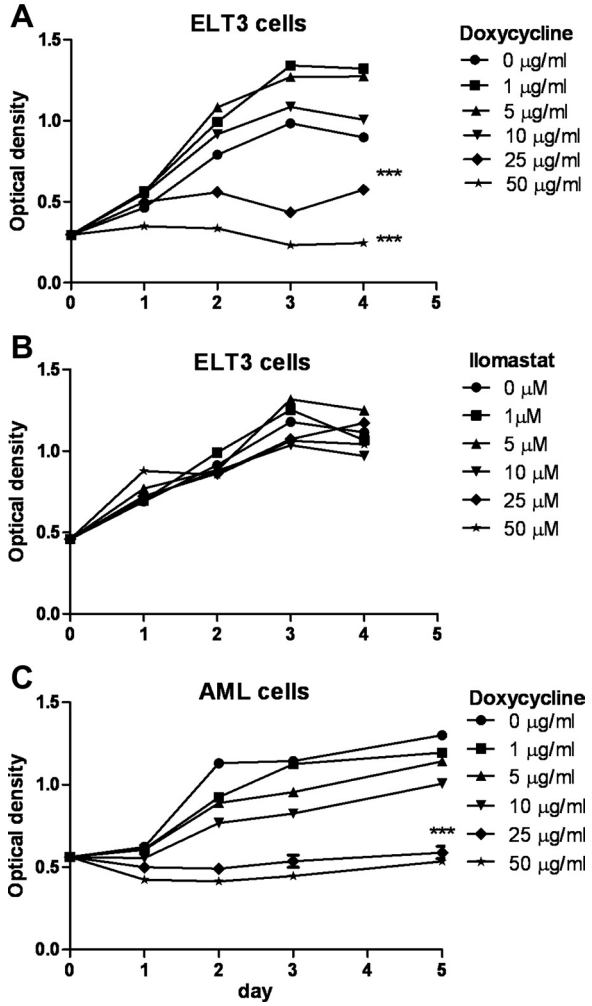

Fig. 1. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay to assess cell proliferation in ELT3 cells at 0, 1, 2, 3, and 4 days cultured in serum-free media with 0–50 μg/ml doxycycline (*A*) and 0–50 μM ilomastat (*B*) and primary angiomyolipoma (AML) cells (*passage 2*) cultured in media with 15% FBS and 10 ng/ml EGF (*C*). These data presented are from single experiments representative of 3 to illustrate the trends seen. ***$P < 0.001$ from *day 2*.

currently insufficient evidence to say whether treatment with doxycycline will have any role in the treatment of LAM. Despite this, a significant number of patients are taking doxycycline treatment for LAM.

In rat vascular smooth muscle cells (7), 104 μM doxycycline (~50 μg/ml) was found to increase cell adhesion and decrease cell migration and proliferation and inhibit MMP-2 and -9 activity in vitro. In human skin keratinocytes, 50 μg/ml doxycycline was found to inhibit MMP-2 expression (39). In our in vitro model of LAM, we sought to determine whether these previously described effects of doxycycline that are the basis for some of the assumed mechanisms of the action of doxycycline in LAM are real or due to nonspecific effects. Additionally, we used an in vivo model to examine the effects of doxycycline on MMP activity and tumor growth. The focus of this study therefore is to examine the previously documented actions of doxycycline in vitro and in vivo on processes relevant to LAM disease.

METHODS

Doxycycline preparation. Doxycycline hydrochloride (also known as doxycycline hyclate; Sigma-Aldrich) is water soluble. All concentrations of doxycycline were prepared freshly for each experiment using tissue culture medium as the vehicle. Therefore, in experiments where 0 μg/ml doxycycline was used, tissue culture medium alone was added.

Cell culture. ELT3 cells are a TSC2-null smooth muscle-derived cell line from an Eker rat spontaneous uterine leiomyoma (20) and have been used as a model for LAM in other studies (12, 13). ELT3 cells were obtained from Dr. Cheryl Walker (MD Anderson Cancer Centre, Smithville, TX), grown in phenol red-free DMEM/F-12 with 10% FBS at 37°C and 5% CO_2, and used between *passages 35* and *40*. For all experiments, cells were seeded at a density of 1×10^4 cells/cm^2. All experiments were performed at subconfluence as ELT3 cells proliferate rapidly and do not growth-arrest easily.

Human airway smooth muscle (ASM) cells were grown from explants from large airways of surgical resection specimens according to a method described by Daykin et al. (5). Ethical permission was received from the Nottingham Research Ethics Committee. Cells were grown in phenol red-free DMEM/F-12 medium with 10% FBS at 37°C and 5% CO_2 and were used between *passages 4* and *8*. For all experiments, cells were seeded at a density of 1×10^4 cells/cm^2 and used at confluency.

Human AMLs from patients with LAM or tuberous sclerosis were collected from excess material obtained for clinical purposes from patients on the United Kingdom LAM database. Ethical approval for the United Kingdom LAM database was obtained from the Trent Multi-Centre Research Ethics Committee, and the use of AML tissue and primary AML cell culture from the Nottingham Research Ethics Committee. Informed consent was obtained. Primary cells were extracted from AMLs and characterized by RT-PCR for a panel of markers including Gp100, Mart1, and estrogen receptor and by Western blotting to demonstrate constitutively active p70 S6 kinase under conditions of prolonged serum withdrawal as previously described (3). Cells were grown in phenol red-free DMEM/F-12 medium with 15% FBS and 10 ng/ml EGF at 37°C and 5% CO_2 and were used between *passages 2* and *3*. For all experiments, cells were seeded at a density of 1×10^4 cells/cm^2 and used at subconfluence after 1 wk.

