Extracellular acidosis triggers a senescence-like phenotype in human melanoma cells
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INTRODUCTION

Malignant melanoma is an aggressive tumor based on its high metastatic potential at early tumor stage (Maguire, Thomas, & Goldstein, 2015). Melanomas show high intratumoral heterogeneity with different subpopulations (Roesch et al., 2013). This phenotypic plasticity within the tumor is strongly influenced by the intratumoral microenvironment (Somasundaram, Villanueva, & Herlyn, 2012). Multiple intratumoral microenvironment signals, such as hypoxia, immune cells, and acidosis, influence the formation of phenotypically distinct malignant cell subpopulations that contribute to metastatic spread and therapeutic overcome. One important characteristic of the microenvironment of solid tumors is acidosis. While normal tissues have an extracellular pH (pHe) of 7.4–7.4, the pHe of the tumor microenvironment ranges between pH 5.5 and 7.0 (Tannock & Rotin, 1989; Wike-Hooley, Haveman, & Reinhold, 1984). Extracellular acidification is caused by deregulation of the cancer cell metabolism. Such metabolic alterations of cancer cells are, for example, upregulation of glycolysis (Warburg, 1956b, 1956a), the pentose phosphate pathway (PPP; Helmlinger, Sckell, Dellian, Forbes, & Jain, 2002), and glutaminolysis (Li, Zhang, Zhao, Ma, & Chen, 2018).
2016), which lead to the extensive production of acidic metabolites, especially lactate and carbon dioxide (CO$_2$). To maintain intracellular pH homeostasis, cancer cells increase the expression and activity of several transporters and channels involved in pH regulation such as the monocarboxylate transporters (MCTs; Wahl et al., 2002) and Na$^+$/H$^+$ exchangers (NHEs; Tannock & Rotin, 1989). Consequently, cancer cells acidify their tumor microenvironment and reciprocally react to this pH alteration with different mechanisms. Melanoma cells respond to acidosis with higher production and secretion of proteases (matrix metalloproteinase (MMP) 9 and 2, cathepsin B and L), which catalytically degrade proteins of the extracellular matrix (reviewed in (Bohme & Bosserhoff, 2016)). In addition, melanoma cells increase the release of proangiogenic factors (VEGF-A, IL-8; Rofstad, Mathiesen, Kindem, & Galappathi, 2006). This results in neoangiogenesis, anchorage-independent growth and genetic instability and contributes to malignant progression, higher invasion and metastasis (Martinez-Zaguilan et al., 1996; Schadendorf et al., 1993). Moreover, studies demonstrated that cancer cells show impaired cell proliferation and accumulating evidences point to cellular dormancy and stemness (Peppicelli et al., 2017; Rovida et al., 2014) associated with resistance to chemotherapy and radiation therapy (Peppicelli, Bianchini, & Calorini, 2014). Malignant melanoma cells upregulate the autophagic flux as a protective and adaptive response to survive acidic stress-induced cell death (Marino et al., 2012; Wojtkowiak et al., 2012). However, studies also showed that intracellular acidosis can activate the apoptotic cascade (Famulski, Macdonald, Paterson, & Sikora, 1999; Matsuyama, Llopis, Deveraux, Tsien, & Reed, 2000). Supporting this, studies in melanoma showed that acidosis results in high cell mortality during acclimation of the tumor to acidic condition, suggesting that acidosis selects for resistant phenotypes (Moellering et al., 2008).

The mechanisms by which an acidic tumor microenvironment drives phenotypic plasticity are poorly understood. We assume that melanoma cells are permanently located to an acidic tumor microenvironment in vivo. Therefore, we examined molecular effects of long-time (LT) acidosis on the development of phenotypic plasticity. Here, we show the direct impact of extracellular acidosis on the formation of a MITF$^{low}$/AXL$^{high}$ and senescence-like phenotype in human melanoma cells.

