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Stable depletion of poly (ADP-ribose) polymerase-1 reduces *in vivo* melanoma growth and increases chemosensitivity

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ABSTRACT

Poly(ADP-ribose) polymerase (PARP)-1, which plays a key role in DNA repair, inflammation and transcription, has recently been shown to be involved in angiogenesis. The aim of this study was to investigate PARP-1 role in melanoma aggressiveness and chemoresistance *in vivo* using clones stably silenced for PARP-1 expression. Whilst the growth characteristics of PARP-1-deficient melanoma cells were comparable to those of PARP-1-proficient cells *in vitro*, their tumourigenic potential *in vivo* was significantly compromised. In fact, mice challenged intra-muscle with PARP-1-deficient cells showed a delayed development of measurable tumour nodules, which were also significantly reduced in size with respect to those of mice inoculated with PARP-1-proficient cells. Moreover, animals challenged intra-cranially with PARP-1-deficient cells, a model that mimics CNS localisation of melanoma, showed an increased survival. Immunohistochemical analyses of PARP-1-depleted melanoma grafts indicated a reduced expression of the angiogenesis marker PECAM-1/CD31 and of the pro-inflammatory mediators TNF-α and GITR. Notably, PARP-1-silenced melanoma was extremely sensitive to temozolomide, an anticancer agent used for the treatment of metastatic melanoma. These results provide novel evidence for a direct role of PARP-1 in tumour aggressiveness and chemoresistance.

1. Introduction

Malignant melanoma is one of the most highly invasive and metastatic cancer. Moreover, the incidence of melanoma and mortality rate from this malignancy are increasing.[1] Whilst the prognosis is excellent for patients diagnosed at an early stage, the outcome of the progressive disease is poor. The appropriate therapy of metastatic melanoma is still

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unsatisfactory. Single-agent therapy with the methylating compound dacarbazine has been the reference treatment for melanoma but no improvement of the patients' overall survival has been achieved.[2] The analogue temozolomide (TMZ) has shown in clinical trials a similar efficacy to dacarbazine but improved quality of life. TMZ, unlike dacarbazine, is an orally available drug and is able to cross the blood–brain barrier thus representing a promising agent for the treatment of brain metastases.[3,4] This pharmacokinetic property is of particular importance since the incidence of brain metastases is about 10–40% depending on clinical studies, and at autopsy about two-third of the patients show CNS involvement.[5] When present, brain metastases eventually lead to death in the majority of cases, with a median overall survival time of 3–4 months.[2] A recent strategy to increase the efficacy of TMZ relies on the use of biomodulators of resistance such as the inhibitors of poly(ADP-ribose) polymerase (PARP), which are currently under evaluation in phase II clinical trials.[6,7]

PARP-1 is a nuclear protein which is activated by single and double strand breaks as an early response to DNA damage.[8] The enzyme shares with the other members of the PARP family the ability to use NAD^+ as a substrate to catalyse the attachment of ADP-ribose polymers to acceptor proteins altering their activity. Most of the cellular poly(ADP-ribosyl)ating activity has been attributed to PARP-1 whose primary targets include PARP-1 itself, histones and a variety of transcription factors. Amongst the members of the PARP family, PARP-1 and the closely related PARP-2 are known to bind to DNA, acting as a sensor of genotoxic damage and co-ordinating repair. Indeed, cells from PARP-1 knockout (KO) and PARP-2 KO mice show an elevated frequency of recombination, gene amplification or sister chromatid exchanges, and are hypersensitive to cytotoxicity mediated by genotoxic agents.[9,10] Nevertheless, PARP-1-deficient mice develop spontaneous mammary tumours at a low incidence and with long latency.[11] Both PARP-1 and PARP-2 are important mediators of the base excision repair, which is devoted to the correction of DNA base lesions including those induced by ionising radiation or methylating agents such as TMZ.[12] Poly(ADP-ribos)ylated PARP-1 also counteracts the action of topoisomerase I poisons by facilitating resealing of DNA strand breaks, and PARP inhibitors have been shown to enhance the efficacy of camptothecin derivatives of clinical use.[13]

Besides DNA repair, PARP-1 is also involved in transcriptional regulation by at least two different mechanisms: changing chromatin conformation and affecting the function of a number of transcription regulators acting as transcriptional co-factor.[14] This activity is accomplished by poly (ADP-ribosyl)ation of the target molecule, direct protein–protein interaction or non-covalent binding of histones with poly(ADP-ribose), as free polymer or attached to proteins.[15–18] PARP-1 also plays a key role in inflammation contributing to cellular energetic failure. In fact, the oxidant species generated during the inflammatory reaction cause DNA damage and PARP-1 hyperactivation. This leads to elevated NAD^+ consumption for the synthesis of ADP-ribose polymers and consequent ATP depletion in an attempt to restore the cellular pool of NAD^+.[19] Moreover, PARP-1 enhances the activities of key transcription factors such as NF-kB which regulates the expression of inflammatory mediators, adhesion molecules and cytokines involved in immune responses.[18,20]

It is recognized that inflammation contributes to the development and progression of a variety of cancer types.[21] Moreover, tumour-infiltrating lymphocytes, macrophages and tumour cells are known to produce several inflammatory cytokines. Cytokines, in turn, can enhance the tumourigenic process by up-regulating mediators of angiogenesis such as vascular endothelial growth factor (VEGF).[22] Noteworthily, it has been recently demonstrated that PARP-1 KO mice show a reduced susceptibility to skin cancer induced by carcinogens due to an impaired oxidative/inflammatory response in the initial step of carcinogenesis through inactivation of transcription factors which are also involved in inflammation.[23] Moreover, impairment of PARP-1 function hampers angiogenesis as indicated by the reduction of blood vessel neo-formation in response to angiogenic stimuli observed in PARP-1 KO mice or in endothelial cells treated with PARP inhibitors.[24] Indeed, many tumours have high basal levels of PARP-1 in the absence of genotoxic damage (including colorectal and hepatocellular carcinoma or melanoma) and it has been reported that PARP-1 might participate in colorectal carcinogenesis, since its expression was found to be increased in carcinomas with respect to the corresponding normal intestinal epithelium.[13,25–28]

Since chemical inhibitors of PARP activity, which target the NAD^+-binding domain, affect both PARP-1 and PARP-2 and likely other members of the PARP family, to investigate the role of PARP-1 in tumour growth and chemosensitivity we have selectively down-regulated its expression by RNA-mediated interference (RNAi). In the present study, we show that the genetic elimination of PARP-1 in malignant melanoma reduces tumour progression and chemoresistance *in vivo*.

2. Materials and methods

2.1. Cell cultures

The murine melanoma B16 cell line of C57BL/6 ($H\text{-}2^b/H\text{-}2^b$) origin (ATCC, Manassas, VA) was cultured in RPMI-1640 containing 10% foetal calf serum (Sigma–Aldrich, Milan, Italy), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma–Aldrich), at 37 °C in a 5% CO_2 humidified atmosphere.

For the stable depletion of PARP-1 in B16 cells, a DNA vector-based RNAi approach was used. In particular, a clone obtained by limiting dilution from B16 cells (hereafter referred to as Clone 1), was transfected with the pBS-U6-SiP912 vector,[29] using the CalPhos Mammalian transfection Kit (Clontech, Palo Alto, CA, USA). The pBABE vector (kindly provided by Dr. Robert Weinberg, Whitehead Institute for Biomedical Research, Cambridge, MA, USA), carrying the puromycin resistance gene, was co-transfected with pBS-U6-SiP912 vector (1:3) to allow the selection of transfected clones. Antibiotic resistant clones were isolated by ring cloning and maintained in the presence of 5 μg/ml puromycin.

