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Restoration of thrombospondin 1 expression in tumor cells harbouring mutant *ras* oncogene by treatment with low doses of doxycycline

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Abstract

Oncogenes act as inducers of tumor neovascularization, at least in part through suppression of endogenous angiogenesis inhibitors, e.g., thrombospondin 1 (TSP-1/THSP1). Therefore, restoration of TSP-1 levels can be viewed as a possible means to inhibit tumor angiogenesis. We observed that low concentrations (0.1–10 μg/ml) of doxycycline (but not those of related tetracycline) restore TSP-1 expression in *H-ras* oncogene-expressing tumor cell lines (528ras1, MT-Ras). Interestingly, this effect was relatively *ras*-specific, as doxycycline did not alter TSP-1 expression in several cell lines (e.g., 528neu2 fibrosarcoma, B16F1 melanoma, and Lewis lung carcinoma) harbouring other types of transforming alterations. Doxycycline-induced reversal of TSP down-regulation was abrogated under hypoxic conditions. Therefore, we conclude that, in vivo, TSP-1 is likely under dual and/or synergistic control of oncogenes and hypoxia-related pathways. Disruption of both components may be necessary for the 'rescue' of TSP-1 expression in *ras*-driven cancers.
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Recruitment of new blood vessels (angiogenesis) is now regarded as a hallmark [1] and an integral part of cancer as a disease [2]. It is believed that the "angiogenic switch" is triggered when the balance between angiogenesis stimulators and inhibitors within the tumor microenvironment is shifted to favour the former over the latter [3,4]. Such a transition from an angiogenically inert to a pro-angiogenic state is tightly linked to genetic tumor progression [4]. Indeed, over 20 different oncoproteins (e.g., Ras, Src, Neu/HER-2, epidermal growth factor receptor (EGFR), Myc, and many others) have been documented to act as inducers of pro-angiogenic growth factor expression [5]. Although the impact of mutant *ras* (pathway) on VEGF up-regulation is amongst the best described events in this category [5–7], it is becoming increasingly clear that this oncogene (as well as others) also impacts tumor angiogenesis through down-regulation of angiogenesis inhibitors, of which TSP-1/THSP1 is a well-described paradigm [8–11].

TSP-1 is a large (450 kDa) trimeric, multifunctional, and ubiquitously expressed extracellular matrix (ECM), i.e., 'matricellular' protein [12], and a potent inhibitor of new blood vessel formation, vascular remodeling, and tumor growth [13]. Anti-angiogenic effects of TSP-1 are thought to be mediated through several possible molecular mechanisms, including binding to the CD36 receptor on microvascular endothelial cells [14], induction of FasL and apoptosis [15], activation of transforming growth factor beta (TGF-β) [16], binding to endothelial integrins [17], and by other complex interactions with various specific angiogenic pathways [18]. As such, TSP-1 is a target of multiple regulatory influences, including growth factors, cytokines,and hypoxia [19,20], as well as tumor suppressor genes [4,21] and oncogenes, e.g., *ras*, *myc*, *src*, *Her-2*, polyoma middle T antigen, and others [8,9,22–25]. Administration of TSP-1 peptidomimetics [11], or constitutive re-expression of full length TSP-1

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[10] results in the inhibition of in vivo growth and neovascularization of tumors driven by oncogenic *ras*, an observation that may suggest a crucial and 'permissive' role of TSP-1 in this context.

