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SHORT COMMUNICATION

Glioblastoma cells inhibit astrocytic *p53*-expression favoring cancer malignancy

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The tumor microenvironment has a dynamic and usually cancer-promoting function during all tumorigenic steps. Glioblastoma (GBM) is a fatal tumor of the central nervous system, in which a substantial number of non-tumoral infiltrated cells can be found. Astrocytes neighboring these tumor cells have a particular reactive phenotype and can enhance GBM malignancy by inducing aberrant cell proliferation and invasion. The tumor suppressor *p53* has a potential non-cell autonomous function by modulating the expression of secreted proteins that influence neighbor cells. In this work, we investigated the role of *p53* on the crosstalk between GBM cells and astrocytes. We show that extracellular matrix (ECM) from $p53^{+/-}$ astrocytes is richer in laminin and fibronectin, compared with ECM from $p53^{+/+}$ astrocytes. In addition, ECM from $p53^{+/-}$ astrocytes increases the survival and the expression of mesenchymal markers in GBM cells, which suggests haploinsufficient phenotype of the $p53^{+/-}$ microenvironment. Importantly, conditioned medium from GBM cells blocks the expression of *p53* in $p53^{+/+}$ astrocytes, even when DNA was damaged. These results suggest that GBM cells create a dysfunctional microenvironment based on the impairment of *p53* expression that in turns exacerbates tumor endurance.

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INTRODUCTION

The influence of the microenvironment on tumor cells' behavior is a subject that has been recognized as an important factor modulating tumor malignancy. Nowadays, it is well accepted that non-malignant cells surrounding tumor masses have an important, and often tumor-promoting role during all the stages of carcinogenesis.[1] In epithelial tumors, cancer-associated fibroblasts are known to secret soluble factors, such as hepatocyte growth factor, which is mitogenic for malignant cells,[2] and also, transforming growth factor beta, which induces epithelial-to-mesenchymal transition and invasion of tumor cells.[3] In this same context, tumor-associated macrophages often exhibit a tumor-promoting cytokine expression profile, such as a high expression of IL-10 and a low expression of IL-12.[4] Besides secretion of soluble factors, the extracellular matrix (ECM) provides, not only a physical scaffold for all cells in the tumor microenvironment but also has a dynamic role during the evolution and spread of cancers.[5] Cancer-associated fibroblasts are known to produce ECM components such as type I and III collagens and fibronectin, which are correlated with a poor prognosis and enhanced metastatic potential.[6]

Glioblastomas (GBM) are malignant tumors of the central nervous system, with a very inexpressive response to current therapeutic approaches.[7] GBM cells are radio- and chemo-resistant; therefore, ways to increase cell death are one of the major targets in GBM research.[8–10] Moreover, the parenchymal infiltration of GBM cells makes total surgical resection an impossible task,[11] rendering the study of tumor invasion mechanisms an issue regarding GBM therapy, besides the study of survival mechanisms of GBM cells.[7]

In surgically resected GBM tissue, a considerable mass of non-transformed cells can be found together with the tumor cells,[12] revealing the expressiveness of tumor cells interaction with non-tumoral cells. Astrocytes surrounding GBM commonly present a particular reactive phenotype with high expression of the glial fibrilary acidic protein and have been shown to facilitate the migration of GBM cells by the expression of metalloproteinase 2.[13] It has also been suggested that astrocytes may induce aberrant proliferation of GBM cells by secretion of the stromal cell-derived factor 1.[12] Thus, the study of GBM and parenchymal astrocytes interaction is relevant concerning tumor therapy.

p53 is a well-known tumor suppressor gene, found mutated in almost 50% of all human cancers, and in 87% of GBM cases.[14] In normal conditions, p53 protein has a very-short half-life.[15] The role of *p53* in DNA damage-induced responses is well studied and established.[16,17] Briefly, healthy cells in the presence of DNA damage, have their p53 stabilized, mostly by the disruption of its interaction with the ubiquitin ligase MDM2, post-translationally modified, and accumulated in the nucleus, where it activates the transcription of genes that lead to growth arrest or cell death.[18,19] DNA damage-induced apoptosis is completely abolished in the $p53^{-/-}$central nervous system during development, and is significantly reduced in *p53* heterozygous mutant, showing that apoptotic response exhibits p53-haploid insufficiency.[20]