### Materials and Methods

#### 2.1 Cell culture

The melanoma cell lines used in this study are Mel Im, SKMel28 (both derived from melanoma metastases) and Mel Juso (derived from primary cutaneous melanoma). Untreated control was maintained in RPMI (1,640) medium with NaHCO$_3$, supplemented with penicillin (400 U/ml), streptomycin (50 μg/ml), and 10% fetal calf serum (FCS), and split at a 1:5 ratio every three to four days. Cells were cultured under a humidified atmosphere of 8% CO$_2$ at 37°C. We analyzed the influence of extracellular acidosis on melanoma cells by using an acidification system at pH 6.7. For acidosis treatment, RPMI medium (R7388) without NaHCO$_3$ was supplemented with 2-(N-morpholino) ethanesulfonic acid MES buffer (pK$_a$ value of 6.15 at 20°C, buffering range: pH 5.5–6.7). We decided to use MES buffer for long-time culturing of melanoma cells because MES is not metabolized by eukaryotic cells and shows minimal salt effects, minimal temperature-dependent change in pK$_a$, high chemical and enzymatic stability (Good et al., 1966), and weakly binding of ions (Ferreira, Pinto, Soares, & Soares, 2015). For monitoring of the pH in cell culture medium, we used RPMI (1,640) with the pH-indicator

| Gene        | Oligonucleotide sequences | T$_m$ (°C) | T$_s$ (°C) |
|-------------|---------------------------|------------|------------|
| CDKN1A/p21  | 5’-CGAGCCACCGGACCTCGAAGG-3’ 5’-CCTGCCCTCCCAACTCATCCC-3’ | 89         | 60–62      |
| PML         | 5’-CAGCAGTGTGCTCCGACGTGC-3’ 5’-GACAGCCCTGGGAGGTAGAT-3’ | 84         | 60         |
| ASNS        | 5’-CTGACAGCTACGGCAGAACAC-3’ 5’-CTAGATCAGGGACCGAGC-3’ | 89         | 60         |
| CCL2        | 5’-AGAGGGTGAGTACCAAACCCAGA-3’ 5’-CCTGGGGAATGAAGGTTCG-3’ | 84         | 60         |
| NLRP1       | 5’-CTCCCCTGCTCTTCTTACTCC-3’ 5’-CTGAGGTGCTTATTGAC-3’ | 86         | 60         |
| ACTB        | 5’-CTACCTGCCTGAGCTTGAGGC-3’ 5’-GATGGAGCCGGCTCCACCG-3’ | 86         | 60–68      |

**TABLE 1** Oligonucleotide sequences and qRT-PCR conditions
phenol red to observe daily pH alterations and we measured the pH₄ using pH meter (LAQUAtwin HORIBA). For investigating LT acidosis-treated melanoma cell after transfer into pH 7.4 (LT to pH 7.4), MES cell culture medium was replaced by RPMI (1,640) medium (pH 7.4) and experimental analyses were performed after 96 hr.

2.2 | RNA isolation, reverse transcription, and analysis of mRNA expression by quantitative PCR

Total cellular RNA was isolated from Mel Im, Mel Juso, and SKMel28 using the RNeasy kit (E.Z.N.A. Omega Bio-Tek). Complementary DNA was generated by a reverse transcriptase reaction (500 ng of total RNA) using the SuperScript II Reverse Transcriptase Kit (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed on a Lightcycler480 device (Roche Mannheim) as already described (see Table 1 for primer sequences) (Schummer, Kuphal, Vardimon, Bosserhoff, & Kappelmman, 2016). The β-actin was used as a reference gene for normalization. The specificity of the PCR amplification reaction was evaluated by analyzing the PCR products on 1.5% agarose gels.

