Role of microenvironment on neuroblastoma SK-N-AS *SDHB*-silenced cell metabolism and function

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Abstract

In solid tumors, neoplastic cells grow in contact with the so-called tumor microenvironment. The interaction between tumor cells and the microenvironment causes reciprocal metabolic reprogramming and favorable conditions for tumor growth and metastatic spread. To obtain an experimental model resembling the *in vivo* conditions of the succinate dehydrogenase B subunit (*SDHB*)-mutated paragangliomas (PGLs), we evaluated the effects of *SDHB* silencing on metabolism and proliferation in the human neuroblastoma cell line (SK-N-AS), cultured alone or in association with human fibroblasts. Silencing caused a 70% decrease in protein expression, an almost complete loss of the complex specific enzymatic activity, and a significant increase in *HIF1 α* and *HIF2 α* expression; it thus resembled the *in vivo* tumor cell phenotype. When compared with WT SK-N-AS cells, *SDHB*-silenced cells showed an altered metabolism characterized by an unexpected significant decrease in glucose uptake and an increase in lactate uptake. Moreover, silenced cells exhibited a significant increase in cell proliferation and metalloproteinase activity. When co-cultured with human fibroblasts, control cells displayed a significant decrease in glucose uptake and a significant increase in cell proliferation as compared with their mono-cultured counterparts. These effects were even more evident in co-cultured silenced cells, with a 70% decrease in glucose uptake and a 92% increase in cell proliferation as compared to their mono-cultured counterparts. The present data indicate for the first time, to our knowledge, that *SDHB* impairment causes metabolic and functional derangement of neural-crest-derived tumor cells and that the microenvironment, here represented by fibroblasts, strongly affects their tumor metabolism and growth capacity.

Key Words

- *SDHB*
- glucose/lactate
- fibroblast
- tumor microenvironment
- pheochromocytoma/paraganglioma

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Introduction

Cancer research on solid tumors has recently focused on two main topics: i) the role played by the micro-environment and ii) altered cell metabolism in tumor growth and progression.

Solid tumors are very complex tissues, comprising not only cancer cells but also non-malignant stromal cells, such as endothelial cells, fibroblasts, immune cells, and extracellular matrix, which together form the so-called

tumor microenvironment. Over the past few years, it has become increasingly evident that the continual interplay between cancer and stromal cells generates a positive loop that leads cancer cells to survive the hostile environment, grow, and spread metastases to healthy tissues (Hu & Polyak 2008, Hanahan & Weinberg 2011, Fiaschi *et al.* 2012, Hanahan & Coussens 2012, Karagiannis *et al.* 2012, Zhang & Liu 2012, Quail & Joyce 2013, Santi *et al.* 2013, Taddei *et al.* 2013).

Tumor microenvironment has thus become a potential target for the therapy of certain tumors (for recent reviews, see Sounni & Noel (2013) and Klemm & Joyce (2014)).

Recently, it has been demonstrated that numerous oncogenic mutations reprogram cellular metabolism to fuel cancer hallmarks, such as growth and proliferation. Indeed, recent findings have indicated that some metabolic enzymes act as tumor suppressors, because, when they are mutated, such enzymes are responsible for metabolism impairment and reprogramming. These findings have renewed and revived interest in cancer metabolism. In this scenario, the relevance of mitochondria as central metabolic organelles that play a pivotal role in cell biochemical functions is once again in vogue. A clear example can be found in some tumors, like pheochromocytomas (PHEO) and paragangliomas (PGL), which harbor mutations in the tricarboxylic acid cycle enzymes, such as fumarate hydratase (FH) (Castro-Vega *et al.* 2014) and succinate dehydrogenase (SDH) (Baysal *et al.* 2000, and for recent reviews, see Dahia (2014) and Martucci & Pacak (2014)).

Impairment of SDH activity causes an accumulation of oncometabolite succinate, which is responsible for a wide spectrum of pathways that range from the pseudo-hypoxia response to epigenetic reprogramming (Dahia *et al.* 2005, Pollard *et al.* 2005, Selak *et al.* 2005, Koivunen *et al.* 2007, Goffrini *et al.* 2009, Letouzé *et al.* 2013; for recent reviews, see Adam *et al.* (2013), Kaelin & McKnight (2013), Semenza (2013), and Morin *et al.* (2014)). Importantly, it has also been reported that mutations in the *SDHB* subunit are associated, unlike mutations in other *SDH* subunits, with a high incidence (up to 80%) of malignant PHEO/PGL (Gimenez-Roqueplo *et al.* 2003).

Therefore, SDH-mutated tumors are an incredibly interesting model that links mitochondria to cancer.

The purpose of the present study is to investigate the changes induced by *SDHB* silencing on cell metabolism, growth, and invasiveness as well as the influence that human fibroblasts, as part of the microenvironment, exert on these functions. To achieve this aim, we used

primary cultures of human fibroblasts and the human neuroblastoma cell line SK-N-AS, which share with Pheo/PGL the same origin in the human neural crest (Takahashi *et al.* 2013).