Given the potent anti-angiogenic activity of TSP-1 and the relative commonality of Ras activation in human neoplasia [26], a better understanding of the molecular interrelationship between these two entities emerged as a question of considerable therapeutic interest. In this regard, a recent comprehensive study by Watnick et al. [10] delineated several critical signaling steps and mediators of TSP-1 down-regulation in human cells over-expressing mutant *H-ras* oncogene. Thus, TSP-1 regulating signals were found to emanate from activated Ras through a unique pathway involving epistasis of phosphatidyl-inositol 3′-OH kinase (PI3K), a small GTPase known as Rho, and its dependent kinase ROCK, finally leading to myc-dependent inhibition of TSP-1 expression [10]. While this analysis unveiled a number of plausible targets (e.g., PI3K) for pharmacological restoration of TSP-1 expression [10], additional modalities of TSP-1 'rescue' may also exist. Here, we report that treatment of *ras*-transformed cells with the common antibiotic doxycycline leads to restoration of TSP-1 expression. This effect was not observed in non-transformed cells, in cells transformed in a manner independent of mutant *ras* (e.g., by expression of activated *neu*/*HER-2*), and was attenuated under hypoxic conditions. Our data suggest that doxycycline may specifically interact with as yet unidentified element(s) of the Ras-activated TSP-1-regulating circuitry.

Materials and methods

Cells and culture conditions. Derivation of the MDF528 (mouse dermal fibroblast) cells, as well as their *V12H-ras*- (528ras1) and *neu*/*HER-2*-transformed (528neu2) counterparts has been described elsewhere in detail [11]. Briefly, MDF528 is a VEGF-deficient (VEGF−/−) spontaneously immortalized and non-tumorigenic fibroblastic cell line, which has been derived from dermal explants of adult chimeric (VEGF+/+ and VEGF−/−) mice. Despite the absence of a functional VEGF gene, both oncogene-transformed sublines derived from MDF528 (528ras1 and 528neu2 cells) readily form tumors in SCID mice [11]. Both Lewis lung carcinoma (LLC) and B16F1 melanoma (B16) were obtained from American Type Tissue Collection (ATCC). All cell lines (except for IEC-18 and MT-Ras) were cultured in DMEM (HyClone), supplemented with 10% fetal bovine serum (FBS), unless otherwise indicated. Rat intestinal epithelium-derived cell lines IEC-18 and MT-Ras were maintained in minimal essential medium (αMEM), supplemented with 5% FBS, 4 mM L-glutamine, 20 mM glucose, and 10 μg/ml insulin (Sigma Chemical, St. Louis, MO). In order to activate metallothionein (MT) promoter driving H-Ras expression in MT-Ras cells, the growth medium was supplemented with 100 μM $ZnCl_2$ and 2 μM $CdCl_2$ (both from Sigma) as described earlier [6]. Both doxycycline and tetracycline hydrochloride were purchased from Sigma (St. Louis, MO) and used at concentrations indicated.

Western blotting. Cells were treated with indicated concentrations of doxycycline (Sigma Chemical, St. Louis, MO) for 48 h (or as specified), after which the medium was removed and the cells were lysed in Cell Culture Lysis Reagent (CCLR) containing: 1% Triton X-100, 10% glycerol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 2 mM dithiothreitol (DTT), and 25 mM Tris-phosphate, pH 7.8 (Promega, Madison, WI), supplemented with a protease inhibitor mix (Roche, Palo Alto, CA). Tumor and normal tissue specimens were minced mechanically, stored at −80 °C, and lysed as above before analysis. Protein content was quantified by Bradford Assay (Bio-Rad, Mississauga, ON) and 50 μg/lane of protein was resolved by SDS–PAGE (8% or 12% gel) and transferred to Immobilon-P membrane (Millipore, Nepean, ON). Membranes were probed with a mixture of primary antibodies (anti-TSP-1 Ab1 and Ab4; each 2 μg/ml; Neomarkers, Fremont, CA). The signal was visualized by using HRP-conjugated anti-mouse secondary antibody, 1/5000 (Bio-Rad), followed by incubation with the ECL reagent (Amersham Biosciences, Baie d'Urfe, Quebec). In order to confirm equal loading, membranes were probed with the anti-ERK1/2 antibody (Upstate, Charlottesville,VA) 1/1000 followed by anti-rabbit secondary antibody 1/5000. The signal intensity was measured and quantified by using Typhoon 9410 scanner and software (Amersham Biosciences). All analyses were performed at least twice (often up to five times) with similar results.