Cell growth of the transfected clones was evaluated by counting viable cells in quadruplicate, every 24 h, during a 3-day culture. Cell viability was determined by trypan blue dye exclusion.

2.2. Analysis of PARP activity and expression

For the analysis of PARP activity cells (5×10^5 cells), untreated or exposed to 20 mM H_2O_2 for 15 min, were permeabilised with digitonin (0.1 mg/ml) in the presence of 0.25 μCi ^{3}H-NAD^+ (Perkin-Elmer, Milan, Italy).[30] Western blot analysis was performed using monoclonal antibodies directed against PARP-1 (clone C2-10; Trevigen, Gaithersburg, MD, USA; 1/2000 dilution), PARP-2 (clone Yuc; Alexis, Florence, Italy; 1/5000 dilution); β-actin (clone AC-15; Sigma–Aldrich 1/2500 dilution). Signals were quantified using a Kodak densitometer (Rochester, NY, USA).

2.3. Colony-formation assay

Cells were seeded in triplicate into a 6-well plate (2×10^2/well) and, after overnight incubation, treated with TMZ (Schering-Plough, Kenilworth, NJ, USA, 15–250 μM), or SN-38 (Alexis, 0.1–5 nM). Stock solutions were prepared by dissolving the drugs in dimethyl sulphoxide. The final concentration of dimethyl sulphoxide was always less than 0.5% (v/v) and did not contribute to toxicity (data not shown). Cells were cultured to allow colony-formation; after 10–14 days colonies were fixed, stained with 2% methylene blue in 95% ethanol and counted. Only colonies comprising >50 cells were scored as survival colonies. All experiments were repeated at least three times in triplicate. Chemosensitivity was evaluated in terms of IC_{50}, i.e. the concentration of the drug capable of inhibiting colony formation by 50%.

2.4. Flow cytometry analysis

Necrosis induced by treatment with H_2O_2 was evaluated by flow-cytometry. Cells were exposed to H_2O_2 and, after 4 h, washed and suspended in PBS containing 2 μg/ml of propidium iodide (PI). Necrotic cells, which have lost plasma membrane integrity, are positive for PI staining.

Cell cycle perturbations induced by TMZ or SN-38 were evaluated by flow-cytometry analysis of the DNA content. Untreated or drug-treated melanoma cells (1×10^6) were washed with PBS and fixed in 70% ethanol at −20 °C for 18 h. Cells were then centrifuged, suspended in 1 ml of a solution containing 0.1% (w/v) sodium citrate, 0.1% Triton-X (v/v), 50 μg/ml PI, 10 μg/ml RNase and incubated in the dark at 37 °C for 15 min. Data collection were gated utilising forward light scatter and side light scatter to exclude cell debris and aggregates. The PI fluorescence was measured on a linear scale using a FACSscan flow cytometer and the CellQuest software. For cell cycle analysis, the Mod-Fit software version 3.0 was used (Becton and Dickinson, San Jose, CA, USA).

2.5. In vivo studies

B16 melanoma cells (2.5×10^5), from control or PARP-1 silenced clones, were inoculated intra-muscularly (i.m.) in the hind leg muscles of histocompatible male C57BL/6N mice (Charles River, Calco, Milan, Italy). Tumours were measured with calliper and volumes were calculated according to the formula: $[(\text{width})^2 \times \text{length}]/2$. Melanoma growth was monitored by measuring tumour nodules every 3 days for 3 weeks.

The intracranial transplantation procedure was performed as previously described.[31] Briefly, cells (10^4 in 0.03 ml of RPMI-1640) were injected intra-cranially (i.c.) through the centre-middle area of the frontal bone to a 2 mm depth, using a 0.1 ml glass microsyringe and a 27-gauge disposable needle. Murine melanoma B16 cells were injected i.c. into male C57BL/6 mice. Before tumour challenge, animals were anesthetised with 2,2,2-tribromoethanol (0.25 mg/g, Sigma–Aldrich). In selected experiments, six- to eight-week-old male and female C57BL/6 wild-types or PARP-1 KO mice, kindly provided by Dr. Z.-Q. Wang (Fritz Lipmann Institute, Jena, Germany),[32] were inoculated i.c. with melanoma cells. Body weight was measured thrice weekly; survivals were recorded for 60 days and median survival times (MST) were determined.

Histological examination of the tumour nodules or of the brains was performed using additional animals that were not considered for the monitoring of tumour growth or survival. Tissues were fixed in 10% (w/v) phosphate-buffered formaldehyde, cut along the axial plane and embedded in paraffin. Histological sections (5 μm thick) were stained with haematoxylin–eosin and analysed by light microscopy.

For *in vivo* treatment, TMZ was dissolved in dimethyl sulphoxide, diluted in saline and administered intraperitoneally (i.p.), one day after tumour challenge, at the dose of 68 mg/kg/day for 5 days. Control mice were always injected with drug vehicle. The percentage of increase in lifespan was calculated as $\{[\text{MST (days) of treated mice/MST (days) of control mice}] - 1\} \times 100$.

All procedures involving mice and care were performed in compliance with national (D.L. No. 116, G.U., Suppl. 40, Feb. 18, 1992 and G.U. 163, July 1994) and international laws (EEC Council Directive 86/609, OJ L 358. 1, Dec 12, 1987; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996).

2.6. Immunohistochemical analysis

Tissue sections were deparaffinised and endogenous peroxidase was quenched with 0.3% (v/v) H_2O_2 in 60% (v/v) methanol for 30 min. Non-specific adsorption was minimised by incubating the sections in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with the following antibodies: anti-PAR (Santa Cruz Biotechnology, CA, USA), anti-PECAM-1/CD31 (Santa Cruz), with anti-tumour necrosis factor-α (TNF-α, Santa Cruz), or with anti-glucocorticoid-induced TNFR-related protein (GITR, R&D Systems, Minneapolis, MN, USA). All antibodies were used 1/100 (v/v) in PBS. Sections were washed with PBS and incubated with secondary antibody. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin–biotin peroxidase complex (DBA). The counterstain was developed with 3,3'-diaminobenzidine (brown colour) and the nuclear fast red (red background).

2.7. Statistical analysis

Statistical analysis was performed using the Primer of Biostatistics Statistical Software Program, (McGraw-Hill, New York,

NY, USA). Survival curves were generated by Kaplan–Meier product-limit estimate, and statistical differences between the various groups were evaluated by log-rank analysis with Yates correction. For multiple comparisons, the results were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni test. A *P*-value of less than 0.05 was considered significant. Student's *t*-test was used for statistical analyses of PARP activity assay. The results were considered to be statistically significant at $P < 0.05$ (two sided).