Materials and methods

Cell transfection and clone selection for *SDHB* silencing

The neuroblastoma cell line SK-N-AS was grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a 5% CO₂ humidified atmosphere. To establish a stable *SDHB*-silenced line, cells were transfected to 90% confluent cell cultures using Lipofectamine reagent according to the manufacturer's instructions, with a specific *SDHB* SureSilencing shRNA vector and a negative control shRNA vector (Qiagen). Cells were maintained under selection with G418 (0.6 mg/ml) for 3 weeks. After selection, clones were grown in media with a lower amount of G418 for maintenance (0.2 mg/ml).

Cell homogenates and lysates preparation

Homogenates and lysates were prepared as previously described (Rapizzi *et al.* 2014) with minor modifications. Clone pellets were homogenized in a solution containing 120 mM KCl, 20 mM HEPES, 2 mM MgCl₂, 1 mM EGTA, and 5 mg/ml BSA. The homogenates were centrifuged at 800 g for 10 min at 4 °C, and the enzyme assays were performed on the supernatant. For western blot analysis, cell pellets were lysed in buffer containing 50 mM Tris-HCl pH=7.5, 120 mM NaCl, 1 mM EGTA, 6 mM EDTA, 15 mM Na₄P₂O₇, 20 mM NaF, and 1% Triton X-100 protease inhibitor cocktail. Lysates were clarified by centrifugation at 10 000 g for 15 min at 4 °C. Supernatants were quantified for protein content (Coomassie Blue reagent, Bio-Rad) (Rapizzi *et al.* 2014). All passages were carried out on ice.

Western blot analysis

Cell lysates (30 µg of proteins) were separated by SDS/PAGE and transferred onto PVDF (Immobilon, Billerica, Millipore, MA, USA), as previously described (Rapizzi *et al.* 2014). Bound antibodies were detected using ECL reagents (Immobilon) and analyzed with a Bio-Rad ChemiDoc Imaging System (Quantity One) for dedicated chemiluminescent image acquisition. The polyclonal anti-*SDHB* was from Atlas Antibodies (Stockholm, Sweden), the monoclonals anti-*SDHA* were from Abcam (Cambridge,

UK), and the anti-*HIF1α* and *HIF2α* were from BD Transduction Laboratories (BD Biosciences, Franklin Lakes, NJ, USA). The polyclonal anti-actin and all of the secondary antibodies, such as the anti-rabbit, anti-mouse, and anti-goat IgG conjugated to HRP, were from Santa Cruz Biotechnology.

SDH activity

Clone homogenates (50 μg) were incubated in a phosphate buffer containing sodium azide, 2,6 dichlorophenolindophenol (DCPIP), sodium succinate, and phenazine methosulfate. Complex II specific activity was evaluated by photometry using the Victor3 1420 Multilabel Counter (Packard Instruments, Perkin-Elmer, Waltham MA, USA) to measure the decrease in absorbance that resulted from the oxidation of DCPIP at 600 nm.

Succinate measurement

Cells (500 000 per well) were cultured in six-well plates for 16 h. Dishes were washed twice with PBS before being lysed with 0.5 ml of cold 100% methanol. Cells were kept on ice during the whole procedure. An internal standard mix was added, and supernatants were collected in tubes and stored at -80°C until further processing was performed. UPLC-MS/MS analysis for the quantification of succinate and fumarate was performed as previously described (Richter *et al.* 2014).

Oxygen consumption analysis

Quantification of oxygen consumption by silenced *SDHB* and the control neuroblastoma cell clones was conducted by means of the Oxygraph system (Hansatech Instruments, Pentney, Norfolk, UK). Cells (300 000) were loaded in the chamber, which contained 300 μl of DMEM with glutamine 2 mM and sodium succinate 20 mM. Oxygen consumption was monitored for 5 min at 37°C .

Glucose and lactate uptake measurement

Briefly, to determine glucose or lactate uptake, tumor cells were seeded into six-well plates in single culture or in co-culture with fibroblasts in a 1:2 ratio. After obtaining permission from the Local Ethical Committee (Prot. N. 2011/0020149), human fibroblasts were isolated from patients undergoing surgery for non-oncological reasons, yielding wild-type tissue for genetic analysis.

In the case of co-cultures, tumor cells were plated in tissue culture inserts for multi-well plates (transparent membrane with 0.4 μm pores, Greiner Bio-One International, Frickenhausen, Germany), with fibroblasts in the plates. They were left to grow for 48 h in serum-free DMEM. Lactate uptake or 2-deoxy-glucose was evaluated in a buffered solution (140 mM NaCl, 20 mM HEPES/Na, 2.5 mM MgSO_4 , 1 mM CaCl_2 , and 5 mM KCl, pH 7.4) containing 0.5 μCi/ml [^3H]deoxyglucose or 1 μCi/ml [$\text{U-}^{14}\text{C}$]lactate. Lactate uptake or glucose was programmed at 37°C for 15 min. Cells were subsequently washed with cold PBS and lysed with 0.1 M NaOH. Incorporated radioactivity was assayed by liquid scintillation counting (Fiaschi *et al.* 2012, Rapizzi *et al.* 2014).

