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 HIF1α and HIF2α 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.

Introduction
Cancer research on solid tumors has recently focused on two main topics: i) the role played by the microenvironment 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 & Poljak 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 subunits, with a high incidence (up to 80%) of malignant SDH mutations (Gimenez-Roqueplo et al. 2003, Letouzé et al. 2013; 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 EDTA, 15 mM Na2HPO4, 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 ml 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 MgSO₄, 1 mM CaCl₂, and 5 mM KCl, pH 7.4) containing 0.5 μCi/ml [3H]deoxyglucose or 1 μCi/ml [U-14C]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 µl 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 µl 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 ± S.D. of at least three experiments.

Results

SDHB silencing reduces SDH expression and activity and enhances HIF1α and HIF2α 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 HIF1α and HIF2α degradation. As expected, in the silenced cells, we found a significant increase in HIF1α and HIF2α 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 ± 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 µl) as compared with control cells (11 nmol/300 µl) (P < 0.05) (Fig. 3A). Nowadays, it is well known that tumor cells undergo profound changes in their metabolism that are aimed at favoring cell growth and invasion.
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% 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±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%
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
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 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 ± S.D. for five independent experiments, *P<0.05, **P<0.001.
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.

Funding
This work was supported by Fondazione Cassa di Risparmio di Pistoia e Pisa (Prot. 2013.0375) to M Mannelli; E Rapizzi was the recipient of an Ente Cassa di Risparmio di Firenze research fellowship (Prot. 2013.0503); E Rapizzi, R Fucci, L Canu, and M Mannelli are members of the ENS@T (European Network for the Study of Adrenal Tumors).

Acknowledgements
We wish to thank Professor Gabriella Nesi for editing the manuscript.

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Received in final form 19 March 2015
Accepted 25 March 2015
Made available online as an Accepted Preprint
25 March 2015