Life Sciences 230 (2019) 121–131

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Theophylline induces differentiation and modulates cytoskeleton dynamics and cytokines secretion in human melanoma-initiating cells

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ARTICLE INFO

Keywords:
Theophylline
Melanoma
Cancer stem cells
Differentiation
Cytokines

ABSTRACT

Aims: Cutaneous melanoma is the most aggressive skin cancer, derived from neoplastic transformation of melanocytes. Since several evidences highlighted the importance of a hierarchical model of differentiation among cancer cells, closely related to resistance mechanisms and tumor relapse, we investigated the effects of theophylline (Theo), a methylxanthine commonly used in treatment of respiratory diseases, on melanoma cells with different degree of differentiation, including patient-derived melanoma-initiating cells.

Materials and methods: The antiproliferative and antimetastatic effects of Theo was demonstrated by cell counting, adhesion and migration assays on A375 and SK-MEL-30 cells. Further, Theo ability to reduce cell growth was highly significant in A375-derived spheroids and in two patient-derived melanoma-initiating cells (MICs). In order to identify pathways potentially involved in the antineoplastic properties of Theo, a comparative mass spectrometry proteomic analysis was used. Then, melanin content, tyrosinase and tissue transglutaminase activities as differentiation markers and actin re-organization through confocal microscopy were evaluated. Furthermore, a secretome profile of MICs after Theo treatments was performed by multiplex immunoassay.

Key findings: Obtained results demonstrate inhibitory effects of Theo on melanoma cell proliferation and migration, mainly in MICs, together with the induction of differentiation parameters. Moreover, our data indicate that the known anti-melanoma effect of Theo is due also to its ability to interfere with cytoskeleton dynamics and to induce the secretion of inflammatory molecules involved in recruitment of immunosuppressive cells in tumor microenvironment.

Significance: Data strongly suggest that Theo supplement, either as drug or as dietary supply, may represent a potent additional weapon against melanoma.

1. Introduction

Despite significant improvements in the oncologic therapeutic strategies, cancer continues to be a major health problem [1]. The most used chemotherapeutic approaches often exhibit limited therapeutic efficacy and can have important side effects. Differentiation therapies are defined as those that allow cancer cells to revert to a more benign phenotype [2,3]. This type of therapeutic approach has been particularly successful for acute promyelocytic leukemia, with the use of all-trans retinoic acid [4]. On the other hand, cancer stem-like cells (CSCs) play a key role in cancers of different origins, as they have been isolated from different types of solid tumors such as melanoma, breast, brain, prostate cancer and others [5,6]. Since CSCs are often responsible for cancer initiation, maintenance, relapse and resistance to therapy, the possibility to target these cells may affect tumor progression and improve patient prognosis [7].

Melanoma is one of the most aggressive skin cancer and its incidence is on the rise in different countries [8]. Similarly to CSCs

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https://doi.org/10.1016/j.lfs.2019.05.050
Received 8 March 2019; Received in revised form 17 May 2019; Accepted 20 May 2019
Available online 21 May 2019
0024-3205/

isolated from various tumors, malignant melanoma-initiating cells (MICs) can be expanded *in vitro* as tumor spheres (melanospheres) and reproduce the original tumor when transplanted in immunodeficient mice [9]. Several studies indicated that the potential for tumor initiation is not restricted to a rare tumor cell sub-population, but may be a common feature in melanoma cells. Moreover, recent data support a model of dynamic stemness, where the stem cell state may be shifted to a differentiated state and *vice-versa*, depending on tumor context or as a consequence of an environmental stimuli or injury [10]. These evidences indicate that melanoma doesn't follow a hierarchical model, but rather a dynamic one also named tumor microenvironment-driven model [11]. Therefore, the induction of differentiation in MICs is an optimal approach to counteract the re-emergence of stem cell features following the short term therapeutic efficacy, improving long term cancer therapy response.

Several lines of evidence suggest that pro-differentiative dietary natural compounds might be extremely useful to prevent or limit cancer development [12,13]. Theophylline (1,3-dimethyl-xanthine; Theo) is a bioactive compound mostly present in *Camellia sinensis* (L.) Kuntze leaves. Theo is a methylxanthine (MX) drug used in therapy for its potent anti-inflammatory activity [14] and it has been also used to treat patients with airways's diseases because of its well-established action as bronchodilator [15]. Methylxanthines have been shown to inhibit growth of several types of cancer cells [16,17], including melanoma ones [18].

The molecular mechanisms of Theo antiinflammatory and anticancer effects are not completely understood, although adenosine receptor (AR) antagonism, activity of phosphodiesterase (PDE) inhibitor, inhibition of nuclear factor kappa B (NF-κB) and inhibition of phosphoinositide 3-kinase (PI3K) have been suggested as its main mechanisms of action [14]. Furthermore, the anti-inflammatory action can be an effect of interaction between Theo and Nuclear Histone Deacetylase (HDAC), involved in the inhibition of transcription of many cytokines genes in pro-inflammatory cells.

The purpose of this study was to elucidate the effect of Theo in melanoma cell differentiation, using a MICs model. In particular, we investigated pathways involved in tumor growth and metastatic behavior to better understand which extent the induction of CSCs differentiation may be relevant to counteract cancer. Furthermore, the recent broad interest in natural compounds and the clinical relevance of drugs repurposing prompted us to deepen the knowledge of the antineoplastic effect of Theo and to investigate its potential immunomodulatory role in tumor microenviroment.

2. Materials and methods

2.1. Materials

Roswell Park Memorial Institute medium (RPMI-1640), Dulbecco's Modified Eagle Medium (D-MEM), foetal calf serum (FCS) phosphate buffer saline (PBS), glutamine, penicillin (10,000 UI/ml) and streptomycin (10,000 µg/ml) were obtained from Gibco-Invitrogen, Carlsbad, *CA*. Theophylline (Theo), bovine serum albumin (BSA), ethylenediaminotetraacetic acid (EDTA), polyethylene glycol hexadecyl ether (Brij35), synthetic melanin, L-dihydroxyphenylalanine (L-DOPA) and all other reagents were from Sigma-Aldrich (St. Louis, MO, USA). Eight µm-sized porous filters were purchased from Millipore S.p.A. (Milano, Italy). Immunopure 5-biotinamidopentylamine (BPA) was from Pierce (Rockford, USA). Matrigel® (MG) was from Becton Dickinson Bioscience (Franklin Lakes, NJ, USA).

2.2. Cell culture

The human metastatic melanoma cell lines SK-MEL-30, SK-MEL-28 and A375 were purchased from the American Type Colture Collection (ATCC, Manassas, VA); human melanoma WM793 cell lines were kindly supplied by Dr. Enrico Proietti (Istituto Superiore di Sanità - ISS, Rome). All cell lines were propagated under standard culture conditions. SK-MEL-30 and SK-MEL-28 cell lines were cultured in RPMI-1640 medium while A375 and WM793 were cultured in D-MEM medium. Culture media contained 10% FCS, 0.05% L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml) and maintained at 37 °C with 5% CO_2 in a humidified atmosphere. The two patient-derived human melanoma-initiating cell lines (MICs), namely Mel1 and Mel3 were isolated as melanospheres and cultured in suitable medium as described [9]. MICs *in vitro* differentiation was obtained by culturing cells in Melanocyte Growth Medium (MGM4, Lonza, East Rutherford, NJ, USA). The A375 spheroids (A375-Spher) were also obtained and expanded as described [19]. For proliferation studies, Theo was dissolved in PBS without Ca^{++}/Mg^{++}. Melanoma cells were seeded and grown in 12-well tissue culture plates in culture medium supplemented as reported above, and treated with Theo (1 and 2 mM) for 24, 48 and 72 h. Melanospheres, A375-Spher, Mel1 and Mel3 were seeded in 12-well non-tissue culture plates in complete culture medium and treated with 1 and 2 mM Theo. Cells were harvested and counted with a Neubauer modified chamber, after Trypan Blue staining for cytotoxicity evaluation.