Interestingly, recent evidences are pointing out that, besides its cell-autonomous function, p53 also exerts a non-cell autonomous function, by the regulation of secreted proteins that can influence the behavior of neighboring cells.[21,22] Consistently, Kiaris *et al.*[23] have shown that xenografted epithelial tumor cells

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exhibited a lower apoptotic rate when injected in a $p53^{-/-}$ host. Inactivation of *p53* also occurs within the tumor microenvironment, including cancer-associated fibroblasts, that are associated with an increased rate of metastases and poor prognosis.[24,25] Thus, stromal p53 may play an important role in the crosstalk between tumor and surrounding non-tumoral cells.

In this work, we investigated the role of astrocytic p53-expression for GBM malignancy.

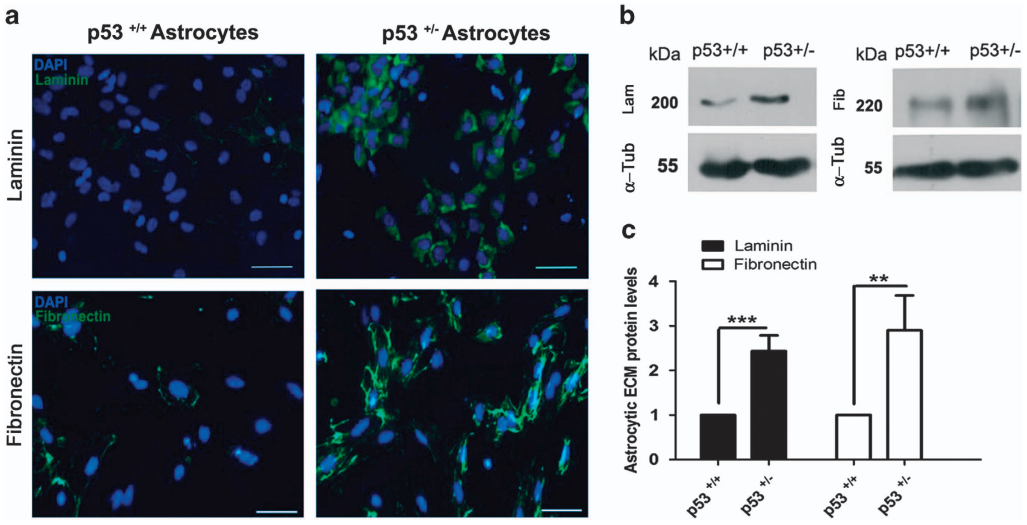

Figure 1. $p53^{+/-}$ astrocytic ECM presents more laminin and fibronectin than $p53^{+/+}$ astrocytic ECM (**a**) Representative images of three independent experiments showing immunofluorescence staining of laminin with anti-laminin 1+2 antibody (abcam, Cambridge, UK), (upper panel-green) or fibronectin with anti-fibronectin antibody (Sigma, St Louis, MO, USA) (lower panel-green) in $p53^{+/+}$ or $p53^{+/-}$ astrocyte ECM. All cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI—blue). Calibration bar: 50 μm. (**b**) Representative images of three independent western blots showing laminin (Lam) and fibronectin (Fib) expression from $p53^{+/+}$ and $p53^{+/-}$ astrocytes. α-Tubulin (α-Tub) was used as loading control by anti- α-Tubulin (Sigma antibody at 1:10 000 dilution). (**c**) Histogram showing the levels of astrocytic laminin (black bars) and fibronectin (white bars) proteins. Data represent the mean and error bars of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.005$ by Tukey test.