Proliferation assay. ELT3 and AML cells were seeded on 6-well tissue culture plates in phenol red-free DMEM/F-12 medium with 10% FBS for 24 h. ASM cells were seeded on 6-well tissue culture plates in phenol red-free DMEM/F-12 medium with 10% FBS and AML cells with phenol red-free DMEM/F-12 medium with 15% FBS and 10 ng/ml EGF on 6 wells of a 96-well plate until subconfluent. Cells were then growth-arrested in serum-free medium for 24 h before

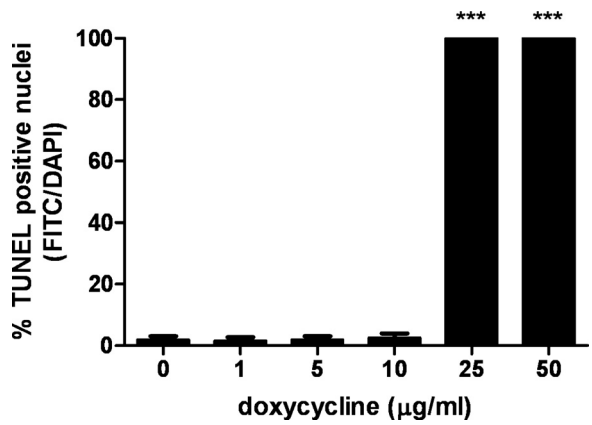

Fig. 2. Apoptosis assay on ELT3 cells cultured in 10% FCS and 0–50 μg/ml doxycycline at 48 h. ***$P < 0.001$. TUNEL, TdT-mediated dUTP nick end labeling; DAPI, 4′,6′-diamidino-2-phenylindole.

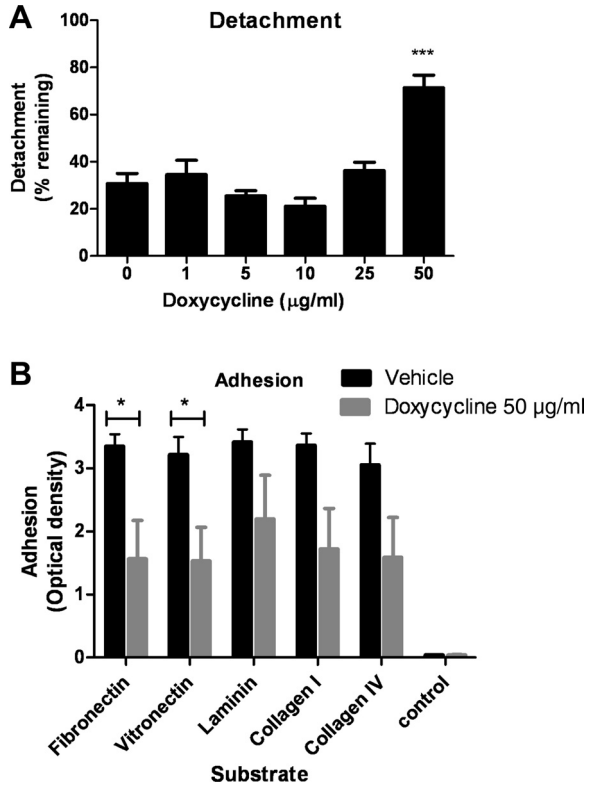

Fig. 3. *A*: percentage of ELT3 cells remaining adherent after 5-min exposure to nonenzymatic detachment solution. Error bars = SE, $n = 3$. ***$P < 0.001$. *B*: cell adhesion following 2-h exposure to 50 μg/ml doxycycline (gray bars) and no doxycycline (black bars). Error bars = SE, $n = 3$. *$P < 0.05$.

exposure to doxycycline for ≤96 h. Proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (28) at 0, 24, 48, 72, and 96 h.

Zymography. Gelatinolytic activity of cell culture supernatants, serum, and cell lysates was analyzed by gelatin zymography as described previously (16) using precast Novex polyacrylamide zymogram gels (Invitrogen, Paisley, United Kingdom). Supernatants were used undiluted; serum and cell lysates were diluted 1:40 with distilled water before use. Semiquantitative analysis of gelatinolytic activity was performed by densitometry using computer imaging software (ImageJ 1.38x; W. S. Rasband, National Institutes of Health, Bethesda, MD; http://rsbweb.nih.gov/ij/).

Cell adhesion assay. Cell adhesion was assessed using the Cytomatrix screening kit (Chemicon) according to the manufacturer's instructions. ELT3 cells were grown to 50% confluence, serum-depleted, and then treated with either 50 μg/ml doxycycline or vehicle for 48 h. Cells were removed using nonenzymatic detachment solution (Sigma-Aldrich), washed twice, resuspended in DMEM without serum containing either 50 μg/ml doxycycline or vehicle (tissue culture medium alone), and plated at a density of 1×10^4 cells/well onto 96-well plates coated with fibronectin, vitronectin, laminin, collagen I, and collagen IV or albumin. After 4 h, the medium was removed, and plates were washed twice in Dulbecco's PBS. Cells were stained using crystal violet solution, gently washed 3 times with PBS, and crystal violet-solubilized, and absorbance was read at 540 nm.

Cell detachment assay. ELT3 cells were seeded onto 12-well plates and used at subconfluence. After 24 h, cells were serum-starved for 24 h before adding medium containing 0, 1, 5, 10, 25, or 50 μg/ml doxycycline. For each 12-well plate, 2 wells of each concentration were used. At 48 h, the medium from 1 well of each concentration was aspirated, and the wells were washed 3 times with Dulbecco's PBS warmed to 37°C. The cells were then stained using 0.2% (vol/wt) crystal violet solution in 18% (vol/vol) ethanol for 5 min followed by 3 washes with PBS. The remaining 6 wells were washed 3 times with warmed Dulbecco's PBS and exposed to nonenzymatic detachment solution (Sigma-Aldrich) for 5 min. The cells were then washed and stained as above. The crystal violet was solubilized using 50 mM NaH_2PO_4 in 25% (vol/vol) ethanol (pH 4.5), and absorbance was read at 540 nm. The data were presented as percentage of absorbance after detachment/absorbance before detachment to give an estimation of cells remaining. All experiments were repeated in triplicate.