3. Results

3.1. *Stable depletion of PARP-1 by RNAi in melanoma cells*

In order to stably silence PARP-1 expression in melanoma cells, the pBS-U6-SiP912 vector, which targets selected PARP-1 sequences, was used.[29] A clone (clone 1) from murine B16 melanoma, with *in vitro* growth rate and *in vivo* tumourigenicity comparable to those of the parental cell line (data not shown), was co-transfected with the pBS-U6-SiP912 vector and the pBabe vector expressing only the puromycin resistance gene. As control, clone 1 was transfected with pBabe only. Drug-selected clones were then analysed for PARP-1 expression and activity. Several clones were found to be devoid of PARP-1 expression (hereafter referred to as SiP clones). Three PARP-1 silenced clones (SiP G, SiP N and SiP O) together with three control clones (clone 1, Babe 3 and Babe 6 clones) with intact PARP-1 expression were then used for further *in vitro* and *in vivo* studies (Fig. 1A). These clones were also analysed for PARP-2 expression and the results indicated that PARP-2, although barely detectable, was present both in control and in PARP-1 silenced clones (Fig. 1A). Analysis of the

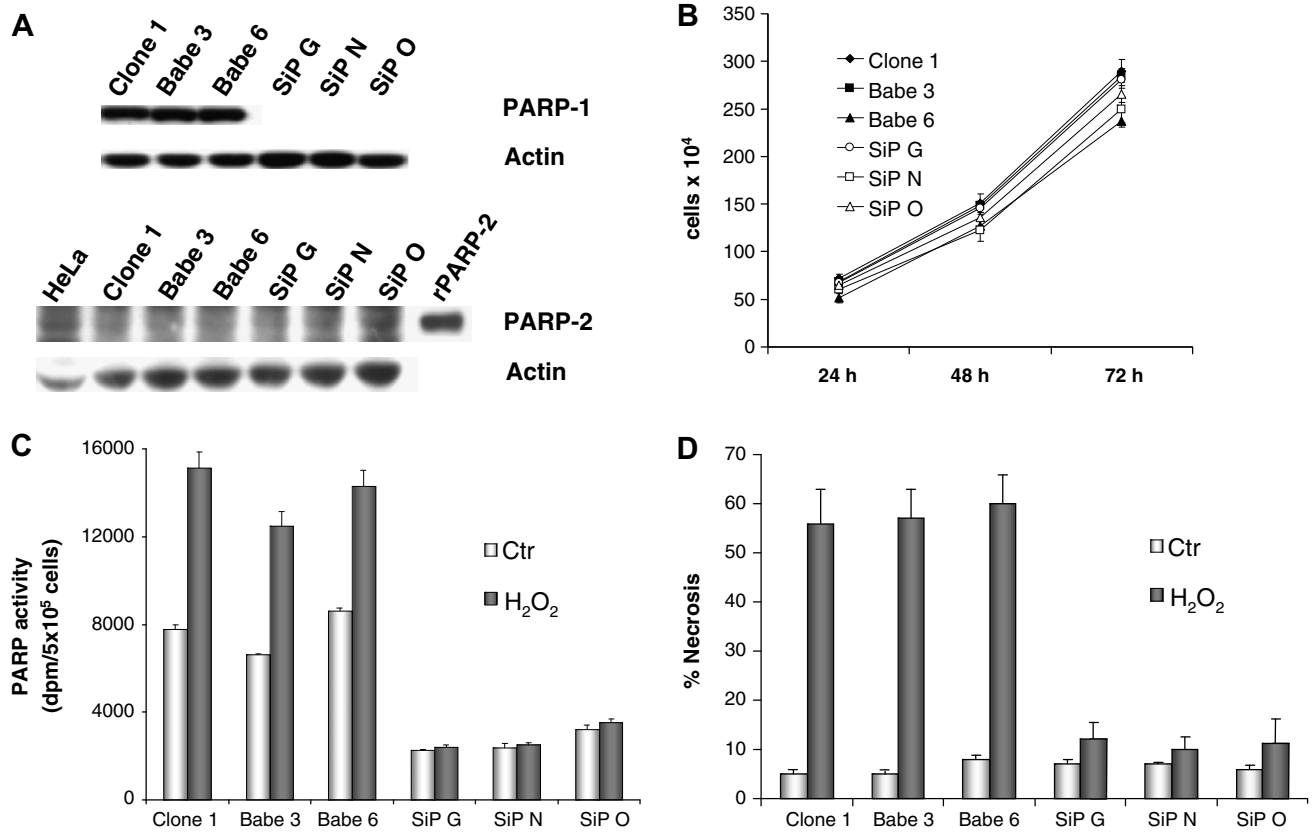

Fig. 1 – Characterisation of PARP-1 silenced melanoma clones. (A) Cell lysates (50 μg) from control clones (Clone 1, Babe 3 or Babe 6) or from clones transfected with pBS-U6-SiP912 vector (SiP) were electrophoresed and analysed for the expression of PARP-1, PARP-2 or actin. Murine recombinant PARP-2 (rPARP-2, 500 ng, Alexis) and cell lysate (50 μg) from the cervical carcinoma HeLa cell line were used as positive control for PARP-2 immunodetection. For rPARP-2, a short exposure of the immunoblot is shown. (B) Cell growth was evaluated by plating each clone in triplicate and counting viable cells in quadruplicate, every 24 h. Values represent the mean number of viable cells ± SD. Mean doubling times ± SD, calculated at the different time points, were as follows: Clone 1, 20 ± 2; Babe 3, 21 ± 1; Babe 6, 25 ± 4; SiP G, 21 ± 1; SiP N, 24 ± 1; SiP O, 22 ± 1. Data are representative of one out of three independent experiments with similar results. (C) Analysis of PARP activation in Clone 1, Babe or SiP clones was assessed by incubating cells, untreated or exposed to 20 mM H_2O_2 for 15 min, with ^{3}H-NAD$^+$ after permeabilisation with digitonin. The results are expressed as dpm of trichloroacetic acid-precipitable ^{3}H-NAD$^+$/5 × 10^5 cells and values represent the mean of three independent experiments. Bars: + SD values. Clone 1 or Babe samples (untreated or treated with H_2O_2) versus SiP clones (untreated or treated with H_2O_2) samples: $P < 0.0001$ (Student's *t*-test). (D) Data are expressed as the percentage of necrotic cells and represent the mean of three independent experiments +SD. Clone 1 or Babe samples treated with H_2O_2 versus SiP clones treated with H_2O_2 samples: $P < 0.05$ (ANOVA for multiple comparisons followed by a Bonferroni test).

doubling times, evaluated on the basis of the growth kinetics illustrated in Fig. 1B, indicated that SiP clones possess doubling times ranging from 20 to 25 h which are in the same range of those observed in PARP-1 proficient clones. SiP clones, when exposed to the oxidant H_2O_2, which is known to generate DNA strand breaks and to induce PARP-1 activation, were unable to trigger PARP activity (Fig. 1C), in accordance with previous findings obtained in different cell types.[29] Since extensive DNA damage, which induces PARP-1 over-activation, leads to necrotic cell death due to NAD^+ consumption and consequent ATP depletion,[33] we have investigated whether PARP-1 silencing might prevent necrosis induced by H_2O_2. The results indicated that SiP clones were protected from the early onset of necrosis, which was instead clearly observed in PARP-1 proficient clones (Fig. 1D).

3.2. *PARP-1 silencing significantly reduces in vivo melanoma growth in an orthotopic model*

We previously demonstrated that the impairment of PARP-1 function reduces blood vessel neo-formation in response to angiogenic stimuli in PARP-1 KO mice.[24] Since angiogenesis is an essential requirement for tumour growth, we investigated whether PARP-1 depletion in melanoma clones might influence their ability to grow *in vivo* using an orthotopic model. Histocompatible C57BL/6 mice were inoculated i.m. with SiP or control clones and tumour volume was monitored. A delay in the development of a tumour mass was observed in mice inoculated with SiP clones, since on day 10 after challenge the nodules were not yet measurable in 30% of the animals. At the same time point nodules were palpable in all mice injected with PARP-1-proficient melanoma clones. On day 22, a significant reduction ($P < 0.05$) in tumour size was observed when mice bearing SiP melanoma clones were compared to animals inoculated with clone 1 or Babe clones (Fig. 2). At this time point animals were sacrificed to avoid unacceptable suffering and according to ethical guidelines.

Histological examination of melanoma nodules performed 15 days after tumour challenge revealed a massive tumour infiltration of muscle tissue in H&E sections from animals inoculated with Babe 3 clone, whereas at this time point the growth of SiP G clone was still limited and confined within the muscle tissue (Fig. 3, panels A and C).