2.3 | Protein analysis in vitro (Western blotting)

Cells were lysed in 150 µl of RIPA buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% N-P40, 0.1% SDS, 0.5% sodium deoxycholate and protease inhibitor) and incubated for 15 min at 4°C. Insoluble fragments were removed by centrifugation at 18,000 g for 10 min, and the supernatant (lysate) was immediately shock frozen and stored at −20°C. Cell lysates (20 µg protein per lane) were loaded and separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, and the proteins were subsequently blotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were then blocked in 5% milk/Tris-buffered saline/0.1% Tween-20 for 1 hr and incubated with primary antibodies: eIF2α (1:1,000, ab5369); eIF2α (phospho) (1:500 (phospho) (1:500 ab32157); AXL (1:100, sc166269); MITF (1:100 sc515925); ATF4 (1:100, sc390063); p21 (1:1,000, ab109199); and β-actin (1:5,000, A5441 SIGMA-ALDRICH) in 5% milk/Tris-buffered saline/0.1% Tween-20 overnight at 4°C. A dilution of 1:3,000 secondary antibody anti-rabbit-horseradish or anti-mouse-horseradish peroxidase in TBS-T (Cell Signaling Leiden) was used and incubated for 1 hr at room temperature. Staining was performed using the ECL Plus Western Blotting Detection Kit (GE Healthcare). To determine the relative protein regulation between LT acidosis treatment (LT pH 6.7) and control (Ctr. pH 7.4), densitometric analysis of proteins intensity was normalized to control, set as 1.

2.4 | Cell cycle analysis

For cell cycle analysis by flow cytometry, 200,000 of control cells and LT acidosis-treated cells were seeded into six-well plates and cultured again with RPMI (1,640) pH 6.7 (LT pH 6.7) or pH 7.4 for control (Ctr. pH 7.4) for 48 hr. Subsequently, the cells were fixed in ice-cold 70% methanol for 1 hr, washed twice with phosphate-buffered saline (PBS) and treated with RNase A for 20 min at 37°C, stained with propidium iodide, and analyzed using a FACS CALIBUR cytometer (BD Biosciences). Flow cytometry data were analyzed using FlowJo Software (LLC).

2.5 | Apoptosis analysis

For apoptosis analysis, 200,000 of control cells and LT acidosis-treated cells were seeded into six-well plates and after 24 hr treated with 100 µM etoposide for 16 hr. Apoptotic cells were investigated by flow cytometry using the Apoptotic/Necrotic Cells Detection Kit (Promokine) according to the manufacturer’s instructions. The flow cytometry analysis was performed in a FACS CALIBUR cytometer (Becton Dickinson). Flow cytometry data were analyzed using CellQuest Pro Software (BD Bioscience).

2.6 | Senescence-associated β-galactosidase staining

For determination of senescent melanoma cells, 100,000 cells were seeded into six-well plates. Cells were fixed and stained by using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology) according to the manufacturer’s instructions. Stained cells were visualized using the inverted microscope IX83 (Olympus Life Science) under bright field. Microscope images were analyzed using CellSens Dimension Software 1.12 (OLYMPUS CORPORATION). To determine the ratio of senescent cells, stained and unstained cells were counted by 10× magnification. The percentage of senescent cells (blue) to total cell number in the field of view were calculated.

2.7 | Immunofluorescence staining

After LT acidosis treatment, 20,000 cells/well were seeded into eight-well chamber slides for 1 day, washed with PBS, and fixed with 4% paraformaldehyde for 15 min. For permeabilization of the cell membrane, the cells were incubated for 5 min with 0.1% Triton-X-100 (Sigma-Aldrich), washed again, and covered with blocking solution 1% bovine serum albumin/phosphate-buffered saline (BSA/PBS) for 1 hr. Subsequently, cells were incubated with anti-PML antibody (1:200; Santa Cruz Biotechnology) overnight at 4°C. After washing, cells on coverslips were incubated with the secondary antibody (1:200, goat anti-mouse IgG secondary antibody, Alexa Fluor 488) for 1 hr at room temperature. After washing with PBS, cells were stained with DAPI for 30 min and mounted with Aqua-Poly/Mount medium (Polysciences, Inc). Images were collected using the inverted microscope IX83 (Olympus Life Science). Microscope images were analyzed using CellSens Dimension Software 1.12 (Olympus Cooperation).