Lactate concentration measurement

Cells were seeded into six-well plates, left to grow to confluence, and washed twice with PBS; the culture media was replaced with fresh media. After 24 h, the media were collected and centrifuged at 10 000 g for 10 min at 4°C , and the supernatants were transferred to clean tubes. Lactate was measured in the cultured media with the Lactate Assay Kit (Bioscience Life Sciences, Milan, Italy) according to the manufacturer's instructions.

Proliferation assay

To evaluate [^3H]thymidine incorporation, tumor cells were seeded into six-well plates, serum-starved for 24 h, and challenged with 1% FCS for 24 h. [^3H]thymidine (0.5 μCi/well) was added for the final 2 h of incubation. Cells were washed twice in ice-cold PBS before the addition of 500 μl of 10% trichloroacetic acid (TCA) for 30 min at 4°C and then washed twice with 250 μl of 5% TCA. The cells were then lysed in 0.25 N (250 mM) NaOH (500 μl/well) for 1 h at 37°C . Incorporation of [^3H]thymidine was measured by scintillation counting using a Tri-Carb2800 TR Liquid Scintillation Analyzer (Perkin-Elmer).

In the case of co-cultures, proliferation was evaluated using the cell trace CFSE cell proliferation kit (Molecular Probes, Eugene, Oregon, USA). Tumor cells were labeled with the dye at a concentration of 2.5 μM. Then cells were plated in co-culture with human primary fibroblasts at a 1:2 ratio for 48 h. The cells were then detached, fixed in 3% paraformaldehyde, and analyzed by flow cytometry. The fluorescence value obtained was analyzed using ModFit software to estimate the proliferation index (Santi *et al.* 2013).

Zymography

SDHB-silenced and control cells at the confluence were washed twice in PBS and serum starved in DMEM containing 0.1% BSA. The culture media were harvested after 24 h, centrifuged at 10 000 *g* for 10 min at 4 °C. Supernatants containing metalloprotease were recovered. Cell lysates were used for protein assay.

For zymography, after the addition of 5 μ l of sample buffer (4% SDS, 100 mM Tris-HCl at pH 6.8, 20% glycerol, and 0.01% blue bromine phenol) without β -mercaptoethanol, samples (45 μ l each) were separated in an 8% acrylamide gel containing 0.1% gelatin. Gels were then washed twice for 30 min with 2.5% Triton X-100 and for 30 min in the reaction buffer (50 mM Tris-HCl at pH 7.5, 200 mM NaCl, 5 mM CaCl₂). Finally, the gels were incubated overnight at 37 °C in reaction buffer. Gels were stained with Coomassie Brilliant Blue R-250 (Amersham-Pharmacia). As a result, visible light bands relating to enzymatic digestion of gelatin by metalloprotease became evident.

Co-cultures

In an attempt to recreate an *in vitro* cellular microenvironment resembling *in vivo* tissue conditions, co-cultures were performed in special plates where tumor cells and fibroblasts are seeded into contiguous wells separated by a membrane, which is permeable to the cell culture media (Greiner Bio-One International).

Statistical analysis

Statistical analysis was performed using a one-way ANOVA followed by Dunnett's *post hoc* test for multiple comparisons; Student's *t*-test was used for comparing the two classes of data. A *P*-value of less than 0.05 was considered significant. Data are reported as means $\pm$ s.d. of at least three experiments.

Results

SDHB silencing reduces SDH expression and activity and enhances HIF1 α and HIF2 α expression

Although PHEO/PGL tissue specimens may yield a great deal of information, several dynamic parameters and functions can be properly evaluated only in living cells.

We first established a stable SDHB-silenced SK-N-AS cell line and subsequently verified the silencing of the

SDHB subunit by western blot and SDH activity analysis. The results, which are shown in Fig. 1, indicate a significant decrease in the SDHB subunit expression level of about 70% (A and B) and an almost total loss of SDH activity (C). It is well known that the alteration of a single enzyme subunit leads to the destabilization of the entire complex, which is then degraded. In this regard, it is not surprising that SDHA subunit expression also slightly, but significantly, decreased (by about 20%). Silencing the SDHB subunit causes loss of activity of the complex, and, as a result, succinate accumulates. Indeed, as shown in Fig. 1D, succinate concentration in SDHB-silenced cells is approximately double that of the control cells. Succinate, by acting as a competitive inhibitor of the enzyme prolyl-hydroxylase, inhibits HIF1 α and HIF2 α degradation. As expected, in the silenced cells, we found a significant increase in HIF1 α and HIF2 α expression (Fig. 1E), which was confirmed by the results of densitometric analysis (Fig. 1F).

