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Coordinate regulation of microenvironmental stimuli and role of methylation in bone metastasis from breast carcinoma

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ABSTRACT

The pathogenesis of bone metastasis is unclear, and much focus in metastatic biology and therapy relays on epigenetic alterations. Since DNA-methyltransferase blockade with 5-aza-2′-deoxycytidine (dAza) counteracts tumour growth, here we utilized dAza to clarify whether molecular events undergoing epigenetic control were critical for bone metastatization. In particular, we investigated the patterns of secreted-protein acidic and rich in cysteine (SPARC) and of Endothelin 1, affected by DNA methyltransferases in tumours, with the hypothesis that in bone metastasis a coordinate function of SPARC and Endothelin 1, if any occurs, was orchestrated by DNA methylation. To this purpose, we prepared a xenograft model with the clone 1833, derived from human-MDA-MB231 cells, and dAza administration slowed-down metastasis outgrowth. This seemed consequent to the reductions of SPARC and Endothelin 1 at invasive front and in the bone marrow, mostly due to loss of Twist. In the metastasis bulk Snail, partly reduced by dAza, might sustain Endothelin 1-SPARC cooperativity. Both SPARC and Endothelin 1 underwent post-translational control by miRNAs, a molecular mechanism that might explain the *in vivo* data. Ectopic miR29a reduced SPARC expression also under long-term dAza exposure, while Endothelin 1 down-regulation occurred in the presence of endogenous-miR98 expression. Notably, dAza effects differed depending on *in vivo* and *in vitro* conditions. In 1833 cells exposed to 30-days dAza, SPARC-protein level was practically unaffected, while Endothelin 1 induction depended on the 3′-UTR functionality. The blockade of methyltransferases leading to SPARC reduction *in vivo*, might represent a promising strategy to hamper early steps of the metastatic process affecting the osteogenic niche.

1. Introduction

The molecular events underlying the tropism for the secondary organs represent an unresolved problem of the metastatic process. Metastasis to bone is one of the most common and devastating complication in patients with advanced cancers of the breast, prostate or lung [1]. Osteomimetic properties contribute to the preference of breast-carcinoma metastasis for the bone, as exemplified by Endothelin 1 axis that orchestrates signalling pathways including Runt-related transcription factor 2 (Runx2) [2]. Also, the tumour-stroma interaction and the composition of bone microenvironment are critical for skeleton metastatization, favouring specific adhesion/recognition and invasion [1,3–5]. The bone marrow is not conditioned by osseous metastatic cells as extensively as stroma in pulmonary metastasis, and the same mechanisms that govern the homing of hemopoietic stem cells in healthy individuals are co-opted by tumour cells [1].

Secreted protein acidic and rich in cysteine (SPARC), a matricellular glycoprotein, is associated with bone remodelling, repair, development, cell turnover, mineralization process, and collagen fibril assembly [3]. The SPARC function in tumorigenesis and tumour progression is still controversial and not fully understood, and the different expression and activity of SPARC depend on cancer type [6]. These diverse patterns of SPARC would be influenced by tumour microenvironment in terms of local composition of matrix, molecules, cytokines and protease profile, while some inconsistencies are due to SPARC proteolytic products (peptide fragments corresponding to different regions of SPARC) [3].

There are only few studies regarding prostate and breast cancer, that try to disclose the roles of SPARC in bone metastasis [3]. Prostate carcinoma PC3 cells on wild type matrices containing SPARC, show decrease of cell proliferation and resistance to radiation-induced cell death, with an increase in cell spreading [7]. SPARC gene is an early marker of poorly differentiated phenotype, and high SPARC expression at the time of prostatectomy is associated with development of metastasis [8]. Data obtained with breast carcinoma MDA-MB231 cells, showing low SPARC level [9] and added exogenous SPARC, lead to suppose its

Abbreviations: Runx2, Runt-related transcription factor 2; SPARC, secreted protein acidic and rich in cysteine; dAza, 5-aza-2′-deoxycytidine; PPARγ, Peroxisome proliferator activated receptor γ; ROI, regions of interest; PTEN, phosphatase and tensin homolog; ME, mice bearing bone metastasis; CHX, cycloheximide.

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favouring role for metastasis to bone, by stimulating indirectly the motility and chemoattraction towards vitronectin [10]. Adenovirally produced SPARC, however, inhibits *in vivo* metastasis of MDA-MB231 cells into lung and bone, reducing the aggregation of tumour cells with platelets [11].

While normal mammary tissue has undetectable or lightly detectable amounts of SPARC, and benign breast lesions are weakly positive, 75% of both *in situ* and invasive breast carcinomas are strongly positive for SPARC in stromal cells (CD-34-negative, α-SMA-positive) [12,13]; breast carcinoma cells also show SPARC signal [14]. In various carcinomas, high SPARC expression is associated with significantly poorer outcomes compared with low SPARC expression [15].

Much focus in cancer biology and therapy deals with epigenetic alterations that are drivers in neoplastic progression, and targeting the molecular events regulated by DNA methylation may be an useful approach for chemoprevention [16,17]. Even if SPARC is methylated in a 300 bp CpG island spanning from exon 1 to intron 1 in many tumours [18], the knowledge of the biological function and regulation of SPARC by methylation in bone metastasis from breast carcinoma is scarce.

The aim of the present paper was to examine whether endogenous SPARC plays a role in the metastatic process of breast carcinoma with tropism for the skeleton, and whether SPARC is regulated by mechanisms dependent on DNA-methyltransferases, to clarify the relevance of methylation for metastasis outgrowth. To this end, a xenograft model was prepared with the metastatic clone 1833 with bone tropism- derived from invasive MDA-MB231 cells [19]- blocking DNA methyltransferases with 5-aza-2′-deoxycytidine (decitabine, dAza). dAza is a chemotherapic directly incorporated in the newly synthesized DNA strands, that is in phase III trial for myeloid monocyte chronic leukaemia, colangiocarcinoma and colon carcinoma [16,20]. Of note, dAza has never been used to fight bone metastasis from breast carcinoma. The significance of our study *in vivo* would be to identify the network of molecular events implicated in SPARC expression both in metastatic cells and microenvironment under DNA methyltransferases, that might include the transcription factors Twist and Snail, and the biological stimulus Endothelin 1. The additional *in vitro* studies were performed to examine the molecular mechanisms of SPARC expression at transcriptional, translational and post-translational levels, with miRNAs involvement: they would clarify the *in vivo* data. The post-translational mechanism was investigated since SPARC as well as Endothelin 1 are genes containing 3′-UTR regulatory sequences [21,22], which bind miR29a and miR98 [23,24]. The miR98 is enhanced by Peroxisome proliferator activated receptor (PPAR)γ activity [24], which is elevated in 1833 cells [25]. By binding to the 3′-UTR, miRNAs may control mRNA degradation or the translational inhibition of cancer associated protein coding genes [26].

We found that SPARC was highly expressed in bone metastasis and in the bone-marrow cells, and the blockade of DNA methyltransferases slowed-down metastasis outgrowth. Notably, dAza reduced not only SPARC but also Endothelin 1 at the invasive front of bone metastasis and in supportive cells of the bone marrow. The transcription factor Twist seemed to be especially involved in the methylation-controlled signalling pathway for SPARC expression *in vivo* and *in vitro*. In 1833 cells exposed to dAza, exogenous miR29a reduced SPARC expression, and Endothelin 1 transactivation depended on the 3′-UTR. Endothelin 1 protein level was reduced under PPARγ activity, being present miR98 in metastatic cells. These features of DNA methylation pertaining to SPARC in bone metastasis from breast carcinoma might have functional and therapeutic relevance.

2. Materials and methods

2.1. Materials

dAza was from Cayman Chemical (Ann Arbor, MI, USA). Human Endothelin 1 Calbiochem® was from Merck Chemicals Ltd. (Nottingham, UK). Anti-SPARC (H-90), anti-Endothelin 1/2/3 (H-38), anti-Twist 1/2 (H-81) and anti-Akt 1/2/3 (H-136) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphoAkt (pAkt, Ser 473) antibody was from Cell Signaling Technology (Beverly, MA, USA). Anti-Snail 1/2 antibody (ab53519) was from Abcam (Cambridge, UK).

2.2. Cell lines

The parental breast carcinoma MDA-MB231 cells, the derived 1833-bone metastatic clone and the 1833 cells, retrovirally transfected with HSV1-tk/GFP/firefly luciferase (1833/TGL), were kindly given by Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New York). The comparative study of the transcriptomic profile of the two cell lines identifies a gene set whose expression pattern is associated with, and promotes the formation of metastasis to bone [19]. 1833 and MDA-MB231 cells were authenticated with the method of short-tandem repeat profiling (STR) of nine highly polymorphic STR loci plus amelogenin on September 2014 (Cell Service from IRCCS-Azienda Ospedaliera Universitaria San Martino-IST-Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy). The cells, routinely maintained in DMEM containing 10% (*v*/*v*) FBS, were used after 2 or 3 passages in culture [27].