Tumor analysis. The impact of doxycycline on tumorigenicity of 528ras1 cells was tested in SCID mice (5 mice per group). Cancer cells (2×10^6/mouse) were injected s.c. (orthopically) in 0.1 ml of phosphate-buffered saline (PBS) and tumor growth was followed by measurements with a vernier caliper, twice a week. Tumor volume was calculated by using the standard formula [9] and plotted as a function of time. Doxycycline was delivered in drinking water (4 mg/ml), the supply of which was changed twice a week. Mice were sacrificed before tumors reached the size of 17 mm in one dimension, autopsied, and tissues were analyzed for TSP-1 expression and vascular density. All endpoints were executed according to the guidelines of the Canadian Council of Animal Care (CCAC) and institutional AREB Committee.

PECAM immunohistochemistry. Tumors were excised at autopsy, immediately rinsed in cold PBS, and cryoprotected in 15% sucrose (1 h; 4 °C) and then in 30% sucrose (O/N; 4 °C). Cryosections (10 μm in thickness) were placed on silanized microscope slides and fixed in ice-cold acetone. Tissues were stained for blood vessel density by using rat anti-mouse CD31 (PECAM) antibody (Pharmingen, BD Biosciences, Toronto, ON, 1/200 at 4 °C overnight) followed by biotinylated rabbit anti-rat secondary antibody (Jackson Labs, Bar Harbor, ME, 1/500; 30 min at RT). The colour reaction was visualized using Histostain Kit (Zymed, South San Francisco, CA).

Results

Selective reversal of ras-dependent TSP-1 down-regulation by 'low dose' doxycycline

In the non-tumorigenic, immortalized mouse dermal fibroblastic cell line called MDF528, as in many other similar cases, expression of TSP-1 transcript [11], and protein (Fig. 1A) is readily detectable and abundant. As can be expected, enforced expression of the mutant *H-ras* oncogene in these cells (e.g., in the 528ras1 variant) leads to overt malignant transformation, as well as acquisition of tumorigenic and angiogenic properties in vivo [11] (see Fig. 3B), along with a marked down-regulation of TSP-1 expression (Fig. 1A, first two lanes). We noticed serendipitously that when 528ras1 cells were incubated for 48–72 h with low concentrations (0.1–10 μg/ml) of doxycycline, the expression of TSP-1 was dramatically up-regulated and reached levels