3.3. *Mice bearing PARP-1 silenced melanoma at the CNS site survive significantly longer than mice challenged with PARP-1-proficient melanoma*

Since the brain is one of the preferred sites of melanoma metastases, it was investigated whether PARP-1 expression in melanoma clones might affect their ability to grow at the CNS site. To this end, SiP G or control Babe 3 clones, which showed identical growth kinetics *in vitro*, were injected i.c. in C57BL/6 mice and survival curves were compared. Lack of PARP-1 expression resulted in the delayed growth of melanoma in the brain, as evidenced by the significantly longer survival of animals challenged with SiP G clone in comparison to that of mice injected with the control melanoma clone (Fig. 4 and Table 1).

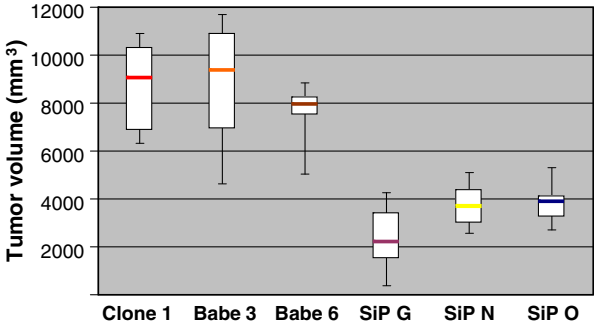

Fig. 2 – *In vivo* growth of SiP and Babe melanoma clones in an orthotopic model. Mice were inoculated i.m. with Clone 1, Babe 3 or Babe 6 clones and with PARP-1 silenced SiP G, SiP N or SiP O clones. Box and Whisker plots refer to tumour volume measured on day 22 after tumour challenge. Data were analysed by ANOVA for multiple comparisons followed by a Bonferroni test and the results indicated that the growth of SiP clones was always significantly different ($P < 0.05$) from that of controls (Clone 1, Babe 3 or Babe 6). No significant differences were observed amongst all control clones. Confidence intervals (95%) were as follows: Clone 1, 7341–10,184; Babe 3, 5777–11,736; Babe 6, 6171–8972; SiP G, 3158–4499; SiP N, 1141–3569; SiP O, 2924–4556.

Microscopic examination of the brain tissues, performed 11 days after tumour i.c. injection, revealed multifocal brain involvement in animals bearing Babe melanoma clone. Moreover, large tumour masses were detected both at the site of injection and in the parenchyma (Fig. 3, panel B). Conversely, analysis of the brain tissues collected from mice challenged i.c. with SiP G clone showed minimal tumour infiltration (Fig. 3, panel D).

In order to investigate whether PARP-1 ablation in the cells of tumour microenvironment (e.g. endothelial, inflammatory cells) might also influence melanoma growth, melanoma cells with intact or silenced PARP-1 were inoculated i.c. in syngeneic PARP-1 KO mice, but no differences in terms of MST were observed between KO and wild-type mice (Table 1). The data suggest that the expression of PARP-1 in tumour cells has a major role in tumour development whereas the surrounding tissues did not seem to influence the growth of melanoma at the CNS site.

3.4. *In vivo growth of PARP-1-silenced melanoma cells is accompanied by reduced PAR staining, tumour-associated vascularisation and inflammatory reaction*

Immunohistological analysis of PAR revealed a positive staining within the tumour and in the peri-tumoural areas only in samples collected from mice challenged with Babe clone (Fig. 5, panels A and B). Conversely, no PAR staining was observed in tissues from animals inoculated with SiP melanoma clone (Fig. 5, panels C and D).

The microvessel neo-formation within the tumour was analysed by immunohistochemical staining for platelet/endothelial cell adhesion molecule-1 (PECAM-1/CD31) as an

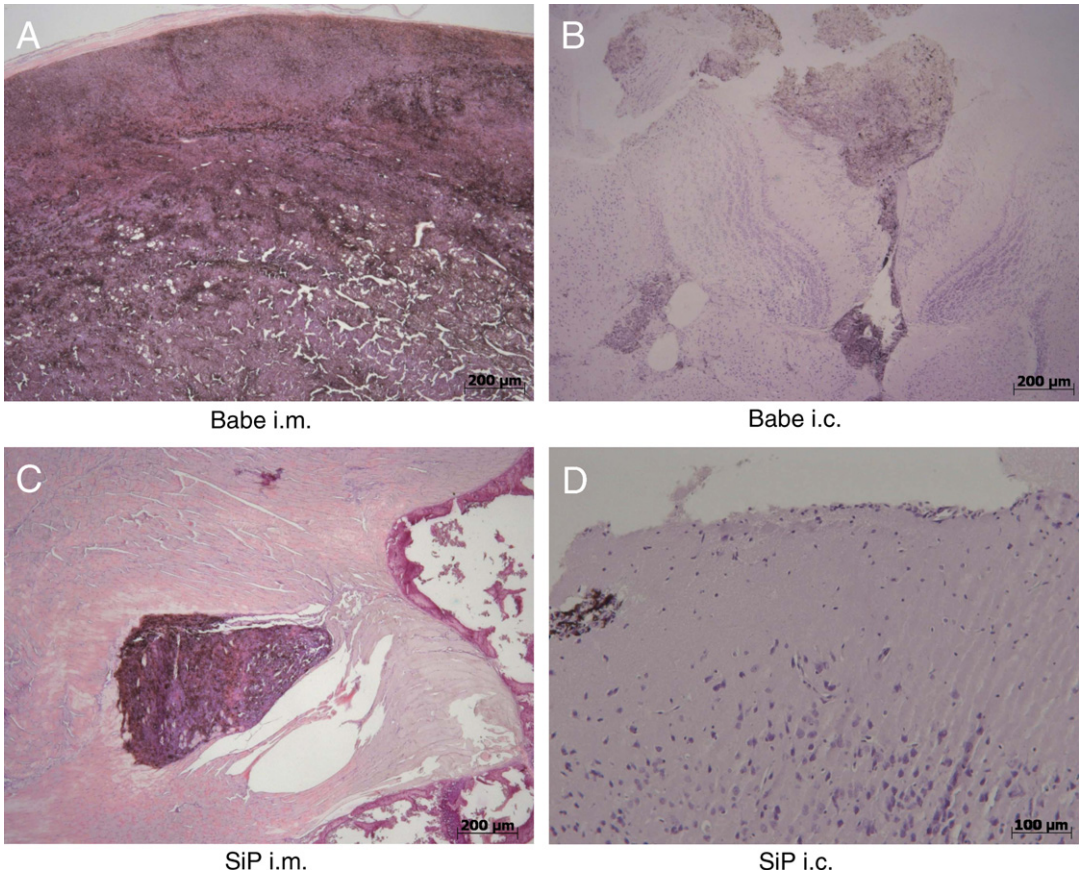

Fig. 3 – Histological examination of the growth pattern of control or PARP-1 silenced melanoma clones. Histological analysis of the muscle hind-limb and of the brain tissue obtained from mice (6 mice/group) challenged with Babe 3 (A, B) or SiP G clones (C, D) was performed by H&E staining of samples collected on day 15 or 11 for the i.m. and i.c. models, respectively. Representative histological sections of melanoma growing in the hind limb muscles (i.m.) (A, C) or in the brain (i.c.) (B, D) are presented. Microscopic examination of the muscle showed a massive tumour infiltration of the tissue in the section from animals inoculated with Babe 3 clone (A). Similarly, analysis of the brain tissues demonstrated multifocal brain involvement in animals bearing Babe melanoma clone. Moreover, large tumour masses were detected both at the site of injection and in the parenchyma (B). Conversely, analysis of the muscle (C) and the brain (D) tissues collected from mice challenged with SiP G clone showed minimal tumour infiltration.

indicator of tumour-associated angiogenesis. The results indicated that melanoma samples from mice challenged with a Babe control clone were highly vascularised (Fig. 6, panels A and B), whereas sections from a SiP clone presented no detectable staining of vessels (Fig. 6, panels C and D).

