



ROCK1-Predicted microRNAs Dysregulation Contributes to Tumor Progression in Ewing Sarcoma

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Abstract

Over the last decade, the rho-associated kinases and several metastasis-associated microRNAs have emerged as important contributors of tumor invasion. However, despite prominence, our understanding of their involvement in the metastatic potential of Ewing Sarcoma (EWS) is incomplete. The expression profiles of ROCK1 or ROCK2 and miR-124-3p, miR-138-5p, miR-139-5p, miR-335-5p and miR-584-5p (all of which were previously predicted or validated to regulate these kinases) were evaluated through qRT-PCR and associated with clinical parameters. In vitro assays to evaluate colony formation and invasion/migration capacities were performed on SK-ES-1 cells transfected with pre-miR mimics. ROCK1 expression was significantly reduced in EWS tissues, though there was no association with pathological parameters. miR-124-3p, miR-139-5p and miR-335-3p were also found significantly downregulated and positively correlated with ROCK1. Stratification indicated an association between lower levels of miR-139-5p and miR-584-5p with disease progression ($p < 0.05$), while reduced expression of the former and miR-124-3p were associated with reduced survival. In vitro miR-139-5p overexpression yielded inconsistent results: while miR-139-5p restoration significantly reduced invasion, the clonogenic capacity of cells was increased. Our study demonstrated that downregulation of miR-124-3p, miR-139-5p and miR-584-5p are associated with disease progression in EWS and may serve as a risk assessment biomarkers though, as seen for miR-139-5p, their specific role remain to be elucidated for considering tailoring treatment options.

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Introduction

Ewing's sarcoma (EWS) is the second most common bone cancer in children and adolescents. This tumor is characterized by undifferentiated, small and round cells of neuroepithelial origin, that in 90% of patients, harbor a reciprocal chromosomal translocation that results in the expression of the oncogenic EWS/FLI1 chimeric protein [1].

Standard treatment consists of pre- and postoperative chemotherapy, followed by surgery and/or radiotherapy. Despite this, in patients with metastatic disease, the 5-year survival diminishes from 60 to 70% to 10–40%. Moreover, the dissemination of tumor cells is such that even in patients without metastasis at diagnosis there may be circulating tumor cells [2].

For metastasis occurring, tumor cells must first detach from their tissue of origin, migrate and invade blood

vessels, be able to adhere, proliferate and colonize a secondary site [3]. Among the regulators of these processes are ROCK1 and ROCK2, effectors of the Rho family of GTPases that are responsible for cytoskeleton control and whose dysregulation aggravates phenotypes associated with cancer and other diseases [4].

Thus, in view of the intrinsic metastatic nature of EWS, the present study aimed to verify the expression profiles of ROCK1 or ROCK2 in tumor samples and to evaluate metastasis-associated microRNAs predicted or validated to regulate these kinases (Online Resource 1).

Materials and Methods

Clinical Samples EWS samples ($n=19$) were obtained from surgeries at the Clinics University Hospital (Ribeirao Preto School of Medicine – University of Sao Paulo), performed from 2006 to 2015 (survival analysis was followed until June 2016). None of the patient received chemotherapy or radiotherapy prior to biopsy. The patients corresponded to 7 women and 12 men with a mean age at diagnosis of 14 years (range: 4–28 years old). Seven patients presented metastasis, and ten had relapse. Non-tumor bone tissues were obtained from malformation bone corrective surgery. All specimens were snap frozen in liquid nitrogen and stored at -80°C for further analysis. Signed statement of informed consent was obtained from each participant or their parents/guardians. This study was approved by the local Ethics Committee that follows the Helsinki convention criteria (Comitê de Ética em Pesquisa da FFCLRP n° 43,619,215.9.0000.5407).