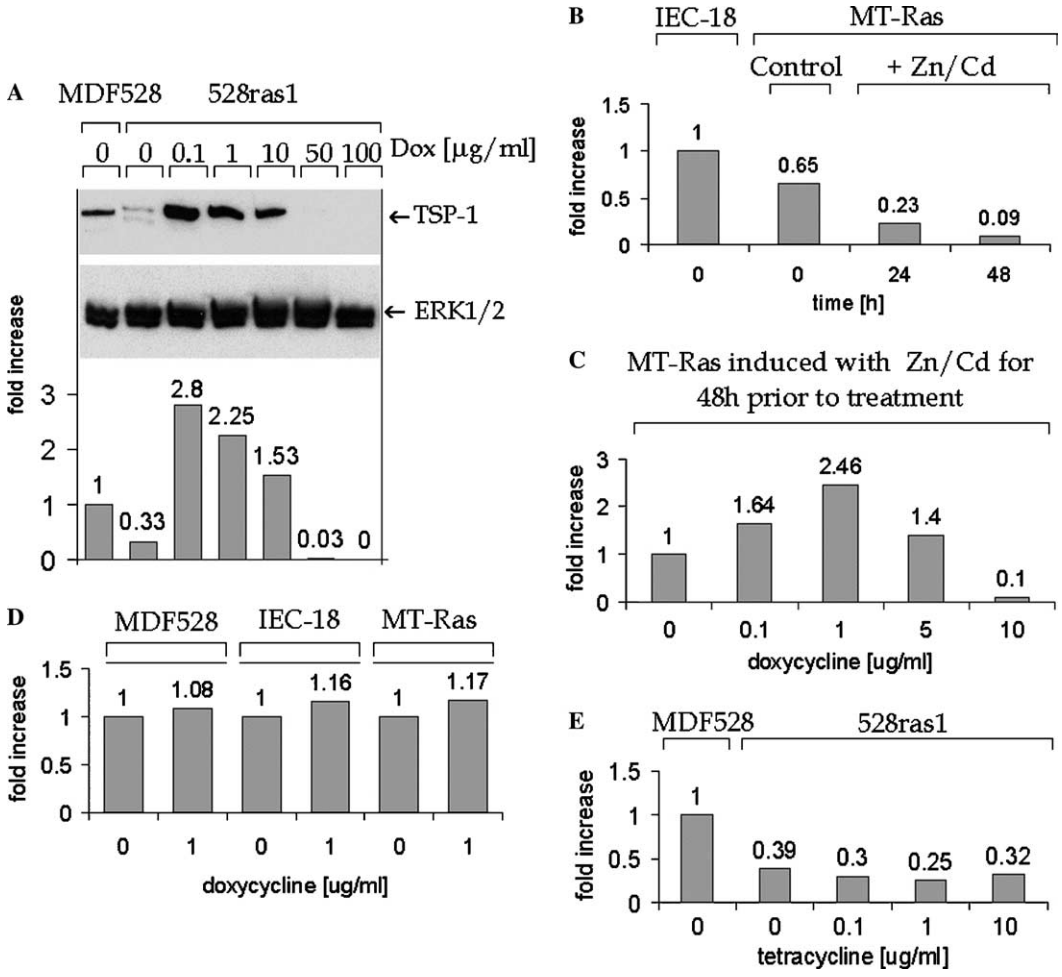

Fig. 1. Doxycycline-induced reversal of TSP-1 down-regulation in tumor cells harbouring mutant *ras* oncogene. (A) Constitutive down-regulation of TSP-1 expression in MDF528 fibroblasts upon expression of mutant *H-ras* (528ras1 cells). This effect can be abrogated, (and TSP-1 expression increased) in the presence of low (0.1–10 μg/ml), but not high (50–100 μg/ml) concentrations of doxycycline. (B) Conditional, Ras-dependent TSP-1 down-regulation in MT-Ras epithelial (IEC-18-derived) cells engineered to express *H-ras* oncogene under control of a Zn/Cd-inducible promoter. (C) Reversal of Ras-dependent TSP-1 down-regulation by incubation of Zn/Cd-treated (Ras-transformed) MT-Ras cells with low concentrations of doxycycline (0.1–1 μg/ml). (D) Unchanged TSP-1 expression in doxycycline (0.1 μg/ml)-treated untransformed cell lines (MDF528, IEC-18, and Zn/Cd-untreated MT-Ras). (E) Tetracycline hydrochloride treatment does not recapitulate the effects on doxycyline on TSP-1 expression by 528ras1 cells.

comparable to, or even exceeding (by up to 2.8-fold) those of parental MDF528 fibroblasts (Fig. 1A).

A similar pattern was also observed in the case of another ras-transformed unrelated cell line called MT-Ras (Figs. 1B and C), in which mutant *H-ras* can be conditionally expressed (under control of the MT promoter) on the background of immortalized intestinal epithelium (IEC-18 cells). In this system, addition of heavy metals (Zn and Cd) reproducibly results in *ras*-dependent cellular transformation and expression of Ras-responsive genes such as VEGF [6]. As shown in Fig. 1B, Zn/Cd treatment of MT-Ras cells triggered a precipitous and sustained (3–6-fold) down-regulation of TSP-1, compared to both their untreated controls and parental IEC-18 counterparts. The residual difference between the latter two cells lines is due to some 'leakiness' of the MT promoter. However, also in this cellular context, Ras-dependent TSP-1 down-regulation can be diminished (by up to 2.46-fold) by addition of doxycycline (Fig. 1C). In contrast, no appreciable doxycycline-related changes in TSP-1 expression were detected in various parental (mutant *ras*-negative) control cell lines (i.e., MDF528, IEC-18, and non-induced MT-Ras), or in normal skin tissue extracts (Figs. 1D and 3C, respectively). These observations suggest that the TSP-1-inducing effect of the drug is conditional upon transformation of target cells (Fig. 1D). It is also of note that higher drug concentrations (e.g., 50–100 μg/ml) were ineffective in (and even suppressed) TSP-1 expression in *ras*-transformed cell lines (Figs. 1A and C), and that a related (but not identical) agent tetracycline hydrochloride did not recapitulate the effects of doxycycline on TSP-1 production (Fig. 1E).