SDHB silencing increases cell growth and invasiveness

The effects of silencing on cell proliferation were assessed by measuring [³H]thymidine incorporation as an index of DNA synthesis. As shown in Fig. 2A, SDHB-silenced cells showed a statistically significant increase in proliferation (22 $\pm$ 5%) as compared with controls (*P* < 0.05). Furthermore, to investigate the potential for invasion of adjacent tissue, we evaluated the ability of silenced cells to digest extracellular matrix. We thus analyzed matrix metalloproteinase (MMP) activity in the culture medium of silenced and control cells; activity was significantly higher in the SDHB-silenced SK-N-AS (Fig. 2B).

SDHB silencing affects cell metabolism

The tricarboxylic acid cycle sustains oxidative phosphorylation carried out by the mitochondrial respiratory chain and ends in oxygen reduction and ATP production. Indeed, a decrease in SDH activity in the Krebs cycle involves a reduction in the amount of reducing equivalents (NADH) and consequently in electrons entering the respiratory chain, which results in lower oxygen consumption and ATP production. In the present study, we observed that oxygen consumption was significantly lower in SDHB-silenced cells (3 nmol/300 μ l) as compared with control cells (11 nmol/300 μ l) (*P* < 0.05) (Fig. 3A). Nowadays, it is well known that tumor cells undergo profound changes in their

Figure 1

Succinate dehydrogenase (SDH) activity, succinate concentration, SDH subunit, and *HIF1α* expression levels. Total protein lysates were assessed for mitochondrial SDH subunits A and B (A) and for *HIF1α* and *HIF2α* (E) by western blot analysis. Blots represent three independent preparations; actin immunoblot was used as a loading control. Corresponding densitometric analysis of western blot bands (B and F), which was performed using imaging and analysis software by Bio-Rad (Quantity One), showed significant differences in protein expression between *SDHB*-silenced cells (gray) and control cells (black). Bars are means $\pm$ s.d. for three

independent preparations, ** $P < 0.01$, *** $P < 0.001$. (C) SDH activity expressed as a percentage of the activity measured in cell homogenates. *SDHB*-silenced cells (gray) showed significantly decreased SDH activity as compared with control cells (black). Bars are means $\pm$ s.d. for five independent experiments (each conducted in duplicate samples), ** $P < 0.01$. Decreased activity led to the doubling of succinate concentration in silenced cells as compared to control (D). Experiments were conducted in triplicate samples, and values are means $\pm$ s.d., * $P < 0.05$.

metabolism. Indeed, even in the presence of oxygen, cancer cells preferentially use aerobic glycolysis, which is also called the Warburg effect, and consists of the production of glycolytic intermediates, such as lactate, that are needed to increase cancer biomass (Vander Heiden *et al.* 2009, Koppenol *et al.* 2011). To establish whether silencing of subunit B might induce modifications of glycolysis, we measured cell glucose uptake and lactate levels in the culturing medium. In *SDHB*-silenced cells, we observed a significant decrease in both glucose uptake (of $41 \pm 5\%$, $P < 0.05$) and concentration of lactate in the medium (2.3 ± 0.1 versus 3.3 ± 0.6 mmol/l) as compared with the controls (Fig. 3B and C, $P < 0.05$). After the addition of labeled lactate, we observed a significant increase in lactate uptake by *SDHB*-silenced cells ($33 \pm 5\%$) as compared with the controls (Fig. 3D, $P < 0.05$).

Role of the microenvironment

Effects of fibroblasts on control and silenced SK-N-AS cells: glucose uptake, lactate uptake, and proliferation After evaluating glucose and lactate uptake in individual tumor cells in which *SDHB* was silenced and in control cells to study changes in their metabolism, the same procedure was repeated with the co-cultures to investigate any possible interactions between cancer cells and primary human fibroblasts. In co-culture experiments, glucose uptake was significantly lower in both the controls and the silenced cells as compared to the results from the single-cultured cells. The decrease was higher in the *SDHB*-silenced co-cultured cells, reaching 70% (Fig. 4A). Lactate uptake significantly increased and was even more pronounced in co-cultured *SDHB*-silenced cells as compared with the controls (352%

Figure 2

Effects of *SDHB* silencing on proliferation and metalloproteinase activity. (A) Labeled thymidine incorporation was measured in 24 h serum-starved cells at approximately 50% confluence and treated with 1% FCS for 24 h. *SDHB*-silenced cells (gray) showed a significant increase in cell proliferation as compared with controls (black). Data are means for three independent experiments performed in triplicate, $*P<0.05$ as compared to controls. (B) Matrix metalloproteinase (MMP) activity as analyzed by zymography. MMP activity is represented by the visible-light bands relating to the enzymatic digestion of gelatin by metalloproteinases.

versus 300%, Fig. 4B). The effect of fibroblasts on cancer cell growth rate was analyzed by CellTrace CFSE. Compared with tumor cells cultured alone, in the presence of human primary fibroblasts, both control cells and *SDHB*-silenced cells proliferated significantly more ($P<0.05$). In co-cultured silenced neuroblastoma cells, the increase was significantly more evident, reaching 92% ($P<0.05$; Fig. 4C).