Since the inflammatory component in tumour development is remarkable and due to the involvement of PARP-1 in the modulation of inflammatory reaction mainly through the transcription regulation of pro-inflammatory cytokines, TNF-α expression was investigated in melanoma histological sections. Moreover, immunohistochemical analysis of GITR, which is also involved in inflammatory responses, was performed.[34] The results indicated that both TNF-α and GITR were highly expressed in Babe melanoma clone growing i.m. whereas no such signs of inflammatory reaction were detectable in the PARP-1 silenced melanoma clone (Fig. 7). Similar results were obtained in the i.c. melanoma model (data not shown).

3.5. *PARP-1 silenced melanoma clones show increased chemosensitivity in vitro and in vivo*

Since pharmacological inhibition of PARP activity has been shown to increase tumour sensitivity to methylating agents and topoisomerase I inhibitors, SiP and control clones were tested for their susceptibility to the anti-proliferative effects of TMZ or SN-38, the active metabolite of the camptothecin derivative irinotecan, by means of colony-formation assay. The results indicated that silenced clones were about twofold more susceptible to the anti-cancer drugs than control clones (Table 2).

Since we previously observed that TMZ in combination with PARP inhibitor increased the percentage of cells in G2/M phase of the cell cycle 24 h after treatment with respect to TMZ used as single agent,[35] control or PARP-1 silenced melanoma cells were treated with TMZ or SN-38 and analysed for cell cycle distribution. The results indicated that drug treatment of SiP cells increased the percentage of cells

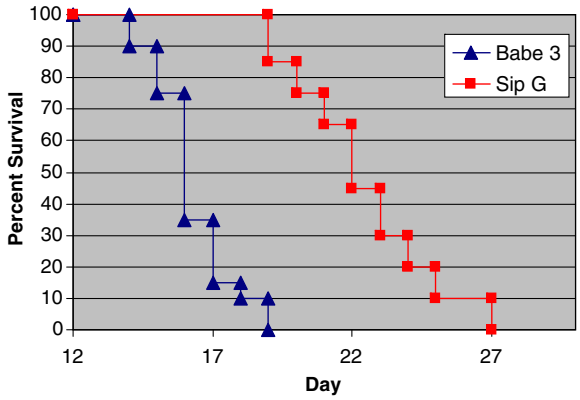

Fig. 4 – Survival of mice bearing intra-cerebral PARP-1-proficient or -deficient melanoma. Survival curves of mice challenged i.c. with Babe 3 or SiP G melanoma clones (n = 20/group) are represented. Curves are generated by Kaplan–Meier product-limit estimate and statistical differences between groups are evaluated by log-rank analysis with Yates correction. A significant increase ($P < 0.0001$) in survival was observed when the mice injected i.c. with SiP G clone was compared to the mice bearing Babe 3 clone.

Table 1 – Survival of wild-type or PARP-1 knockout mice bearing intra-cerebral Babe or Sip melanoma clones

Mice	Clones	MST (range)	P[a] (SiP versus Babe)	P[b] (KO versus WT)
WT	Babe 3	16 (14–19)	–	–
WT	Sip G	22 (19–27)	<0.05	–
KO	Babe 3	16 (14–18)	–	NS
KO	Sip G	22 (20–27)	<0.05	NS

a Statistical differences between groups are evaluated by log-rank analysis with Yates correction. P was calculated comparing the survival curves of SiP G versus Babe 3 groups.

b P was calculated comparing the survival curves of wild-type animals (WT, i.e. PARP-1-proficient) challenged with Babe 3 or SiP G cells with the survival curves of PARP-1 KO mice injected with Babe 3 or SiP G cells, respectively.

in the G2/M phase with respect to PARP-1-proficient cells (Fig. 8).

Then, it was investigated whether *in vivo* administration of TMZ in mice bearing PARP-1-deficient B16 melanoma at the CNS site was more effective than that in mice challenged with PARP-1-proficient melanoma. SiP G or control Babe 3 clones were injected i.c. in C57BL/6 mice; subsequently, animals were treated with five doses of TMZ (68 mg/kg/i.p./day). Comparison of survival curves of animals receiving TMZ with those of the untreated groups indicated that TMZ significantly ($P < 0.0001$) increased survival both in Babe and in SiP clones (Fig. 9 and Table 3). Notably, the increase in lifespan induced by TMZ in mice injected with SiP clone was about 2.5-fold higher than that provoked by the drug in animals challenged with control Babe clone (Table 3).

4. Discussion

In the present study, we demonstrate for the first time that the abrogation of PARP-1 expression by stable gene silencing reduced the aggressiveness of melanoma as indicated by the delay in tumour progression observed *in vivo*. In fact, mice challenged with syngeneic PARP-1 silenced melanoma developed measurable tumour nodules later and of smaller size with respect to mice inoculated with PARP-1-proficient melanoma. The decreased *in vivo* growth of PARP-1-deficient cells was also demonstrated by the significant increase in the survival time of mice bearing intracranial tumour that mimics CNS localisation of malignant melanoma.

The melanoma clones used for this study were stably transfected with pBS-U6-SiP912 vector and did not express detectable PARP-1 protein. Silenced melanoma clones showed *in vitro* growth kinetics characterised by doubling times in the same range of those observed in PARP-1 proficient clones. Moreover, upon treatment with H_2O_2, which generates DNA strand breaks through the formation of hydroxyl radicals and induces PARP-1 activation, they did not respond to the genotoxic agent synthesising significant amount of PAR. Interestingly, the abrogation of PARP-1 expression protected cells from the early occurrence of necrosis in accordance with previous studies.[33] It should be noted that the modest expression of PARP-2 detected in B16 melanoma cells did not cause in SiP clones additional NAD^+ incorporation in newly synthesized PAR after H_2O_2 treatment, at least within the short time frame of the assay, suggesting a prominent role of PARP-1 in response to oxidative DNA damage. These data are in accordance with a previous study in PARP-1 silenced fibroblasts in which H_2O_2 did not cause PAR synthesis.[29] The residual PARP activity detected in untreated SiP clones could derive from other members of the PARP family, whose activity appears, so far, to be independent on DNA damage.

The analysis of PARP activation and vessel formation within the tumour grafts indicated an evident vascularisation and PAR formation in the histological sections of control melanoma grafts, whilst in the PARP-1 SiP G silenced clone, collected at the same time point, no evidence of vascularisation or the synthesis of PAR was found. It is likely to hypothesise that the lack of PARP-1 mediated regulation of gene transcription and the reduced availability of PAR might influence the expression and function of proteins necessary for the establishment of a vascular network which is an essential requirement for the *in vivo* tumour growth. These data are in line with the recent reports demonstrating that the impairment of PARP-1 function by gene deletion or pharmacological inhibition hampered angiogenesis in response to vascular growth factors.[24,36,37] Indeed, it has been shown that PARP inhibition may also exert antitumour activity likely through the reduction of tumour-associated vessel neo-formation, independently on its effect on DNA repair.[38] A role for PARP-1 in tumour formation and angiogenesis has been recently suggested by Martin-Oliva and colleagues, showing that the inhibition of PARP resulted in a delay in tumour development and a decrease of tumour size during skin carcinogenesis induced by an anthracene derivative. This effect has been attributed to the modulation of transcription factors involved in cell responses, including proliferation, inflammation and