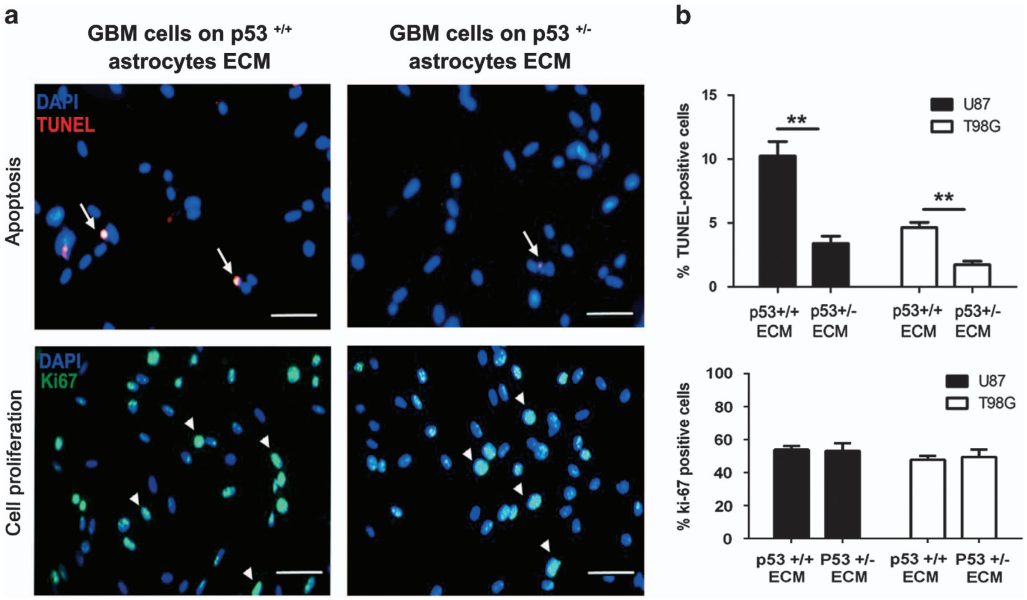

Figure 2. $p53^{+/-}$ astrocytic ECM promotes an increase of GBM cell survival compared with $p53^{+/+}$ astrocytic ECM. (**a**) Representative photomicrographs of three independent experiments of TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay (red, upper panels) and immunofluorescence staining of Ki67 (green, lower panels) of U87MG cells cultured on $p53^{+/+}$ or $p53^{+/-}$ astrocytic ECM for 24 h. Freshly immobilized ECMs from astrocytes were obtained as previously described.[31] TUNEL assay was performed as described by Borges and co-workers.[47] Ki-67 antibody used was purchased from BD Pharmingen, San Diego, CA, USA. All cell nuclei were stained with 4′,6-Diamidino-2-phenylindole (DAPI, blue). TUNEL and ki-67 quantification was done using the percentage of TUNEL or ki-67-positive cells, respectively, relative to total cells (DAPI). Experiments were carried out in duplicates and for every experimental condition at least 500 cells were counted. Cell counting was done by using the Embryonic Stem Cell Counter—ESCC software.[48] Arrows indicate TUNEL-positive cells, and arrowheads indicate Ki67-positive cells. Calibration bar: 50 μm. (**b**) Histogram showing the percentage of U87MG (black bars) and T98G (white bars) TUNEL or Ki67-positive cells, when cultured on $p53^{+/+}$ and $p53^{+/-}$ astrocytic ECM. Data represent the mean and error bars of three independent experiments. $^{**}P < 0.01$ by Tukey test.

RESULTS AND DISCUSSION

$p53^{+/-}$ astrocytic extracellular matrix presents more laminin and fibronectin than the one from $p53^{+/+}$

The importance of microenvironment for the growth of tumor has been observed during tumorigenesis. For example, in fibroblasts, p53 is able to modulate the composition of their own ECM.[26] Thus, within the GBM microenvironment, we asked whether, in astrocytes, p53 was able to modulate the composition of their own ECM, which could imply some advantage for GBM cells. To answer these questions, we used astrocytes from mice *heterozygous* for *p53 gene* (*Trp53*) $p53^{+/-}$ [27] and control ones ($p53^{+/+}$), as a way to analyze the role of reduced or full p53 expression in astrocytes for GBM.

First, we compared extracellular matrices produced by cerebral cortex astrocytes from newborn $p53^{+/+}$ and $p53^{+/-}$ littermate mice. Our results showed, by immunofluorescence (Figure 1a) and western blot (Figure 1b) that the expression of the ECM components, fibronectin and laminin, were increased in $p53^{+/-}$ astrocytes, compared with $p53^{+/+}$ astrocytes, a phenotype similar to the observed in reactive astrocytes.[28,29]

$p53^{+/-}$ astrocytic ECM promotes GBM cells survival

Since ECM components are able to modulate the malignancy of tumor cells,[30] we tested whether GBM cells would have a growth advantage when cultured on ECM produced by $p53^{+/-}$ astrocytes, compared with GBM cells cultured on ECM produced by astrocytes from wild-type littermates. Freshly immobilized ECMs from astrocytes were obtained as previously described.[31] Briefly, astrocytes in confluent monolayers were disrupted with cold lysis buffer (PBS-Ca^{2+}, pH 7.4, containing 0.1% Triton X-100, 0.1 M NH_4OH, 40 μM leupeptine and 1 mM PMSF) and cellular debris was washed twice with cold PBS-Ca^{2+}.