Interestingly, the 'rescue' of TSP-1 expression by doxycycline shows considerable specificity for oncogenic

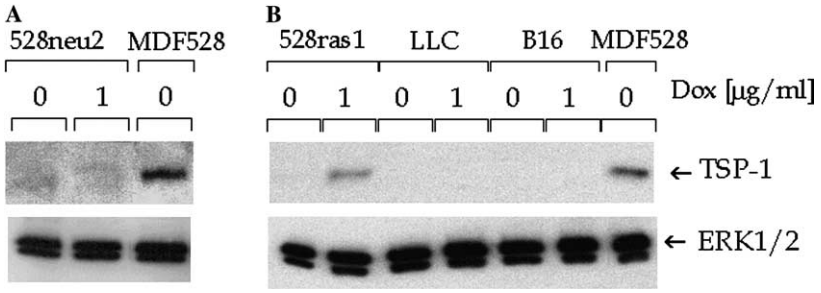

Fig. 2. Mutant *ras*-specific impact of doxycycline treatment on TSP-1 expression in cancer cells. (A) Absence of TSP-1 up-regulation in doxycycline-treated 528neu2 cells harbouring *neu*/*HER-2* oncogene (instead of mutant *ras*). (B) Contrasting effects of doxycycline treatment on the 'rescue' of TSP-1 expression in 528ras1 cells and two unrelated 'spontaneous' mouse tumors (B16F1 melanoma and LLC carcinoma).

H-ras (Figs. 2A and B). Indeed, unlike in 528ras1 cells, no doxycycline-dependent changes in TSP-1 levels were observed in 528neu2 cells, which were also derived from MDF528 fibroblasts, express similar tumorigenic and angiogenic properties, and have profoundly reduced TSP-1 levels, but were engineered to express activated *neu*/*HER-2* rather than *ras* [11] (Fig. 2A). Likewise, TSP-1 remained largely unaffected in two other unrelated cancer cell lines derived from spontaneous murine tumors (B16F1 melanoma and Lewis lung carcinoma, LLC), the malignant properties of which are not known to depend on expression of mutant *H-ras* oncogene (Fig. 2B).

Inability of doxycycline to reverse ras-dependent TSP-1 down-regulation in hypoxia

The restoration of TSP-1 expression in 528ras1 cells exposed to doxycycline in vitro naturally leads to the idea that this drug may possess unsuspected (i.e., TSP-1-mediated) anti-tumor and anti-angiogenic properties in vivo. To examine whether this may in fact be the case, 528ras1 cells were injected into SCID mice and tumor growth was monitored in the presence or absence of doxycycline supplemented in the drinking water. Somewhat surprisingly, such an in vivo treatment resulted in no significant change in tumor growth (Fig. 3A) or vascular density (Fig. 3B). Moreover, as compared to protein lysates obtained from the adjacent normal (unaffected) skin, material extracted from 528ras1 tumor masses contained extremely low quantities of TSP-1 protein, regardless of whether the mice had received doxycycline treatment or not (Fig. 3C).