2.3. Adhesion and migration assays

The adhesion assay was performed on 24-wells plates coated with MG (50 µg/well). After MG polymerization, 80 µl of cell suspension (1×10^6 cells/ml) of control and Theo-treated SK-MEL-30 and A375 cells were added to each well. The plates were incubated for 1 h at 37 °C. The adherent cells were detached with trypsin/EDTA and counted. Attachment to Matrigel (at 1 h) was expressed as percentage of cells adhered, and the percentage of the control was taken as 100%.

Migration assays were performed in a modified Boyden chamber. A total of 2×10^5 SK-MEL-30, A375, Mel1 and Mel3 cells were added to the upper compartment in serum-free medium; FCS or MICs complete medium, for cell lines and MICs respectively, were added to the lower chamber. Polyvinylpyrrolidone-free polycarbonate filters (8 µm pore size, 10 mm diameter) were placed between the two compartments. Chambers were incubated in a humidified 5% CO_2 atmosphere for 8 h at 37 °C. Non migrated cells were carefully cleared away from the upper surface of the filter. Migrated cells were counted under light microscopy after fixing by 100% methanol, staining with Giemsa solution and mounting filters on glass slides. Migration was expressed as percentage of migrating cells, and the percentage of the control was fixed as 100%.

2.4. Mass spectrometry analysis

Postnuclear cell lysates were prepared as described with few modifications [20]. Briefly, the cell extracts from Mel1 and Mel3, treated or not with Theo for 72 h, were analyzed on 4–15% Mini-PROTEANs TGX™ pre-cast polyacrylamide gel (Bio-Rad). In order to perform protein identification, mass spectrometry analysis was performed as described [21] with few modifications. In particular, digested peptides were analyzed by nanoflow reversed-phase liquid chromatography tandem mass spectrometry (RP-LC-MS/MS) using HPLC Ultimate 3000 instrument (DIONEX, Sunnyvale, CA) connected on line with a linear Ion Trap (LTQ, ThermoElectron, San Jose, CA). Peptides were desalted by means of a trap column (Acclaim PepMap 100 C18, LC Packings, DIONEX) and then discriminated in a reverse phase column, a 10 cm long fused silica capillary (Silica Tips FS 360–75- 8, New Objective, Woburn, MA), slurry-packed in-house with 5 mm, 200 Å pore size C18 resin (Michrom BioResources, CA). Peptides were eluted using a linear gradient from 96% A (H_2O with 5% acetonitrile and 0.1% formic acid) to 50% B (acetonitrile with 5% H_2O and 0.1% formic acid) in 72 min, at 300 nl/min flow rate. Analyses were performed in positive ion mode and the HV Potential was set up around 1.7–1.8 kV. Full MS spectra ranging from m/z 400 to 2000 Da were acquired in the LTQ mass spectrometer operating in a data-dependent mode in which each full

MS scan was followed by five MS/MS scans where the five most abundant molecular ions were dynamically selected and fragmented by collision-induced dissociation (CID) using a 35% normalized collision energy. Target ions already fragmented were dynamically excluded for 30 s. Then, tandem mass spectra were matched against UNIPROT database and through SEQUEST algorithm incorporated in Proteome Discoverer software (version 1.4, Thermo Fisher) using fully tryptic cleavage constraints with 2 miss-cleavage allowed, static carbamidomethylation on cysteine residues and methionine oxidation as variable modification. Data were searched with 2 Da and 0.8 Da tolerance respectively for precursor and fragment ions. A peptide was considered legitimately identified when it achieved cross correlation scores of 1.8 for [M + H]1+, 2.5 for [M + 2H]2+, 3 for [M + 3H]3+, and a peptide probability cut-off for randomized identification of $p < 0.001$. Furthermore, the search filters included a minimum of two peptide identification per protein and a FDR < 0.01 for peptide identification and protein identification.

2.5. Bioinformatic analysis

Proteins identified by proteomic analysis were further analyzed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) software (http://david.abcc.ncifcrf.gov/). DAVID provides a comprehensive set of functional annotation tools for investigators to understand biological meaning behind a large list of proteins. DAVID functional annotation cluster analyses were performed on the list of specific proteins commonly expressed by Mel1 and Mel3 cells treated or not with 2 mM Theo for 72 h. Only those terms with a $p < 0.005$ were selected for DAVID analysis. The GeneOntology term of cellular component and biological processes (GOTERM_CC_FAT and GOTERM_BP_DIRECT) sections in DAVID were used.

2.6. Measurement of spheroids growth

Spheroids formation assay was carried out as described [19]. Briefly, A375-Spher cells were plated in ultralow attachment plates at a density of 1000 cells/ml and exposed to Theo for 7 days. MICs were plated in ultralow attachment plates at a density of 1000 cells/ml and exposed to Theo. Spheroids were photographed (Olympus BH-2 LH50A) and spheroids number and size (percentage area covered by spheroids) were quantified with Image Processing and Analysis in Java (Image J®, v. 1.5d) software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Data are expressed as % of the control (100%) [22].

2.7. Evaluation of melanin content

For melanin content evaluation, melanotic Mel1 and Mel3 cells were cultured in the presence of Theo for 72 h, washed twice in PBS, collected by centrifugation and counted. Melanin was extracted from cells with 1 ml of 1 M NaOH at 75 °C for 1 h. Melanin content was determined by absorption at 475 nm, using the standard curve obtained from a solution of synthetic melanin (0–100 μg/ml) and a spectrophotometer (Uvikon 860 Instrument, Kontron, Zurich, CH) with polystyrene cuvettes.

2.8. DOPA-staining (tyrosinase zymography) and DOPAchrome tautomerase assay

DOPA-staining assay to quantify tyrosinase (TYR) activity was performed as described [23]. Briefly, Mel1 and Mel3 cells were lysed in 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, and resolved by 8% SDS polyacrylamide gel electrophoresis under non-denaturing conditions. At the end, gels containing tyrosinase were transferred into 200 ml of a staining solution containing 5 mM L-DOPA, and incubated in the dark for 3 h at 37 °C. Tyrosinase activity was visualized in the gels as dark melanin-containing bands. Densitometric quantification for each of the gelatinase bands of the digitalized images were measured by FluorChem System (Cell Biosciences, CA). DOPAchrome tautomerase (DCT) activity assay was performed according to a published method [24].

2.9. Cellular transamidation activity assay

In vitro transamidating activity of transglutaminase was performed as described [25] and data have been presented as percentage of transglutaminase activity (control 100%).

2.10. Gelatin zymography

Secretion and activities of matrix metalloproteinase-2 (MMP-2) from Theo treated cells was analyzed by gelatin zymography according to a published method [26] with a few modifications. Briefly, MMP-2 activity was determined in MICs Mel1 and Mel3 conditioned medium after 2 mM Theo for 72 h. Samples were loaded and separated under non-reducing conditions into 7% polyacrylamide gel polymerized with 1% gelatin. At the end of the electrophoretic separation, the gel was washed in 2.5% Triton X-100 for 30 min, incubated overnight at 37 °C in the substrate buffer (0.5 M Tris–HCl, pH 7.5, 50 mM $CaCl_2$, 2 M NaCl and 0.2% Brij35), stained in Coomassie Blue (G-250) and destained. Densitometric quantification for each of the gelatinase bands of the digitalized images was achieved by Image J® (v1.5d).

2.11. Real-time PCR

Total RNA from Mel1 and Mel3 cell lines was extracted using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany). Total RNA from Mel1 and Mel3 cell lines was retrotranscribed by the High Capacity RNA-to-cDNA kit (P/A 4387406, Applied Biosystems). Quantitative RT-PCR (qPCR) was performed using the 7900HT Fast System (Applied Biosystems, Foster City, CA, USA) and Sybr Green methodology targeting exon-exon junction region. The amount of mRNA was calculated using the 2–ΔΔCt method and expression values were normalized to the internal control gene GAPDH. The specific oligonucleotide primer pairs for qPCR were: GAPDH, 5′-ACCCACTCCTCCACCTTTGA-3′ (sense), 5′-CTGTTGCTGTAGCCAAATTCGT-3′ (antisense); RAP1GAP, 5′-AAGC ACTTTCTCGGCAAGGA-3′ (sense), 5′-AGTGAGAAGACAAGGTGGCC-3′ (antisense); DOCK7, 5′-CTCGGGACTGCAGAACTCTT-3′ (sense), 5′-ACAGTCCTCAACATGTGGATCCA-3′ (antisense).