Figure 3

Study of O_2 consumption, glucose uptake, lactate concentration, and uploading. (A) Oxygen consumption was measured by the Oxygraph system (Hansatech Instruments, Pentney, Norfolk, UK). Cells were loaded into a chamber containing 300 μ l of DMEM and 10% FCS. Oxygen consumption was monitored for about 5 min at 37 $^{\circ}$ C. Oxygen consumption significantly decreased in *SDHB*-silenced cells (gray bars) as compared with control cells (black bars). (B) Glucose uptake was measured in cells that had been serum-starved in DMEM with a low glucose concentration (1 g/l) overnight. Incorporated radioactive 2-deoxy-glucose was assayed by liquid scintillation counting. Silenced cells showed a significant decrease in glucose uptake. (C) Culture media were replaced with fresh media once

Effects of tumor cells on fibroblast metabolism

To investigate whether tumor cells could affect fibroblast metabolism, we measured glucose uptake in primary human fibroblasts co-cultured with control or *SDHB*-silenced cells. Interestingly, we found that glucose uptake was significantly increased in fibroblasts conditioned with tumor cells and that this effect was even more evident in fibroblasts co-cultured with *SDHB*-silenced tumor cells as compared with the corresponding single-cultured fibroblasts or fibroblasts co-cultured with control cells (Fig. 5).

Discussion

The correlation between SDH mutations and the onset of PHEOs/PGLs has been well established (Baysal et al. 2000, Eng et al. 2003, and, for recent reviews, see Dahia (2014) and Martucci & Pacak (2014)). Nevertheless, the whole molecular mechanism leading from loss of SDH enzymatic activity to occurrence of a tumor is still only partially known.

In the present paper, we report for the first time, to our knowledge, the effects of *SDHB* silencing on cellular metabolism and the role of the tumor microenvironment in tumor progression.

For the present study, we decided to use the human neuroblastoma cell line SK-N-AS for the following reasons: SK-N-AS cells share the same origin (neural crest) with

cells reached 100% confluence. After 24 h, media were collected and centrifuged, and supernatants were transferred into clean tubes. Lactate was immediately measured in the culture media by a Lactate Assay kit (Bioscience Life Sciences, Milan, Italy) according to the manufacturer's instructions. Silenced cells showed a significant decrease in lactate concentration. (D) Radioactive lactate uptake was assayed by liquid scintillation counting. Silenced cells showed a significant increase in lactate uptake in this case. In all of the experiments, bars are the means $\pm$ s.d. of three independent experiments, $*P<0.05$. For glucose and lactate uptake, each experiment was conducted in triplicate samples.

Figure 4

Effects of fibroblasts on tumor cell proliferation, glucose uptake, and lactate uptake. Tumor cells and fibroblasts were co-cultured in contiguous wells separated by a membrane, which was permeable to the common culture medium. In co-culture, glucose uptake (A) significantly decreased both in controls (black) and silenced cells (white) as compared with the results for the corresponding single-cultured cells (dark gray and light gray respectively). In contrast, lactate uptake (B) significantly increased both in controls (black) and silenced cells (white) co-cultured with fibroblasts as

compared to the results for the corresponding single-cultured cells (dark gray and light gray respectively). (C) The effect of fibroblasts on cancer cell growth rate was analyzed using CellTrace CFSE. Once again, in comparison with tumor cells alone (dark gray for controls and light gray for *SDHB*-silenced cells), in the presence of human primary fibroblasts, both control cells (black) and *SDHB*-silenced cells (white) proliferate significantly more. Bars are means $\pm$ s.d. for five independent experiments, * $P < 0.05$, ** $P < 0.01$.

ganglionic cells; they derive from a rather immature human neural phenotype (it should be remembered that *SDHx* as well as other mutations are germline and therefore modify cell functions beginning at a very early embryonic stage); and they are WT for all of the currently known PGL susceptibility genes (as opposed to animal cell lines such as PC12, MPC, and MTT) (Greene & Tischler 1976, Powers et al. 2000, Martiniova et al. 2009). Finally, the only human PHEO cell line (hPheo1) described to date (Ghayee et al. 2013) is not commercially available and was obtained from a primary culture of an adrenergic cluster 2-pertaining PHEO that is immortalized by hTERT viral transfection, which therefore drives its growth potential.

We have previously demonstrated that *SDHB*-mutated cells have a phenotype closely resembling the *in vivo* phenotype of *SDHB*-mutated PHEOs/PGLs (Rapizzi et al. 2012) in that they show reduced SDH expression levels and activity. These results are confirmed even more clearly in the present study, where *SDHB* was silenced. Moreover, as expected from the finding that succinate accumulation inhibits prolyl-hydroxylase activity and reduces HIF degradation (Dahia et al. 2005, Pollard et al. 2005, Selak et al. 2005, Koivunen et al. 2007, Favier et al. 2009, and, for recent reviews, see Richter et al. (2013) and Jochmanová et al. (2014)), we found a significant increase in *HIF1α* and *HIF2α* expression. Consistent with a reduction of SDH activity in the Krebs cycle, which leads to a decrease in reducing equivalent production, we also observed a significant decrease in oxygen consumption in *SDHB*-silenced cells.