2.3. Western blot analysis

We used total extracts (100 μg of protein) from cells exposed to dAza, Endothelin 1, or troglitazone. dAza (1 μM) was added to the cells at the time of seeding (1 day) and after 24 h (2 days). For 30 days treatment, dAza (0.1 μM) was added in concomitance with cell splitting (every 4 days) [28]: for comparison some cells were cultured for 30 days (c30) without treatments. When present, cycloheximide (100 μg/ml) was added to 1-day or 30-days dAza exposed cells, and to the respective controls. Then, the protein extracts were prepared over a 24-h time course. Endothelin 1 (50 ng/ml) was added to starved cells [2]. Troglitazone was used at the final working concentration of 20 μM [25]. Some cells were transfected with 25 nM siRNASnail (ON-TARGETplus Human SNAI1, SMART pool) or siRNA control (ON-TARGETplus Non targeting Pool) (Dharmacon, Lafayette, CO, USA) [29], with 400 ng/ml of the expression vectors for Twist (pCMV-TWIST) and for Snail, or with 30 nM miR29a-3p mimic or miRcontrol (Ambion/Life Technologies, Carlsbad, CA, USA) [30]. The antibodies used for immunoblotting were: anti-SPARC (1:200), anti-Akt (1 μg/ml), anti-pAkt (1:1000), anti-Endothelin 1/2/3 (1:200), anti-Twist (1:200) and anti-Snail 1/2 (1:500). SPARC level was also evaluated on the conditioned medium: 20 x 10^6 cells were seeded in T75 flasks, were treated or not for 30 days with dAza, were maintained without serum for 48 h, and the supernatants from two flasks was pooled [2]. The conditioned medium was harvested, lyophilized and, then, reconstituted in the loading buffer.

The densitometric analysis of Western blots was performed after reaction with ECL plus chemiluminescence kit from Thermo Fisher Scientific (RockFord, IL, USA).

2.4. Plasmids, siRNA and miRNA transfection, and luciferase activity assay

The cells seeded in 24-multiwell plates, were transfected with 400 ng/ml of the gene reporter for the Endothelin 1 promoter 650(+UTR)Luc (Dr. F. Rodriguez-Pascual, Madrid, Spain), for the Endothelin 1 promoter 650Luc [31], or for the SPARC promoter (SPARCLuc, GoClone n° 32001, Active Motif, La Hulpe, Belgium). The cells transfected with the gene reporters for Endothelin 1, were co-transfected with *Renilla* luciferase plasmid, and Firefly/*Renilla* luciferase activity ratios were calculated by the software. The SPARCLuc activity was evaluated by following the manufacturer's protocol. Cells transfected with SPARCLuc in 96 multiwell, were co-transfected with 2 mg/ml of the dominant negative for Runx2 (ΔRunx2), Ets1 (ΔEts1,

ΔEBets1) or Twist (ΔTwist, pEMSV/TwistA/GΔCOOH), or with 25 nM siRNASnail and siRNA control, and were treated or not with dAza for 2 or 30 days, or with Endothelin 1. ΔRunx2, ΔEts1 and ΔTwist were from Drs. O. Broux (Lille, France), J. Ghysdael (Orsay, France) and E.-M. Füchtbauer (Aarhus, Denmark), respectively. The transfections of the gene reporters alone or in the presence of the expression vectors were performed with a mixture (3:1) of DNA and Fugene 6. DharmaFECT 4 reagent (Dharmacon) was used for 48 h transfection of siRNASnail and siRNA control, as well as for 72 h transfection of miR29a-3p mimic and miRcontrol. Some cells in 96 multiwell were transfected with 100 ng of the reporter construct containing the 3′-UTR of SPARC (S810986, LightSwitch, GoClone, Active Motif), using DharmaFECT Duo (Dharmacon), in the presence of 80 nM miR29a or miRcontrol. The luminescence was measured with Glomax Discover System (Promega, Madison, WI, USA).

2.5. Quantitative RT-PCR detection of mRNAs and miR98

For mRNA measurement, total RNA was extracted from 1833 and MDA-MB231 cells following manufacturer's instructions (TRIzol, Life Technology, Monza, Italy). Reverse transcription was performed using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Monza, Italy). Gene expression was measured using TaqMan Gene Expression Assays (Applied Biosystems, Monza, Italy; Endothelin1, Assay ID: Hs00174961_m1; SPARC/Osteonectin, Assay ID Hs00234160_m1). GAPDH was used as internal control to normalize the mRNA levels. For miR98 assay, total RNA was extracted from 1833 cells using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. Reverse transcription and expression were assayed using mercury LNA™ Universal RT microRNA PCR Starter Kit (Exiqon, Woburn, MA, USA; hsa-miR-98-5p). SNORD was used as internal control to normalize the miRNA levels.

2.6. Xenograft model preparation and treatment

The xenograft model was prepared with 1833/TGL cells, injecting $5x10^5$ cells in the left ventricle of 20 nu/nu mice under anaesthesia [32]. One group of 12 mice underwent concomitant administration of 1833/TGL cells and dAza (5 mg/kg, i.p.), and these mice were subsequently treated with dAza once a week until the sacrifice [33]; the other group of 8 mice was administered with the vehicle. The animals were monitored and sacrificed to prevent the suffering, following the Institutional Guide for Care and Use of Laboratory Animals, and the International Laws. Firefly D-luciferine (150 mg/kg) was given intraperitoneally under anaesthesia, and metastasis formation was monitored using Xenogen IVIS 200 System-Perkin Elmer at Istituto San Raffaele, Milano Italy. Acquisition time for bioluminescence was 5 min at the beginning of the experiment, i.e. 1 h and 24 h after xenografting; for the following observations, the acquisition time was reduced to 1 min in accordance with the signal strength, to avoid saturation. Bioluminescence all over the skeleton (total burden) takes into consideration chosen regions of interest (ROI). The normalization of the data for each animal was done. For this, the values of ROI at 24 h were evaluated, and used to normalize the bioluminescence signals detected at each time point thereafter, a procedure that avoids variations affecting extravasation and homing due to the treatment.

2.7. Immunohistochemistry

Analyses were performed on five mice 25 days after 1833 cell injection, in the presence or the absence of dAza treatment. Femora and tibiae were fixed and decalcified before preparation of serial sections [27]. Immunostaining of five serial sections for each specimen was performed using anti-SPARC (1:200), anti-Twist (1:200), anti-Snail (1 μg/ml) and anti-Endothelin 1 (1:50) antibodies. Negative controls were performed without the specific antibody. The slides were examined under Eclipse 80i microscope (Nikon, Milano, Italy).

2.8. Statistical analysis

Densitometric values for protein levels, as well as the values for PCR, luciferase activities and bioluminescence were analysed by analysis of variance with $P < 0.05$ considered significant. Differences from controls were evaluated on original experimental data.

3. Results

3.1. Effect of dAza on SPARC expression in invasive and metastatic breast carcinoma cells

SPARC protein and mRNA levels and their regulation were studied in 1833-bone metastatic clone, compared with parental breast carcinoma MDA-MB231 cells, to clarify whether SPARC expression depended on the cell phenotype (Fig. 1). In particular, we evaluated the involvement of the epigenetic mechanisms, considering whether DNA methylation orchestrated the transcriptional and/or post-translational control of SPARC expression. This experimental approach was suggested because the software analysis of SPARC promoter, used in our study, indicated the presence of numerous putative-methylation sites, beyond binding sites for the transcription factors Runx2, Ets1, Twist and Snail (Fig. 1A). For these studies we blocked DNA methyltransferases with dAza and we assayed the short- and long-term effects. As shown in Fig. 1B, the 1 or 2 days dAza treatment of both the cell lines increased SPARC-protein levels, while only in 1833 cells the 30-days culture (c30) enhanced SPARC-protein level (4-fold), that was almost unchanged after 30-days dAza. Differently, in c30-MDA-MB231 cells the SPARC-protein level was very low, i.e. under the control (c) value, increasing after 30-days dAza. Thus, both under basal and dAza conditions the 1833 cells with bone tropism showed two bands for SPARC of 32–35 and 43 kDa, as reported for the bone [3]; the SPARC basal level was higher in 1833 than in MDA-MB231 cells.

In the next experiments, we deepened the knowledge of the molecular mechanisms responsible for SPARC expression. First, we evaluated the SPARC mRNA levels by RT-PCR (Fig. 1C). In 1833 cells SPARC mRNA level doubled after 2-days dAza in respect to the control; the 30 days culture induced SPARC mRNA, that was practically unchanged by 30-days dAza treatment, consistent with the protein levels. In MDA-MB231 cells, concomitant inductions of SPARC transcript and protein occurred after short- and long-term dAza treatment.