2.12. FACS analyses

The effects of Theo on cell cycle distribution were performed after propidium iodide (PI) staining as described [9]. Apoptotic cells were analyzed by Annexin V conjugated with fluorescein isothiocyanate (FITC) combined with 7-Aminoactinomycin (7AAD), using flow cytometry as described [9].

For markers studies through FACS analysis, cells were fixed with 3% paraformaldehyde, permeabilized with 0.01% saponin (in PBS/3% BSA/HEPES), and stained with the following primary antibodies: rabbit polyclonal anti-CD39 (1:50; Cat#33558) and mouse monoclonal anti-Rac1 (1:50; Cat#610650) (BD Biosciences). Primary Abs were followed by goat anti-mouse isotype-specific Ab or goat anti-rabbit Ab labeled with Alexa Fluor 488 (Invitrogen). For F-actin analysis, Mel1 cells were treated with 2 mM Theo for 72 h and cells were analyzed in suspension and after adhesion on MG for 1 h with phalloidin-FITC. The samples were acquired using a FACSCalibur (Becton and Dickinson). Results were analyzed using the FlowJo Pro software (Tree Star, Inc.).

2.13. Confocal microscopy

For cytoskeleton studies, F-actin analysis was performed. Mel1 cells,

treated or not with 2 mM Theo, were seeded into microchambers (Lab-Tek Chamber coverglass Nunc, France), previously coated with MG. After MG adhesion for 1 h, MICs were fixed with 3% paraformaldehyde, permeabilized with Triton X-100 (in PBS/3% BSA/HEPES), and stained with phalloidin-FITC (1:100, Cat#A12379) (ThermoFisher). Cells were analyzed using a LSM 510 confocal microscope (Zeiss) with a 63× Plan-Apochromat objective (1.4 oil) at 37 °C and 5% CO_2. Images were processed with ImageJ software.

2.14. Secretome profile in melanospheres supernatants

Conditioned media of Mel1 and Mel3 untreated and treated with Theo were collected and centrifuged. The protein concentration was then measured by Bradford's procedure as above reported. Twenty five micrograms of proteins from total cell supernatants were analyzed with a magnetic-bead based multiplex immunoassay, *i.e.* xMAP technology on Luminex platform, carried out according to manufacturer guidelines (Bio-Plex, BioRad Laboratories, Milan, Italy). Cytokines/chemokines were measured with a custom Bio-Rad Bio-Plex human cytokine reagent kit for granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interferon-inducible protein 10 (IP-10 or CXCL10), monocyte chemoattractant protein-1 (MCP-1 or CCL2), macrophage inflammatory protein 1-α (MIP-1α or CCL3), MIP-1β (CCL4), regulated and normal T cell expressed and secreted (RANTES), vascular endothelial growth factor (VEGF), interleukin 1β (IL-1β), interferon-γ (IFN-γ) and IL-12. Data were acquired on the BioRad Bio-Plex X200 reader equipped with Bio-Plex Pro™ Wash Station and analyzed using Bio-Plex Software Manager™ 6.1 (Bio-Rad).

2.15. Statistical analysis

Results are expressed as means of three independent experiments ± standard deviation (SD). The statistical significance of differences was determined by Student's *t*-test, the significance threshold was set at $p \leq 0.05$.

3. Results

3.1. Theophylline decreases melanoma cells proliferation and metastatic potential

In order to investigate Theo anti-cancer properties, we first evaluated its ability to affect the growth of human A375 and SK-MEL-30 melanoma cell lines. Cells were treated with 1 or 2 mM Theo for 24, 48 and 72 h. As shown in Fig. 1A-B, Theo moderately reduced melanoma cell proliferation in a time- and dose-dependent manner. In particular, cells treated with 2 mM Theo showed a significant decrease of proliferation rate that was particularly evident at 72 h of treatment. The antiproliferative activity of Theo was also evaluated in two additional cell lines, SK-MEL-28 and WM793 (Fig. 1C). No significant changes in cell viability (evaluated by Trypan Blue staining) were detected in all cell lines analyzed (data not shown). Since more significant anti-proliferation results were observed with 2 mM Theo, this concentration was used for subsequent experiments. Theo capability to induce melanoma cell biochemical and morphological differentiation and to reduce metastatic potential was previously demonstrated in the murine B16-F10 melanoma model [12]. Our results confirmed and extended those reported data to a wider panel of cell lines. As shown in Fig. 1D, Theo-treated melanoma cells acquired a starry-dendritic morphology characterized by cytoplasmic protrusions, typically found in melanocytes, possibly suggesting an action of Theo on membrane/cytoskeleton dynamics. To investigate Theo impact on metastatic capability of A375 and SK-MEL-30 melanoma cells, adhesion (Fig. 1E) and migration (Fig. 1F) assays were performed. The adhesion assay on MG indicates a significant reduction ($p < 0.05$) of adhesion capability of melanoma cells treated with 2 mM Theo for 72 h. Moreover, Theo was able to reduce the ability of human melanoma cell lines to migrate as evaluated using Boyden chambers.

3.2. Theophylline activity on melanoma spheroids

Since it has been previously reported [12,18] that Theo can act as a melanoma cells differentiation-inducer, we also used a less differentiated human melanoma cell model [27] for our study. We first evaluated Theo capability to reduce cancer cell proliferation. Melanoma spheroids (A375-Spher) were obtained from A375 cell line (Fig. 2A) as described in Materials and Methods section. A375-Spher cells were treated with 1 and 2 mM Theo for 24, 48 and 72 h. In particular, cells treated with 2 mM Theo showed a decrease of proliferation both at 48 and 72 h of treatment (by 34.9% and 35.3% respectively) compared to control (Fig. 2B). To extend the reliability of our results, we evaluated also Theo ability to affect the growth of the non-adherent 3D spheroid bodies, such as A375-Spher. Spheroid growth has been calculated by analyzing the size and the number of spheroids following Theo exposure. As shown in Fig. 2C-D, Theo is able to inhibit spheroid growth (up to 75% of inhibition) when compared to controls.

3.3. Effects of theophylline on MICs proliferation and melanospheres formation

MICs represent a dynamic subpopulation of cancer cells probably responsible both for the generation and the perpetuation of a continuously growing tumor and for cancer recurrence. Thus, we evaluated Theo ability to affect MICs proliferation and spheroids formation. For these experiments, two primary melanoma stem cell lines (an undifferentiated melanoma model) previously established from human metastatic melanoma specimens were used (Mel1 and Mel3) [9], As shown in Fig. 3A, obtained results indicate that Theo (1 mM) reduces proliferation of Mel3 cells after 72 h of treatment by 31.8%, while 2 mM Theo reduces proliferation rate both of Mel1 and Mel3 cells by 45.7% and 40.9% respectively, compared to control. Moreover, as shown in Fig. 3B-C, melanospheres growth analysis following Theo exposure confirmed its ability to reduce melanosphere growth and spheroid formation of > 60% in both cell lines when compared to control. To further confirm Theo growth inhibition, melanospheres were analyzed for cell cycle distribution and apoptosis. Exposure to 2 mM Theo resulted in an alteration of cell cycle distribution with an accumulation of cells in sub-G1 and G2/M phases. Further, an induction of cell death was also observed (Supplementary Fig. 1).

3.4. Proteomic analysis of MICs after theophylline treatment reveals the expression of proteins involved in cell differentiation

To further investigate the anti-cancer activity of Theo, a proteomic analysis of MICs' extracts was carried out. To this end, mass spectrometry analysis of cell extracts from Mel1 and Mel3 treated with Theo was performed allowing to identify a large number of proteins from each sample. Mel1 control and treated cells expressed 197 and 178 specific proteins respectively and 716 common proteins (Supplementary Table 1) while Mel3 control and treated cells express 180 and 200 specific proteins respectively and 724 common proteins (Supplementary Table 2). The total number of proteins revealed by mass spectrometry analysis is summarized in Fig. 4A.