We reasoned that such a disparity between the outcomes of in vitro and in vivo doxycycline treatments might either be of a pharmacological nature (i.e., due to unfavourable drug distribution, rapid metabolism, and/or poor cellular uptake), or else might result from influences of the tumor microenvironment (e.g., hypoxia) on the Ras-dependent TSP-1 regulation. Results presented in Fig. 3D suggest that the latter may in fact be the case. Thus, in order to recapitulate hypoxic conditions that may be present within tumors, 528ras1 cells or their MDF528 counterparts were cultured under low oxygen conditions (0.3% instead of normoxic 20% O_2) and at the same time treated with 0.1 μg/ml of doxycycline or vehicle. In hypoxia, TSP-1 levels were somewhat up-regulated in non-tumorigenic parental MDF528 fibroblasts, but no such effect was observed in 528ras1 tumor cells. In contrast, and unlike in normoxia (compare Fig. 1A), doxycycline failed to 'rescue' TSP-1 expression in *ras*-transformed cells cultured under hypoxic conditions (Fig. 3D). This suggests that TSP-1 expression may come under dual control of the Ras-related (doxycycline-sensitive) and hypoxia-related (doxycycline-insensitive) pathways, the combined influence of which does not appear to be reversed by the drug.

Discussion

Our study describes a previously unknown activity of doxycycline, namely the reversal of *ras*-dependent down-regulation of TSP-1 expression. This observation may have several interesting and potentially important clinical, biological, and experimental implications. With respect to the latter, doxycycline is one of the most common agents used to achieve conditional gene expression in various "tet-on" and "tet-off" vector and transgenic systems [27]. This elegant approach is used extensively to reversibly activate/inactivate the expression of various molecular entities of possible importance to tumor angiogenesis, including oncogenes, e.g., *H-ras* [28], potent angiogenic growth factors, e.g., VEGF [29] or angiogenesis inhibitors, such as TSP-1 [30]. Our study does not dispute these various specific results, but rather offers a general cautionary note as to the possibility of inadvertent induction of doxycycline-dependent changes in TSP-1 expression.

The biological implications of our results stem from the fact that the action of doxycycline amounts to a partial reversal of an event (perhaps more than one) associated with *ras*-dependent cellular transformation. Indeed, doxycycline up-regulates TSP-1 in 528ras1 cells but not in