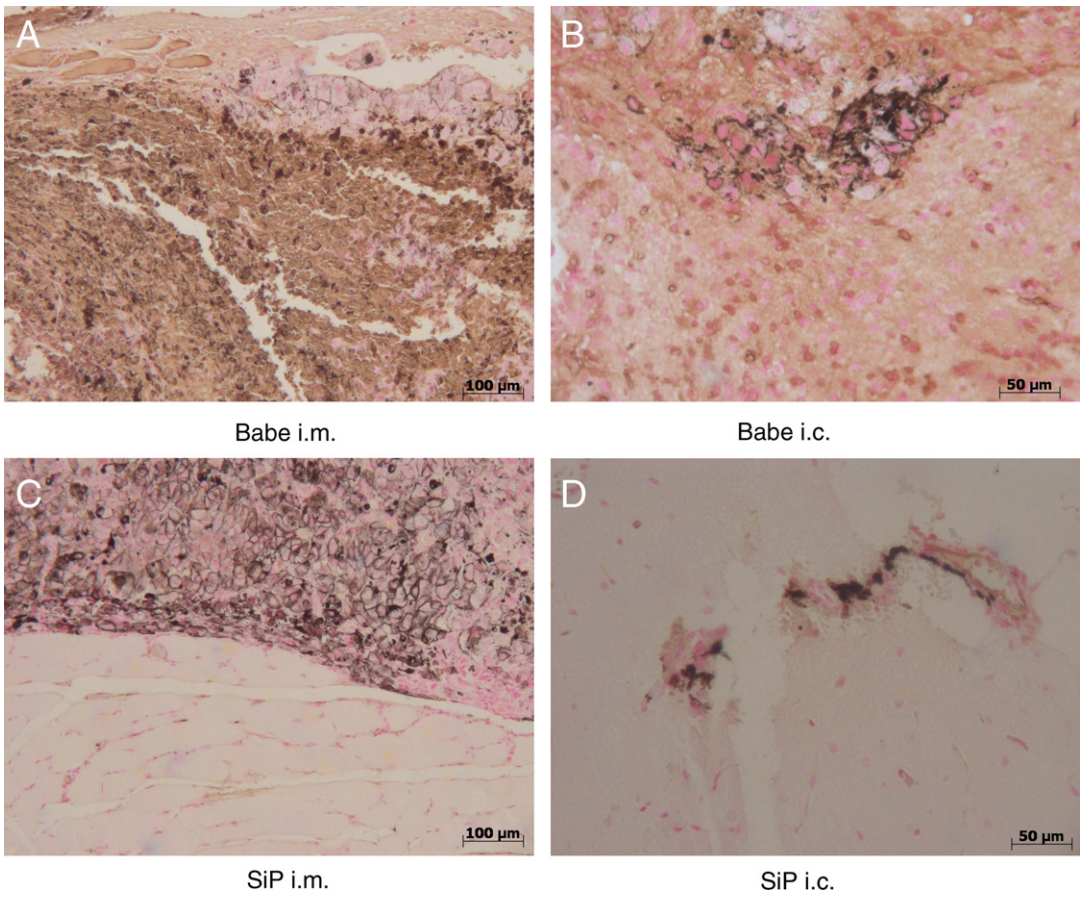

Fig. 5 – Immunohistochemical staining of tumour sections for poly-(ADP-ribosyl)ated proteins. Immunohistochemical staining for PAR formation, as an indicator of PARP activation, was performed in tumour samples collected on day 15 or 11 for the i.m. and i.c. models, respectively, from mice (6 mice/group) challenged with Babe 3 (A, B) or SiP G (C, D) clones. Representative histological sections of melanoma growing in the muscle hind limb (i.m.) (A, C) or in the brain (i.c.) (B, D) are presented. The evidence of PAR accumulation can be seen as a brownish staining in Babe groups. In particular, positive PAR staining was found in the tumour and in the peri-tumoural areas in tissues from mice challenged with Babe clone (A, B). Conversely, no PAR positive staining was observed in tissues from animals inoculated with SiP melanoma clone (C, D). Similar immunohistochemical profiles were seen in other five samples per group.

angiogenesis during the tumour promotion phase.[23] The involvement of PARP in angiogenesis has been shown at different levels and with conflicting functions. In fact, whilst pharmacological inhibition of PARP reduced endothelial cell migration in response to VEGF,[24,36,37] post-transcriptional modification of VEGF by PAR inhibited VEGF activity.[39] Moreover, the insulin-like growth factor-1, which is known to promote angiogenesis, down-regulated PARP function by phosphorylation and this effect would contribute to increase VEGF transcription.[40] It should be noted that in the present model of PARP-1 silenced melanoma, the expression of VEGF was not decreased in SiP G clone, as assessed by ELISA analysis (data not shown). Thus, the reduced aggressiveness in melanoma clones derived from the lack of PARP-1 expression may depend on the modulation of targets which still need to be identified.

The *in vivo* growth of PARP-1-proficient melanoma clone is accompanied by an intense positive staining for TNF-α. This finding is in agreement with previous studies showing that there is a significant infiltration of cells of the immune system in melanoma, particularly macrophages and mast cells, both of which are known to secrete TNF-α and other cytokines.[41] Moreover, TNF-α plays a critical role in various cancers as indicated by the resistance of TNF- α null mice to skin carcinogenesis.[42] Consistent with the results obtained with TNF-α, also the expression of GITR resulted to be elevated in histological sections from B16 melanoma. GITR is a receptor belonging to the TNFR superfamily which is expressed in T lymphocytes as well as in macrophages and neutrophils.[34] Besides its involvement in immune responses, GITR also exerts a pro-inflammatory role in a number of experimental models.[34,43,44] It should be noted that PARP-1-deficient melanoma grafts analysed at the same time point resulted to be negative for the expression of both TNF-α and GITR in accordance with the limited tumour aggressiveness observed *in vivo*. The inflammatory reaction was remarkably less represented in PARP-1-deficient clones which showed a reduced expansion and invasion of the surrounding tissues. *PARP-1* gene disruption is known to provide protection from tissue damage in a number of models of acute or chronic inflamma-