We focused our attention on the specific proteins that resulted commonly expressed (both in Mel1 and in Mel3) both in untreated cells (42 proteins) and in treated ones (33 proteins) (Fig. 4A; Supplementary Table 3). These proteins were analyzed using Gene Ontology tools. As shown in Fig. 4B-C, Gene Ontology classification for Biological Process (BP) and Cellular Component (CC) of control cells proteins, indicates that proteins mainly expressed in Control (untreated) MICs are involved in cell proliferation and survival with a negative regulation of apoptotic pathways and are often related to cellular membrane. On the other

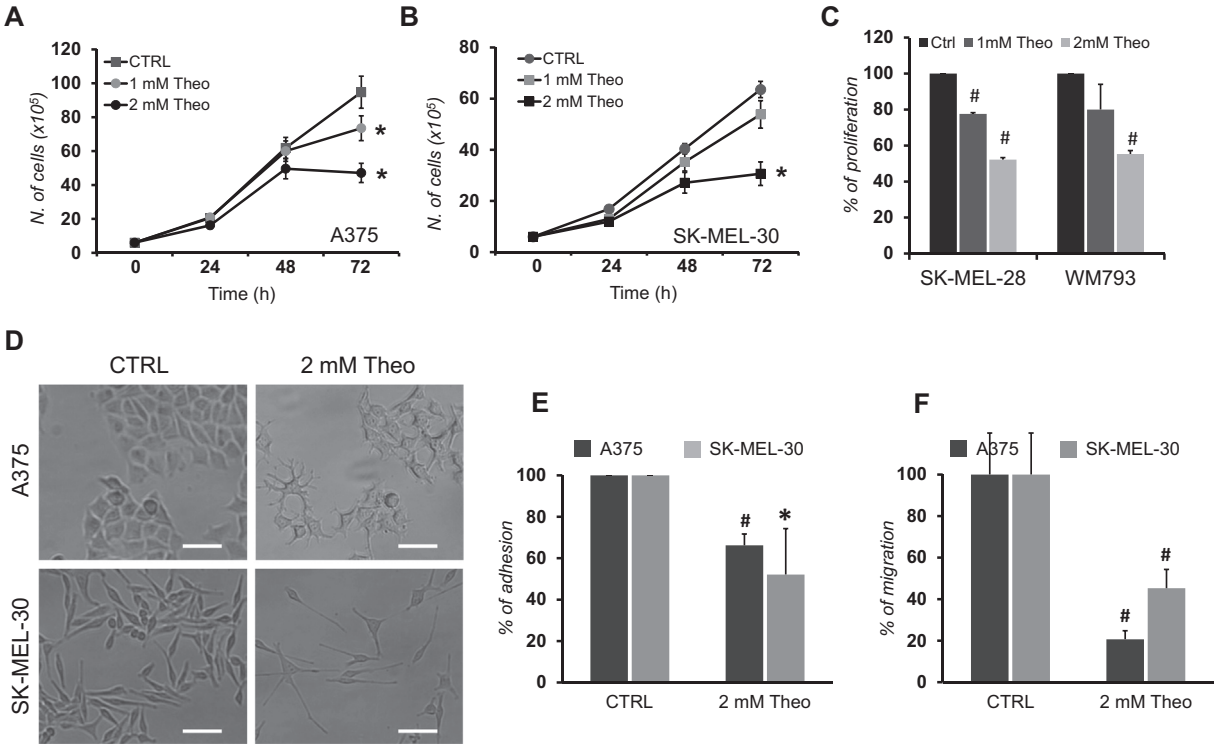

Fig. 1. Theophylline (Theo) affects growth and metastatic potential of human melanoma cells. Cell proliferation curve of A375 (**A**) and SK-MEL-30 (**B**) cells treated with 1 and 2 mM Theo for 24, 48 and 72 h. (**C**) Per cent of proliferation of SK-MEL-28 and WM793 cells treated with 1 and 2 mM Theo for 72 h. (**D**) Light microscopical appearance of 2 mM Theo treated A375 and SK-MEL-30 melanoma cells after 72 h of exposure compared to controls. Typical morphological signs of melanocyte differentiation appear upon Theo treatment (dendritic shape; scale bar: 30 μm). A375 and SK-MEL-30 showed a reduction of metastatic potential evaluated by adhesion (**E**) and migration (**F**) assays after 2 mM Theo treatment for 72 h. Control cells were incubated with vehicle only. Data represent the mean ± S.D. of three experiments carried out in triplicates (statistical significance *versus* control: * $p < 0.05$; # $p < 0.005$).

hand, the analyses for BP and CC of proteomic profiles obtained from Theo-treated cells (Fig. 4D-E) revealed that the proteins mainly expressed by these cells are those involved in dendrites formation and cytoskeleton dynamics, strongly suggesting an induction of differentiation pathways on MICs by Theo. In particular, these data suggest the induction of actin cytoskeleton re-modeling genes, a result in line with other data presented in this study (see Fig. 1D). This observation supports the hypothesis that Theo might have effects on multiple biological functions including cell migration, phagocytosis, vesicular transport, apoptosis, and proliferation [28].

3.5. Effects of theophylline on differentiation state and metastatic potential of MICs

Melanoma-initiating cells are, among tumor cell populations, the less differentiated and the more resistant to drugs. Based on our proteomic results and previous studies [12], the differentiation activity of Theo on MICs was investigated.

Melanin production is considered a main differentiation marker in melanoma models. In fact, melanoma cells derive from oncogenic transformation of melanocytes, whose physiological differentiation is achieved through a progressive acquisition of melanin biosynthesis. Melanin content after Theo treatment was evaluated both in Mel1 and Mel3 cells. As shown in Fig. 5A, melanin production increased by 1.3 and 1.8 fold after 72 h of 1 mM Theo treatment and by 1.8 and 3.5 fold after 72 h of 2 mM Theo treatment for Mel1 and Mel3 respectively. The increase of melanin levels is also supported by the assessment of the activity of tyrosinase (TYR; E.C. 1.14.18.1) and dopachrome tautomerase (DCT; E.C. 5.3.2.3), two key enzymes of melanin biosynthesis [29]. DOPA staining assay indicates a significant increase in TYR activity after 2 mM Theo treatment for 72 h both in Mel1 and Mel3 (Fig. 5B). In agreement with previous data, DCT activity was increased after treatment (Fig. 5C). Further, the antiproliferative and differentiative ability of Theo was also confirmed in Mel1 under other differentiative stimuli (*in vitro* differentiation with MGM4 medium) (Supplementary Fig. 2).

To better analyze the differentiative state of melanospheres after Theo treatment, transamidase activity of tissue transglutaminase (TG2; EC 2.3.2.13), that represents one of the methods to evaluate cell differentiation [30], was explored. The Fig. 5D indicated an increase in TG2 activity after 2 mM Theo treatment for 72 h, with respect to control. Further, CD39 (ectonucleoside triphosphate diphosphohydrolase 1) expression was studied as another melanoma differentiation marker [31]. FACS analyses indicated a significant increase of CD39 expression in Mel1 after Theo treatment, compared to control (Fig. 5E). These data represent the first evidence in literature regarding the pro-differentiation activity of Theo on MICs using some of the most universally accepted differentiation markers for melanoma cells.

Melanoma cell metastatic potential was also studied by evaluating the ability of MICs to migrate after Theo treatment. Migration studies, carried out using a Boyden chambers assay, showed a significant decrease of migration both in Mel1 and in Mel3 after 2 mM Theo treatment for 72 h (by about 21% and 31% respectively) compared to control (100%) (Fig. 5F). Considering that MMPs are responsible of extracellular matrix degradation during invasive processes, we performed a gelatin zymography. Fig. 5G shows a slight reduction of MMP2 activity after 2 mM Theo treatment for 72 h, when compared to control in both cell lines. Proteomic analysis revealed also that both MICs treated with Theo specifically express the dedicator of cytokinesis protein 7 (DOCK7) and Rap-1 GTPase activating protein 1 (RAP1GAP), proteins related to melanocytes function and aggressiveness. To evaluate DOCK7 and RAP1GAP gene expression, quantitative RT-PCR