In MDA-MB231 cells exposed to dAza SPARC regulation seemed to occur at transcriptional level, while the data suggested a complex regulation of SPARC expression in 1833 cells. To deepen the knowledge in 1833 cells, we performed a second series of experiments in the presence of cycloheximide (CHX) (Fig. 1D). CHX is an inhibitor of new protein synthesis, that permits to evaluate the degradation rate of a specific protein; a possible stabilizing effect of a chemical substance might be, therefore, evidenced [34]. CHX treatment under 30-days and 1-day dAza exposure, increased SPARC-protein levels (3- to 5-fold), in respect to CHX added alone to the respective controls: these findings suggested that dAza enhanced SPARC half-life leading to the stabilization (Fig. 1D graphic). The data suggested that in dAza-treated 1833 cells different mechanisms might underlie the increases in SPARC protein at 1 and 2 days, that were due to protein stabilization (at 1 day) and to enhanced transcription (at 2-days).

Notwithstanding the increase of SPARC protein half-life after 30-days dAza (Fig. 1D), the steady-state protein level of SPARC did not augment (Fig.1B), compared with c30. This was explained because of SPARC release from 30-days dAza-treated 1833 cells, as demonstrated by SPARC accumulation in the conditioned medium (Supplementary Fig. S1A).

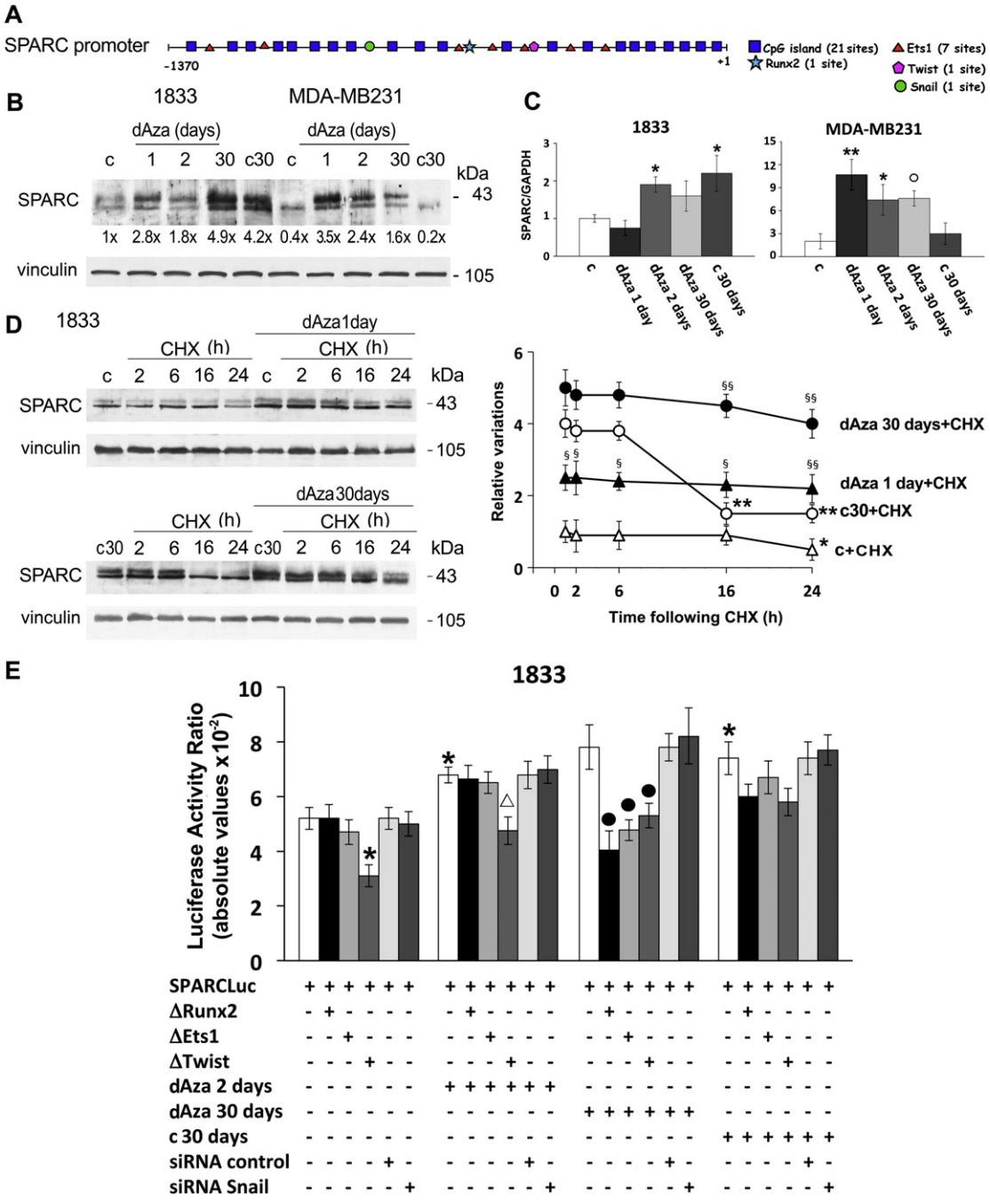


Fig. 1. Effect of dAza treatment on SPARC expression in 1833 and MDA-MB231 cells. (A) transcription-factor binding sites and methylation islands in SPARC promoter are shown. DNA methylation is characterized by an enzymatic addition of a methyl group at the carbon 5 position of cytosine in the context of the sequence 5′ cytosine-guanosine (CpG) through DNA methyltransferase activity. CpG dinucleotides are clustered in regions, named CpG islands. (B) representative images of Western blots repeated three times are shown; vinculin was used for normalization. The numbers at the bottom indicate the fold variations versus control (c) value of 1833 cells, considered as 1. (C) RT-PCR of total RNA was normalized for GAPDH values. The data are the means ± S.E. of three independent experiments performed in triplicate. *P < 0.05, **P < 0.005, versus the control value for each cell line; °P < 0.05, versus c30. (D) representative images of Western blots performed with proteins from cells treated or not with cycloheximide (CHX), in the presence or the absence of dAza. Vinculin was used for normalization. The data shown in the graphic are the means ± S.E. of three independent experiments. §P < 0.05, §§P < 0.005 versus the value at the same time of c + CHX or of c30 + CHX; *P < 0.05, **P < 0.005 versus the respective control values at time 0. (E) the histogram indicates the absolute values of SPARCLuc, in respect to Cypridina-luciferase internal control, under various experimental conditions. The data are the means ± S.E. of three independent experiments performed in triplicate. *P < 0.05, versus basal SPARCLuc activity value (first white column); ΔP < 0.05, versus SPARCLuc value under 2-days dAza; •P < 0.05, versus SPARCLuc value under 30-days dAza.

To further clarify the transcriptional control of SPARC in 1833 cells exposed or not to dAza, we studied SPARC transactivation and the transcription factors involved. To this purpose, we used the dominant negatives for the transcription factors, with consensus sequences in the promoter [35–37], and siRNA Snail [29] (Fig. 1E). The dominant negatives forms of the transcription factors, are partially deleted proteins maintaining only the DNA-binding domain, and they have been previously used in our papers to evaluate the involvement of Twist, Runx2 and Ets1 in gene transactivation [2,5,38]. Because of the high affinity of the dominant negatives for the specific consensus sites, without showing transactivating activity, they inhibit the promoter activity depending on their functional involvement. For Snail, the dominant negative does not exist and, therefore, we used a specific siRNA to reduce the protein level of Snail in 1833 cells (Supplementary Fig. S1B).

siRNAs are small pieces of double-stranded RNA, that can be used to interfere with the translation of proteins by binding to and promoting the degradation of the specific mRNAs [29].

SPARCLuc was activated in the cells maintained in culture for 30 days (c30) or under 2-days dAza, in respect to SPARCLuc basal value (first white column); these data were consistent with the mRNA levels. ΔTwist reduced basal SPARCLuc activity, indicating a role of this transcription factor in SPARC transactivation. Runx2, Ets1 and Twist were involved in the transactivation of SPARC under 30-days dAza. siRNASnail was ineffective on SPARCLuc activity, even if it reduced the Snail protein level in 1833 cells (Supplementary Fig. S1B). The effectiveness of dAza in 1833 cells was evaluated by studying the Akt-signalling pathway, that is downstream of phosphatase and tensin homolog (PTEN): the latter is a target gene of methyltransferases and is activated by dAza [33] (Supplementary Fig. S1C).