GBM cell lines T98G (*p53 mutant*) and U87MG (*p53 wild-type*) were cultured on ECM produced by $p53^{+/-}$ astrocytes for 24 h. GBM cells cultured on ECM from $p53^{+/-}$ astrocytes showed reduced apoptotic rate when compared with GBM cells cultured on ECM produced by $p53^{+/+}$ astrocytes. However, no difference was observed in the proliferation of GBM cells in these two culture conditions (Figure 2). Our results show that ECM from $p53^{+/-}$ astrocytes favors GBM cells survival by reducing apoptosis.

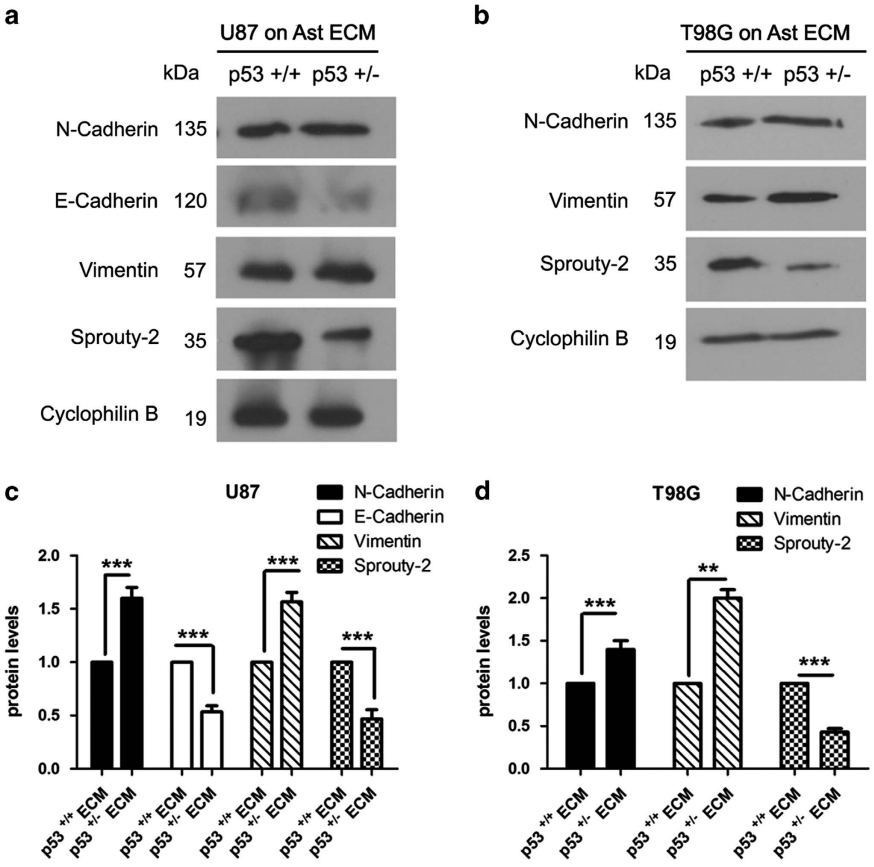

Figure 3. $p53^{+/-}$ astrocytic ECM favors a mesenchymal phenotype of GBM cells. (**a**) Representative images of three independent western blots showing levels of mesenchymal (N-Cadherin and Vimentin) and epithelial (E-Cadherin and Sprouty-2) phenotype markers of U87MG cells cultured on $p53^{+/+}$ or $p53^{+/-}$ astrocytic ECM for 24 h. Cyclophilin B was used as loading control. Antibodies used were: anti-N-cadherin (1:1000—BD Pharmingen), anti-E-cadherin (1:1000—BD Pharmingen), anti- Sproty 2 (1:1000—abcam) and anti-vimentin (1:1000—DAKO, Glostrup, Denmark). (**b**) Representative images of three independent western blots showing levels of mesenchymal (N-Cadherin and Vimentin) and epithelial (Sprouty-2) phenotype markers of T98G cells cultured on $p53^{+/+}$ or $p53^{+/-}$ astrocyte ECMs. T98G E-cadherin expression is not shown because it was not detectable, as already observed by Mikheeva and co-workers.[49] Cyclophilin B was used as loading control. (**c**) Histogram showing levels of U87MG epithelial-to-mesenchymal transition proteins when GBM cells were cultured on $p53^{+/+}$ or $p53^{+/-}$ astrocyte ECMs. Data represent the mean and error bars of three independent experiments. $^{***}P < 0.005$ by Tukey test. (**d**) Histogram showing levels of T98G epithelial-to-mesenchymal transition proteins when GBM cells were cultured on $p53^{+/+}$ or $p53^{+/-}$ astrocyte ECM. Data represent the mean and error bars of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.005$ by Tukey test.

$p53^{+/-}$ astrocytic ECM increases mesenchymal markers in GBM cells

