Acidic extracellular pH and V-ATPase activity modulate cancer survival and stemness of Ewing’s sarcoma

ABSTRACT INTRODUCTION
It was already at the early 20th century that most cancers appeared to have an intense anaerobic glycolysis and, as a consequence, a high lactate production that leads to an acidic microenvironment. More recently, the extracellular acidification activity in cancer cells has been associated with increased aggressiveness, extracellular proteolytic activity, chemoresistance, expression of pro-angiogenic factors, and immunotolerance, therefore appearing as a promising therapeutic target for anticancer innovative approaches. In Ewing’s sarcoma, the commonly observed high circulating levels of LDH, cytoplasmic PAS staining of cytoplasmic glycogen, and the positive signal of PET-FDG imaging are strongly suggestive for these phenomena. Different ion/proton pumps can compartmentalize or directly pump excessive cytosolic H⁺ into the extracellular microenvironment. Among these, the Na⁺/H⁺ exchanger, the carbonic anhydrases IX, or the vacuolar ATPase (V-ATPase) have been associated with cell survival, malignant transformation, and tumor progression. In this study, in a panel of Ewing’s sarcoma cell lines, we investigated the role of extracellular acidification activity and of proton pump/transporters, with particular reference to V-ATPase, with the aim to identify a new therapeutic target to be used in combination with conventional therapies. Cancer stem cells are currently emerging as a fundamental model to identify effective anti-cancer therapies. They are widely considered as a rare biologically distinct population of tumor-initiating cells, and as the real responsible for the development, maintenance, and drug-resistance in several malignancies. Accordingly, in this study we also investigated on how an acidic extracellular pH can modulate the stemness of Ewing’s sarcoma cells.

METHODS
In this study, the A-673, SK-N-MC, SK-ES-1, and RD-ES cell lines were used as Ewing’s sarcoma cell models, whereas MRC-5 cells or primary cultures of fibroblasts from the healthy donors were used as controls. First, the ability to acidify the supernatant and to survive at different acidic pH were evaluated. Then, the presence of lysosomes was analyzed by using the neutral red, lysosensor, and acridine orange staining. The presence of vesicles and the morphology of mitochondria were also evaluated by transmission electron microscopy (TEM). After exposure to different pH, the level of expression of the stemness related genes OCT4 and Nanog, and the proton pump/transporter Na⁺/H⁺ exchanger, the carbonic anhydrases IX, and the V-ATPase were measured by Real Time PCR. The protein expression and localization of V-ATPase were also analyzed by immunofluorescence and Western blotting. To investigate the role of V-ATPase in the survival of Ewing’s sarcoma cells, increasing concentrations of Bafilomycin a1, of omeprazole, or capillary electroporation for the delivery of a specific anti-V-ATPase siRNA were used.

RESULTS SECTION
Ewing’s sarcoma cells showed highest proliferation rates at a more acidic pH (6.5) in respect with normal human fibroblasts (7.1), 60.5% of Ewing’s sarcoma cell cultures still surviving at pH 5.6 vs 38.7% of normal human fibroblasts. Moreover, Ewing’s sarcoma cells induced a significant reduction of extracellular pH (0.23 units) after only three hours of incubation. Moreover, an inverse correlation between medium pH and the level of mRNA OCT4 and Nanog expression in Ewing’s sarcoma cells was observed. Since the acidification activity is usually related to the number of lysosomes, we unexpectedly observed very few and not so acidic vesicles in Ewing’s sarcoma cells. A reduced vacuolar compartment was also confirmed by TEM analysis (Figure 1). Notably, abnormal mitochondria with very few cristae were observed, suggesting an altered oxidative phosphorylation activity. Among the proton pump/transporters, we observed a very high expression of V-ATPase, that was mainly localized on the plasma membrane (Fig. 2). Moreover, both treatment with Bafilomycin 1A, omeprazole, or capillary electroporation with siRNA anti-V-ATPase significantly impaired tumor survival at acidic pH 6.5. In particular, omeprazole significantly inhibited the SK-ES-1 cell line, especially in unbuffered conditions (EC50 in unbuffered condition 20.2 mM, p = 0.03204; in buffered condition 111.23 mM, p = 0.0445).

DISCUSSION
We confirmed that Ewing’s sarcoma cells are able to strongly acidify the extracellular space and survive in acidic conditions, in contrast to normal human fibroblasts. In addition, as a new concept, we showed that an acidification environment can induce the expression of stem cell-like properties, implying the selection of a more aggressive phenotype. Then, we found that this acidification activity is mainly related to the activity of V-ATPase on the plasma membrane, and that Ewing’s sarcoma cells can extrude excessive protons by directly pumping them out in the extracellular space. Notably, in other cancers the plasma membrane localization of V-ATPase has been associated with a higher invasiveness and ability to develop metastases, and these results are in agreement with the very aggressive behavior of Ewing’s sarcoma cells. Lastly, we demonstrated that the V-ATPase activity can be impaired by siRNA technology or by using specific inhibitors that are already available for chlorthalidone, such as omeprazole. In conclusion, we suggest proton pump/transporters inhibitors for the treatment of Ewing’s sarcoma to be used in combination with standard anticancer agents.

SIGNIFICANCE
This study demonstrates that stemness and survival of Ewing’s sarcoma cells are modulated by the ability of tumor cells to acidify the extracellular space, and that this activity can be impaired by targeting the proton pump V-ATPase. These data can be helpful to identify new effective therapeutic regimens for Ewing’s sarcoma patients.

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REFERENCES