In the present study, *SDHB* silencing was associated with an increase in cell proliferation and invasion, as demonstrated by increased thymidine incorporation

and metalloproteinase assays. We proposed the hypothesis that these increases might be the consequences of altered metabolism in these cells. It was reasonably assumed that adaptation to this metabolism could lead to increased cell division rate and migration. In a previous study (Rapizzi et al. 2012), we demonstrated that in each SK-N-AS *SDHB*-mutated clone, reduced glucose uptake and reduced lactate concentration in the medium were

Figure 5

Effects of tumor cells on fibroblast metabolism. Glucose uptake in primary human fibroblasts co-cultured with control (dark gray) or *SDHB*-silenced cells (light gray) were significantly increased as compared with corresponding single-cultured fibroblasts (black). Bars are means $\pm$ s.d. for three independent experiments, * $P < 0.05$, ** $P < 0.0001$.

coupled with an increase in cell proliferation and migration. These results have been confirmed in the present study, where we also demonstrated that *SDHB*-silenced cells increase the uptake of lactate, thus proving that SDH impairment itself induces metabolic reprogramming.

Recently, Fiaschi *et al.* (2012) demonstrated that prostate cancer cells that had been exposed to cancer-associated fibroblasts gradually became independent of glucose use, but lactate uploading increased. Moreover, they showed that prostate cancer cells secrete soluble factors that activate human prostate fibroblasts, which increases their glucose uptake and lactate secretion. The present results, consistent with the results of Fiaschi *et al.* (2012), indicate not only that *SDHB* silencing causes a metabolic and functional derangement of tumor cells but also that the microenvironment, here represented by fibroblasts, strongly affects tumor metabolism and growth capacity. In particular, we demonstrated that primary fibroblasts and tumor cells establish reciprocal metabolic changes. When co-cultured with human fibroblasts, control cells showed a significant decrease in glucose uptake and a significant increase in cell proliferation as compared with their mono-cultured counterparts. These effects were even more striking in co-cultured silenced cells. Moreover, in the present experimental model, tumor cells also promoted a Warburg-like glycolytic metabolism in the fibroblasts, which thus increased glucose uptake and its conversion into lactate. These results fit a model in which neuroblastoma cells and fibroblasts exert a reciprocal influence on their metabolism and function, and they indicate that lactate extruded by fibroblasts is uploaded by tumor cells and most probably used for fueling the Krebs cycle as well as anabolic processes and cell proliferation.

The present experimental model allowed us to dynamically evaluate the functional consequences of impaired SDH activity at the cellular level and reciprocal conditioning in cells of the tumor microenvironment. In this study, the metabolic changes and increase in proliferation induced by fibroblasts on neuroblastoma cells were found to be further increased by *SDHB* silencing, which thus indicates that fibroblast-derived factors and SDH enzymatic impairment play an additive role in tumor progression.

To the best of our knowledge, the present study is the first to evaluate metabolic and functional cell modifications induced by both *SDHB* silencing and fibroblast/tumor cells cross-talk.

We believe that comprehension of the mechanisms driving this cross-talk between tumor cells and the

microenvironment might represent not only an attempt to understand the novel molecular mechanisms that lead to tumor onset and progression but also the first step toward the development of possible pharmacological approaches aimed at limiting the proliferative effect and invasive/metastasizing potential of these tumors.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Adam J, Yang M, Soga T & Pollard PJ 2013 Rare insights into cancer biology. *Oncogene* **33** 2547–2556. (doi:10.1038/onc.2013.222)
- Baysal BE, Ferrell RE, Willett-Brozick JE, Lawrence EC, Myssiorek D, Bosch A, van der Mey A, Taschner PE, Rubinstein WS, Myers EN *et al.* 2000 Mutations in *SDHD*, a mitochondrial complex II gene, in hereditary paraganglioma. *Science* **287** 848–851. (doi:10.1126/science.287.5454.848)
- Castro-Vega LJ, Buffet A, De Cubas AA, Cascón A, Menara M, Khalifa E, Amar L, Azriel S, Bourdeau I, Chabre O *et al.* 2014 Germline mutations in *FH* confer predisposition to malignant pheochromocytomas and paragangliomas. *Human Molecular Genetics* **23** 2440–2446. (doi:10.1093/hmg/ddt639)
- Dahia PL 2014 Pheochromocytoma and paraganglioma pathogenesis: learning from genetic heterogeneity. *Nature Reviews Cancer* **14** 108–119. (doi:10.1038/nrc3648)
- Dahia PL, Ross KN, Wright ME, Hayashida CY, Santagata S, Barontini M, Kung AL, Sanso G, Powers JF, Tischler AS *et al.* 2005 A HIF1 α regulatory loop links hypoxia and mitochondrial signals in pheochromocytomas. *PLoS Genetics* **1** 72–80. (doi:10.1371/journal.pgen.0010008)
- Eng C, Kiuru M, Fernandez MJ & Aaltonen LA 2003 A role for mitochondrial enzymes in inherited neoplasia and beyond. *Nature Reviews Cancer* **3** 193–202. (doi:10.1038/nrc1013)
- Favier J, Brière JJ, Burnichon N, Rivière J, Vescovo L, Benit P, Giscos-Douriez I, De Reyniès A, Bertherat J, Badoual C *et al.* 2009 The Warburg effect is genetically determined in inherited pheochromocytomas. *PLoS ONE* **4** e7094. (doi:10.1371/journal.pone.0007094)
- Fiaschi T, Marini A, Giannoni E, Taddei ML, Gandellini P, De Donatis A, Lanciotti M, Serni S, Cirri P & Chiarugi P 2012 Reciprocal metabolic reprogramming through lactate shuttle coordinately influences tumor-stroma interplay. *Cancer Research* **72** 5130–5140. (doi:10.1158/0008-5472.CAN-12-1949)
- Ghayee HK, Bhagwandin VJ, Stastny V, Click A, Ding LH, Mizrahi D, Zou YS, Chari R, Lam WL, Bachoo RM *et al.* 2013 Progenitor cell line