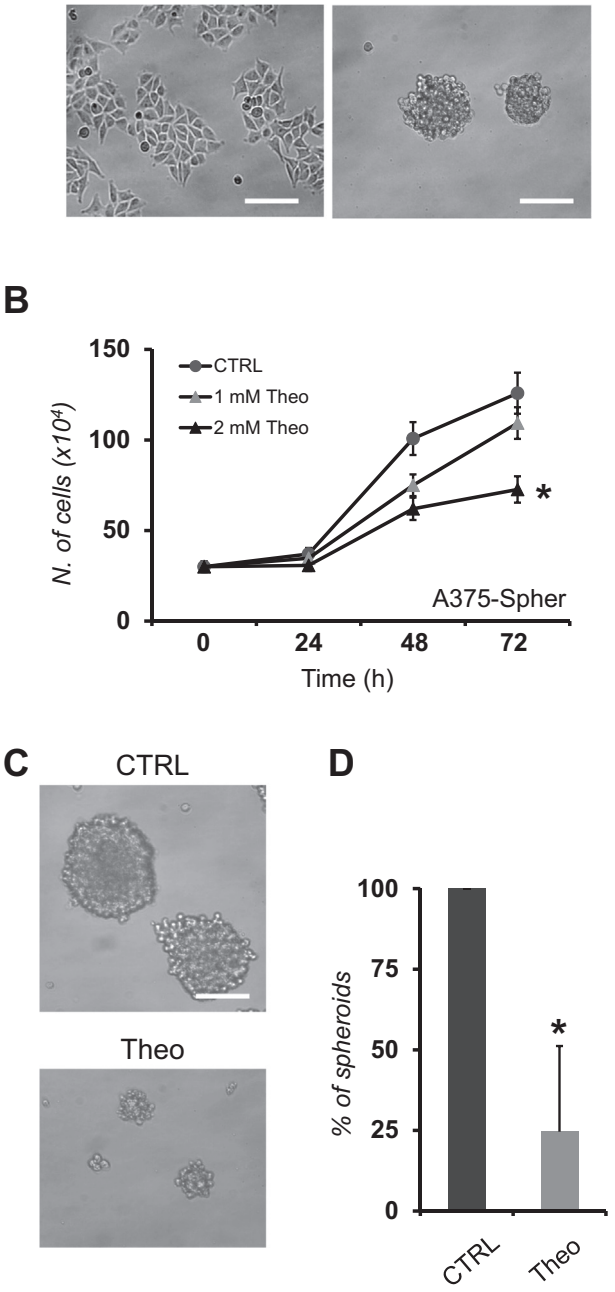

Fig. 2. Theophylline (Theo) affects the proliferation and growth of A375-Spher cells. (A) Light microscopical appearance of untreated A375 and A375-Spher (spheroids) cells. (**B**) Proliferation curve of A375-Spher cells treated with 1 and 2 mM Theo for 24, 48 and 72 h. (**C**) Theo significantly inhibits spheroids formation and growth in 3D culture; spheroids formation was quantified (**D**) with Image J®. Data are expressed as % of the control (100%). Control cells were incubated with vehicle (PBS). Data represent the mean ± S.D. of three experiments carried out in duplicates (statistical significance *versus* control: * $p < 0.05$; # $p < 0.005$).

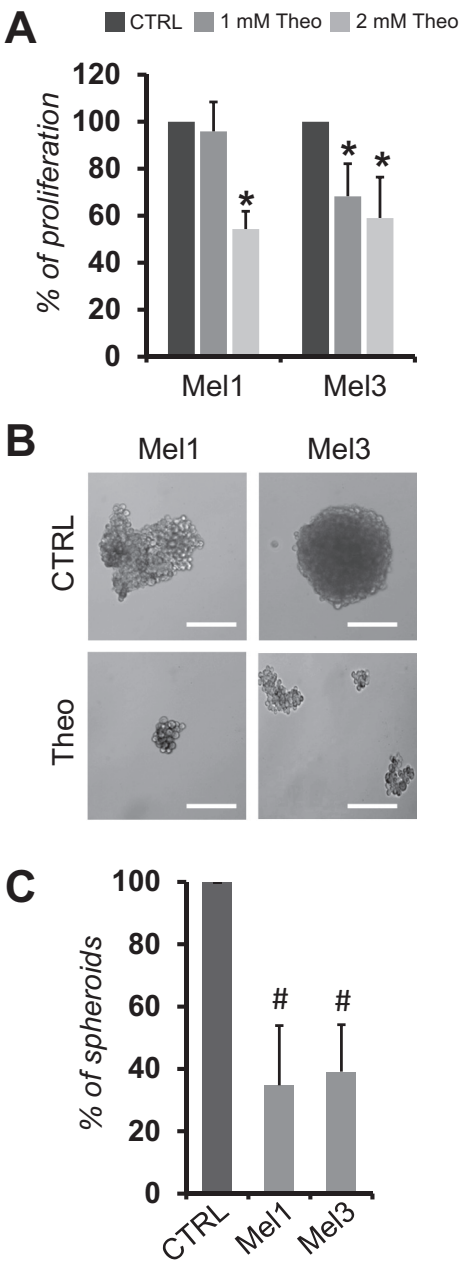

Fig. 3. Theophylline (Theo) inhibits the proliferation of MICs and reduces melanospheres formation. (**A**) 1 and 2 mM Theo for 72 h affect Mel1 and Mel3 proliferation (**B**) Light microscopical appearance of untreated and 2 mM treated MICs spheroids after 72 h of exposure. Images show the reduction of spheroids growth after Theo exposure (scale bar: 30 μm). (**C**) Mel1 and Mel3 spheroids were quantified with Image J®. Data are expressed as % of the control (100%).

analysis was performed. Interestingly, no significant differences in gene expression were found (data not shown).

3.6. Theophylline interferes with cytoskeleton dynamics in MICs

The protein profiles obtained from proteomic analysis (together with data reported in Fig. 1D) indicate a possible involvement of Theo in the cytoskeleton reorganization of MICs. To elucidate MICs cytoskeleton dynamics, confocal microscopy and FACS analyses were carried out. As shown in Fig. 6A, confocal microscopy experiments allowed to analyze F-actin cytoskeleton through phalloidin-FITC in Mel1 both in suspension and after MG adhesion for 1 h. Confocal laser-scanning microscopy analysis of MICs growth in suspension did not show any significant difference among control and treated cells. Conversely, after MG induction of adhesion, a modification of actin cytoskeleton between control and treated cells was clearly detectable. In fact, Mel1 after Theo treatment showed the presence of stress fibers absent in Mel1 control cells. Moreover, FACS analyses with phalloidin-FITC indicate a significant increase of F-actin in treated cells when compared to control cells both in suspension (Growth) and after the induction of adhesion on MG for 1 h (Fig. 6B). In line with these observations, we found that Mel1 cells treated with Theo express higher Rac1 levels than control