2.8 | Statistical analysis

All experiments were performed on at least three independent occasions. Results were expressed as the mean ± SD (range). Results of densitometric analysis of protein intensity were expressed as
mean ± SEM (range). Comparisons between groups were made using unpaired t test. A p-value of ≤.05 was considered statistically significant. Statistical significance of p-values was represented. *p ≤ .05, **p ≤ .01, ***p ≤ .001, ****p ≤ .0001. All calculations were performed using the GraphPad Prism software package (GraphPad Software Inc).

3 | RESULTS

3.1 | Extracellular acidosis leads to reduced proliferation and G1/G0 cell cycle arrest

As we assume that melanoma cells are exposed permanently to an acidic microenvironment in vivo, we focused to examine effects of extracellular long-time (LT) acidosis. Here, we treated three melanoma cell lines (Mel Im, SKMel28, and Mel Juso) with RPMI medium acidified with MES to pH 6.7 for more than 2 months. Strikingly, after LT acidosis, the melanoma cell lines developed morphological alterations, such as enlarged cell bodies and partially branched cellular extensions (Figure 1a, arrows). All investigated melanoma cell lines Mel Im, Mel Juso, and SKMel28 showed a strong reduction of proliferation under LT treatment, indicating a slow-cycling phenotype (Figure 1b, LT pH 6.7). We detected no induction of cellular apoptosis during LT acidosis (Figure S1A,B). To investigate whether the inhibition of proliferation is based on cell cycle arrest, we analyzed the cell cycle by flow cytometry using propidium iodide (PI) staining. An

**FIGURE 1** Long-time acidosis mediates morphological alterations, reduction of proliferative activity, and G1/G0 cell cycle arrest. (a) Morphological alterations after LT acidosis treatment of Mel Im, Mel Juso, and SKMel28 melanoma cells in RPMI medium acidified to pH 6.7 (LT pH 6.7) compared to untreated control cells cultured in RPMI medium with pH 7.4 (Ctr. pH 7.4). Representative images are shown. Arrows indicate melanoma cells with enlarged cell bodies in Mel Im, Mel Juso, and SKMel28. (b) Proliferation curves of Ctr. pH 7.4 and LT pH 6.7 of the indicated melanoma cell lines were analyzed by cell counting after 24 hr, 48 hr, 72 hr and 96 hr. (c) Cell cycle analyses by flow cytometry using PI staining were performed of the indicated melanoma cell lines. (d) Percentage of cells in cell cycle phases of the indicated melanoma cell lines are shown. (*p < .05, **p < .01)
increase of LT pH 6.7-treated Mel Im, Mel Juso, and SKMel28 cells in G1/G0 phase and a significant reduction of cells in S-phase compared to control cells demonstrated an induction of G1/G0 cell cycle arrest (Figure 1c, d).

These data indicated that acidosis is an environmental stress factor to melanoma cells resulting in reduced cell proliferation and G1/G0 cell cycle arrest. The interesting phenotype was in focus of our next experiments.

FIGURE 2 Long-time acidosis induces senescence-associated-(SA)-β-galactosidase activity. (a) Light microscopic examination of SA-β-galactosidase staining in Mel Im, Mel Juso, and SKMel28 cell lines after LT acidosis treatment (LT pH 6.7) compared to untreated control cells (Ctr. pH 7.4). For positive control (+) Ctr., melanoma cells were treated with etoposide. (b) Quantification of SA-β-galactosidase staining of LT acidosis-treated (LT pH 6.7) and untreated control cells (Ctr. pH 7.4) counted by 10× magnification. The percentage of senescent cells (blue) to total cell number were calculated. (*: p < .05, **: p < .01; ****: p < .0001)