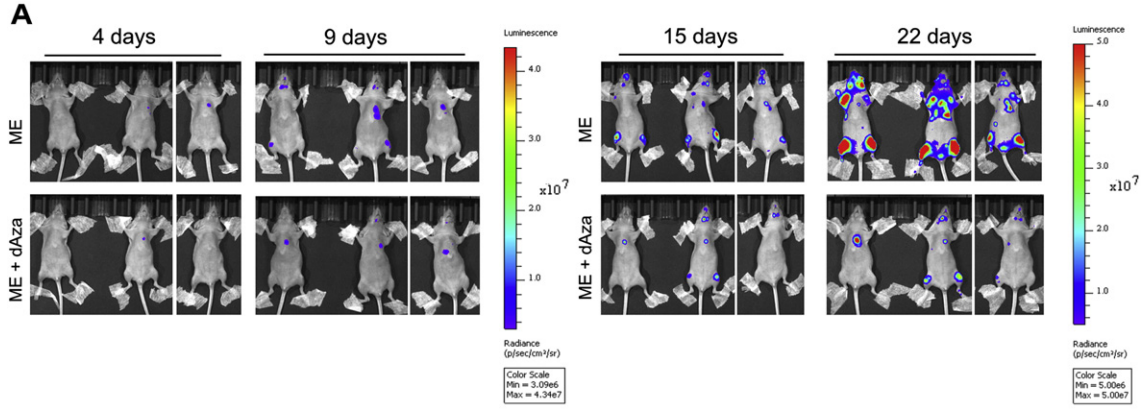
3.2. Effect of dAza on bone metastasis outgrowth and SPARC expression in the xenograft model

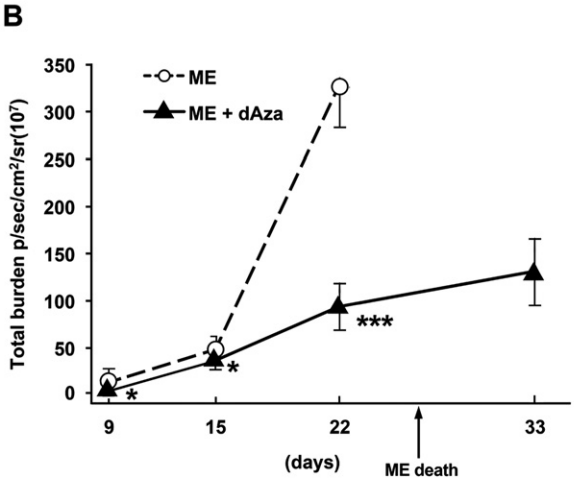
To give new insight into the effect of DNA-methylation status on survival of mice bearing bone-metastasis, related to the molecular characteristics, we treated the 1833-xenograft mice with dAza. To this purpose, we studied the cooperativity of SPARC with the molecular network important for epithelial–mesenchymal transition, that includes Twist and Snail transcription factors and the other biological stimulus Endothelin 1 [2,5].

As shown in Fig. 2A, we monitored the metastatic development in real-time, exploiting the time-course of bioluminescence of mice bearing bone metastasis (ME), compared with ME treated with dAza. All the data were normalized versus bioluminescence at 24 h, and the injection efficiency was controlled by monitoring the bioluminescence signal 1 h after xenografting (Supplementary Fig. S2A).

As shown in Fig. 2B, dAza treatment reduced the total burden starting from 9 days reaching the highest inhibitory effect at 22 days, when the percent of inhibition was 74% (see Table). ME mice died at 26 days on average [32], and dAza treatment positively influenced mice survival, so that ME + dAza mice were sacrificed between 31 and 38 days.

Fig. 3 shows the immunohistochemical analyses of SPARC in control bone, and in the bone of ME and ME + dAza. In control bone, SPARC expression was found in chondrocytes of calcified cartilage (c) and in osteoblasts (o), according to the literature [3]. In osteoblasts, the signal





Total burden: p/sec/cm²/sr (10^7)

	4 days	9 days	%	15 days	%	22 days	%
ME	0.38±0.05	2.75±0.31§§§		35.4±3.78§§§		314.2±15.89§§§	
ME+dAza	0.28±0.04	1.99±0.28*	- 27	25.5±2.99*	- 27	80.7±11.21***	- 74

Fig. 2. dAza affected bone-metastasis outgrowth. (A) representative bioluminescence images of three xenograft mice for each experimental group (n = 5). (B) The time-course of the changes in total burden is shown for ME mice, until their death at 26 days, and for ME + dAza mice until 33 days from xenografting. The Table reports the total burden data, including those at 4 days, and the percentages of inhibition. The data are the means ± S.E. of the values for five mice. §§§P < 0.001, versus ME value at 4 days; *P < 0.05, ***P < 0.001, versus ME value at the corresponding time.

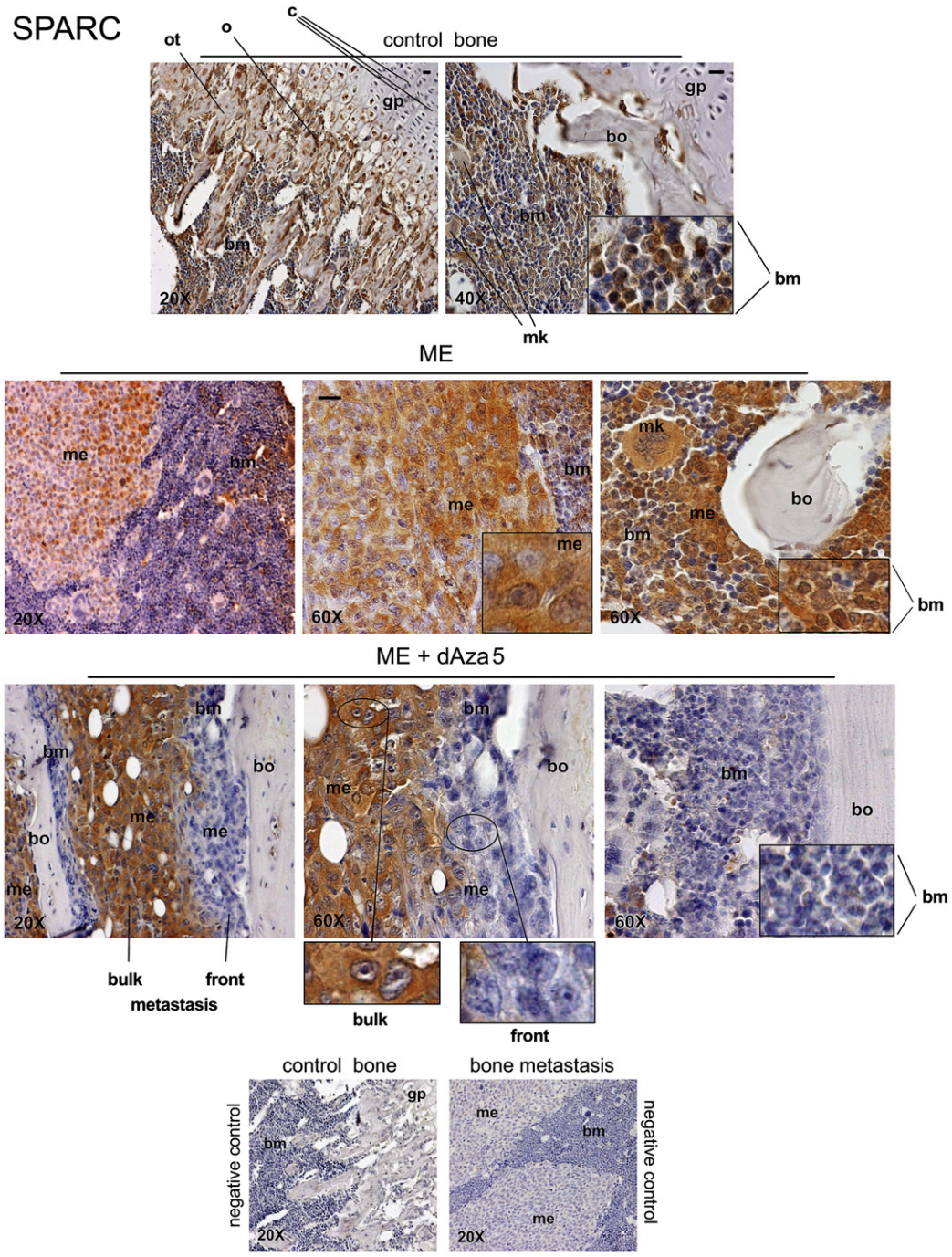


Fig. 3. SPARC signal in the bone metastasis of the xenograft model, and effect of dAza treatment. We show representative images of control bone, and of bone-metastasis tissue from mice treated or not with dAza. Five serial sections were examined for each specimen from five mice, obtaining similar results, and the original magnification is shown. c, chondrocytes; o, osteoblasts; ot, osteoid tissue; gp, growth plate; bm, bone marrow; bo, bone; mk, megakaryocytes; me, metastasis. Scale bar = 120 μm (reported in exemplificative Panels 20×, 40× and 60×).

was stronger than in the osteoiod tissue (ot). SPARC signal in the bone marrow was observed in the megakaryocytes (mk) and in the mesenchymal-stromal cells. In ME mice, SPARC signal was highly expressed in metastatic cells, both in the cytosol and the nuclei, but showing different intensity throughout the bone metastasis. In nuclei, SPARC may influence the cell cycle [39]. In the bone marrow of ME mice, the SPARC signals of megakaryocytes and the other supportive cells were higher than those found in control bone. After dAza treatment, the metastatic SPARC signal remained elevated in the metastasis bulk, but disappeared from the front lining the bone and from the bone marrow. Negative controls did not show specific signal. All the animals showed similar responsiveness to dAza treatment as regards SPARC signal (data not shown), consistent with the findings reported for mice dAza5.