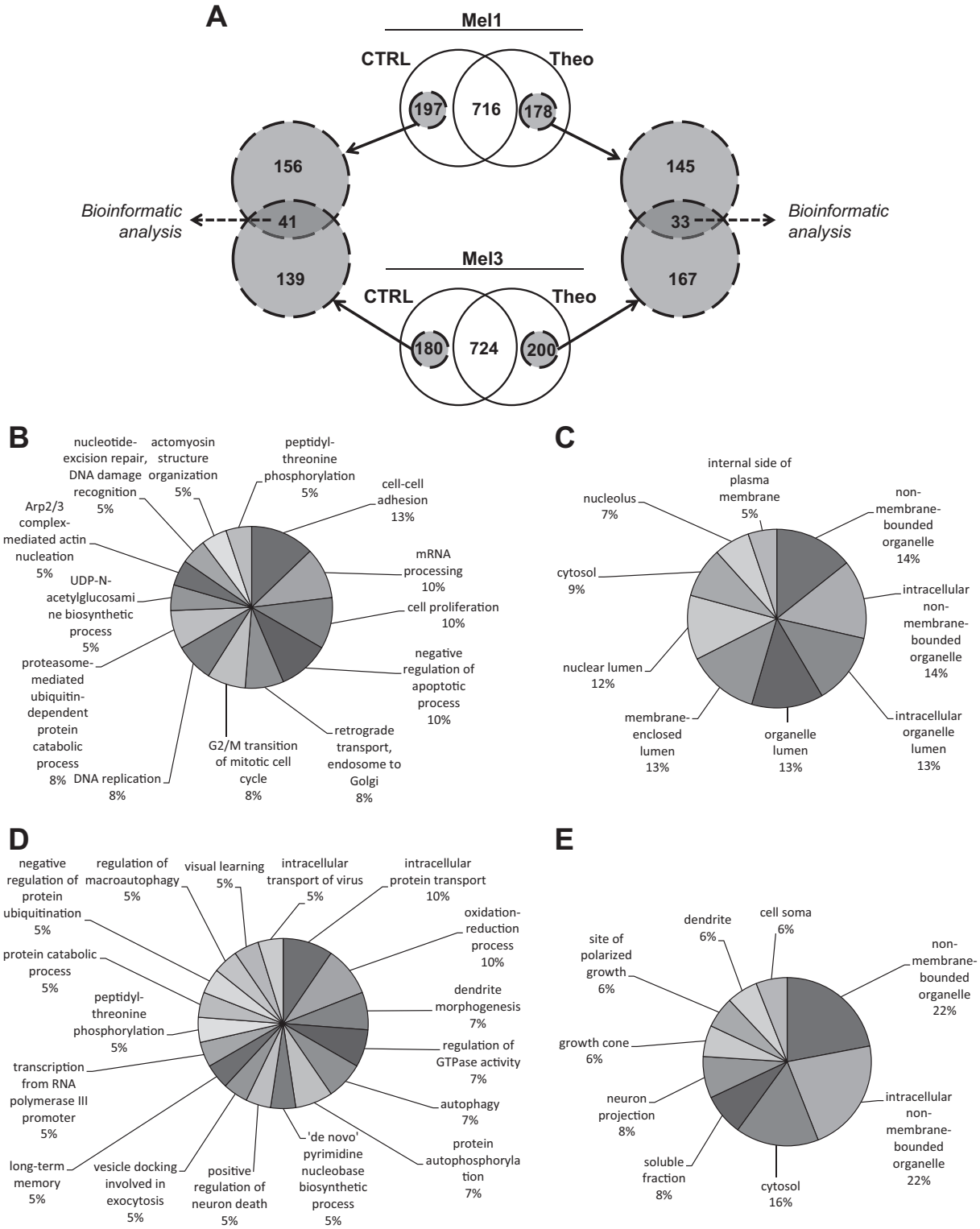

Fig. 4. Proteomic analysis of Mel1 and Mel3 cell lysates. (A) Proteomic workflow analysis of Mel1 and Mel3 protein profiles. Data obtained from mass spectrometry analysis were studied through GeneOntology tools. DAVID classification based on biological processes (BP) (GOTERM_BP_DIRECT) and cellular components (CC) (GOTERM_CC_FAT) were carried out respectively for common specific proteins (42 proteins) in control cells (**B-C**) and common specific proteins (33 proteins) for treated ones (**D-E**).

cells (Supplementary Fig. 3).

3.7. Cytokines and chemokines secretion after theophylline treatments

Among Theo's mechanisms of action, adenosine receptor (AR) antagonism, phosphodiesterase inhibition (PDE) [32], inhibition of phosphoinositide-3-kinase-δ (PI3Kδ) and its ability to increase histone deacetylase-2 (HDAC) levels are able to modulate the expression of genes that control production of soluble factors and pro/anti-inflammatory molecules [33]. We thus evaluated the profile of cytokines

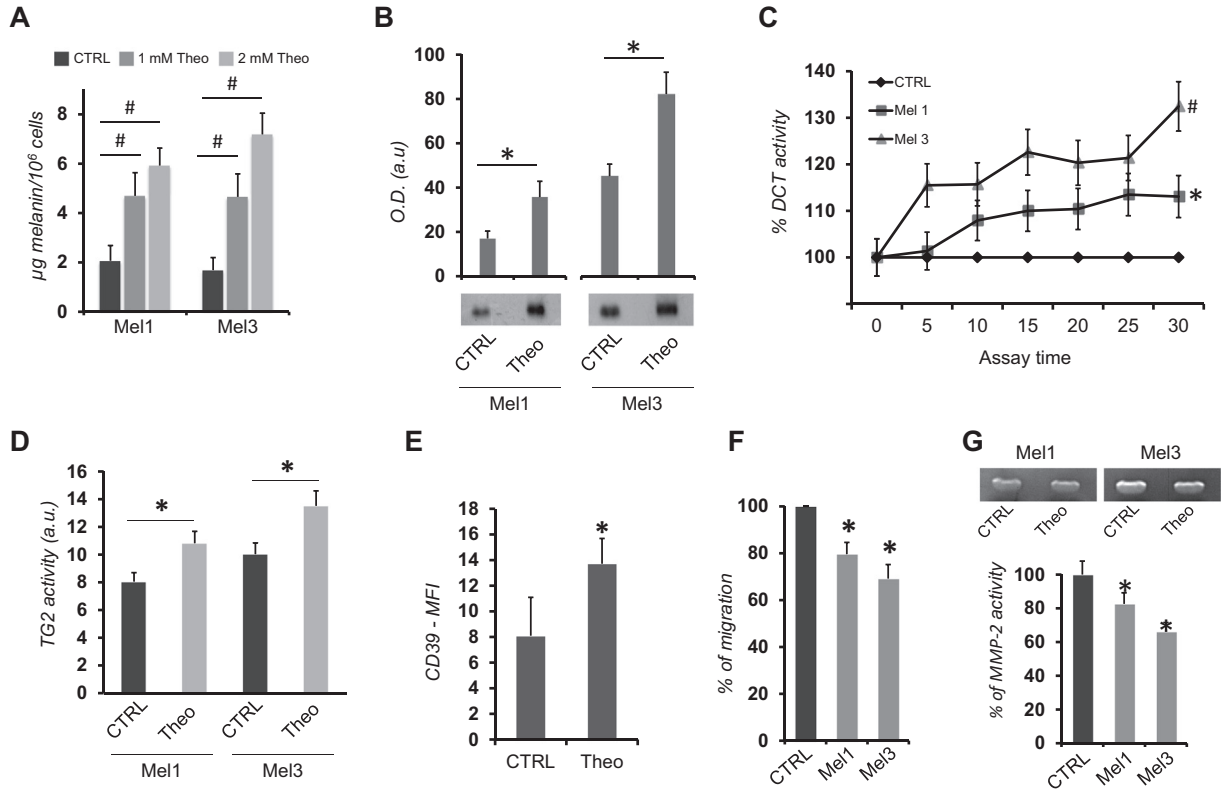

Fig. 5. Theophylline (Theo) induces MICs differentiation and affects their metastatic potential.
(**A**) Theo induces a significant dose-dependent increase of melanin production in Mel1 and Mel3 melanotic cells. (**B**) Theo increases tyrosinase (TYR) activity in MICs. To observe tyrosinase activity, DOPA-staining was performed on polyacrylamide gels. Results are expressed as arbitrary units of optical density (O.D.). (**C**) DOPAchrome Tautomerase (DCT) activity assay indicates a time-dependent increase of DCT activity in Mel1 and Mel3 cells. Results are expressed as per cent of activity (control 100%). Theo induces an enhancement of transglutaminase transamidating activity in MICs and *in vitro* assay results were expressed as arbitrary units (**D**). FACS analysis of CD39 expression (**E**) indicate a significant increase of this surface marker expression on MICs after Theo treatment (mean fluorescent intensity, MFI). Theo induces a significant reduction of migration capability (**F**) of MICs and a reduction of matrix metalloproteinases 2 (MMP2) activity (**G**) in conditioned media. Quantitative evaluation of MMP2 activity after 2 mM Theo treatment for Mel1 and Mel3 cells were revealed by gelatin zymography. A representative zymogram to detect the activity of secreted MMP-2 using conditioned medium from MICs melanoma cells is shown. The bar graph shows results of densitometric analysis that are expressed as a percentage of the control (100%). Control cells were incubated with vehicle only. Data represent the mean ± S.D. of three experiments (statistical significance *versus* control: * $p < 0.05$; # $p < 0.005$).

