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Phenotypic Switch and Growth of Melanoma Spheroids in the Presence of Mast Cells: Potential Impact of Nutrient-starvation Effects

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Abstract. *Background/Aim: Mast cells are abundant in melanoma tumors, and studies suggest that they can be either detrimental or protective for melanoma growth. However, the underlying mechanisms are not fully understood. Materials and Methods: Here, we adopted an established hanging-drop spheroid system to investigate how mast cells influence melanoma growth and phenotype in a 3-D context. To address the underlying mechanism, we conducted transcriptomic and pathway analyses. Results: In the presence of mast cells or mast cell-conditioned medium, growth of melanoma spheroids was profoundly reduced. Transcriptomic analysis revealed that mast cell-conditioned medium had extensive effects on the gene-expression patterns of melanoma. Pathway analyses revealed profound effects on the expression of genes related to amino acid and protein metabolism. The conditioned medium also induced up-regulation of cancer-related genes, including adhesion molecules implicated in metastatic spreading. In line with this, after transfer to a Matrigel extracellular matrix milieu, spheroids that had been developed in the presence of mast cell-conditioned medium displayed enhanced growth and adhesive properties. However, when assessing the possible impact of nutrient starvation, i.e., reduced nutrient content in mast cell-conditioned medium, we found that the observed effects on growth of melanoma spheroids could potentially be explained by such a scenario. Conclusion: Our findings suggest that the phenotypic alterations of melanoma spheroids grown in the presence of mast cells or mast cell-conditioned media are, at least partly, due to nutrient starvation rather than to the action of factors secreted by mast cells. Our findings may provide insight into the effects on gene-expression events that occur in melanoma tumors under nutrient stress.*

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Mast cells (MCs) are immune cells with an important role in the innate defense against various external insults such as toxic substances released from venomous animals (1), but they are mostly known for their detrimental impact in allergic conditions, including asthma (2). In addition, MCs are associated with a range of other types of pathological conditions, including arthritis, diabetes and cardiovascular complications, as well as being implicated in cancer (3). A role of MCs in cancer was originally suggested based on clinical observations of extensive MC infiltration in tumor settings such as breast, lung, pancreatic and prostate cancer, Hodgkin's lymphoma, and malignant melanoma (4-8). In these conditions, MCs typically accumulate in large numbers in the tumor stroma, but they can also be found, although usually in lower numbers, in the tumor parenchyma (4-8).

In many malignant settings, MC infiltration has been linked to poor clinical outcome, and this has been interpreted as a detrimental impact of MCs under such circumstances. However, there are also numerous studies showing the opposite, *i.e.* that the presence of MCs is associated with good prognosis, suggesting a beneficial role of MCs. Intriguingly though, in some types of malignancies, such as melanoma and prostate cancer, there are reports supporting both detrimental and protective functions of MCs (4-8).

The role of MCs in cancer has also been addressed by adopting various animal models of MC deficiency and, in such studies, MCs have been suggested to promote tumor growth (4, 5). However, several of these studies were performed in mice in which MC deficiency is accompanied by defects in

other cellular niches in addition to their MC deficiency, and it is therefore not certain that the observed effects were indeed due to MC deficiency and not to effects on or of other cell populations (9). However, new-generation MC-deficient mice in which only MCs are targeted have been developed (10). By evaluating such mice in a lung colonization melanoma model, we showed that the absence of MCs led to reduced melanoma colonization of the lung, *i.e.* supporting a detrimental role of MCs in melanoma dissemination (11). However, when assessing mice in which MCs lack the expression of a panel of major granule-localized MC proteases (tryptase, chymase and carboxypeptidase A3), we found that lung metastasis of melanoma was enhanced (12). In agreement with the latter findings, we found that mice lacking tryptase displayed enhanced melanoma growth in a subcutaneous tumor model (13). Intriguingly, these data collectively suggest that although MCs overall may support tumor growth, individual compounds expressed by MCs can have a dampening effect on tumor progression.