Next, we performed experiments to identify molecular events responsible for the down-regulation of SPARC under dAza. Notably, dAza induces miR29a [30], that targets the 3′-UTR of SPARC [23]. Since miR29a in 1833-parental cells is very low [40], we transfected a synthetic miRNA mimic to overexpress the mature form of miR29a in 1833 cells. This experiment permitted to evaluate the biological function of miR29a. As shown in Fig. 4, the transfection of the specific miR29a mimic strongly reduced SPARC protein levels in controls (c and c30)

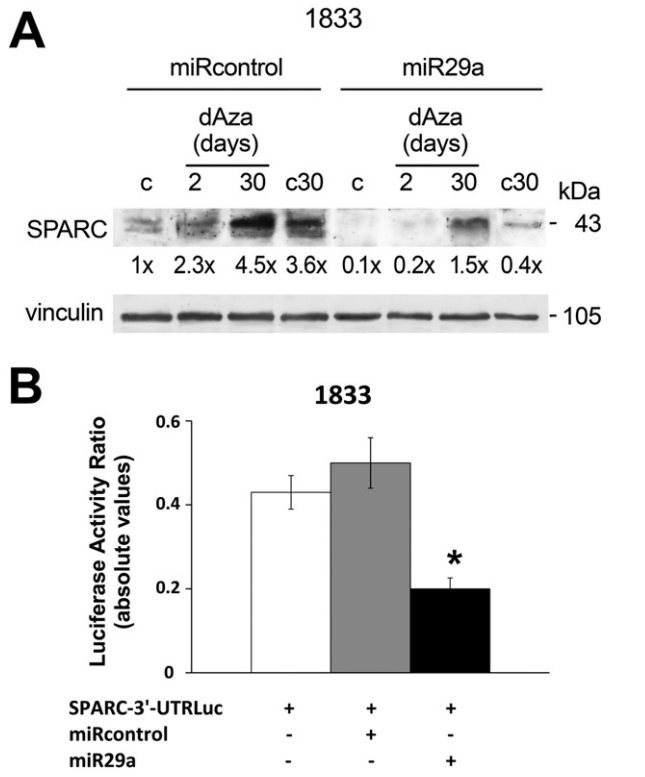


Fig. 4. Effect of miR29a on SPARC protein levels, and on the 3′-UTR luciferase activity. (A) miR29a mimic was transfected into 1833 cells, untreated or treated with 1 μM dAza for 2 days, or exposed to 0.1 μM dAza for 30 days. Representative images of Western blots repeated three times are shown; vinculin was used for normalization. The numbers at the bottom indicate the fold variations versus control (c) value of miRcontrol transfected cells, considered as 1. (B) the SPARC-3′-UTRLuc was transfected together with miRcontrol or miR29a for 24 h. The data are the means ± S.E. of three independent experiments performed in triplicate. *$P < 0.05$, versus the value of SPARCLuc in the presence of miRcontrol.

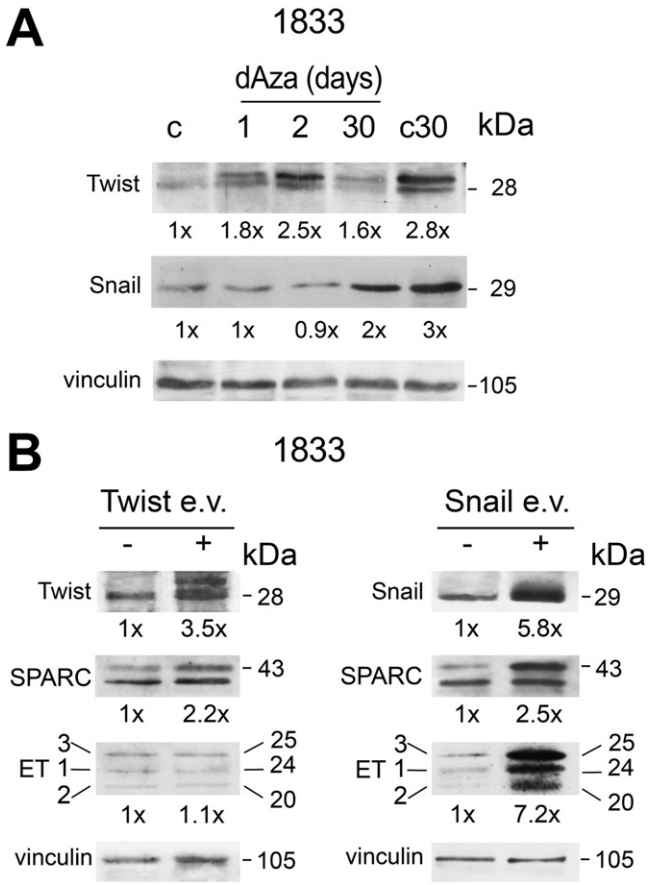


Fig. 5. Effects of dAza on Twist and Snail protein levels, and influence of their overexpression on SPARC and Endothelin protein levels. The cells were (A) treated with dAza or (B) transfected with Twist and Snail expression vectors (e.v.). Representative images of Western blots repeated three times are shown; vinculin was used for normalization. The numbers at the bottom indicate the fold variations versus the first lane, considered as 1. When multiple bands for a specific protein were present, they were considered altogether in the densitometric evaluation, used to calculate the fold-variations.

and in dAza-treated cells for 2 or 30 days, when compared to the corresponding miRcontrol transfected cells (Fig. 4A). The specificity of miR29a for the regulatory sequence of SPARC promoter was demonstrated by measuring the SPARC-3′-UTR activity. The luciferase activity of SPARC-3′-UTR decreased (−60%) in 1833 cells transfected with miR29a, in agreement with the diminutions of SPARC protein level. miRcontrol was ineffective on SPARC-3′-UTR activity (Fig. 4B).

3.3. Effect of dAza on the expression of Twist and Snail in vitro and in bone metastasis of the xenograft model

In further experiments we evaluated whether Twist and Snail were implicated in SPARC expression depending on the methylation status. These transcription factors, showing consensus sequences in SPARC promoter, are regulated by DNA methyltransferases in breast cancer [41,42].

Fig. 5A shows that in 1833 cells, short-term exposure to dAza enhanced only Twist protein level. Importantly, the 30-days culture *per se* caused the accumulation of Twist and Snail proteins, that decreased of about 45% at 30 days of dAza exposure, a treatment similar to that performed *in vivo*. Thus, it seemed interesting to evaluate the effects of Twist and Snail overexpression on the biological stimuli examined in the present paper (Fig. 5B), and experiments were performed with the expression vectors for Twist and Snail. Under the forced expression of Twist and Snail, SPARC steady-state protein level doubled; only Snail expression vector strongly enhanced the three forms of Endothelin.

Altogether, the *in vitro* studies suggested to examine Twist and Snail signals in the xenograft model under dAza. As shown in Fig. 6, Twist and Snail appeared remarkably expressed in bone metastasis, in all the compartments of the metastatic cells, and in the bone marrow cells. The effect of dAza treatment is shown for the mice 1 and 5. In both these ME + dAza mice, Twist signal was impaired in the metastatic tissue, including the bone marrow. The Snail signal decreased more in the bone metastasis of dAza5 than of dAza1, the latter mouse showing major metastasis outgrowth. For both the dAza mice, Snail signal seemed more persistent in the bulk of metastasis than in the front, and diminished in the bone marrow. Negative controls did not show specific signal. To support these results, in Supplementary Fig. S3 we report Twist and Snail immunohistochemical assays for the bone metastases of the other animals examined. Also for the mice dAza 3, 6 and 8 we showed down-regulation of Twist and Snail signals in metastasis and bone marrow, compared to ME mice.

The *in vivo* data of Fig. 6 and Supplementary Fig. S3 indicated that in bone metastasis Twist expression was mainly affected by methylation status, being largely reduced by DNA methyltransferase blockade *in vivo*, consistent with the *in vitro* data (Fig. 5).