Cell Lines and Reagents The EWS cell lines SK-ES-1 and RD-ES were purchased from Rio de Janeiro Cell Bank (BCRJ, Federal University of Rio de Janeiro, Brazil). MRC-5 (normal fibroblast) was obtained from the ATCC (American Type Culture Collection, Rockville, MD). Cells were cultivated with recommended media supplemented with 10% of fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µg/mL) and kept at 37°C in a humidified 5% CO_2 incubator.

RNA Isolation, Reverse Transcription and Quantitative Real-Time PCR of mRNA and microRNA Total RNA of tumor and non-tumor samples was extracted using TRIzol® Reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's protocol. The quality and quantity of the RNA was evaluated by a ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies). cDNA was synthesized using the High Capacity kit (Applied Biosystems, MA, USA) according to the manufacturer's instructions. For microRNA quantification, the complementary strands were obtained using microRNA-specific primers. qRT-

PCR was performed using Taqman® microRNA and gene assays [ROCK1 (Hs01127699-m1), ROCK2 (Hs00178154-m1), miR-124-3p (001182), miR-138-5p (002284), miR-139-5p (002289), miR-335-5p (000546) and miR-584-5p (001624)] according to the manufacturer's protocol on a 7500 Real Time PCR System (Applied Biosystems, Waltham, MA, USA). As internal controls, small nuclear RNU6B and RNU48 were used to normalize the microRNAs level. GAPDH and GUSβ were used to normalize the gene levels. The MRC5 cell line was used as calibrator. Relative expression was calculated by $2^{-\Delta\Delta\text{CT}}$ analysis method.

Transfection of microRNA The SK-ES-1 cell line was transfected with pre-microRNA miR-139-5p (mirVana™ microRNA mimic ID: MC11749, Ambion®) and control (mirVana™ microRNA mimic negative control, Ambion®) using Lipofectamine® RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. microRNA transfection efficiency was monitored by qRT-PCR after 48 h.

Colony Formation Assay Single cell suspensions of 500 cells were seeded in 6-well plates 24 h after the transfection and incubated at 37°C for 10 days. Colonies were then washed, fixed with methanol and stained with Giemsa 3%. Only colonies with >50 cells were counted by direct visual inspection with a stereo microscope at $20\times$ magnification. Assays were performed in triplicate.

Invasion Assay Cell invasion was measured by invasion of the cells through Matrigel-coated Transwell inserts (Becton Dickinson & Co., NJ, USA). Cells were harvested after 24 h of transfection, re-suspended in serum-free medium and seeded on the top of coated invasion 8 µm pore size chambers (5×10^5 cell/insert). The bottom chamber was filled with media containing 10% of FBS. After 24 h of incubation, noninvasive cells were removed from the membrane upper surface with swabs. The invaded cells attached to the lower side of the membrane were fixed with 100% methanol and stained with Giemsa 3%. The membranes were removed from the inserts and placed on microscope slides with Entellan (Merk, NY, USA) and photographed at $20\times$ magnification (ten random fields of each membrane). The invaded cells were counted with the ImageJ software (Schneider et al., 2012). Assays were performed in triplicate.

Boyden Migration Assay Migration was assessed using a 48-wells Boyden Chamber containing a polycarbonate membrane with 8 µm pores (Neuroprobe Inc., MD, USA). Each membrane was coated with 5 mg/mL of fibronectin (Sigma-Aldrich, St. Louis, MO, USA) overnight before the assay. The bottom chambers were filled with FBS-free medium or

medium containing FBS. Cell lines were harvested after 24 h of transfection, re-suspended in serum-free medium and seeded on the upper chambers (2.5×10^4 cells/well). After 24 h of incubation, non-migrating cells were scraped from the upper surface of the membrane. Cells on the lower surface were fixed with 100% methanol and stained with hematoxylin. The membrane was placed on microscope slides and photographed at $20\times$ magnification (four random fields of each well). The invaded cells were counted with the ImageJ® software. Assays were performed in triplicate.