FIGURE 3 Long-time acidosis induces senescence characteristics in human melanoma cells. (a) PML immunofluorescence staining (green) of Mel Im, Mel Juso, and SKMel28 melanoma cell lines after LT acidosis treatment (LT pH 6.7) and control (Ctr. pH 7.4). Merged panels are overlays of PML (green) and DAPI (blue) staining. (b) The PML-NB fluorescence intensity was determined using the microscope software. (c) and (d) Quantitative analysis of mRNA expression of indicated cell lines after LT acidosis treatment (LT pH 6.7) compared to control cells (Ctr. pH 7.4). Relative mRNA expression of PML (c) and p21 (d) was normalized to ACTB. (e) Western blot of p21 and β-actin protein production after LT acidosis (LT) compared to untreated control cells (Ctr.). The β-actin protein was used as loading control. (f) Quantification of multinucleated cells of indicated melanoma cells lines. The percentage of multinucleated cells to total cell number in the field of view were calculated. (*: p < .05, **: p < .01, ****: p < .001, ****: p < .0001)
3.2 | LT acidosis induces a senescence-like phenotype in melanoma

To investigate whether cell cycle arrest of Mel Im, Mel Juso, and SKMel28 melanoma cells seen after LT acidosis occurs due to induction of senescence, we performed senescence-associated (SA)-β-galactosidase staining. Interestingly, the SA-β-galactosidase staining was strongly induced after LT acidosis (LT pH 6.7) in Mel Im, Mel Juso, and SKMel28 melanoma cell lines compared to control cells (Ctr. pH 7.4) (Figure 2a, b), suggesting a strong formation of a senescence-like phenotype under acidosis. Since it is well known that etoposide induces premature senescence (Petrova, Velichko, Razin, & Kantidze, 2016), etoposide was used as positive control (+ Ctr.).

Senescence is experimentally also defined by the promyelocytic leukemia protein (PML). PML is a core component of nuclear substructures termed PML nuclear bodies (PML-NB) and is functionally implicated in cellular senescence in melanoma (Bernardi & Pandolfi, 2007; Pearson et al., 2000). To confirm the senescent phenotype, we additionally performed measurements for PML-NB in Mel Im, Mel Juso, and SKMel28 via immunofluorescence staining. LT acidosis (LT pH 6.7)-cultured melanoma cells showed a strong increase of PML-NB size compared to control cells (Ctr. pH 7.4) (Figure 3a) as well as significant increase of PML-NB fluorescence intensity (Figure 3b). We further determined a higher PML expression at mRNA level after LT acidosis in all investigated melanoma cell lines (Figure 3c). Additionally, the cyclin-dependent kinase inhibitor 1A (p21/CDKN1A), a regulator of cell cycle progression at G1-cell cycle phase, was upregulated after LT acidosis at mRNA level in all investigated melanoma cell lines (Figure 3d) and at protein level in Mel Im and Mel Juso melanoma cell lines (Figure 3e). Due to a p53 mutation, SKMel28 cell line showed weak p21 protein expression, which was reported previously (Daveri, Valacchi, Romagnoli, Maellaro, & Maioli, 2015; Haapajarvi, Pitkanen, & Laiho, 1999).

Furthermore, Mel Im, Mel Juso, and SKMel28 cells developed further characteristics of cellular senescence under LT acidosis in comparison with control cells, including enlarged cell bodies (Figure 1a, arrows) and an increase of multinucleated cells (Figure 3f). Taken together, these results suggest that LT acidosis induced a senescence-like phenotype in human melanoma cells.

3.3 | LT acidosis induces the development of a MITFlow/AXLhigh phenotype in human melanoma

It has previously been shown that acidosis induces endoplasmic reticulum (ER) stress and unfolded protein response (UPR) in several cell types, which results in an integrated stress response (ISR) (John et al., 2012; Tang et al., 2012; Visioli et al., 2014). This ISR leads to phosphorylation of eIF2α, resulting in the inhibition of eIF2β followed by an inhibition of protein translation and in the activation of the transcription factor ATF4 (Zhang & Kaufman, 2008). Here, we demonstrate that LT acidosis induces the phosphorylation of eIF2α at serine 51 (eIF2α-P) (Figure 4a, b) and the activation of ATF4 (Figure 4a, c). Besides the ATF4 upregulation at protein level, we supported the transcriptional activity of ATF4 by analysis of ATF4 target gene expression of CCL2 (Liu, Chen, Xi, Zhu, & Gao, 2017), ASNS (Falletta et al., 2017), and NLRP1 (D’Osualdo et al., 2015) using qRT-PCR. Our data revealed an upregulation of all investigated ATF4 target genes after LT acidosis in all investigated melanoma cell lines (Figure 4f). The inhibition of eIF2β and the activation of ATF4 indicate that extracellular acidosis stimulates ISR in human melanoma cell lines.