Altogether, the findings described above suggest a highly complex impact of MCs on tumor cell populations, but the mechanisms underlying these effects have not been fully delineated. Hence, more detailed insight is needed to explain how MCs can affect various aspects of tumor progression. One strategy for gaining such mechanistic understanding can be to implement detailed analyses of the direct interaction between MCs and tumor cells in a milieu reminiscent of the *in vivo* context. Here we developed a spheroid-based 3-D system for this purpose. Our findings reveal that MCs have a dampening effect on melanoma cell growth, which is accompanied by the induction of gene-expression patterns that may promote a metastatic phenotype of melanoma cells.

Materials and Methods

Bone marrow-derived MCs. Bone marrow-derived MCs (BMMCs) were obtained by culturing bone marrow cells from the femur and tibia of either wild-type, serglycin-null (14) or triple-knockout [mast cell proteases 4 and 6 (*Mcpt4*/Mcpt6) and, carboxypeptidase A3 (*Cpa3*)] (15) mice (all on C57BL/6J genetic background) as previously described (16) but with the following modifications: Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM non-essential amino acids, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 ng/ml recombinant interleukin 3 (IL3) and 20 ng/ml stem cell factor (SCF; Peprotech Nordic, Stockholm, Sweden).

Preparation of conditioned medium from human skin MCs. Human MCs were isolated from healthy skin as described previously (17), and cultured for 5 days in RPMI (Gibco/Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 4 mM L-glutamine, stem cell factor (SCF) (100 ng/ml), IL4 (20 ng/ml) and antibiotics. After incubation, cells were pelleted by centrifugation and removed from the medium.

Development of spheroids. The mouse melanoma cell line B16F10 (American Type Culture Collection; CRL-6475) was a gift from A.R. Thomsen (Copenhagen University, Denmark) and the human melanoma cell line MM253 was purchased from CellBank (CBA-1347; Westmead, NSW, Australia). B16F10 and MM253 cells were cultured in Dulbecco's modified Eagle's medium or RPMI medium, respectively, both supplemented with 10% fetal bovine serum (Thermo Scientific, Waltham, MA, USA), 2 mM glutamine (Merck KGaA, Darmstadt, Germany) and 1% penicillin and streptomycin solution (Merck KGaA). At 60-90% confluency, the cells were trypsinized. B16F10 cells were then resuspended in RPMI medium with 20 ng/ml recombinant IL3 and 20 ng/ml SCF, whereas MM253 were resuspended in RPMI medium with 20 ng/ml recombinant IL4 and 100 ng/ml rh SCF, and counted using trypan blue in order to adjust the cell density to 80,000 cells/ml. The cell suspensions were supplemented with 0.25% methyl cellulose (Merck KGaA), and with BMMCs in RPMI medium (with 20 ng/ml rm IL3 and 20 ng/ml SCF) or with conditioned medium from BMMCs, or with conditioned medium from human skin MCs. A volume of 25 μl of the prepared cell suspensions, corresponding to 2,000 B16F10/MM253 cells, were pipetted on a Petri dish lid, which was then inverted onto a Petri dish filled with sterile phosphate-buffer solution (PBS). Petri dishes with hanging drops were placed in an incubator with 5% CO_2 for 3 or 5 days. At chosen time points, images of spheroids in hanging drops were taken with a Nikon 300630 TMS-F inverted phase-contrast microscope using a 2× objective and a Nikon D3300 camera (Nikon, Tokyo, Japan). Spheroids were transferred into 1.5 ml centrifuge tubes; 100 μl of trypsin-EDTA solution (Merck KGaA) was added and the samples were trypsinized on a benchtop shaking incubator (Corning LSE, Solna, Sweden) at 37˚C, 190 rpm, for 1 h. Before counting the cells with a hemocytometer, the spheroids were additionally disrupted by pipetting and 10 μl of trypan blue (Gibco, Paisley, UK) was added to the samples. When the cells were subjected to the flow cytometry, 1 ml of RPMI medium with 10% FCS was added to the samples after 1-h trypsinization.