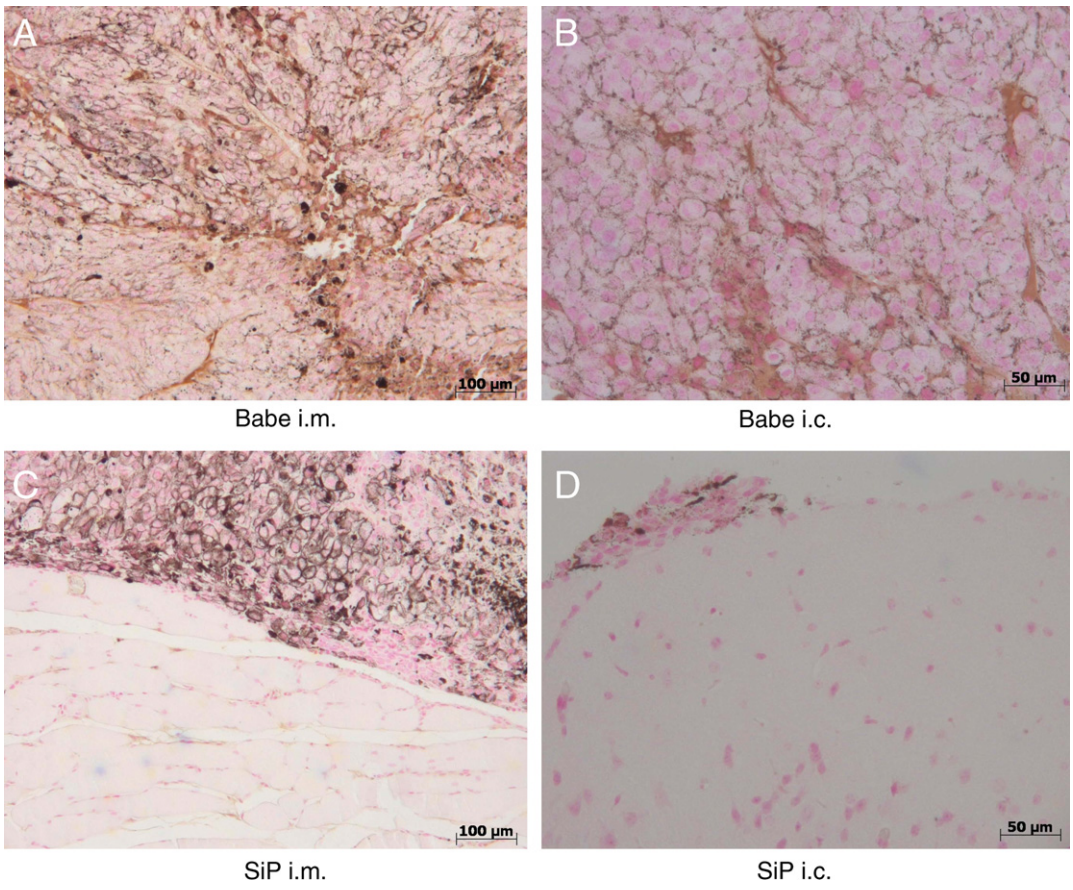

Fig. 6 – Immunohistochemical of PECAM-1/CD-31 expression in control or PARP-1 silenced tumour grafts. Vessel formation was analysed by immunohistochemical staining performed in tumour samples, collected on day 15 or 11 for the i.m. and i.c. models, respectively, from mice (6 mice/group) challenged with Babe 3 (A, B) or SiP G (C, D) clones. Representative histological sections of melanoma growing in the muscle hind-limb (i.m.) (A, C) or in the brain (i.c.) (B, D) are presented. In particular, positive PECAM-1/CD31 staining, an index of highly vascularised tumour, was found in the tissues from mice challenged with Babe clone. Blood vessels are evidenced as branched structures and little vascular lacunas which positively stain for PECAM-1/CD31 (A, C). On the contrary, melanoma samples from mice challenged with SiP clone presented no detectable positive staining of vessels (B, D). Similar immunohistochemical profiles were seen in other five samples per group.

tion, and PARP inhibitors have been shown to be effective in various models of inflammatory diseases. In particular, PARP inhibitors reduced the infiltration of neutrophils into inflammatory sites, the production of pro-inflammatory cytokines and the severity of the disease. These effects have been related to the ability of PARP-1 to modulate the transcription of adhesion molecules and of proteins involved in the preservation of endothelial integrity.[20]

Our findings on the reduced *in vivo* aggressiveness of PARP-1-deficient melanomas are consistent with a delayed recruitment of inflammatory components which would favour tumour development and progression. On the other hand, PARP-1 deficiency in the cells of tumour microenvironment and surrounding tissues did not influence the growth of melanoma, as evidenced by the comparable tumour behaviour of melanoma clones regardless of whether they are inoculated in PARP-1 KO or in PARP-1-proficient wild-type mice. These data suggest that the expression of PARP-1 in the tumour plays a major role in its behaviour *in vivo*, emphasising the importance to selectively target PARP-1 in tumour cells for cancer therapy.

PARP-1-deficient melanoma clones were more sensitive to the methylating agent TMZ and to the topoisomerase I inhibitor SN-38 with respect to proficient clones, as a result of the lack of PARP-1 intervention in the repair of DNA strand breaks induced by these anticancer agents. Moreover, a G2/M accumulation after drug treatment was observed in PARP-1-deficient cells, in accordance with previous studies showing a stronger G2/M checkpoint response in the absence of PARP-1 after exposure to methylating agents, topoisomerase I poisons or ionising radiations.[35,45–47] Similarly to our previous studies in B16 cells treated with TMZ in combination with a PARP inhibitor,[35] cell cycle modifications induced by the methylating agent were evident as early as 24 h after the treatment of PARP-1 silenced melanoma cells. In fact, genotoxic damage derived from the interruption of base excision repair process after the initial removal of the N-methylated purines (i.e., N3-methyladenine or N7-methylguanine) by the N-methylpurine-DNA glycosylase would likely take place within the time frame of the first cell replication.[12]

Noteworthily, PARP-1 silenced melanoma showed an extremely high sensitivity to TMZ in the *in vivo* model of CNS

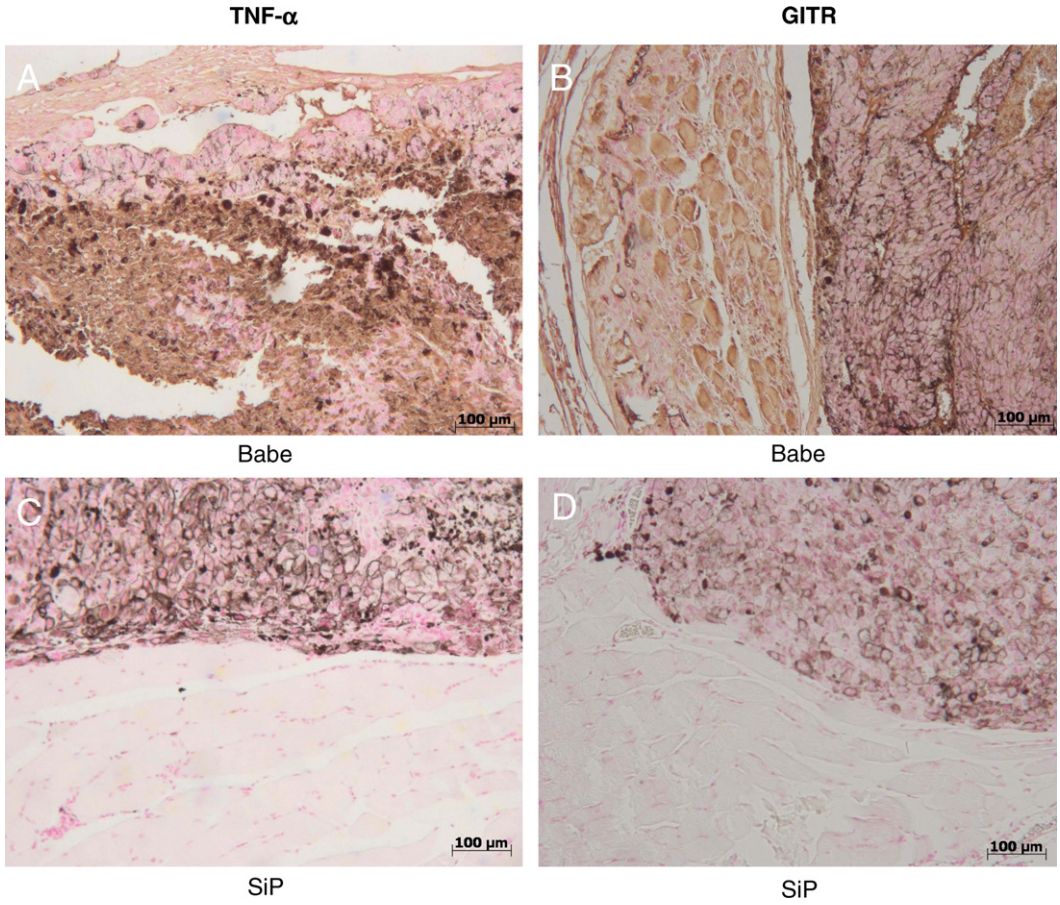

Fig. 7 – Immunohistochemical analysis of TNF-α and GITR expression in control or PARP-1 silenced tumour grafts. Inflammatory reaction was analysed by staining the histological sections of melanoma nodules collected on day 15, from mice (6 mice/group) challenged with Babe 3 (A, B) or SiP G (C, D) clones with TNF-α (A, C) and GITR (B, D) antibodies. Representative histological sections of melanoma growing in the hind limb muscles are presented. A positive staining for TNF-α and GITR is detectable in Babe 3 melanoma clone. Moreover, GITR expression is also evident in association to vascular structures (A, B). On the contrary, no such positive staining for TNF-α and GITR was detectable in the tissues from mice challenged with PARP-1 silenced melanoma clone (C, D). Similar immunohistochemical profiles were seen in other five samples per group.