Previous studies on melanoma showed that ATF4 transcriptionally represses MITF and the reduced MITF level leads to the activation of AXL, resulting in an MITFlow/AXLhigh drug-resistant melanoma phenotype (Falletta et al., 2017; Müller et al., 2014). Due to the observed ATF4 activation after LT acidosis, we determined the MITF and AXL protein expression under acidic conditions in melanoma. The Mel Juso cell line showed a significant AXL induction, the SKMel28 cells a weaker induction of AXL expression. AXL protein was not detectable in Mel Im cells (Figure 4a, d). Besides the regulation of AXL expression, we also found a strong repression of MITF in all cell lines under acidosis (Figure 4a, e) demonstrating that the MITFlow/AXLhigh phenotype is induced by acidic conditions. Taken together, these data indicated an extracellular acidosis-mediated formation of a MITFlow/AXLhigh cellular phenotype and a translational reprogramming via eIF2α.

3.4 | LT acidosis leads to enhanced resistance of melanoma cells against etoposide

To investigate whether the acidosis-induced senescence-like phenotype protects melanoma cells against cytotoxic agents and mediates resistance, we treated LT acidosis cells with etoposide and determined cell apoptosis using Annexin V/PI staining flow cytometry. Etoposide is a chemotherapeutic topoisomerase II inhibitor (TOP2) which is used as anticancer drug for the treatment of numerous types of cancer (Nitiss, 2009) and was also used in recent studies (Ruzzolini et al., 2017). We determined that untreated control cells (Ctr. pH 7.4) showed a distinct increase of apoptotic cells after etoposide exposure, while LT acidosis-treated cells (LT pH 6.7) showed a smaller fraction of apoptotic cells as well as an enhanced population of living cells (Figure 4g, h), indicating resistance against etoposide due to LT acidosis.

3.5 | Transfer of LT acidosis-treated melanoma cells into physiological pH 7.4 results in re-proliferation

Due to the observed G1/G0 cell cycle arrest, we were interested to investigate whether the cell arrest and the senescence-like phenotype is irreversible. We transferred the cells into physiological pH 7.4 (LT to pH 7.4) after LT acidosis treatment. Here, we revealed a significant decrease of SA-β-galactosidase activity of these cells (Figure 5a, b, LT to pH 7.4) in comparison with the LT acidosis-treated cells (LT pH 6.7) (Figure 5b) and no induction of apoptosis (Figure S2A, B). Surprisingly, after transfer into pH 7.4, all three melanoma
cell lines showed a re-induction of proliferation (Figure 5c, LT to pH 7.4). Moreover, SKMel28 and Mel Juso cell lines showed an even higher proliferative activity than control cells (Figure 5c). These data suggest that the cells reduce the senescence-like phenotype, can re-enter into an active cell cycle after acidosis-induced G1/G0 arrest, and can increase their proliferative activity compared to melanoma cells without acidosis treatment. Further, protein regulation of ATF4, AXL, and MITF was analyzed after transfer into pH 7.4 (Figure 5d LT-7.4, E LT to pH 7.4) presenting a reduction of the ATF4 and AXL protein and a slight upregulation of the MITF protein in the cell line Mel Juso compared to the protein level of untreated control cells. Interestingly, the cell line Mel Im showed an upregulation of AXL and the cell line SKMel28 exhibited a maintained MITF reduction after switch into neutral pH 7.4 (Figure 5d, e) demonstrating that the molecular phenotype developed under acidic conditions is partially preserved.