Epithelial-to-mesenchymal transition is a process by which tumor cells acquire a mesenchymal and migratory phenotype.[32] This phenomenon can be triggered by growth factors and ECM components like fibronectin and laminin.[32,33] Therefore, we investigated whether $p53^{+/-}$ astrocytes ECM could favor the mesenchymal phenotype of GBM cells, besides favoring their survival. To test this, we compared the levels of epithelial (E-cadherin and sprouty 2) and mesenchymal (vimentin and N-cadherin) markers in GBM cells cultured on the ECM from $p53^{+/+}$ or $p53^{+/-}$ astrocytes. Our results confirmed that, on ECM from $p53^{+/-}$ astrocytes, GBM cells express higher levels of mesenchymal associated proteins than on ECM from $p53^{+/+}$ astrocytes (Figure 3).

The mesenchymal phenotype of tumor cells is linked to resistance to apoptosis [34] and could also indicate an increase in migratory and invasive potentials. Indeed, it has already been shown that fibronectin and laminin increase migration and growth of GBM.[35,36]

To test whether increased levels of mesenchymal markers, induced by $p53^{+/-}$ ECM, were enough to trigger a change in migration or motility, we performed assays of spontaneous migration and motility in GBM cells cultured on ECM produced by either $p53^{+/+}$ or $p53^{+/-}$ astrocytes. Migration and motility assays were performed as described previously.[37] Briefly, for migration, U87MG cells were cultured on a non-adherent plate for 48 h to form GBM spheres, which were then seeded onto $p53^{+/+}$ or $p53^{+/-}$ astrocytic ECM. The area of spheres at 6 and 24 h showed no differences in migration (data not shown).

The motility assay was performed using a time-lapse video microscopy. U87MG cells were seeded onto $p53^{+/+}$ or $p53^{+/-}$ ECM in 96-well plates. Images of live cells were acquired on the Operetta High Content Imaging System equipped with Harmony software (PerkinElmer, Waltham, MA, USA) using a $\times 20$ long wide distance objective in a digital phase contrast mode at a temperature of 37 °C and 5% CO_2. Cell motility was monitored by time-lapse image sequence for 90 min at intervals of 2 min. Interestingly, the percentage of migrating U87MG cells on $p53^{+/-}$ ECMs (62.3±6.3% s.e.m.) was increased compared with the cells on