Apoptosis assay. Apoptosis was assessed using a TdT-mediated dUTP nick end labeling (TUNEL)-based fluorescein in situ cell death detection kit (Roche) according to the manufacturer's instructions (38). In brief, ELT3 cells were seeded on an 8-well chamber slide in DMEM with 10% FCS. After 24 h, cells were serum-starved for 24 h, and the medium was replaced with DMEM containing 0, 1, 5, 10, 25, or 50 μg/ml doxycycline. At 48 h, the cells in wells were air-dried and fixed with 3.7% formaldehyde for 1 h at room temperature and then washed twice with Dulbecco's PBS. The cells were permeabilized

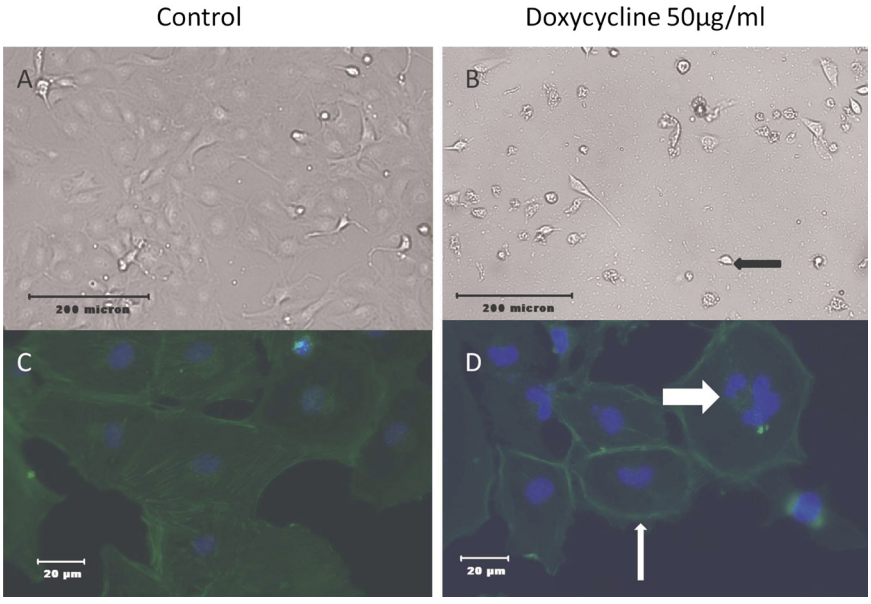

Fig. 4. ELT3 cells cultured in serum-free DMEM (*A* and *C*) and with additional 50 μg/ml doxycycline (*B* and *D*) for 48 h where the cells have become small and rounded (black arrow). *C* and *D* illustrate fluorescence for actin and nuclei using phalloidin and DAPI, respectively. In *D*, multinucleate cells (thick white arrow) and disrupted stress filaments (thin white arrow) can be seen.

with 0.1% (vol/vol) Triton X-100 and 0.1% (vol/wt) sodium citrate in PBS for 2 min at 4°C. The chambers were treated with TUNEL reaction mixture and left to incubate in the dark at 37°C. A negative control was provided by a well treated with the addition of label solution in the absence of terminal transferase, and a positive control was provided by a well pretreated with DNase I (Qiagen, Crawley, United Kingdom). After incubation, cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) and viewed under an inverted microscope (Nikon Diaphot 300 with epifluorescent capabilities; Nikon, Kingston Upon Thames, United Kingdom). Six random fields were counted for FITC and DAPI staining, and the number of apoptotic cells was calculated as a percentage of FITC/DAPI cells.

Staining for actin. ELT3 cells were seeded directly onto glass coverslips at a density of 1×10^4 cells/ml. The cells were serum-starved for 24 h, and the medium was changed to contain 0, 1, 5, 10, 20, or 50 μg/ml doxycycline. At 48 h, the samples were fixed using 3.7% (vol/vol) formaldehyde at room temperature for 10 min and then washed twice with PBS and permeabilized using 0.1% Triton X-100 (vol/vol) in PBS for 5 min followed by 2 further washes in PBS. The cells were then preincubated with 1% (vol/wt) bovine serum albumin for 30 min at room temperature. Cells were stained for actin using FITC-labeled phalloidin (Sigma-Aldrich), counterstained with DAPI, and imaged using an inverted microscope (Nikon Diaphot 300 with epifluorescent capabilities) and Spot-Cam software.

Real-time RT-PCR. Relative quantification of ELT3 and MMP-2, -9, and -14 mRNA was performed using a SYBR Green quantitative PCR method relative to β_2-microglobulin. mRNA was extracted from the experimental samples using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Contaminating DNA was removed using DNase I (Qiagen), and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen). PCR was performed in triplicate with appropriate negative controls on a Stratagene Mx3005P QPCR System. PCR comprised 5 μl of cDNA (prediluted 25-fold with sterile distilled water), 12.5 μl of Brilliant SYBR Green QPCR Master Mix (Stratagene), 6.5 μl of sterile distilled water (Baxter), and 1 μl of 5 mM primer mix using the rat β-actin upstream primer 5′-GGGAAATCGTGCGTGACATT-3′ and downstream primer 5′-GCGGCAGTGGCCATCTC-3′ (6), the rat GAPDH upstream primer 5′-ATGATTCTACCCACGGCAAG-3′ and downstream primer 5′-CTGGAAGATGGTGATGGGTT-3′, the rat β_2-microglobulin upstream primer 5′-CGTGATCTTTCTGGTGCTTGTC-3′ and downstream primer 5′-TTCTGAATGGCAAGCACGAC-3′ (9), the rat MMP-2 upstream primer 5′-GACACGATGAAGGCTATGAGG-3′ and downstream primer 5′-CTGGATGCAGAAAGTGATCTC-3′, the rat MMP-9 upstream primer 5′-CTGGCAGAGGATTACCTGTACC-3′ and downstream primer 5′-CTGGCAGAGGATTACCTGTACC-3′, and the rat MMP-14 upstream primer 5′-GTTACACACCTGACCTGGACC-3′ and downstream primer 5′-GTTACACACCTGACCTGGACC-3′. Each PCR reaction generated only the expected amplicon as demonstrated by the melting temperature profiles of the final products. Results [threshold cycle (CT) values] were normalized against β_2-microglobulin and expressed as $2^{-\Delta CT}$ and $2^{-\Delta\Delta CT}$ (25). Identical results were obtained using GAPDH for normalization.