4 Discussion

Malignant melanoma is an aggressive cancer with a highly heterogeneous cancer cell population. The heterogeneous phenotypic plasticity within the tumor is influenced by the intratumoral microenvironment (Somasundaram et al., 2012), with acidosis as one important intratumoral microenvironmental cue. Our results demonstrate that LT acidosis results in inhibited cell proliferation and G1 cell cycle arrest in melanoma cells, confirmed by the increased expression of the cell cycle inhibitor p21.
In the current study, we revealed for the first time a significant induction of a senescence-like phenotype by LT acidosis indicated by numerous senescence characteristics, such as induction of SA-β-galactosidase activity, PML-NB formation, enlarged cell body size as well as cellular multinucleation.

While some cells showed senescence characteristics, other cells had an unchanged cell morphology displaying the heterogeneity in the melanoma cell population. Interestingly, our results show that melanoma cells actively proliferate after re-culturing in physiological pH 7.4 following LT acidosis. Thus, we suggest that melanoma cells have the ability to overcome the arrest and can subsequently show an even stronger proliferation than without pH changes. These observations are supported by the study of Moellering et al. which describes higher invasion and higher motility of acidosis-treated cells following...
re-acclimation of these cells to physiological pH (Moellering et al., 2008).

Actually, the state that cellular senescence is an irreversible and permanent cell cycle arrest is reconsidered. Melanoma studies indicated that senescence might be a key feature of tumor progression and tumor cells may arise from senescent tissue (Braig et al., 2005; Collado et al., 2005; Michaloglou et al., 2005). More recent investigations showed that melanoma cells use the senescence state as trigger for tumor transformation and generate post-senescent tumor-initiating cells with stem cell-like properties (Haferkamp, Becker, Scurr, Kefford, & Rizos, 2008; Leikam et al., 2015). Moreover, during chemotherapy, melanoma cells become senescent and are able to remodel the composition of their secretome toward pro-inflammation. Thus, these senescent cells release factors and send signals to naïve neighboring melanoma cells and induce the development of a melanoma-initiating cell phenotype that could favor chemotherapy resistance (Krayem et al., 2018; Ohanna et al., 2013). Based on our data, we suggest that extracellular acidosis triggers via eIF2α phosphorylation and ATF4 activation the development of MITFlow/AXLhigh phenotype, that is described to be correlated with therapy resistance in melanoma (Figure 6; Müller et al., 2014).

Our data also demonstrate that acidosis-adapted cells show an increased resistance against the anticancer agent etoposide, which is in accordance with previous studies (Ruzzolini et al., 2017). Etoposide is a topoisomerase II inhibitor and targets cells with an active cell cycle, whereas slow-cycling cells do not respond to such therapeutics. Therefore, chemotherapeutic agents, which target rapidly growing tumor cells, are unable to affect the acidosis-mediated slow-proliferating cells resulting in therapeutic overcome and resistance. Consequently, these slow-cycling, drug-resistant cells residually remain in the tissue during cancer therapy and might be responsible for tumor recurrence. Based on our data, tumor relapse is supported by a sometimes even enhanced proliferative potential of the melanoma cells after transfer of LT acidosis cell to pH 7.4. Further, the upregulation of AXL in the cell line Mel Im and the preserved MITFlow phenotype in the cell line SKMel28 after transfer of acidosis-treated melanoma cells into neutral pH 7.4 indicate that the acidosis-induced molecular phenotype is partially preserved at least for some time after acidosis exposure.

Taken together, our study reveals that extracellular acidosis induces a senescence-like phenotype with MITFlow/AXLhigh signature and cellular translation reprogramming. These alterations might enable the survival during chemotherapeutic treatment and might be responsible for tumor recurrence after therapeutic medication, thereby proving an important role of acidosis in the tumor microenvironment on tumor progression.

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CONFLICT OF INTEREST

The authors declare that the manuscript presents original research, has not been previously published, and is not being considered for publication elsewhere. The authors declare that there are no conflicts of interest.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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