Western Blot Total protein samples were extracted from transfected (control and pre-miR-139-5p) SK-ES-1 cells after 48 h with RIPA buffer (Thermo Scientific, CA, USA) according to manufacturer instructions. Heat-denatured protein samples (50 mg per lane) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, EUA). The membranes were incubated for 1 h in TBS containing 0.1% of Tween 20 and 5% of blocker solution followed by incubation for 12 h at 4 °C with primary rabbit monoclonal anti-ROCK1 (Ab45171 at 1:500 dilution, Abcam, Cambridge, United Kingdom) and primary rabbit monoclonal anti-ROCK2 (Ab125025 at 1:10,000 dilution, Abcam) antibodies. The GAPDH expression level was measured as a loading control, using rabbit monoclonal anti-GAPDH antibody (Ab128915 at 1:10,000 dilution, Abcam) incubated for 1 h. The membrane was then incubated with goat anti-rabbit secondary antibody (Ab6721 at 1:5000 dilution, Abcam) for 1 h, and detected with the ECL Western Blotting Substrate Kit (Abcam, Cambridge, United Kingdom) and visualized in a ChemiDocBioimaging System (Bio-Rad, Hercules, California, EUA). Expression levels were quantified using ImageJ® software and normalized to loading control. Assays were performed in triplicate.

Statistical Analysis The association between the following variables: age (< 14 years versus 14 years old); sex; EWS/FLI1 status; necrosis stage after chemotherapy – HUVOS levels 1 and 2 (less than 90% of necrotic areas) versus HUVOS levels 3 and 4 (more than 90% necrotic areas); metastasis (presence versus absence); relapse (presence versus absence); disease progression (relapse/metastasis versus complete remission), death (alive versus deceased); and expression levels of each microRNA and genes was determined by Mann-Whitney tests. Survival analysis was carried out based on Log-Rank test represented on Kaplan-Meier curves using the median of the microRNA/gene expression observed as cutoff. Correlations between gene and miRNA expression were evaluated by Spearman's test and presented as a heatmap using the online platform Plotly 2.0. The functional assays data were statistically analyzed by Student's two-tailed t-test or One-Way Repeated Measures Analysis of Variance (ANOVA)

followed by the Bonferroni Pairwise Multiple Comparison. All tests were carried out for $\alpha=0.05$. All analyses were performed using the SPSS 21.0 software (SPSS Inc., IL, USA) and expressed as the mean \pm standard deviation.

Results

ROCKs and ROCK-Associated microRNAs are Dysregulated in Ewing Sarcoma

qRT-PCR was performed on nineteen EWS samples and twelve non-tumor bone samples from paired-age individuals to evaluate the expression levels of ROCKs and ROCK-associated microRNAs. As seen in Fig. 1a, ROCK1 expression was significantly reduced in EWS tissues compared with the control. Among the selected set of microRNAs, three of them (miR-124-3p, miR-139-5p and miR-335-3p) were found significantly downregulated compared to the control.

Similar expression patterns were observed for both EWS cell lines with decreased expression of ROCK1, miR124a-3p and miR139-5p. Conversely, no significant alterations were found to ROCK2, miR138-5p and miR584-5p (Fig. 1a).

miR-124a-3p, miR139-5p and miR-584-5p Expression Levels are Associated with EWS Progression

Associations between ROCKs and microRNA expression in EWS samples and clinical features were also investigated (Table 1). Contrary to the expected, our results showed a significant positive correlation between ROCK1 and miR-124a-3p, miR139-5p and miR-584-5p expression (Fig. 1b). Moreover, lower miR-139-5p and miR-584-5p expression was found in tumors from patients who presented relapse after treatment or any signals of tumor progression such as metastasis or death (Fig. 1c, d). Event-free survival analysis also showed that lower expression levels of miR-139-5p and miR-124a-3p are also correlated with worse prognosis (Fig. 1c, e).