3.4. Involvement of Endothelin 1 in the regulation of SPARC expression, and response of Endothelin 1 to DNA methyltransferase blockade

To evaluate whether the cellular expression of SPARC was coordinated with that of Endothelin 1, another biological stimulus important for the osteomimetic phenotype and for tumour-stroma cross-talk [2,43], we studied SPARCLuc activity and the steady-state protein levels of

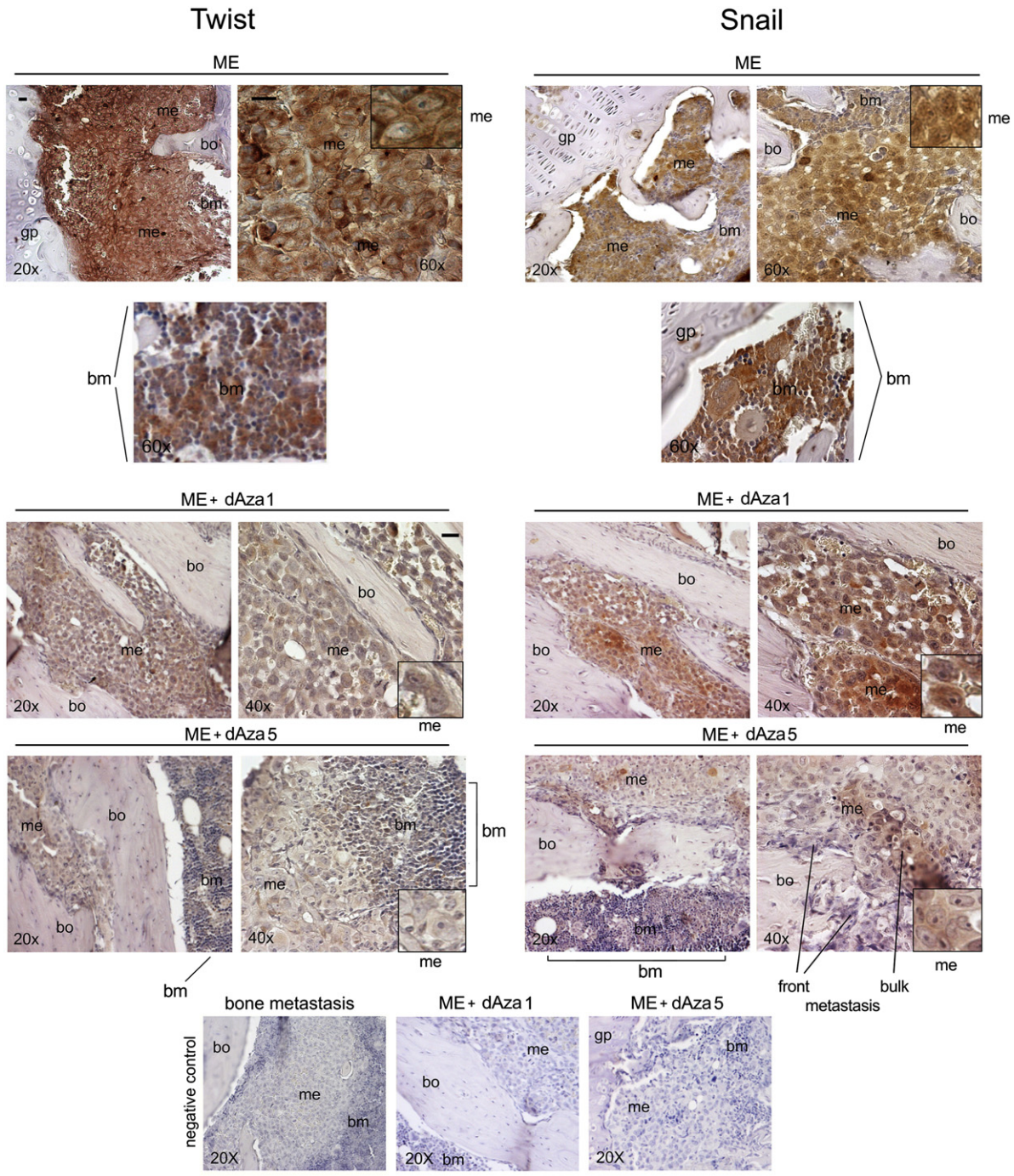


Fig. 6. Twist and Snail signals in the bone metastasis of the xenograft model, and effect of dAza treatment. Representative images of bone-metastasis tissue from mice treated or not with dAza are shown. Five serial sections were examined for each specimen from five mice, obtaining similar results, and the original magnification is shown. gp, growth plate; bm, bone marrow; bo, bone; me, metastasis. Scale bar = 120 μm (reported in exemplificative Panels 20×, 40× and 60×).

SPARC in response to Endothelin 1. As shown in Fig. 7A, Endothelin 1 enhanced SPARCLuc activity in 1833 but not in MDA-MB231 cells, and ΔRunx2 as well as ΔTwist prevented the luciferase activation. siRNA Snail and siRNA control did not affect Endothelin 1-stimulated SPARCLuc activity. siRNA control was ineffective also on basal SPARCLuc activity (data not shown). In 1833 cells, Endothelin 1 increased SPARC-protein level between 6 and 16 h, diminishing thereafter towards the control level, while SPARC protein level was unaffected by Endothelin 1 in MDA-MB231 cells. Twist and Snail oppositely responded to Endothelin 1. In 1833 cells, Endothelin 1 increased Twist at 24 h while reducing Snail; opposite patterns were observed in MDA-MB231 cells (Fig. 7B).

From the analysis of Endothelin 1 promoter, wide CpG islands with 49 putative methylation sites, and 2 Snail without Twist consensus sequences were found (Fig. 8A), leading to suppose a possible implication of DNA methyltransferases in the expression of Endothelin 1, shown to be important for SPARC induction. Fig. 8B shows the effect of dAza on the steady-state protein levels of the members of Endothelin family: the relative fold-variations, calculated using the densitometric values, are reported in the histograms. The Endothelin family consists in three isoforms [43]. While 30-days culture enhanced especially Endothelin 3 protein level in 1833 and MDA-MB231 cells, 30-days dAza strongly augmented Endothelins 1 and 2 only in 1833 cells.

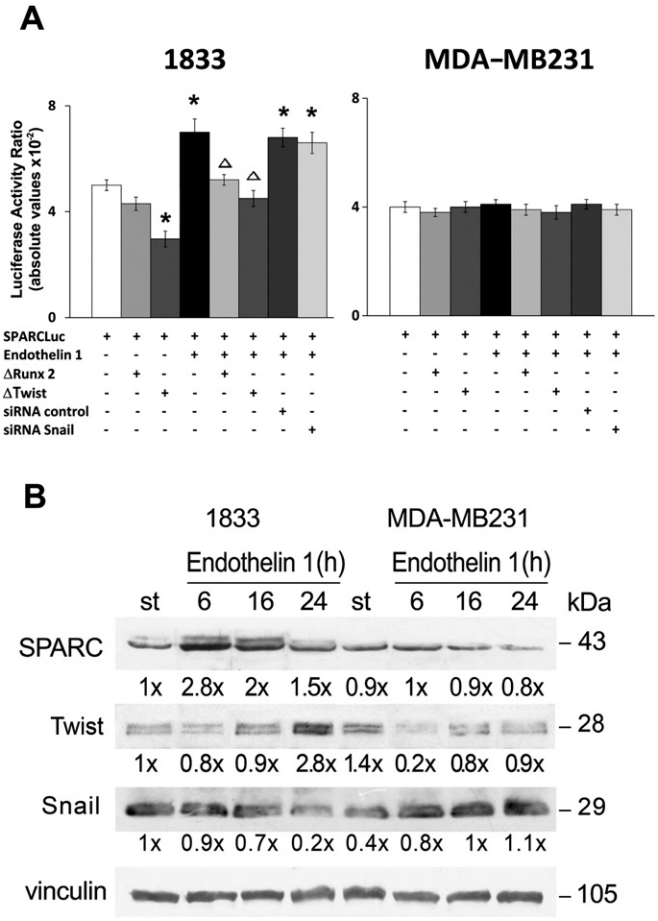


Fig. 7. Effects of exogenous Endothelin 1 on SPARC transactivation as well as on SPARC, Twist and Snail expression. (A) the histograms indicate the absolute values of SPARCLuc, in respect to Cypridina-luciferase internal control, under various experimental conditions. The data are the means ± S.E. of three independent experiments performed in triplicate. *$P < 0.05$, versus basal SPARCLuc value; $^{\Delta}P < 0.05$, versus the luciferase value under Endothelin 1 treatment. (B) representative images of Western blots repeated three times are shown; vinculin was used for normalization. The numbers at the bottom indicate the fold variations versus 1833-starvation value, considered as 1.

We deepened the regulation of Endothelin 1 expression to make a molecular basis to devise the following *in vivo* experiments. First, the messenger level of Endothelin 1 almost tripled under 30-days dAza (Fig. 8C), indicating that the enhancement of the protein level depended on transcription. These data on the enhancement of endogenous Endothelin 1 under blockade of DNA methyltransferases, supported an effective mimicking function of exogenous Endothelin 1 on SPARC expression.

Second, due to the presence of a 3′-UTR sequence, flanking Endothelin 1-promoter (see Fig. 8D, Scheme) [22], we examined whether a post-transcriptional control might occur under our experimental conditions; this regulatory sequence is important for mRNA degradation or inhibition of translation [26]. We used the gene reporters containing the Endothelin 1 promoter, with or without the 3′-UTR. As shown in Fig. 8D (histogram), while the activity of Endothelin 1 luciferase construct containing the 3′-UTR decreased under 30-days dAza, in the absence of the 3′-UTR the same dAza treatment gave a stimulation. The latter finding, which was in agreement with the Endothelin 1 protein induction, also indicated that the 3′-UTR was offside in 1833 cells exposed to 30-days dAza. Of note, the removal of 3′-UTR in the construct decreased c30 luciferase activity.