and chemokines secreted by MICs to study the potential effects of Theo on human melanoma stem cells. MICs conditioned media were collected and analyzed after Theo treatment for 48 and 72 h. As shown in Fig. 7, the secretion of six cytokines/chemokines (namely IL-1β; IP-10; MCP-1; MIP-1α; MIP-1β; RANTES) was significantly modified after Theo treatment both in Mel1 and Mel3 cells. Moreover, other soluble factors were investigated such as cytokines (IL-12 and IFN-γ) and three growth factors (GM-CSF, G-CSF and VEGF). Interestingly, IL-12 and VEGF levels increased in both cellular models following Theo treatment but the only significant results were observed for IL-12 after Mel3 Theo treatment for 72 h and for VEGF after Mel3 Theo treatment for 48 and 72 h, while IFN-γ, GM-CSF and G-CSF secretion was not significantly

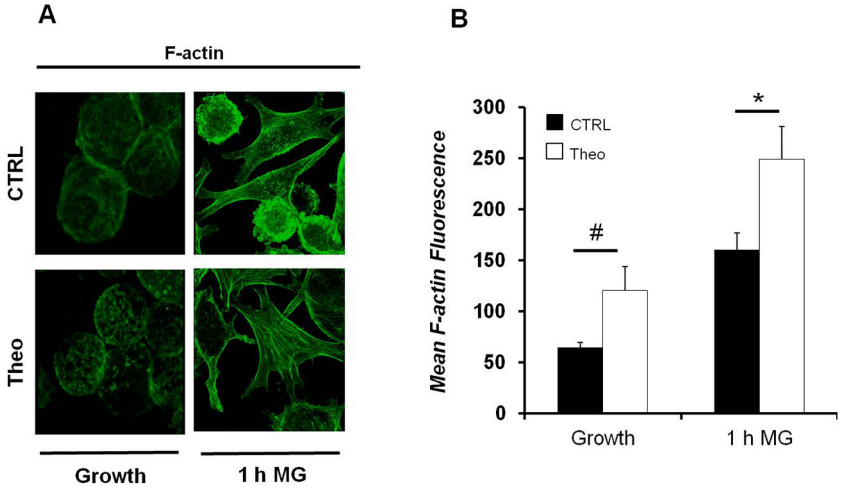

Fig. 6. Theophylline (Theo) induces actin polymerization and the assembly of stress fibers. Theo induces the formation of stress fibers in Mel1 cells compared to control ones. Confocal light microscope (**A**) appearance of actin cytoskeleton of Mel1 grown in suspension (Growth) and after MG adhesion. F-actin formation was evaluated also through FACS analysis (**B**) through phalloidin-FITC. Data are expressed as mean of fluorescence ± S.D. of three experiments (statistical significance *versus* control: * $p < 0.05$; # $p < 0.005$).

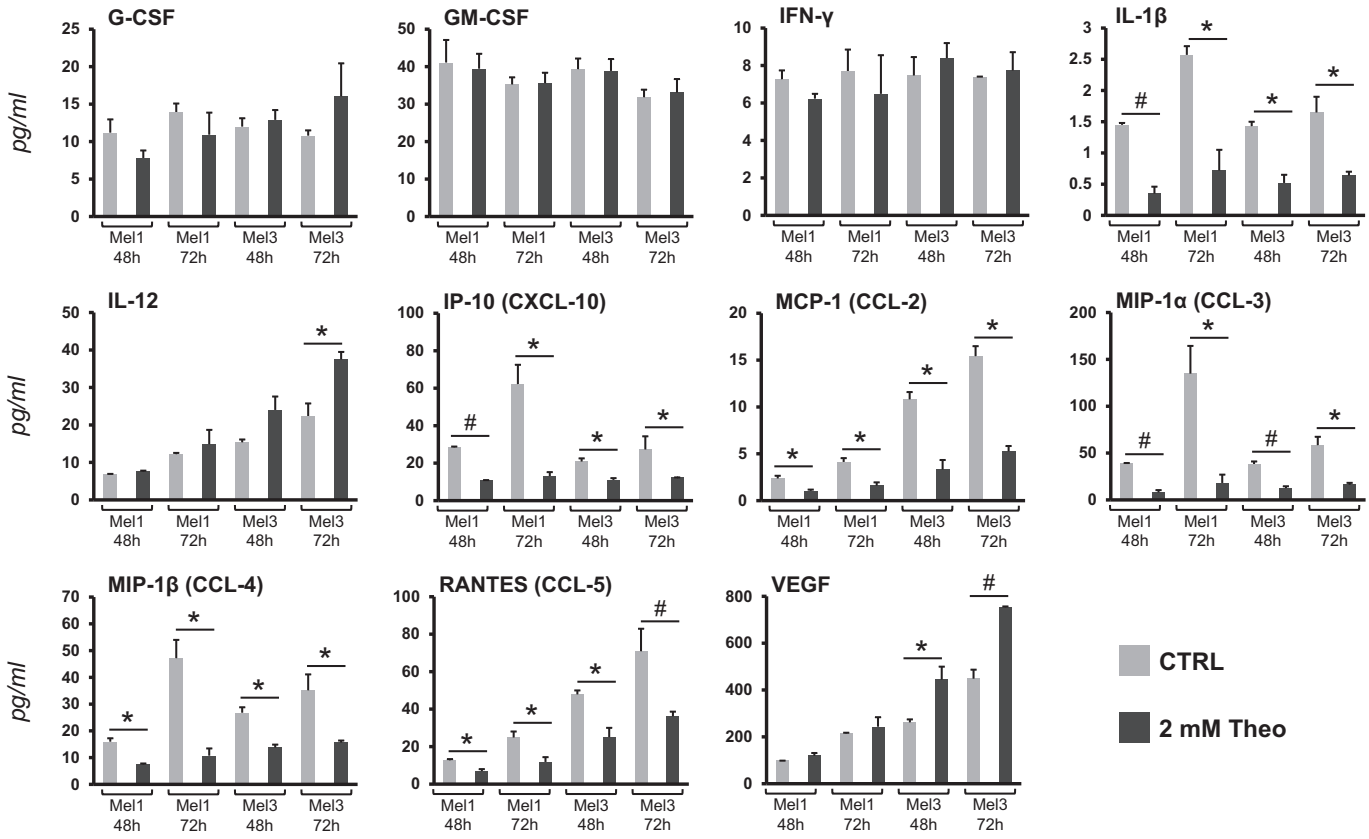

Fig. 7. Theophylline (Theo) modulates cytokines/chemokines secretion by Mel1 and Mel3. Conditioned media were analyzed to investigate levels of the indicated molecules, after 48 and 72 h of Theo exposure, using multiplexed immunoassay (Bio-Plex kits). Data are mean ± SD from three independent determinations (statistical significance *versus* control: * $p < 0.05$; # $p < 0.005$).

modified.

4. Discussion

In the last years diet and nutrition have been thoroughly investigated with the aim to find new tools in the field of cancer prevention. In particular, the consumption of coffee, tea or cocoa and other MX-containing beverages and foods is widely diffused throughout the world at the present time [34]. Hence, this study aims to elucidate the ability of Theo, one of the most representative naturally-occurring MXs, to affect melanoma growth and its potential immunomodulatory activity. In particular, the results presented here clearly indicate that Theo reduces proliferation and induces differentiation of different melanoma cell models including MICs.

Theo significantly reduces proliferation and metastatic potential of melanoma cell lines (A375 and SK-Mel-30), through the induction of differentiation that can be appreciated thanks to the change of cell morphology after treatment. In fact, cytoplasmic protrusions are recognized as a morphological indicator of melanocyte/melanoma cell differentiation [35]. Since MXs are known to act as differentiative agents [36], the anti-cancer activity has been assessed on less differentiated melanoma cell models. Therefore, the obtained results on A375-Spher by Theo treatment led us to investigate the effects on an even more undifferentiated cancer cells population, *i.e.* two MICs that are highly tumorigenic *in vivo* and resistant to chemotherapeutic drugs and to the majority of pathway inhibitors. Interestingly, Theo has proved to be able to significantly affect proliferation of MICs reaching values of anti-proliferation similar to those obtained with chemotherapy drugs as cisplatin [9]. This finding suggest the possibility to use MXs as adjuvant drugs in combination with common chemotherapy drugs to achieve better therapy results, as previously suggested [37]. Mass spectrometry-based proteomic screening of MICs extracts leads to the discovery that the Theo-treated cells differentially express some pathways directly related to dendrites formation and cytoskeleton dynamics. These proteomic results prompted us to investigate in deep MICs differentiation.