Restoration of miR-139-5p Stimulates EWS Clonogenic Capacity but Inhibits Invasion and Migration

Once miR-139-5p was differentially expressed and related with tumor progression, we selected it for further functional analysis. In order to elucidate its contribution on cell growth and metastasis, we induced miR-139-5p expression in SK-ES-1 cell line. Our results suggested that this microRNA affects cell growth and invasion in a different manner. Overexpression of miR-139-5p leads to an increase on colony formation while reduces the cell line invasion capacity, and such results were obtained from 3 independent experiments

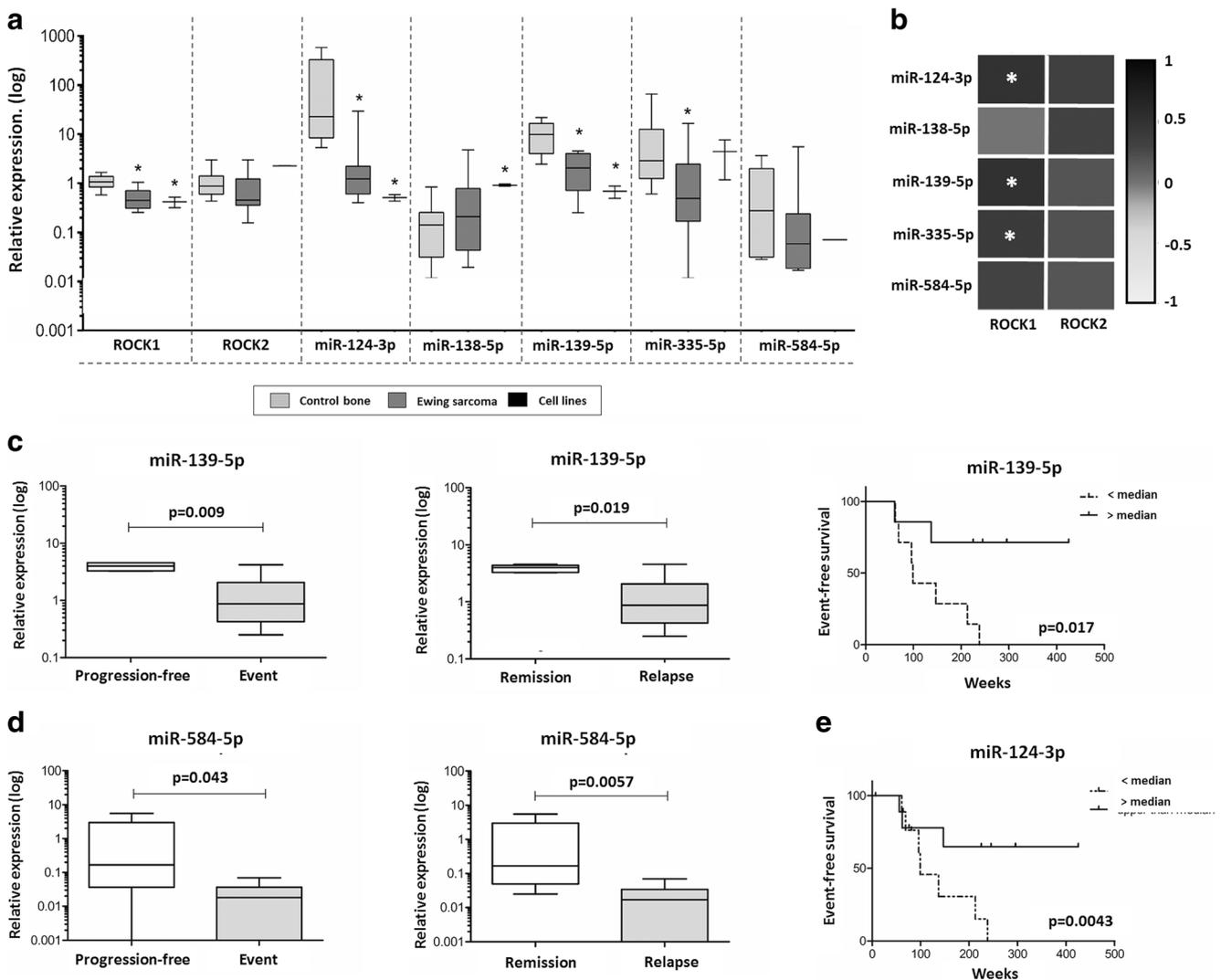


Fig. 1 Analysis of ROCK-1 and -2 and microRNAs related expression in Ewing Sarcoma. **a** Dysregulation of ROCK1, miR-124a-3p, miR-139-5p and miR-335-5p expression in EWS tissue and cell lines compared to non-tumor tissue. **b** Spearman Correlation showed positive correlations between ROCK1 and the following miRNAs: miR-124a-3p, miR-139-5p and miR-335-5p. Downregulation of miR-139-5p associated with tumor progression, relapse and shorter event-free survival. **d** Downregulation of miR-584-5p associated with tumor progression and relapse. **e** Lower expression of miR-124-3p associated with shorter event-free survival

Table 1 Association between clinical variables and microRNAs expression levels in OS tissue samples as determined by Mann–Whitney test

Clinical features (p value)	miR-124a	miR-138	miR-139	miR-335	miR-584	ROCK1	ROCK2
Age	0.870	0.102	0.053	0.568	0.215	0.683	0.624
Sex	0.612	0.933	0.028	0.933	0.281	0.735	0.398
EWS/FLI1 status	0.424	0.920	0.855	0.764	0.926	0.617	0.368
Metastasis	0.176	0.735	1.000	0.554	0.192	0.554	0.933
Death	0.072	0.424	0.572	0.057	0.353	0.230	0.057
Relapse	0.191	0.683	0.020	0.414	0.006	0.165	0.221
HUVOS grade	0.121	0.302	0.606	0.197	0.897	0.157	0.396
Disease progression	0.221	0.744	0.009	0.514	0.043	0.870	0.744
Overall survival	0.342	0.302	0.420	0.054	0.420	0.342	0.170
Event-free survival	0.043	0.591	0.017	0.948	0.117	0.654	0.487