Xenograft model. In vivo studies were performed under United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines (United Kingdom Home Office project license no. 40/2323) using nude mice (Harlan) and SV7^{tert} AML cells [CRL-2461; American Type Culture Collection (ATCC)] as previously described (3). In brief, subcutaneous tumors were induced by subcutaneous inoculation of 2×10^6 cells in 100 μl of PBS in the left flank of female nude mice. They were then either given 30 mg/kg doxycycline daily or vehicle only by oral gavage. Tumor size was measured three times a week using calipers in two perpendicular dimensions. Mice were terminated when the tumor cross-sectional area reached 250 mm^2. On termination, tumors were excised, weighed, snap-frozen in liquid nitrogen, and stored at −70°C. Serum was also isolated and stored at −70°C.

Tumor lysates were prepared by homogenizing ~40-mg samples from the tumors in 200 μl of 0.1% SDS. The samples were pelleted, and the protein content of the supernatants was determined before zymography using the Bio-Rad assay. Serum samples were also analyzed by zymography after protein quantification.

RESULTS

Doxycycline inhibits cell proliferation at high dose. Treatment of ELT3 cells with increasing doses of doxycycline resulted in a decrease in cell proliferation at doses ≥ 25 μg/ml (Fig. 1*A*; $P < 0.01$ from *day 2* by 2-way ANOVA). There was no statistically significant difference at doses lower than this. To examine whether this effect was dependent on MMP activity, we used the MMP inhibitor ilomastat at doses shown to inhibit MMPs in tissue culture (16). Ilomastat did not effect cell proliferation at the doses shown (Fig. 1*B*). We next examined the affect of doxycycline in primary AML cells. Again, a decrease in cell proliferation was seen at doses ≥ 25 μg/ml (Fig. 1*C*; $P < 0.01$ from *day 2* by 2-way ANOVA).

Doxycycline induces apoptosis at high doses. To see whether the antiproliferative effects of doxycycline were due to

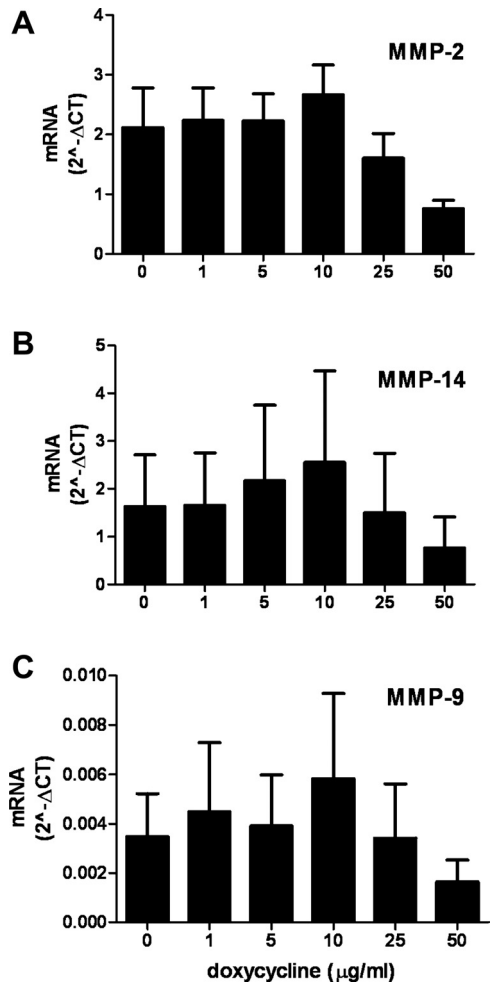

Fig. 5. Quantitative RT-PCR for matrix metalloproteinases MMP-2 (*A*), MMP-14 (*B*), and MMP-9 (*C*) in ELT3s cultured with serum-free DMEM and 0–50 μg/ml doxycycline at 48 h using β_2-microglobulin as the normalizing gene. Similar data were seen when normalized against GAPDH. There was no statistically significant difference seen, although there was a trend to decreased expression with ≥20 μg/ml doxycycline. CT, threshold cycle.