- (hPheo1) derived from a human pheochromocytoma tumor. *PLoS ONE* **8** e65624. (doi:10.1371/journal.pone.0065624)
- Gimenez-Roqueplo AP, Favier J, Rustin P, Rieubland C, Crespin M, Nau V, Khau Van Kien P, Corvol P, Plouin PF, Jeunemaitre X *et al.* 2003 Mutations in the *SDHB* gene are associated with extra-adrenal and/or malignant pheochromocytomas. *Cancer Research* **63** 5615–5621.
- Goffrini P, Ercolino T, Panizza E, Giachè V, Cavone L, Chiarugi A, Dima V, Ferrero I & Mannelli M 2009 Functional study in a yeast model of a novel succinate-dehydrogenase subunit B gene germline missense mutation (C191Y) diagnosed in a patient affected by a glomus tumor. *Human Molecular Genetics* **18** 1860–1868. (doi:10.1093/hmg/ddp102)
- Greene LA & Tischler AS 1976 Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *PNAS* **73** 2424–2428. (doi:10.1073/pnas.73.7.2424)
- Hanahan D & Coussens LM 2012 Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* **21** 309–322. (doi:10.1016/j.ccr.2012.02.022)
- Hanahan D & Weinberg RA 2011 Hallmarks of cancer: the next generation. *Cell* **144** 646–674. (doi:10.1016/j.cell.2011.02.013)
- Hu M & Polyak K 2008 Microenvironmental regulation of cancer development. *Current Opinion in Genetics & Development* **18** 27–34. (doi:10.1016/j.gde.2007.12.006)
- Jochmanová I, Zelinka T, Widimský J Jr & Pacak K 2014 HIF signaling pathway in pheochromocytoma and other neuroendocrine tumors. *Physiological Research* **63** (Suppl 2) S251–S262.
- Kaelin WG Jr & McKnight SL 2013 Influence of metabolism on epigenetics and disease. *Cell* **153** 56–69. (doi:10.1016/j.cell.2013.03.004)
- Karagiannis GS, Poutahidis T, Erdman SE, Kirsch R, Riddell RH & Diamandis EP 2012 Cancer-associated fibroblasts drive the progression of metastasis through both paracrine and mechanical pressure on cancer tissue. *Molecular Cancer Research* **10** 1403–1418. (doi:10.1158/1541-7786.MCR-12-0307)
- Klemm F & Joyce JA 2014 Microenvironment regulation of therapeutic response in cancer. *Trends in Cell Biology* **25** 198–213. (doi:10.1016/j.tcb.2014.11.006)
- Koivunen P, Hirsilä M, Remes AM, Hassinen IE, Kivirikko KI & Myllyharju J 2007 Inhibition of hypoxia-inducible factor (HIF) hydroxylases by citric acid cycle intermediates: possible links between cell metabolism and stabilization of HIF. *Journal of Biological Chemistry* **282** 4524–4532. (doi:10.1074/jbc.M610415200)
- Koppenol WH, Bounds PL & Dang CV 2011 Otto Warburg's contributions to current concepts of cancer metabolism. *Nature Reviews Cancer* **11** 325–337. (doi:10.1038/nrc3038)
- Letouzé E, Martinelli C, Lorient C, Burnichon N, Abermil N, Ottolenghi C, Janin M, Menara M, Nguyen AT, Benit P *et al.* 2013 SDH mutations establish a hypermethylator phenotype in paraganglioma. *Cancer* **23** 739–752. (doi:10.1016/j.ccr.2013.04.018)
- Martiniova L, Lai EW, Elkahoul AG, Abu-Asab M, Wickremasinghe A, Solis DC, Perera SM, Huynh TT, Lubensky IA, Tischler AS *et al.* 2009 Characterization of an animal model of aggressive metastatic pheochromocytoma linked to a specific gene signature. *Clinical & Experimental Metastasis* **26** 239–250. (doi:10.1007/s10585-009-9236-0)
- Martucci VL & Pacak K 2014 Pheochromocytoma and paraganglioma: diagnosis, genetics, management, and treatment. *Current Problems in Cancer* **38** 7–41. (doi:10.1016/j.currprobcancer.2014.01.001)
- Morin A, Letouzé E, Gimenez-Roqueplo AP & Favier J 2014 Onco-metabolites-driven tumorigenesis: from genetics to targeted therapy. *International Journal of Cancer. Journal International du Cancer* **135** 2237–2248. (doi:10.1002/ijc.29080)