To obtain more data on this post-transciptional regulation, dependent on the 3′-UTR activity, we evaluated Endothelin expression and miR98 level under troglitazone, an activator of PPARγ [25]. In fact, miR98 is known to be enhanced by PPARγ activity, and to bind directly to the 3′-UTR of Endothelin 1 [24]; PPARγ is functional in 1833 cells [25]. Fig. 8E shows that in 1833 cells treated with troglitazone for 24 and 48 h, Endothelin 1 protein level decreased of 60 and 80% at the two times, while the isoforms 2 and 3 diminished of 70 and 40% only at 48 h. Under the same experimental condition in the presence of troglitazone, miR98 relative value was 6.39, in respect to the expression of the internal control SNORD.

3.5. Expression of Endothelin 1 in bone metastasis and effect of dAza

To examine the interaction of Endothelin 1 and SPARC *in vivo*, and the role of methylation status, we studied the effect of dAza treatment of the xenograft mice on Endothelin 1. In control bone, Endothelin 1 signal was found in mesenchymal-stromal cells of the bone marrow (Fig. 9A). In ME mice, the metastatic cells strongly expressed Endothelin 1, and the bone marrow signal was similar to that observed in control bone. After dAza treatment, Endothelin 1 signal disappeared from metastatic cells lining the bone, but not from the bulk of metastasis, as observed for SPARC pattern in the same samples. Endothelin 1 signal disappeared under dAza treatment also in the bone marrow. Negative controls did not show specific signal. All the animals showed similar responsiveness to dAza treatment as regards Endothelin 1 signal (data not shown), consistent with the findings reported for mice dAza5.

In conclusion, the key roles of 3′-UTR function as well as of the expression of mi98 might explain why *in vivo* Endothelin 1 decreased while it augmented in 1833 cells: even if the schedule of five administrations of dAza *in vitro* was similar to that performed *in vivo* the network of signalling is different.

4. Discussion

The present paper deals for the first time with the expression and regulation at epigenetic level of endogenous SPARC, and with its role in the establishment of bone metastasis from breast carcinoma. The approach to use dAza to block DNA methyltransferases *in vivo*, was a tool to clarify the biological function of SPARC regulated by methylation in the pathogenesis of bone metastasis. These findings with 1833-xenograft model were corroborated by *in vitro* experiments. In fact, we clarified molecular mechanisms underlying aberrant SPARC expression depending on bone metastatic versus invasive phenotype, the surrounding-environmental signals such as Endothelin 1, and the DNA-methyltransferase activity. Notably, the microenvironment may influence the methylation status of tumour cell DNA [44].

The 1833-bone metastatic cells constitutively expressed two molecular weight forms of SPARC as the bone, possibly due to glycosylation [45], that might confer osteomimetic properties and permit the interaction with platelets, at a difference with MDA-MB231 cells [11]. Since the timing of SPARC expression is important for metastatization, these molecular characteristics of 1833 cells might favour bone-metastasis outgrowth, in contrast to MDA-MB231 cells with low level of endogenous SPARC protein. MDA-MB231 cells are mesenchymal and invasive, with scarce metastatic power and tropism for different organs [11], giving metastasis to bone less efficiently and more slowly than 1833 cells with epithelial phenotype [5,19,27].

It is worth noting that in the 1833-xenograft model, SPARC expression was elevated both in bone metastasis and in the bone-marrow supportive cells. These data led to suppose that SPARC might play a critical role in the cross-talk between metastatic and microenvironmental cells likely by triggering signals for outgrowth and bone-matrix remodelling [21]. This hypothesis is under investigation, since the host stroma has crucial roles in each step of the metastatic process, and the bone marrow cells are important in the establishment of overt, immune-suppressed metastatic lesions [1].

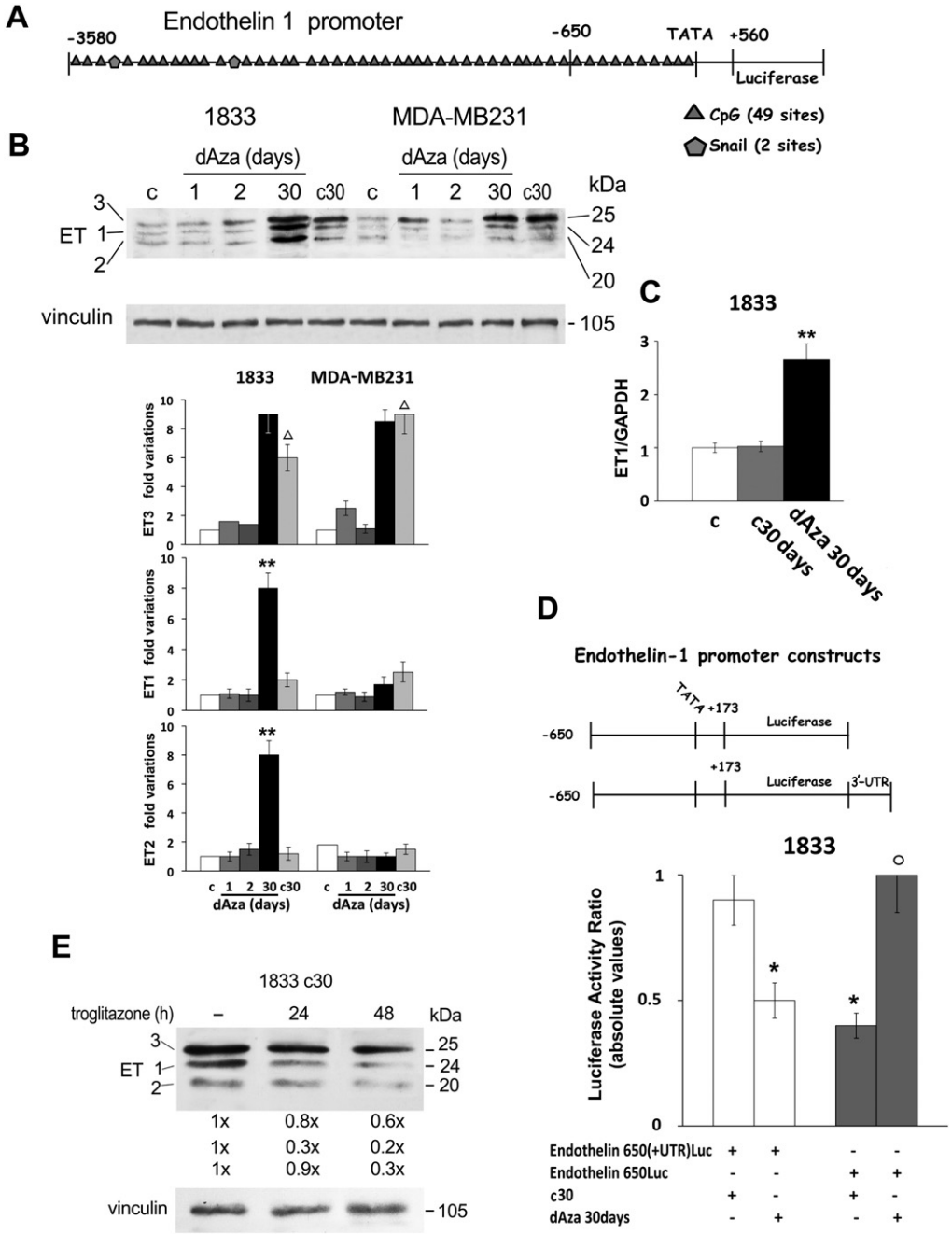


Fig. 8. Transcriptional and post-transcriptional control of Endothelin 1 expression by dAza. (A) Snail binding sites and methylation islands in the entire Endothelin 1 promoter are shown. (B) representative images of Western blots repeated three times are shown; vinculin was used for normalization. The data reported in the histograms were calculated by using the densitometric values, and were the means ± S.E. of three independent experiments. $^{\Delta}P < 0.05$, versus control (c) value; $^{**}P < 0.005$, versus c30 value. (C) RT-PCR of total RNA was normalized for GAPDH values. The data are the means ± S.E. of three independent experiments performed in triplicate. $^{**}P < 0.005$, versus control (c) value. (D) scheme of Endothelin 1 promoter constructs of 650 bp with or without the 3′-UTR. The histogram shows the absolute values of luciferase activity for the two constructs transfected in c30 cells or in the cells exposed to dAza for 30 days. The data are the means ± S.E. of three independent experiments performed in triplicate. $^{*}P < 0.05$, versus the value of Endothelin 650(+UTR)Luc in c30 cells; $^{o}P < 0.05$, versus the value of Endothelin 650Luc in c30 cells. (E) samples of proteins (100 μg) from 1833-c30 cells, exposed or not to troglitazone, were used for Western blot assay. The experiment was repeated three times, and a representative image is shown; vinculin was used for normalization. The numbers at the bottom indicate the fold variations for each Endothelin form versus the first lane, considered as 1.

As shown for SPARC also Wwox, another so called "tumour suppressor", is highly expressed in human bone metastasis and is regulated by methylation [25,46]. SPARC in tumours influences metalloproteinase production and focal adhesion regulation [6]: these functions of SPARC might be important for metastasis engraftment by forcing the endothelial junctions at secondary bone site, and for an intermediate state of adhesion. Moreover, SPARC enhances VEGF expression [47] and Wwox regulates the activity of HIF-1, the transcription factor that transactivates VEGF [48].