(Fig. 2a, b). Moreover, miR-139-5p seems to suppress chemoattractive-independent migration but does not affect this process when stimulated by FBS (Fig. 2c). Also, despite the predicted positive correlation, after miR-139-5p overexpression, ROCK1 and ROCK2 levels were not significantly altered (Fig. 2d).

Discussion

The high metastatic potential of EWS remains a major challenge in clinics and, despite aggressive treatment, 80% of patients with metastatic disease die due to cancer progression [3].

The Rho-associated kinases, ROCK1 and ROCK2, frequently associated with the regulation of invasion through their ability to control cytoskeleton, cell adhesion and motility, have frequently been described as dysregulated in cancer over recent years [5]. Both kinases were found significantly increased and associated with poor differentiation, metastasis and short survival in primary bladder tumors [6]. Similarly, ROCK1 has been described as overexpressed in metastatic breast cancer and related with overall patient survival [7]. ROCK2, was also found hyperexpressed in intrahepatic metastasis [8]. Nonetheless, ROCK involvement in EWS remains elusive.

Our results showed downregulation of ROCK1 in EWS samples. Similar results were previously observed in vulvar cancer, in which ROCK1 levels were also related with low survival rates [9]. Comparatively, ROCK1 was

described as poorly expressed in invasive human melanomas and cutaneous metastases [10], suggesting a tumor dependent participation of Rho-associated kinases in cancer development. More importantly, the absence of associations between either ROCK1 or ROCK2 expression with clinical features or their expression pattern itself do not obligatorily reflect their activity, since they are controlled by several positive and negative regulators and that mainly occur on the posttranslational level [11].

Thus, in our study, we also investigated the expression of five microRNAs (miR-124a-3p, miR-138-5p, miR-139-5p, miR-335-5p and miR-584-5p) already validated to target ROCK1 or ROCK2. Our results showed that among them, miR-124a-3p, miR-139-5p and miR584-5p were also found downregulated EWS tumor samples.