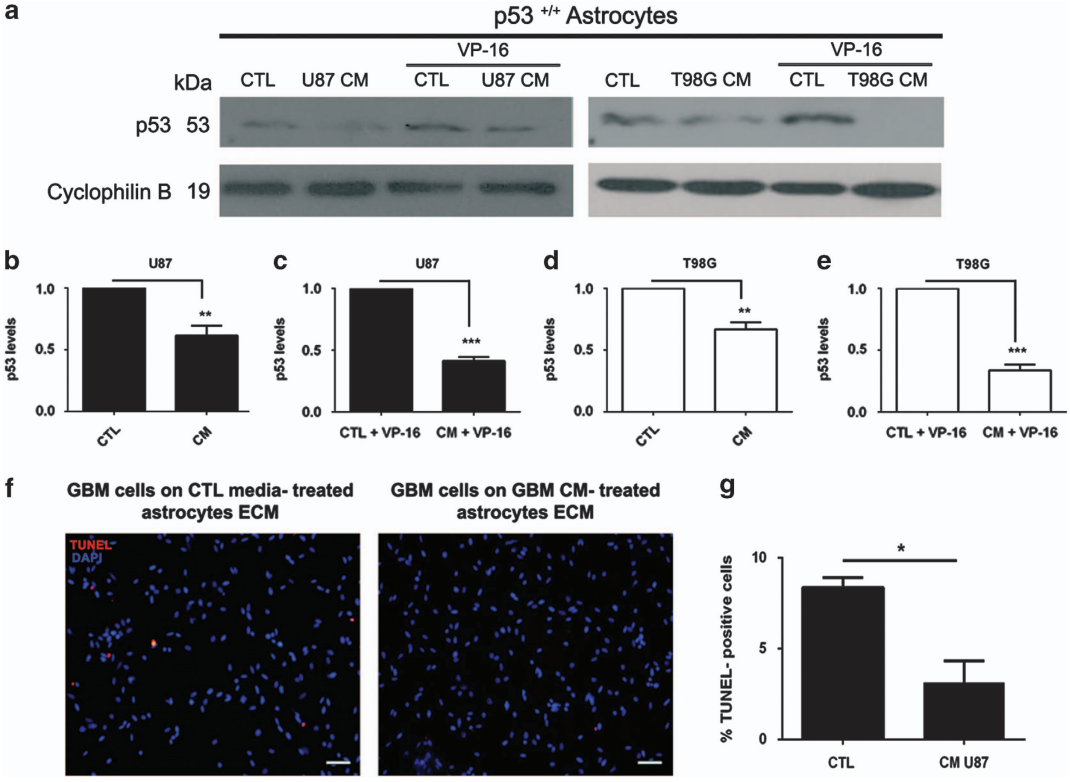

Figure 4. GBM inhibits astrocytic p53-expression favoring GBM survival. (**a**) Representative images of three independent western blots showing p53 levels of astrocytes from wild-type cerebral cortex of newborn mice cultured in conditioned medium (CM) made from U87MG (*p53 wild type*) or T98G (*p53 mutant*) GBMs cell lines for 24 h. Astrocytes were also cultured in control culture medium (CTL) in the presence or absence of VP-16 (1 μM) for 24 h. Cyclophilin B was used as loading control. (**b**) Histogram showing the levels of astrocytic p53 protein, when cultured in CM from U87MG or in CTL. Data represent the mean and error bars of three independent experiments. $^{**}P < 0.01$ by *t*-test. (**c**) Histogram showing the levels of astrocytic p53 protein, when cultured in CM from U87MG or in CTL in the presence of VP-16 (1 μM), for 24 h. Data represent the mean and error bars of three independent experiments. $^{***}P < 0.005$ by *t*-test. (**d**) Histogram showing the levels of astrocytic p53 protein, when cultured in CM from T98G or in CTL. Data represent the mean and error bars of three independent experiments. $^{**}P < 0.01$ by *t*-test. (**e**) Histogram showing the levels of astrocytic p53 protein, when cultured in CM from T98G or in CTL in the presence of VP-16 (1 μM), for 24 h. Data represent the mean and error bars of three independent experiments. $^{***}P < 0.005$ by *t*-test. (**f**) Representative photomicrographs of three independent TUNEL assays (red) of U87MG cells cultured over ECM from $p53^{+/+}$ astrocytes in CTL. For producing ECMs, $p53^{+/+}$ astrocytes were incubated in CTL or in the presence of CM of U87MG cells by 72 h, when cell lyses were performed. U87MG cells were then incubated by 24 h over the ECM produced with (right image) or without CM of GBM treatment (left image). Experiments were carried out in duplicates and for every experimental condition at least 500 cells were counted. All nuclei were stained with DAPI (blue). Cell counting was done by using the Embryonic Stem Cell Counter—ESCC software.[44] Calibration bar: 50 μm. (**g**) Histogram showing the percentage of U87MG TUNEL-positive cells cultured in CTL medium for 24 h. U87 cells were cultured over $p53^{+/+}$ astrocytic ECM that were produced in control media or in the presence of U87MG CM. Data represent the mean and error bars of three independent experiments ($^{*}P < 0.05$ by *t*-test).