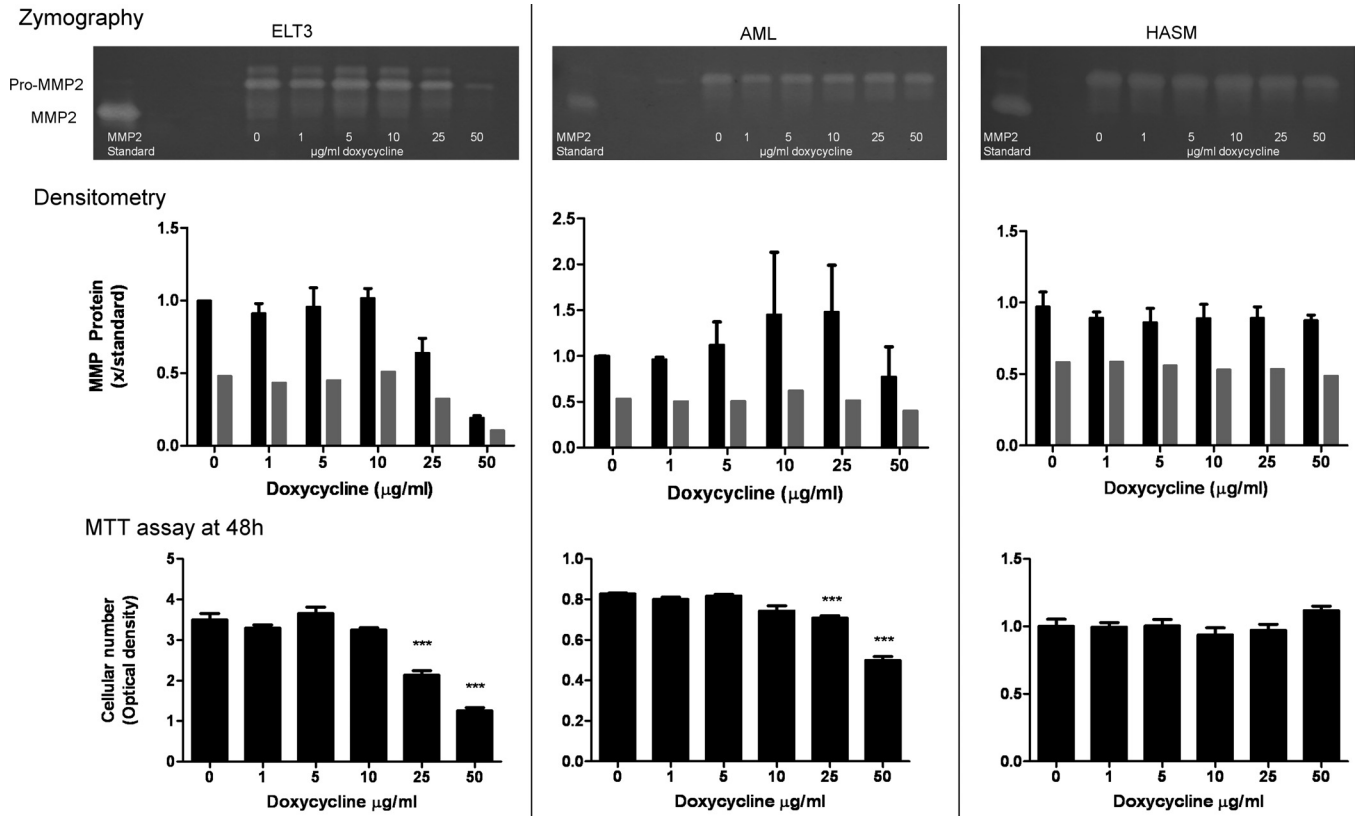

Fig. 6. MMP expression in ELT3s (*A*; $n = 3$) and AMLs (*B*) and human airway smooth muscle cells (HASM; *C*) ($n = 4$). The *top* row shows an example zymogram. The *middle* row shows densitometry (expressed as x/standard, where x = optical density) for total (black bars) and active (gray bars) MMP-2. Error bars = SE. ***$P < 0.001$.

cell death, we next examined whether doxycycline induced apoptosis in ELT3 cells. At concentrations of doxycycline <25 μg/ml, levels of apoptosis were low and did not differ from vehicle-treated cells. Exposure to ≥25 μg/ml doxycycline led to apoptosis in almost all cells ($P < 0.001$, 1-way ANOVA; Fig. 2).

Doxycycline increases cell adhesion. In previous experiments, it was noted that detachment of cells using both trypsin and nonenzymatic detachment solution was significantly prolonged following treatment with 50 μg/ml doxycycline. This effect was observed as early as 2 h after exposure to doxycycline and was not associated with a change in cell morphology. We went on to examine this formally. Cell attachment was significantly enhanced following exposure to 50 μg/ml doxycycline ($P < 0.01$, 1-way ANOVA; Fig. 3*A*). However, once cells had been detached, treatment with doxycycline resulted in

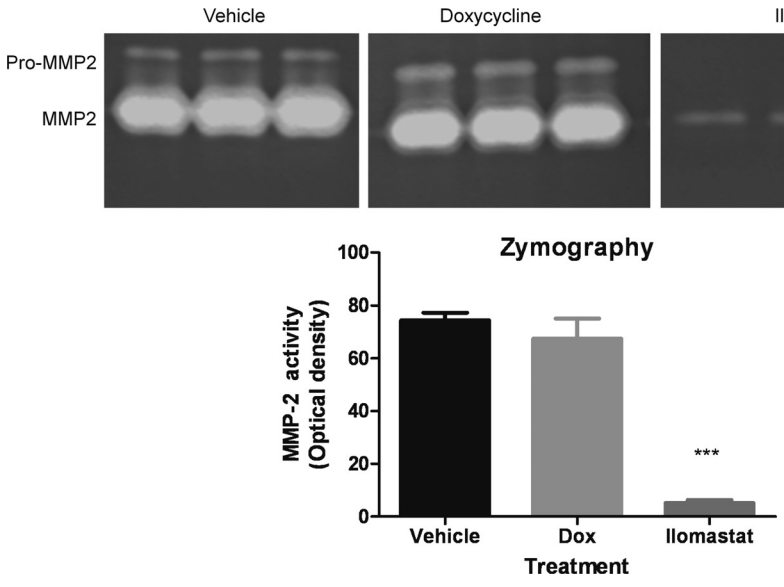

Fig. 7. Effect of ilomastat and doxycycline (Dox) on ELT3-derived MMP-2 gelatinolytic activity. Error bars = SE, $n = 3$. ***$P < 0.001$.

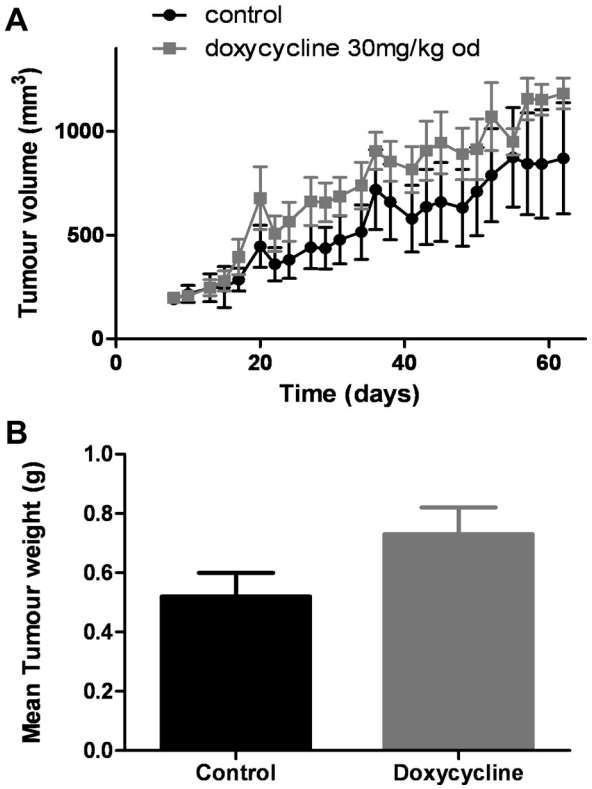

Fig. 8. Effect of 30 mg/kg per os (p.o.; orally) doxycycline daily on mean tumor volume over time (*A*) and end mean tumor weight at *day 62* (*B*) in a mouse xenograft model. Control $n = 5$, treatment $n = 6$, error bars = SE. od, Omne in die (once daily).

reduced adhesion on all ECM substrates tested other than the control (albumin) with statistical significance obtained for fibronectin ($P = 0.049$) and vitronectin ($P = 0.049$) (Fig. 3*B*).