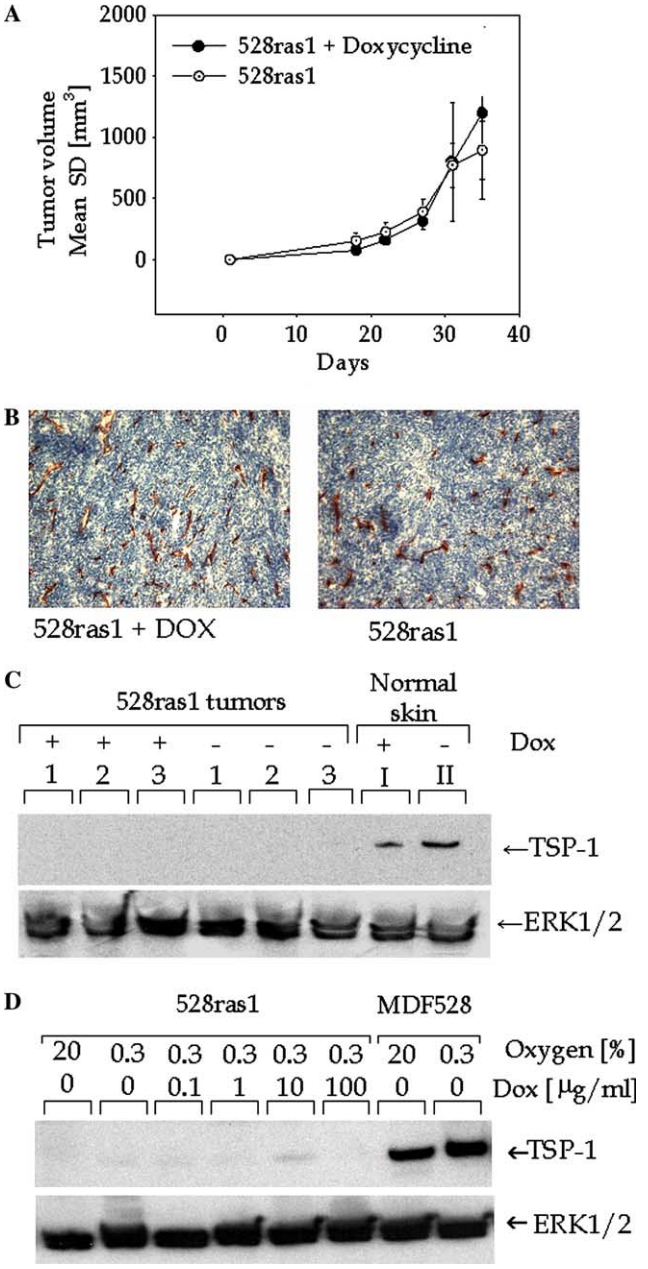

Fig. 3. Attenuation by hypoxic conditions of the doxycycline-dependent TSP-1 restoration in tumor cells harbouring oncogenic *ras*. (A) Insensitivity of large hypoxic 528ras1 tumors to doxycycline treatment (see text). (B) PECAM/CD31 staining indicating unchanged blood vessel density in tumors treated with doxycycline. (C) Absence of TSP-1 up-regulation in 528ras1 tumors treated with doxycycline in vivo (arabic numerals indicate protein extracts from three independent tumors, roman numerals indicate representative protein samples extracted from normal skin of tumor bearing mice). (D) Attenuation of the doxycycline-induced TSP-1 'rescue' in 528ras1 cells cultured under hypoxic conditions (0.3% O_2, see text).

their related tumor cell line 'utilizing' a different oncogene (528neu2). This particular pattern of doxycycline activity may suggest that this drug could interact with elements of the ras/rho/ROCK/myc pathway mediating TSP-1 down-regulation, as described originally by Watnick et al. [10]. Alternatively, doxycycline may interfere with more indirect or secondary aspects of the *ras*-induced cellular transformation, or events that may be necessary (permissive) for maintaining low levels of TSP-1 in cancer cells harbouring this activated oncogene. In this regard, identification of the molecular doxycycline target(s) is a subject of our ongoing efforts.

It is thought-provoking that in hypoxic conditions TSP-1 expression becomes 'locked' in the *ras*-induced, suppressed but doxycyline unsusceptible state (as compared to the MDF528 profile). This observation reinforces the notion of an interdependence between oncogene- and hypoxia-regulated signaling pathways [19,31–33], and suggests that therapeutic agents targeting Ras-pathways may not retain their full biological activity under 'tumor-like,' i.e., hypoxic conditions. It is noteworthy that the TSP-1 gene is not known to be a direct target of the classical hypoxia inducible factor 1 (HIF-1)-mediated transcriptional regulation [20] with which Ras/MAPK-pathway is tightly connected [33].

The molecular mechanisms and biological consequences of the effect that doxycycline exerts on TSP-1 expression in *ras*-transformed cells remain unclear at the moment. However, tetracyclines can alter several cellular properties, e.g., are known to attenuate the activity of matrix metalloproteinases (e.g., MMP-2 and MMP-9), inhibit proliferation, migration, and survival of various cell types (including cancer cells), reduce production of extracellular matrix molecules, as well as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), and inducible nitric oxide synthase (iNOS) [34–36]. While many of these activities remain poorly understood, the main body of work on tetracyclines in cancer concentrates around their ability to inhibit MMPs, particularly at high doses. This activity is believed to be responsible for various degrees of therapeutic activity displayed by these agents in inflammatory conditions, e.g., arthritis, tumor invasion, bone metastasis [37,38], and most notably in tumor angiogenesis [39]. With regards to the latter, certain tetracycline derivatives, including minocycline, doxycycline, and COL-3, have been a subject of particularly extensive laboratory and clinical investigations [39,40].

Overall, our study suggests that TSP-1 may be a relevant target for at least some tetracyclines and under specific (i.e., near normoxic) conditions. If so, a more effective approach to using these drugs in cancer could perhaps involve greater selectivity with respect to tumor types (*ras*-dependent tumors), careful choice of specific drugs (doxycline rather than tetracycline), and treatment regimens (low doses rather than high).

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