First of all, we demonstrated that Theo is able to induce an accumulation of melanin, due to increase of TYR and DCT activities, typical differentiation marker in melanoma. It is well established that Theo is a nonselective inhibitor of PDE isoenzymes, which break down intracellular concentrations of cAMP [14]. Therefore, Theo-mediated differentiation may be ascribed to an increase of intracellular levels of cAMP. In fact, some studies pointed to the involvement of cAMP in the differentiation of melanocytes and in their ability to produce melanin [38]. The increase of melanin content, and also CD39 expression, which some studies suggest is directly related to increased levels of intracellular cAMP and to melanoma differentiation [31,39], confirm this hypothesis.

Previous studies indicate that one of the biochemical effects of MX on B16-F10 murine melanoma cells consists of the increase of enzymatic activity of transglutaminase [12] that is considered a good differentiation marker in melanoma models, but also to play a tumor-suppressor activity [40]. Our results indicate an increase of transglutaminase activity, a further confirmation of Theo differentiation induction on MICs. Moreover, the increase in transglutaminase activity correlates with a significant reduction of migration capability and of MMP2 release in conditioned media. On the basis of the data obtained with mass spectrometry experiments, the cytoskeleton dynamics has been investigated. In particular, proteomics analysis indicated that both Mel1 and Mel3 treated with Theo specifically express DOCK7 (see Supplementary Table 3) closely related to some aspect of melanoblasts and/or melanocytes functions. DOCK7 is a guanine nucleotide

exchange factor and it exerts its activity on small GTPase of Rho family as Cdc42, RhoA and Rac1 which are involved in processes like the restructure of the actin cytoskeleton and for subsequent effects on multiple biological functions including cell migration, phagocytosis, vesicular transport, apoptosis, and proliferation [28]. In particular, *in vitro* studies established that DOCK7 activates Rac and Cdc42 and promotes axon formation as well as Schwann cell migration [41]. Moreover, Blasius and colleagues indicate that DOCK7 plays an important role in pigmentation and may be involved in melanocyte ontogeny and function [42]. Intriguingly, no differences in DOCK7 gene expression were found, suggesting a possible regulatory mechanism [43]. Proteomic analysis also suggests a possible involvement of RAP1GAP in Theo mechanism of action. RAP1GAP represents a negative regulator of Rap1 (Ras associated protein 1). It has been demonstrated that overexpression of RAP1GAP is able to induce differentiation and apoptosis in leukemia cells [44], whereas its down-regulation promotes melanoma proliferation and migration [45]. The induction of differentiation and apoptosis, and the reduction of aggressive phenotype mediated by Theo, corroborates these findings. Quantitative T-PCR analysis revealed that RAP1GAP gene expression was not-significantly modified by Theo treatments of MICs, suggesting the occurrence of a post-transcriptional modulation. To better elucidate these aspects, further investigations are needed.

Our results show the induction of a higher expression of Rac1 small Rho GTPase by Theo compared to the control cells. Furthermore, the actin cytoskeleton dynamics are the basis of many cellular processes such as morphogenesis, development, differentiation, migration, invasion and metastatic spreading [46], mediating the formation of cellular structures like filopodia, lamellipodia, stress fibers and focal adhesion. Abnormal regulation of these cytoskeleton dynamics is often a process involved in many diseases, including cancer. The highly motile cells are characterized by fewer, thinner and more dynamic stress fibers compared to non-motile ones [47]. Adhesion of Theo-treated cells to a reconstituted ECM (MG) remarkably increased stress fibers organization that is often related to a lower cell motility and it is a clear sign of the impairment of migration process. This result is further confirmed by the data obtained for migration assay and validated also by functional analysis (zymography data).

The Theo AR antagonism capability and the PDE inhibitory activity suggest also a potential immunomodulatory role. Efficacy of current therapies for human cutaneous melanoma is limited in time due to the development of drug resistance, to dynamic population of cells with stemness features with tumor immune escape capability. Many soluble factors are able to promote the atypical differentiation of myeloid lineage cells towards the development of different immunosuppressive populations [48]. Melanoma patients in advanced stage of disease show high serum levels of IL-1β, IFN-γ, CXCL10, CCL5, CCL11, IL-4, IL-5, IL-10 and myeloid cells with immunosuppressive capability (MDSCs, Dendritic Cells or DCs, CD4 + CD25hiFoxP3 + T cells or Treg cells, Tumor Associated Macrophages or TAMs) [49]. In physiological conditions, the secretion of these soluble factors is related to an acute inflammation in which the recruitment of CTL and other immune cells to establish an immune reaction is necessary. However, a long-term secretion of these inflammatory molecules induces a chronic inflammation which supports the activation of immunosuppressive cells increasing tumor progression [50]. Cekic and colleagues demonstrated that aminophylline (theophylline ethylenediamine) is able to exert antitumor activity in bladder cancer by blocking adenosine $A_{2b}R$ [16]. Our data indicate that Theo induces a strong time-dependent reduction of inflammatory molecules involved in recruitment of immunosuppressive cells in tumor microenvironment as CCL2, CCL3, CCL4 and CCL5 by MICs. In fact, these chemokines are strongly correlated with increase of intra-tumor myeloid cells. Moreover, also tumor-infiltrating monocytic-MDSCs (MO-MDSCs) produce high levels of the CCR5 ligands, CCL3, CCL4, and CCL5 and directly attract Treg cells in a CCR5-dependent manner [49,51]. Furthermore, it has been demonstrated that a selective $A_{2b}R$ antagonist is able both to limit melanoma progression, due to its ability to revert MDSCs-mediated immune suppression, and to reduce IL-10 and MCP-1 concentration in tumor-bearing mouse [52]. Moreover, also IL-1β and CXCL10 levels in MICs conditioned media are significantly reduced after MX exposure. Several studies demonstrated that high serum levels of IL-1β, CXCL10 and IFN-γ are related to advanced disease and high cells metastatic potential [53–55]. Taken together, these observations indicate that the investigation of cytokine/chemokines production by melanoma cells can be helpful to understand the molecular mechanisms of melanoma cell resistance to immune cell surveillance.

5. Conclusions

Our data suggest that differentiative molecules, such as Theo, may be particularly important in the reduction of proliferation and aggressiveness of MICs. This aspect is reinforced by the immunomodulatory activity of Theo that might act as perturbing agent in the cross-talk between MICs and tumor microenvironment. Moreover, that the induction of tumor cell differentiation mediated by Theo may represent an advantageous therapeutic strategy to be coupled to other therapeutical approaches. Interestingly, epidemiologic studies suggest promising benefits of MXs consumption in chemoprevention [56]. However, further studies are requires to provide answers on the role of MXs in melanoma and other cancer types.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.05.050.

Authors' contribution to study

MC, CT and FF conceived and designed the study. MC, CT, CS, SR, SM and CL conducted the experiments. MC, CT, SV and FF analyzed the data. MC, CT and FF wrote the manuscript. AE and DD provided some material and technical support. AE, SV and AF reviewed and revised the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgments

Cordella M. was recipient of an ISS-Sapienza University PhD Fellowship and of a short-term fellowship funded by the European Molecular Biology Organization (EMBO) that is gratefully acknowledged. Tabolacci C. was supported by Fondazione Umberto Veronesi that is gratefully acknowledged. The authors are grateful to the Proteomic Facility for Complex Protein Mixture (CPM) Analysis at Istituto Superiore di Sanità for technological support. Dr. Alessandra Boe (Istituto Superiore di Sanità, Rome, Italy) is kindly acknowledged for her valuable assistance in FACS analysis.

This work was supported by Ministry of Health, Italy (Targeted Research Funding in Public Researchers GR-2009-1570296; Oncotechnologic Program, Project “Biobanca sieroproteomica”; Italy–USA Oncoproteomic Program; Ricerca Corrente) and by Telethon Foundation (Grant GTF08002) to Facchiano F. Valitutti S. was supported by grants from Region Occitanie (contracts RCLE R14007BB, No 12052802, and RBIO R15070BB, No 14054342), the Laboratoire d'Excellence Toulouse Cancer (TOUCAN) (contract ANR11-LABX) and Fondation Toulouse Cancer Santé (contract 2014CS044).

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