Lower levels of miR-124a-3p were also related with shorter event-free survival in our cohort. In glioma samples, this microRNA was also underexpressed and negatively correlated with malignancy grade [12]. Dysregulation of miR-124a-3p has been reported in liver and cervical cancer, as well as medulloblastoma and acute leukemia [13–16]. Several studies showed that miR-124a-3p pre-treatment inhibits cell proliferation and invasion in vitro [17, 18]. Mostly, the tumor suppressor effects of this microRNA were explained by its capacity to target ROCK1 [18, 19]. Nonetheless, our results showed a positive correlation between these genes, discouraging further analysis.

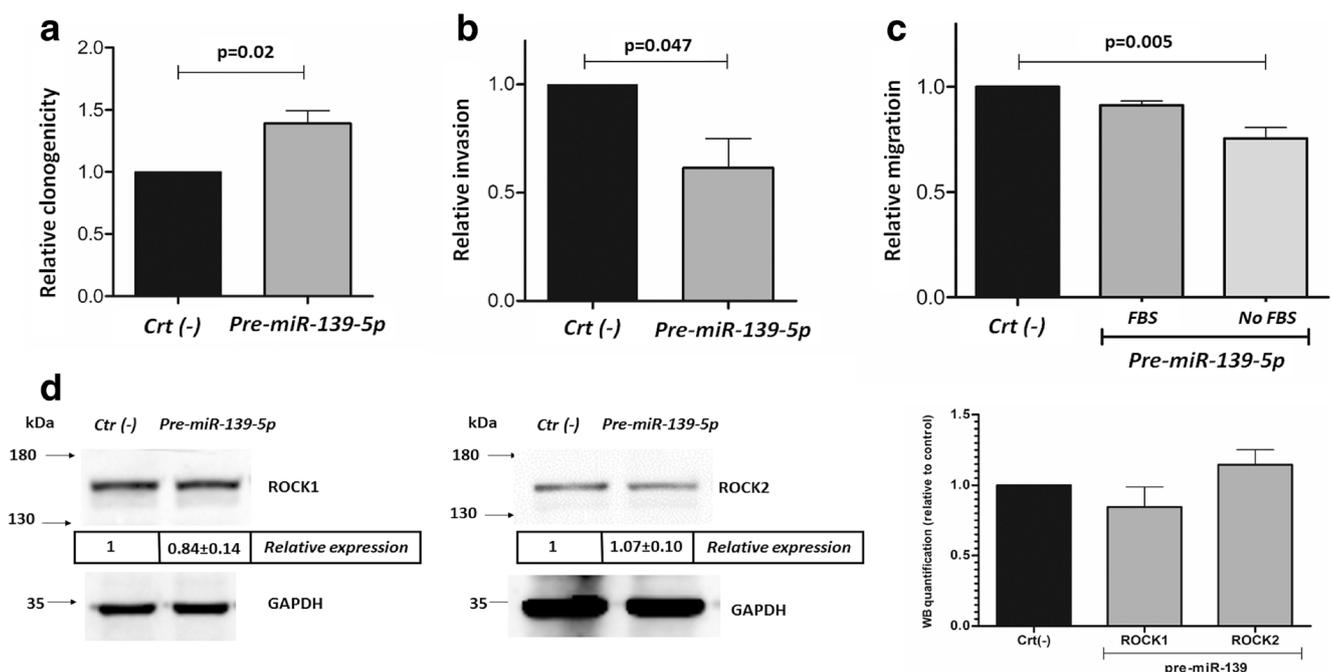


Fig. 2 Effects of miR-139-5p overexpression in SK-ES-1 cell line. **a** Overexpression of miR-139-5p stimulates the clonogenic capacity in vitro. **b** Restoration of miR-139-5p expression suppresses SK-ES-1 cells invasion through Matrigel towards chemoattractant. **c** In the

absence of chemoattractant (FBS), miR-139-5p overexpression decreases SK-ES-1 cells migration ability. **d** Forced miR-139-5p expression does not alter ROCK1 and ROCK2 protein levels

Even though there are few articles in the literature, miR-584-5p anti-invasive properties have also been associated with ROCK1 [20–22]. The expression of this microRNA was found downregulated in renal cell and papillary thyroid carcinoma cell lines [20, 21]. Additionally, Xue et al. [22] reported higher postoperative survival in glioma patients with high miR-584-5p expression. Those results are in agreement with our findings in which low miR-584-5p expression levels were associated with tumor progression (metastasis, relapse or deceased). Nonetheless, we were unable to find any correlations between this microRNA and its predicted target in EWS samples.