RNA extraction, quantitative reverse transcriptase–polymerase chain reaction (PCR) and droplet digital PCR (ddPCR) analysis. RNeasy Micro Kit (Qiagen GmbH, Hilden, Germany) was used to isolate total RNA from the spheroids (containing from 5,000 to 20,000 cells). Total RNA concentration and purity were measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and the ND-1000 V3.7.0 program. cDNA synthesis and quantitative PCR analysis was performed as described elsewhere (13). Gene-expression levels are presented relative to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and relative to nontreated B16F10 spheroids. ddPCR analysis was performed with EvaGreen Supermix (Bio-Rad, Solna, Sweden) and a Bio-Rad ddPCR QX200 system (Bio-Rad) according to the manufacturer's instructions. Briefly, 12.5 μl of Eva-Green master mix (Bio-Rad), 1 μl high-fidelity *Hind*III (NEB, Hitchin, UK), 10.5 μl of cDNA (5 ng/μl per reaction), and 1 μl of respective primer pairs (200 nM final concentration) were mixed and distributed into a 96-well qPCR plate (Bio-Rad). Droplets were generated in an Automated Droplet Generator (Bio-Rad). PCR was performed with a hot-start/enzyme activation at 95˚C for 5 min, denaturation at 94˚C for 30 s, and amplification at 58˚C for 1 min over 40 cycles, followed by signal stabilization at 4˚C for 10 min and 90˚C for 5 min. For all steps, a ramp rate of 2˚C/s was used. The droplets were analyzed in a QX200 droplet reader (Bio-Rad) and the data were analyzed with QuantaSoft Analysis Pro1.0.569 (Bio-Rad). Results were presented as number of copies of a transcript of interest per microliter.

The following primer pairs were used: *Gapdh*, forward primer (5'-3'): CTC CCA CTC TTC CAC CTT CG, reverse primer (5'-3'): CCA CCA CCC TGT TGC TGT AG; L-dopachrome tautomerase (*Dct)*, forward primer (5'-3'): TCC TCC ACT CTT TTA CAG ACG, reverse primer (5'-3'): ATT CGG TTG TGA CCA ATG GG; glycoprotein 100 (*Gp100)*, forward primer (5'-3'): AGC ACC TGG AAC CAC ATC TA, reverse primer (5'-3'): GTT CCA GAG GGC TGT GTA GT; and β-actin (*Actb*), forward primer (5'-3'): AGA CAG CAC TGT GTT GGC ATA GAG, reverse primer (5'-3'): AGG TCA TCA CTA TCG GCA ATG AGC.

Transcriptome analysis. Transcriptome analysis was performed using an Ion AmpliSeq™ Transcriptome Mouse Gene Expression Kit (Life Technologies, Carlsbad, CA, USA), followed by analysis as described elsewhere (18). R (version 4.1.2) was used. gene-expression patterns were evaluated using Kyoto Encyclopedia of Genes and Genomes pathway and Gene Ontology (GO) Biological Processes analyses.

Flow cytometric analysis. After trypsinization, cells were rinsed with a cold fluorescence-activated sorting (FACS) buffer (PBS supplemented with 2% FCS). For apoptosis analysis, cells pooled from several spheroids were recovered in a cold binding buffer (BD Biosciences, Franklin Lakes, NJ, USA) and stained with 3 μl of fluorescein isothiocyanate-conjugated annexin V (BD Biosciences) and 3 μl of Draq7 which stains nuclei of permeabilized cells (Biostatus, Shepshed, UK). Samples were incubated at room temperature, in the dark for 15 min. Subsequently, cells were fixed in 1% paraformaldehyde (PFA) (Santa Cruz Biotechnology, Dallas, TX, USA) in FACS buffer at 4˚C in the dark and for 15 min. Cells were rinsed with and recovered in FACS buffer and filtered through a 70 μm pluristrainer (PluriSelect Life Science, Leipzig, Germany) before being acquired by a BD Accuri C6 Plus flow cytometer (BD Biosciences). Data from 15,000 events/sample were analyzed by FlowJo software (Ashland, OR, USA). For cell-cycle analysis, cells pooled from several spheroids were fixed in 1% PFA in FACS buffer (as described above), rinsed with FACS buffer and permeabilized in 0.5% saponin (Merck KGaA) in PBS, at room temperature for 10 min. Subsequently the cells were stained with Draq7 in 0.5% saponin in PBS at room temperature in the dark and for 15 min. The cells were rinsed with 0.5% saponin in PBS, recovered in FACS buffer and filtered through 70 μm pluristrainer (PluriSelect Life Science). The samples were acquired and analyzed as described above.