Explanations at molecular level for SPARC expression in bone metastasis would be given by DNA methylation (hyper- or hypo-methylation), that leads to aberrant gene transcription, consistent with the control of suppressor-genes promoters by methyltransferases and indicating an interactive link between aberrant-promoter methylation and cancer metastasis [16,49]. Even if the role of DNA methylation in bone metastasis from breast cancer has been scarcely investigated [41], we were encouraged to undertake the present study because DNA methyltransferase blockade with dAza affects miRNA activity [30], and miRNAs are

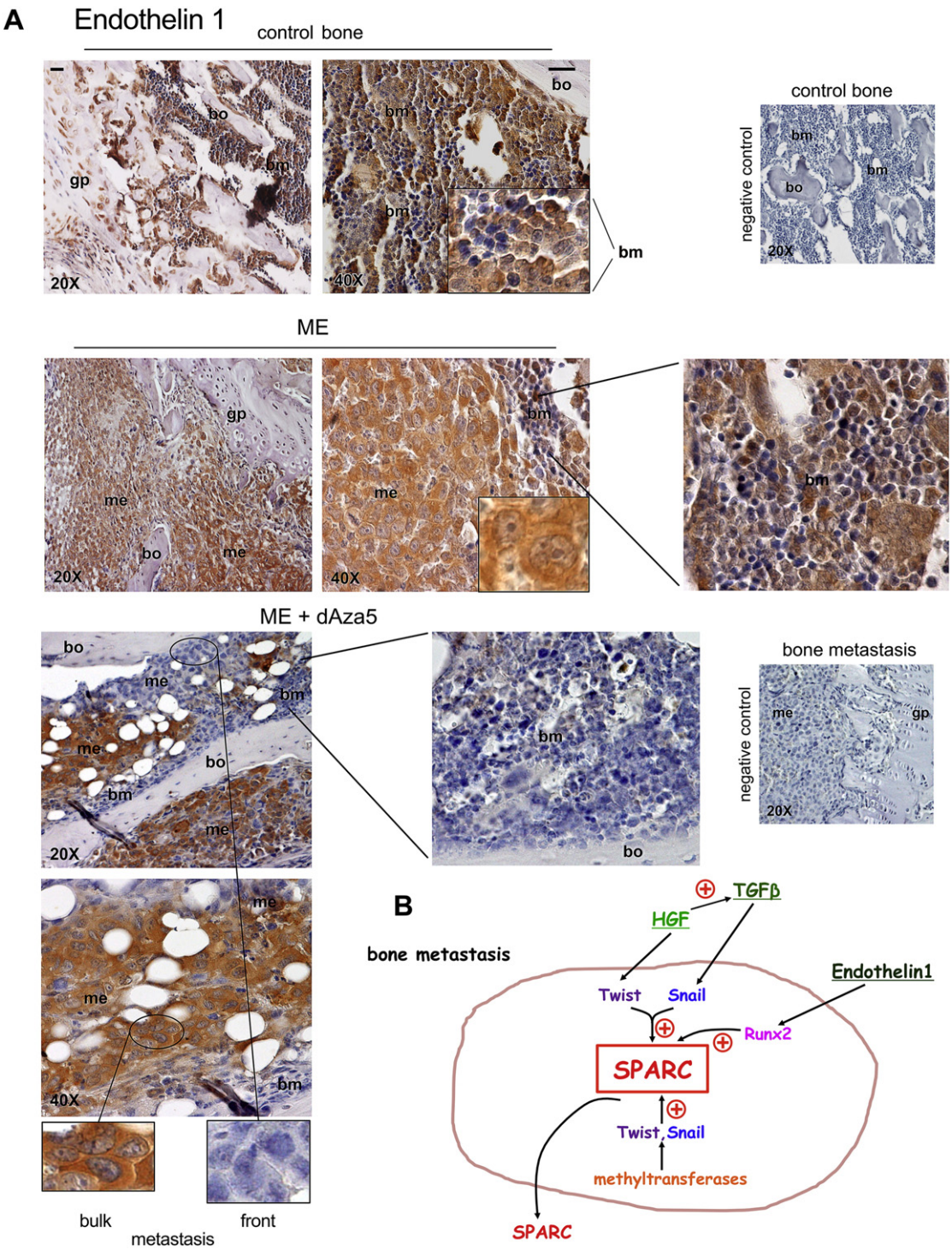


Fig. 9. Endothelin 1 signal in the bone metastasis of the xenograft model, and effect of dAza treatment. (A) we show representative images of control bone, and of bone-metastasis tissue from mice treated or not with dAza. Five serial sections were examined for each specimen from five mice obtaining similar results, and the original magnification is shown. gp, growth plate; bm, bone marrow; bo, bone; me, metastasis. Scale bar = 120 μm (reported in exemplificative Panels 20× and 40×). (B) schematic representation of SPARC regulation in bone metastasis.

relevant players in deregulated mechanisms of DNA hyper-methylation [50].

We observed that dAza exposure slowed-down metastasis outgrowth starting from 9 days of xenografting until the end of the observation period, with the prolongation of mice survival until 38 days in respect to 26 days of metastasis-bearing mice. The complete prevention of SPARC expression in the bone marrow supporting the metastatic tissue, would contribute to the favourable outcome under dAza. Increasing evidence accumulates on the contribution of the host-bone microenvironment to metastatic progression [1]. The present results with dAza given to the xenograft mice represent an advancement in respect to 5′-Azacytidine treatment of the 1833 cells *in vitro*, that changes 1833-bone tropism towards lung after xenografting [25] and that is converted to dAza for incorporation into DNA [20].

The mechanisms of communication between the tumour cells and microenvironment are complex, but fall into two main categories: contact-dependent mechanisms that involve cell-cell and cell-ECM adhesion molecules [51], and contact-independent mechanisms carried out by soluble molecules such as growth factors, chemokines and cytokines, and soluble cellular organelles including microvesicles and exosomes [17]. In our conditions, microenvironmental stimuli like Endothelin 1 might ensure SPARC up-regulation in bone osteogenic niches with successful metastasis formation. Consistently, exogenous Endothelin 1 induced SPARC through Runx2, conferring osteomimicry to bone-metastatic cells.

Altogether, endogenous Endothelin 1 as SPARC disappeared from the front of bone metastasis and in the bone marrow of the xenograft mice under dAza, supporting their coordinate function. The partial resistance of Snail to DNA-methylation blockade might explain the Endothelin 1-SPARC interaction in the bulk of metastasis, in agreement with the role of Snail in orchestrating the supportive pathways from the microenvironment, but in an opposite way to Twist [5].

In bone metastatic cells, Snail is a target gene of TGF-β1 while HGF triggers a Twist program [5], and also TGF-β1 up-regulates and HGF down-regulates Endothelin 1 expression and release [2]. Thus, metastatic SPARC is in a wider context of biological stimuli of bone-metastasis microenvironment including Endothelin 1, and is responsive to methyltransferases, through a network of transcription factors including Twist, Snail and Runx2, as shown in Fig. 9B. Endothelin 1 activates not only Runx2 [2] but also Twist, as reported in the present paper.

Extensive studies were performed to clarify the patterns of Endothelin 1 *in vivo* and *in vitro* under long-term dAza exposure. In particular, we evaluated the function of the 3′-UTR of the gene and the regulation by miR98, since a post-transcriptional control might be explanatory. miR98 interacts with Endothelin 1-3′-UTR [24]. We showed that the regulatory function of the 3′-UTR was fundamental for the transactivating activity of Endothelin 1, and the induction of Endothelin 1 occurred when the 3′-UTR was offside, while the presence of miR98 expression was consistent with Endothelin 1 down-regulation in the xenograft model. Also, SPARC down-regulation under dAza administration seemed to involve a post-transcriptional control, based on *in vitro* experiments using miRNA 29a mimic for a functional analysis.

In conclusion, transcriptional, post-transcriptional and post-translational (stabilization, glycosylation) mechanisms were responsible for SPARC expression in bone metastasis. The acquisition of stromal traits by bone metastasis is a negative predictive factor, but may also be a potential therapeutic target [46,52]. Our findings suggest that targeting SPARC expression by DNA methyltransferases blockade, and the combined treatment with miRNAs is a promising strategy to affect the invasive front of bone metastasis. Methylation of gene promoters is, in fact, reversible influencing gene expression, metastasis phenotype, and the outgrowth of bone metastasis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.10.010.

Authors' contributions

MAD conceived the project and was responsible for the research and the design of the experiments. MAD, PB and PM analysed the data. PM prepared the xenograft model with PB, and PM performed immunohistochemistry; A.D. performed the quantitative RT-PCR assays of mRNA and miRNA; EM performed cell cultures, constructs preparation and transfection, Western blots and luciferase activity assays.

Conflict of interest

No potential conflicts of interest were disclosed.

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