$p53^{+/+}$ ECMs (32.5±12.9% s.e.m.) ($n=3$, $P=0.0285$, one-tailed paired *t* test). The speed of migration and the averages of accumulated distance of the migrating population were similar, regardless of source of ECM. In addition, U87MG cells seem to spread faster over $p53^{+/-}$ ECMs, as suggested by their ratio of width to length and by time-lapse images (Supplementary Information). However, these differences were not statistic significant.

For GBM cells T98G (*p53 mutant)*, however, the percentage of migrating cells, as well as, of all other motility parameters tested by Harmony software in time-lapse images were not different regardless of the ECM tested (not shown). As recently described, an increase in mesenchymal markers is not necessarily translate to increase migration or motility.[38] It remains to be tested whether an enhancement of migration phenotype would be more easily observed using ECMs from $p53^{-/-}$ or from $p53^{+/-}$ astrocytes in combination with migration-stimulating factors.

GBM inhibits astrocytic p53-expression to improve cancer survival

In 2009, Bar *et al*.[39] showed that conditioned medium from lung cancer cells suppress p53 expression of fibroblasts. To test whether GBM cells influence the p53 expression of astrocytes, conditioned medium from T98G and U87MG cells were collected after 48 h, as described previously.[40] Cerebral cortex astrocytes from newborn wild-type mice were then cultured in these serum-free conditioned media (CM) or in fresh serum-free culture medium (control; CTL), in the presence or absence of 1 μM of the DNA-damaging agent etoposide (VP-16) for 24 h. As expected, etoposide increased p53 expression in wild-type astrocytes ($p53^{+/+}$), whereas GBM CM reduced p53 expression in astrocytes even after DNA damage. A decrease in p53 levels in astrocytes was also observed in control conditions (without etoposide) when astrocytes were incubated with GBM CM (Figure 4). These results indicate that GBM cells are able to modulate p53 in astocytes.

We then tested whether ECM produced by astrocytes, under the influence of GBM CM, increases GBM survival. $p53^{+/+}$ Astrocytes were cultured with control medium or with CM of U87MG cells for 3 days. U87MG cells were then seeded onto ECM produced by these astrocytes that had been incubated previously with control or GBM CM. GBM cells were then allowed to grow in control medium for 24 h. As shown in Figure 4, CM of GBM made $p53^{+/+}$ astrocytes more permissive to GBM growth. Moreover, ECM produced by astrocytes under the influence of GBM CM reduced the percentage of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive GBM cells. Therefore, GBM CM was able to make astrocytes to secrete ECM that increases GBM survival.

Using microarray analysis, Katz *et al*.[41] have shown that astrocytes surrounding GBM have a specific expression pattern, which is different from the pattern of astrocytes not surrounding the tumor. Gagliano *et al*.[42] have also shown that the co-culture of astrocytes with GBM cells increases the expression of MMP-2 and a decrease in TIMP-2 (tissue inhibitor of metalloproteinase) in astrocytes. Our work shows that GBM cells are able to modulate p53 expression of astrocytes, confirming that GBM is able to modulate protein expression of surrounding astrocytes as a way to favor malignancy. Moreover, DNA damage is well known to be present in tumor microenvironment because of hypoxia-reoxygenation cycles and is induced by chemotherapy treatment,[43] which makes very interesting to note the reduction of astrocytic p53 expression even after DNA damage.

Recently, it was shown that the loss of p53 in surrounding fibroblasts increases epithelial tumor growth.[44–46] The authors suggest that this phenomenon is dependent on the expression of growth factors[44,45] or by the shift of the tumor-associated macrophage phenotype from M1 (classically activated) to M2 (alternatively activated).[46] Our work is the first to show that the loss of p53 expression in astrocytes is able to modulate ECM composition and to provide advantages for GBM cells, favoring the expression of mesenchymal markers and cell survival. Our results strengthen the concept that, in a tumor microenvironment, p53 acts as a tumor suppressor not only in the tumor cell itself, but also in the parenchymal cell.

Altogether, we have elucidated a very important crosstalk between GBM cells and surrounding astrocytes. We have shown that GBM cells decrease the expression of p53 in astrocytes, which in turn, modulates ECM composition and favors tumor malignancy. Finally, our data point out an important role of p53 in the interaction between GBM cells and astrocytes, a discovery with potential to conceive new approaches to treat GBM.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogenesis website (http://www.nature.com/oncsis).