Doxycycline alters cell morphology at high dose. ELT3 cells were treated with doxycycline (0–50 μg/ml) for 48 h. After 48 h, a change in cell morphology with the cells becoming rounded was observed with doses of doxycycline ≥ 25 μg/ml (Fig. 4*B*) compared with vehicle-treated cells (Fig. 4*A*). To determine whether this was an effect on the cytoskeleton, staining for F-actin with fluorescently labeled phalloidin was performed. In vehicle-treated cells, stress filaments were arranged along the long axis of the cell (Fig. 4*C*). In doxycycline-treated cells, staining was distributed adjacent to the cell membrane (Fig. 4*D*). In addition, counterstaining with DAPI revealed many multinucleate cells.

There is a trend to decreased expression of MMP-2 at ≥25 μg/ml doxycycline, but doxycycline does not significantly decrease MMP expression when corrected for cell number.

After 48-h exposure to doxycycline, there was a trend to decreased expression of MMP-2 mRNA with concentrations of ≥25 μg/ml doxycycline on quantitative RT-PCR using β_2-microglobulin for normalization. This was not of statistical significance, however (Fig. 5). Similar results were seen using GAPDH for normalization. Initial examination of MMP-2 and pro-MMP-2 gelatinolytic activity in ELT3 cells by zymography suggested a decrease in MMP-2 expression with doses of doxycycline ≥ 25 μg/ml (Fig. 6). However, this correlated with a decrease in total cell number, using the MTT reduction assay as a surrogate marker (Fig. 6). ELT3 cells expressed no MMP-9 protein, and, consistent with this, transcript levels of MMP-9 mRNA were 500-fold lower than MMP-2 mRNA (Fig. 5). A similar trend was seen with AML cells (Fig. 6). When the experiment was repeated with confluent, contact-inhibited human ASM cells, there was no effect on MMP-2 activity or cell number (Fig. 6).

Doxycycline does not directly inhibit MMP gelatinolytic activity. We next determined whether doxycycline could affect the activity of preformed MMP-2 in supernatants from ELT3 cells cultured in the absence of doxycycline by gelatin zymography. When doxycycline (50 μg/ml) was added to the zymogram developing buffer, there was no effect on MMP-2 gelatinolytic activity. However, when the MMP inhibitor ilomastat was added at a concentration of 50 μM, there was a strong reduction in gelatinolytic activity (Fig. 7; $P < 0.01$, 1-way ANOVA).

Doxycycline did not affect subcutaneous tumor volume or weight or tumor MMP activity in AML xenografts. To determine whether doxycycline is effective against a LAM-related lesion in vivo, we used a previously validated AML xenograft model (3). Over a 60-day period, there was no significant difference in tumor volume between the control and doxycy-

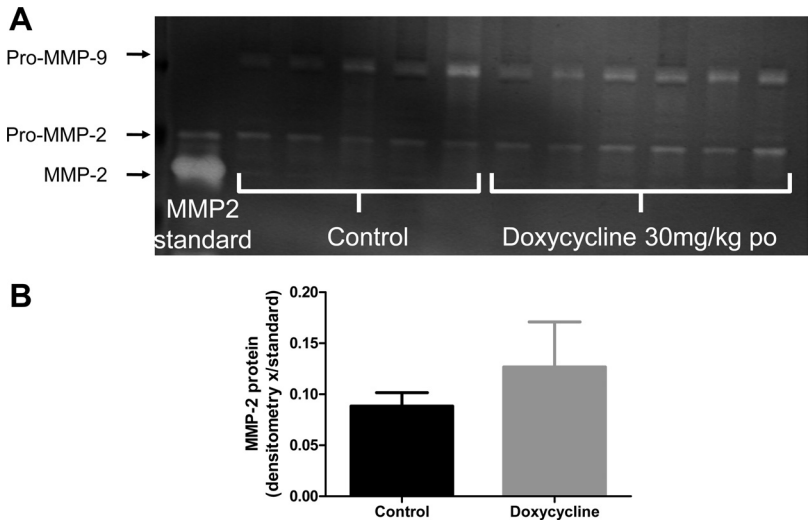

Fig. 9. Effect of 30 mg/kg p.o. doxycycline daily on gelatinase activity from tumor lysates. *A* shows zymography for gelatinase expression, and *B* the analysis via densitometry. No significant difference is seen for either MMP-2 or pro-MMP-2.

cline-treated mice (Fig. 8*A*) nor was there any significant difference in end tumor weight (Fig. 8*B*). Zymography of tumor lysates obtained at the end of the study also showed no significant difference in MMP-2 or -9 expression between treated and untreated mice (Fig. 9).

DISCUSSION

Our findings show that doxycycline decreased cell number, increased apoptosis, and caused changes in cell morphology at ≥25 μg/ml concentrations. The small effect of reduced MMP-2 production by doxycycline is likely to be due to this antiproliferative effect. Consistent with our observations, previous studies suggest that doxycycline inhibits cell proliferation in vitro. One study showed a 58% decrease in thymidine incorporation in rat vascular smooth muscle cells with 104 μmol/l (~50 μg/ml) doxycycline (7). Our data suggest that the antiproliferative effects of doxycycline are unlikely to be directly due to MMP inhibition as cell proliferation was not inhibited by the potent MMP inhibitor, ilomastat. Doxycycline toxicity may be a possible explanation for these findings, which would be consistent with the strong increase in apoptosis seen at these doses of doxycycline. Doxycycline had no effect on tumor volume or lysate MMP-2 or -9 expression in our tumor model, possibly because a previous study has suggested that mean plasma concentrations of humans treated with 100-mg doxycycline twice daily are in the range of 1.8–9.4 μg/ml (30), indicating that the positive results we have seen in vitro may only occur at supraphysiological doses. These findings are in keeping with those of Lee et al. (23), who showed that 10 mg/kg doxycycline did not decrease tumor volume in another xenograft model in nude mice.

The changes in cell morphology we have observed at concentrations of doxycycline ≥ 25 μg/ml have also been observed by other groups in fibroblasts (19) and rat vascular smooth muscle cells (7). In rat vascular smooth muscle cells treated with 31 μmol (~15 μg/ml) doxycycline, this was associated with increased adherence to tissue culture plates, increased areas of focal contact, reorganization of the actin cytoskeleton, and the formation of peripheral focal adhesions (7).