In parallel, miR-139-5p presented the most remarkable results. Low levels of this microRNA were not only evinced in EWS tumor compared with non-neoplastic tissue, but also associated with tumor progression and reduced event-free survival. miR-139-5p displays multifunction in human tumorigenesis, being described as relevant in several tumor types and significantly downregulated in breast, and laryngeal carcinomas [23–26]. Moreover, this microRNA was positively related with overall survival in colorectal cancer [25] and described as a potent tumor suppressor in acute myeloid leukemia [27]. In vitro, we showed that restoration of miR-139-5p was able to impair EWS migration and invasion. Several reports have indicated cancer metastasis as the most affected process by miR-139-5p. Its forced expression leads to reduced rates of invasion and migration in colorectal cancer [25], in laryngeal and hepatocellular carcinomas [24]. However, its involvement in ROCKs regulation might be variable. On hepatocellular carcinoma, miR-139-5p suppresses metastasis by downregulating ROCK2 [8]. Our data showed a positive correlation between miR-139-5p and ROCK1 but not with ROCK2, nonetheless these protein levels were not altered after miR-139-5p overexpression, suggesting that other targets must be implicated on miR-139-stimulated invasion reduction. In gastric cancer, for instance, miR-139-5p downregulation is responsible for the increased expression of its target CXCR4, a transmembrane G-protein-coupled receptor also associated with cell invasion and metastasis [28]. On colorectal cancer, AMFR and NOTCH1 were suppressed by miR-139-5p reducing cell invasion [25]. Moreover, in non-small cell lung cancer, ectopic expression of miR-139-5p inhibited matrix metalloproteinases (MMP)-7 and -9, but also cyclin D1 and BCL2, which resulted on tumor growth inhibition and apoptosis [26]. Conflictingly, our results demonstrated that miR-139-5p expression, instead of inhibiting, stimulated the clonogenic capacity of cells.

One of the most challenging attributes of cancer is the extreme intratumoral heterogeneity at genomic, epigenomic and proteomic levels, which is also a common feature of EWS [29]. This heterogeneity might in turn, be reflected in derivative cell lines. In fact, side populations (SP) with stem

cell characteristics cells have been identified in several tumor cell lines including EWS. Komuro et al., [30] demonstrated that SP cells constituted up to 5.5% of the cells in 5 EWS cell lines. The SK-ES-1 cell lines is believed to possess 1.2% of such cells and functional analysis characterized them as the more clonogenic and invasive within the culture [31].

Tumor plasticity also implies the coexistence of several distinct cell types, including highly proliferative cells and highly invasive cells. Nonetheless, proliferation and invasion seem to be mutually incompatible processes, and were shown to involve different signaling pathways [32]. Experimental data shows that different cell clones behave differently under different environmental conditions: while the proliferative phenotype exhibits the highest performance rate under a normal or high resource level, the invasive phenotype shows the highest success under low levels of nutrients and oxygen [33].

In the absence of FBS cells with restored expression of miR-139-5p lost motility and invaded through matrigel. No alteration on cell movement was observed in the presence of the supplement. Nonetheless, whether our contrasting results are a consequence of different subclones within the culture or whether miR-139-5p is part of the genetic makeup that influences through its innumerable targets a phenotypic proliferation-invasion dichotomy remains to be unveiled in detail.

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Compliance with Ethical Standards

Conflict of Interest None declared.

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