- Pollard PJ, Brière JJ, Alam NA, Barwell J, Barclay E, Wortham NC, Hunt T, Mitchell M, Olpin S, Moat SJ *et al.* 2005 Accumulation of Krebs cycle intermediates and over-expression of HIF1 α in tumours which result from germline *FH* and *SDH* mutations. *Human Molecular Genetics* **14** 2231–2239. (doi:10.1093/hmg/ddi227)
- Powers JF, Evinger MJ, Tsokas P, Bedri S, Alroy J, Shahsavari M & Tisler AS 2000 Pheochromocytoma cell lines from heterozygous neuro-fibromatosis knockout mice. *Cell and Tissue Research* **302** 309–320. (doi:10.1007/s004410000290)
- Quail DF & Joyce JA 2013 Microenvironmental regulation of tumor progression and metastasis. *Nature Medicine* **19** 1423–1437. (doi:10.1038/nm.3394)
- Rapizzi E, Ercolino T, Canu L, Giachè V, Francalanci M, Pratesi C, Valeri A & Mannelli M 2012 Mitochondrial function and content in pheochromocytoma/paraganglioma of succinate dehydrogenase mutation carriers. *Endocrine-Related Cancer* **19** 261–269. (doi:10.1530/ERC-11-0263)
- Rapizzi E, Ercolino T, Fucci R, Zampetti B, Felici R, Guasti D, Morandi A, Giannoni E, Giachè V, Bani D *et al.* 2014 Succinate dehydrogenase subunit B mutations modify human neuroblastoma cell metabolism and proliferation. *Hormones & Cancer* **5** 174–184. (doi:10.1007/s12672-014-0172-3)
- Richter S, Qin N, Pacak K & Eisenhofer G 2013 Role of hypoxia and HIF2 α in development of the sympathoadrenal cell lineage and chromaffin cell tumors with distinct catecholamine phenotypic features. *Advances in Pharmacology* **68** 285–317. (doi:10.1016/B978-0-12-411512-5.00014-2)
- Richter S, Peitzsch M, Rapizzi E, Lenders JW, Qin N, de Cubas AA, Schiavi F, Rao JU, Beuschlein F, Quinkler M *et al.* 2014 Krebs cycle metabolite profiling for identification and stratification of pheochromocytomas/paragangliomas due to succinate dehydrogenase deficiency. *Journal of Clinical Endocrinology and Metabolism* **99** 3903–3911. (doi:10.1210/jc.2014-2151)
- Santi A, Caselli A, Paoli P, Corti D, Camici G, Pieraccini G, Taddei ML, Serni S, Chiarugi P & Cirri P 2013 The effects of CA IX catalysis products within tumor microenvironment. *Cell Communication and Signaling* **11** 81. (doi:10.1186/1478-811X-11-81)
- Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD, Pan Y, Simon MC, Thompson CB & Gottlieb E 2005 Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF- α prolyl hydroxylase. *Cancer Cell* **7** 77–85. (doi:10.1016/j.ccr.2004.11.022)
- Semenza GL 2013 HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. *Journal of Clinical Investigation* **123** 3664–3671. (doi:10.1172/JCI67230)
- Sounni NE & Noel A 2013 Targeting the tumor microenvironment for cancer therapy. *Clinical Chemistry* **59** 85–93. (doi:10.1373/clinchem.2012.185363)
- Taddei ML, Giannoni E, Comito G & Chiarugi P 2013 Microenvironment and tumor cell plasticity: an easy way out. *Cancer Letters* **341** 80–96. (doi:10.1016/j.canlet.2013.01.042)
- Takahashi Y, Sipp D & Enomoto H 2013 Tissue interactions in neural crest cell development and disease. *Science* **341** 860–863. (doi:10.1126/science.1230717)
- Vander Heiden MG, Cantley LC & Thompson CB 2009 Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **324** 1029–1033. (doi:10.1126/science.1160809)
- Zhang J & Liu J 2012 Tumor stroma as targets for cancer therapy. *Pharmacology & Therapeutics* **137** 200–215. (doi:10.1016/j.pharmthera.2012.10.003)

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