Although our data cast doubt on some of the proposed mechanisms of action of doxycycline, they do not explain the effects of doxycycline seen in vivo. Our in vitro models used a monoculture of TSC2-null cells, and in vivo there is likely to be an interplay between LAM cells and other cells in the lung parenchyma. In addition, interpretation of our data from our in vivo animal model must take into account the fact that the induced subcutaneous tumors were poorly vascularized and therefore may not have been exposed to significant concentrations of doxycycline. In addition, a subcutaneous xenograft will not exactly model the lung parenchyma in LAM. Unfortunately, there are currently no animal models of LAM lung disease in which we can test our hypothesis. However, prolonged administration of 100-mg doxycycline twice daily in patients with small asymptomatic abdominal aortic aneurysms has been associated with a reduction in plasma MMP-9 levels (1). Similarly, 20-mg doxycycline twice daily (a subantimicrobial dose) has been found to significantly reduce collagenase activity, thought to be predominantly due to MMP-8, in gingival crevicular fluid in postmenopausal osteopenic women (10). Doxycycline may therefore have a variety of other mechanisms of action that we have not examined here. Our intention was to examine its effects on the gelatinases MMP-2 and -9 based on previous studies, however, its mechanism of action could involve other MMPs, particularly the collagenases MMP-1, -8, and -13, as tetracyclines have been reported to inhibit collagen degradation (7). In addition, effects on angiogenesis (37) and mitochondrial function (33) have been reported and could be of relevance to LAM.

We therefore have not been able to find a clear mechanism for the doxycycline-sensitive elevation of MMPs seen in LAM, and further studies are needed in vivo and in vitro to establish a mechanism for these effects and to determine whether there is a therapeutic role for the use of doxycycline in LAM and other lung diseases characterized by increased MMP activity.

GRANTS

This work was supported by the British Lung Foundation, LAM Action, and the University of Nottingham.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

1. **Baxter BT, Pearce WH, Waltke EA, Littooy FN, Hallett JW Jr, Kent KC, Upchurch GR Jr, Chaikof EL, Mills JL, Fleckten B, Longo GM, Lee JK, Thompson RW.** Prolonged administration of doxycycline in patients with small asymptomatic abdominal aortic aneurysms: report of a prospective (Phase II) multicenter study. *J Vasc Surg* 36: 1–12, 2002.
2. **Carsillo T, Astrinidis A, Henske EP.** Mutations in the tuberous sclerosis complex gene TSC2 are a cause of sporadic pulmonary lymphangioleiomyomatosis. *Proc Natl Acad Sci USA* 97: 6085–6090, 2000.
3. **Clements D, Asprey SL, McCulloch TA, Morris TA, Watson SA, Johnson SR.** Analysis of the oestrogen response in an angiomyolipoma derived xenograft model. *Endocr Relat Cancer* 16: 59–72, 2009.
4. **Crawford HC, Matrisian LM.** Tumor and stromal expression of matrix metalloproteinases and their role in tumor progression. *Invasion Metastasis* 14: 234–245, 1994.
5. **Daykin K, Widdop S, Hall IP.** Control of histamine induced inositol phospholipid hydrolysis in cultured human tracheal smooth muscle cells. *Eur J Pharmacol* 246: 135–140, 1993.
6. **Depreter M, Vandesompele J, Espeel M, Speleman F, Roels F.** Modulation of the peroxisomal gene expression pattern by dehydroepiandrosterone and vitamin D: therapeutic implications. *J Endocrinol* 175: 779–792, 2002.
7. **Franco C, Ho B, Mulholland D, Hou G, Islam M, Donaldson K, Bendeck MP.** Doxycycline alters vascular smooth muscle cell adhesion, migration, and reorganization of fibrillar collagen matrices. *Am J Pathol* 168: 1697–1709, 2006.
8. **Ginns LC, Roberts DH, Mark EJ, Brusch JL, Marler JJ.** Pulmonary capillary hemangiomatosis with atypical endotheliomatosis: successful antiangiogenic therapy with doxycycline. *Chest* 124: 2017–2022, 2003.
9. **Goff LA, Bowers J, Schwalm J, Howerton K, Getts RC, Hart RP.** Evaluation of sense-strand mRNA amplification by comparative quantitative PCR. *BMC Genomics* 5: 76, 2004.
10. **Golub LM, Lee HM, Stoner JA, Sorsa T, Reinhardt RA, Wolff MS, Ryan ME, Nummikoski PV, Payne JB.** Subantimicrobial-dose doxycycline modulates gingival crevicular fluid biomarkers of periodontitis in postmenopausal osteopenic women. *J Periodontol* 79: 1409–1418, 2008.
11. **Golub LM, Ramamurthy NS, McNamara TF, Greenwald RA, Rifkin BR.** Tetracyclines inhibit connective tissue breakdown: new therapeutic implications for an old family of drugs. *Crit Rev Oral Biol Med* 2: 297–321, 1991.
12. **Goncharova EA, Goncharov DA, Eszterhas A, Hunter DS, Glassberg MK, Yeung RS, Walker CL, Noonan D, Kwiatkowski DJ, Chou MM, Panettieri RA Jr, Krymskaya VP.** Tuberin regulates p70 S6 kinase activation and ribosomal protein S6 phosphorylation. A role for the TSC2 tumor suppressor gene in pulmonary lymphangioleiomyomatosis (LAM). *J Biol Chem* 277: 30958–30967, 2002.