Table 2 – In vitro chemosensitivity of PARP-1-proficient or -deficient melanoma clones

Clone	TMZ	SN-38
	IC_{50} (μM)[a]	IC_{50} (nM)[a]
Clone 1	117 ± 5	3.8 ± 0.5
Babe 3	134 ± 16	4.3 ± 0.3
Babe 6	120 ± 8	3.6 ± 0.6
SiP G	72 ± 5	1.8 ± 0.3
SiP N	70 ± 3	1.6 ± 0.2
SiP O	65 ± 6	2.0 ± 0.1

a Data represent the mean values of at least three independent experiments ± SD. Statistical analysis using ANOVA for multiple comparisons followed by a Bonferroni test indicated that the differences between SiP and Babe clones were all statistically significant ($P < 0.05$).

localisation. In fact, the increase in lifespan obtained with TMZ treatment was more than twofold higher than that obtained in mice challenged with PARP-1-proficient melanoma and treated with the methylating agent. These data indicate that PARP-1 is a major target for the chemosensitisation induced by pharmacological inhibitors, which are known to inhibit also other members of the PARP family. Interestingly, chemosensitisation achieved by stable silencing of the PARP-1 gene appeared to be superior to that obtained by pharmacological inhibition,[12,31,35] suggesting that a prolonged and complete abrogation of PARP-1 activity during the treatment with TMZ is required for an optimal chemopotentiation. In addition, unlike pharmacological inhibitors, the lack of PARP-1 expression would hinder PARP-1 functions mediated by protein–protein interactions and this effect might also contribute to the increased chemosensitivity of SiP melanoma clones. These results suggest the importance of developing

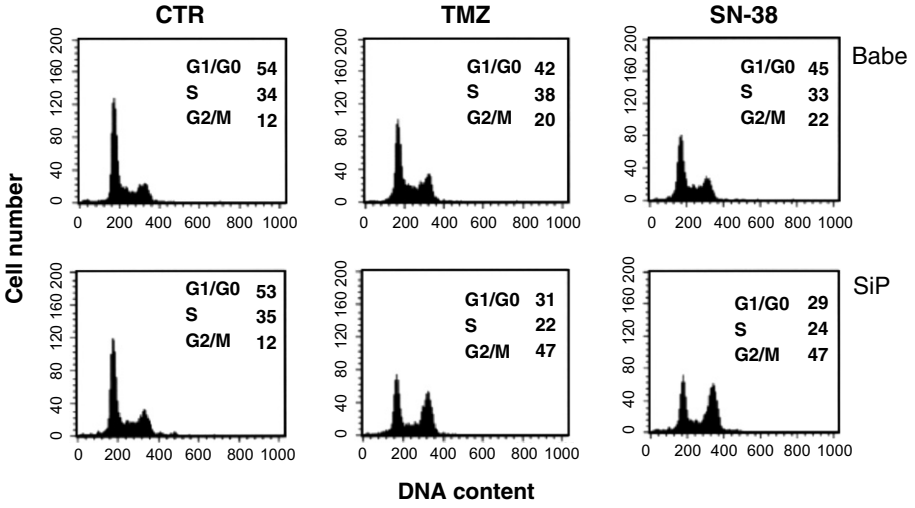

Fig. 8 – Cell cycle analysis of PARP-1-proficient or -deficient melanoma clones treated with TMZ or SN-38. Babe 3 or SiP G tumour cells were exposed to TMZ (250 μM) or SN-38 (5 nM) and the percentages of cells in the different phases of cell cycle were evaluated by flow cytometry 24 h after drug exposure. The profiles of cell cycle distribution and the percentage values are representative of one out of three independent experiments with similar results.

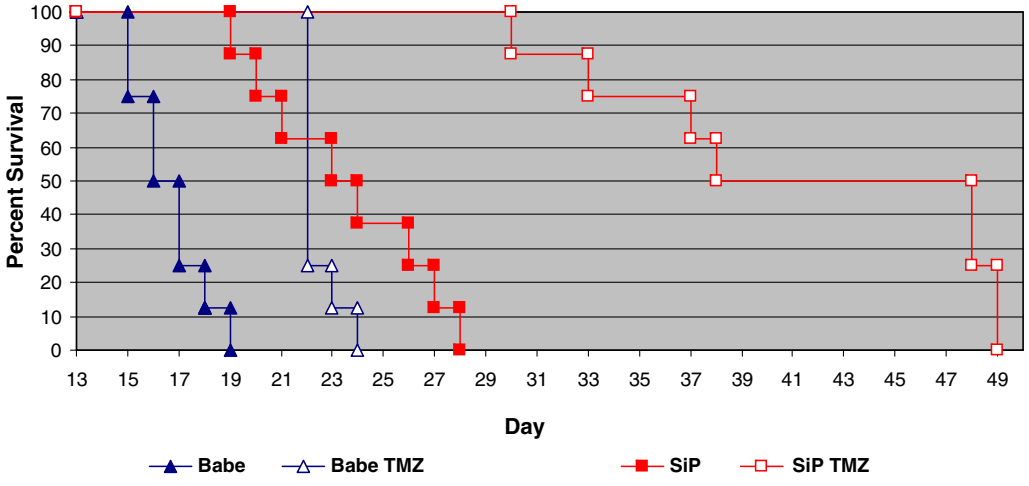

Fig. 9 – *In vivo* chemosensitivity to TMZ of PARP-1-proficient or -deficient melanoma clones in the intra-cerebral model. Mice (n = 8/group) were challenged i.c. with Babe 3 or SiP G melanoma clones and treated with TMZ as indicated in Section 2. Statistical analysis using ANOVA for multiple comparisons followed by a Bonferroni test indicated that all the differences between the groups were statistically significant ($P < 0.05$) with the exception of the differences between TMZ treated Babe 3 and untreated SiP G groups.

Table 3 – In vivo efficacy of TMZ in mice challenged i.c. with control or PARP-1 silenced melanoma clones

Clone	Treatment	MST (range)	ILS[a]
Babe 3	None	16.5 (15–19)	–
	TMZ (68 mg/kg)	22 (22–24)	33.3%
SiP G	None	23.5 (19–28)	–
	TMZ (68 mg/kg)	43 (30–49)	82%

a Increase in lifespan (ILS) of drug-treated mice was calculated comparing their MST with those of their controls injected with drug vehicle only.

inhibitors specific for PARP-1 that do not target the NAD^+-binding domain, which is conserved across the PARP family members. Novel inhibitors could be directed, for instance, against the recently characterised third zinc finger domain that mediates interdomain contacts required for DNA-dependent PARP-1 activation.[48]

In conclusion, these findings provide a novel implication for PARP-1 in cancer development and underscore the importance of targeting PARP-1 for cancer therapy. Further studies are required to elucidate the molecular mechanisms involved in the control of melanoma progression by PARP-1 and to unravel whether PARP-1 expression within the tumour might represent a prognostic factor.

Conflict of interest statement

None declared.

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