13. **Goncharova EA, Krymskaya VP.** Pulmonary lymphangioleiomyomatosis (LAM): progress and current challenges. *J Cell Biochem* 103: 369–382, 2008.
14. **Hanemaaijer R, Visser H, Koolwijk P, Sorsa T, Salo T, Golub LM, van Hinsbergh VW.** Inhibition of MMP synthesis by doxycycline and chemically modified tetracyclines (CMTs) in human endothelial cells. *Adv Dent Res* 12: 114–118, 1998.
15. **Hayashi T, Stetler-Stevenson WG, Fleming MV, Fishback N, Koss MN, Liotta LA, Ferrans VJ, Travis WD.** Immunohistochemical study of metalloproteinases and their tissue inhibitors in the lungs of patients with diffuse alveolar damage and idiopathic pulmonary fibrosis. *Am J Pathol* 149: 1241–1256, 1996.
16. **Henderson N, Markwick LJ, Elshaw SR, Freyer AM, Knox AJ, Johnson SR.** Collagen I and thrombin activate MMP-2 by MMP-14-dependent and -independent pathways: implications for airway smooth muscle migration. *Am J Physiol Lung Cell Mol Physiol* 292: L1030–L1038, 2007.
17. **Henske EP.** Metastasis of benign tumor cells in tuberous sclerosis complex. *Genes Chromosomes Cancer* 38: 376–381, 2003.
18. **Hill CS, Wynne J, Treisman R.** The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* 81: 1159–1170, 1995.
19. **Ho AT, Voura EB, Soloway PD, Watson KL, Khokha R.** MMP inhibitors augment fibroblast adhesion through stabilization of focal adhesion contacts and up-regulation of cadherin function. *J Biol Chem* 276: 40215–40224, 2001.
20. **Howe SR, Gottardis MM, Everitt JI, Goldsworthy TL, Wolf DC, Walker C.** Rodent model of reproductive tract leiomyomata. Establishment and characterization of tumor-derived cell lines. *Am J Pathol* 146: 1568–1579, 1995.
21. **Johnson S.** Rare diseases. 1. Lymphangioleiomyomatosis: clinical features, management and basic mechanisms. *Thorax* 54: 254–264, 1999.
22. **Johnson SR, Tattersfield AE.** Decline in lung function in lymphangioleiomyomatosis: relation to menopause and progesterone treatment. *Am J Respir Crit Care Med* 160: 628–633, 1999.
23. **Lee N, Woodrum CL, Nobil AM, Rauktys AE, Messina MP, Dabora SL.** Rapamycin weekly maintenance dosing and the potential efficacy of combination sorafenib plus rapamycin but not atorvastatin or doxycycline in tuberous sclerosis preclinical models. *BMC Pharmacol* 9: 8, 2009.
24. **Lee PS, Tsang SW, Moses MA, Trayes-Gibson Z, Hsiao LL, Jensen R, Squillace R, Kwiatkowski DJ.** Rapamycin-insensitive up-regulation of MMP2 and other genes in tuberous sclerosis complex 2-deficient lymphangioleiomyomatosis-like cells. *Am J Respir Cell Mol Biol* 42: 227–234, 2010.
25. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C_T)$ method. *Methods* 25: 402–408, 2001.
26. **Matsui K, Takeda K, Yu ZX, Travis WD, Moss J, Ferrans VJ.** Role for activation of matrix metalloproteinases in the pathogenesis of pulmonary lymphangioleiomyomatosis. *Arch Pathol Lab Med* 124: 267–275, 2000.
27. **Moses MA, Harper J, Folkman J.** Doxycycline treatment for lymphangioleiomyomatosis with urinary monitoring for MMPs. *N Engl J Med* 354: 2621–2622, 2006.
28. **Mosmann T.** Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55–63, 1983.
29. **Parsons SL, Watson SA, Brown PD, Collins HM, Steele RJ.** Matrix metalloproteinases. *Br J Surg* 84: 160–166, 1997.
30. **Prall AK, Longo GM, Mayhan WG, Waltke EA, Fleckten B, Thompson RW, Baxter BT.** Doxycycline in patients with abdominal aortic aneurysms and in mice: comparison of serum levels and effect on aneurysm growth in mice. *J Vasc Surg* 35: 923–929, 2002.
31. **Ramamurthy NS, Vernillo AT, Greenwald RA, Lee HM, Sorsa T, Golub LM, Rifkin BR.** Reactive oxygen species activate and tetracyclines inhibit rat osteoblast collagenase. *J Bone Miner Res* 8: 1247–1253, 1993.
33. **Rubins JB, Charboneau D, Alter MD, Bitterman PB, Kratzke RA.** Inhibition of mesothelioma cell growth in vitro by doxycycline. *J Lab Clin Med* 138: 101–106, 2001.
34. **Ryan ME, Ashley RA.** How do tetracyclines work? *Adv Dent Res* 12: 149–151, 1998.
35. **Ryu JH, Moss J, Beck GJ, Lee JC, Brown KK, Chapman JT, Finlay GA, Olson EJ, Ruoss SJ, Maurer JR, Raffin TA, Peavy HH, McCarthy K, Taveira-Dasilva A, McCormack FX, Avila NA, Decastro RM, Jacobs SS, Stylianou M, Fanburg BL; NHLBI LAM Registry Group.** The NHLBI lymphangioleiomyomatosis registry: characteristics of 230 patients at enrollment. *Am J Respir Crit Care Med* 173: 105–111, 2006.
36. **Smith GN Jr, Brandt KD, Hasty KA.** Activation of recombinant human neutrophil procollagenase in the presence of doxycycline results in fragmentation of the enzyme and loss of enzyme activity. *Arthritis Rheum* 39: 235–244, 1996.
37. **Tamargo RJ, Bok RA, Brem H.** Angiogenesis inhibition by minocycline. *Cancer Res* 51: 672–675, 1991.
38. **Timm EA Jr, Stewart CC.** Discrimination between dead and viable apoptotic cells using two-cool TdT assay and surface labeling as detected by flow cytometry. *Biochemica (Indianapolis)* 1: 44–47, 1996.
39. **Uitto VJ, Firth JD, Nip L, Golub LM.** Doxycycline and chemically modified tetracyclines inhibit gelatinase A (MMP-2) gene expression in human skin keratinocytes. *Ann NY Acad Sci* 732: 140–151, 1994.
40. **Vignola AM, Paganin F, Capieu L, Scichilone N, Bellia M, Maakel L, Bellia V, Godard P, Bousquet J, Chanez P.** Airway remodelling assessed by sputum and high-resolution computed tomography in asthma and COPD. *Eur Respir J* 24: 910–917, 2004.
41. **Visse R, Nagase H.** Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 92: 827–839, 2003.
42. **Zhe X, Yang Y, Jakkaraju S, Schuger L.** Tissue inhibitor of metalloproteinase-3 downregulation in lymphangioleiomyomatosis: potential consequence of abnormal serum response factor expression. *Am J Respir Cell Mol Biol* 28: 504–511, 2003.