Immunostaining of spheroids and confocal analysis. Hanging drops with melanoma spheroids were transferred into 8-chamber culture slides (Cellvis, Sunnyvale, CA, USA). The spheroids were covered with 100 μl Matrigel (Corning, Bedford, MA, USA) and allowed to solidify in an incubator with 5% CO_2 at 37˚C for 40 min. Samples were fixed with 300 μl 4% PFA in PBS for 20 min at room temperature, washed once with IF buffer (PBS with 0.2% Triton and 0.05% Tween 20) and permeabilized with a permeabilization solution (PBS with 0.5% TritonX-100) for 20 min at room temperature. Samples were rinsed with IF buffer for 5 min and blocked with a blocking solution (1% bovine serum albumin in IF buffer) for 30 min at room temperature. The primary antibody, rabbit anti-Ki67-Alexa647 (cat. #NB110-89717AF647; Novus Biological, Littleton, CO, USA), was diluted in blocking solution (1:250) and 300 μl of the antibody solution was applied to spheroids in Matrigel. The staining was performed overnight at 4˚C in the dark. The next day, samples were rinsed three times with IF buffer (5 min/rinsing), counter-stained with NucBlue (Invitrogen, Eugene, OR, USA) according to the manufacturer's instructions for 45 min at room temperature, rinsed three times with IF (5 min/rinsing) and covered with IF. The chambers were kept at 4˚C until analysis with a laser-scanning microscope equipped with ZEN 2009 software (LSM700; Carl Zeiss, Berlin, Germany). The area of the nucleus (NucBlue+) was determined with Image J software (National Institutes of Health, Bethesda, MA, USA), as well as the intensity of Ki67-staining of the nucleus. The results are presented as the intensity of Ki67-staining in the nuclear compartment divided by the area of the nucleus (as determined by 4',6-diamidino-2-phenylindole staining).

Growth of spheroids in Matrigel. Spheroids were transferred in hanging drops into 8-chamber culture slides precoated with 100 μl Matrigel. Matrigel (100 μl) was then layered on the top of the hanging drops and left to solidify in an incubator with 5% CO_2 at 37˚C for 40 min. Samples were covered with 500 μl of complete RPMI medium (supplemented with 10% FCS, 2 mM glutamine and 1% penicillin and streptomycin solution, 50 μM β-mercaptoethanol (Gibco), 1% sodium pyruvate, 1% minimal essential medium and 1% HEPES (all Merck KGaA). The medium was changed every second day. Pictures of spheroids were taken with a Nikon D3300 camera connected to a Nikon TMS-F 300630 inverted phase-contrast microscope, using a 2× objective. The area of spheroids was measured with Image J software and the data are presented as the percentage of growth, calculated using the following equation:

$$Growth\ (\%) = \frac{\text{area day 0} - \text{area day 0}}{\text{area on day 0}} \times 100$$

Statistical analyses. Statistical analyses were conducted using the Mann–Whitney test, one-way analysis of variance, Tukey's multiple comparisons test and Dunnett's multiple comparisons test, as specified in the legends to the figures.

Results

MCs impair the growth of melanoma spheroids. To create a 3-D environment reminiscent of the *in vivo* milieu found in the parenchyma of melanoma tumors, B16F10 mouse melanoma cells were cultured in a hanging drop system. Under these conditions, the melanoma cells cluster into spheroids (Figure 1A), with features that partly can replicate properties of the tumors found *in vivo* (19, 20). To assess the impact of MCs, spheroids were developed either in the absence or presence of bone marrow-derived MCs, followed by an assessment of spheroid growth. As seen in Figure 1A and B, when MCs were present at a 1:1 ratio to the melanoma cells, spheroid growth was profoundly reduced, hence indicating that MCs have the ability to interfere with melanoma expansion. In agreement with this, measurements of total RNA content revealed that the presence of MCs led to a trend for reduction of the RNA content of melanoma cells (Figure 1C). Next, we asked whether the reducing

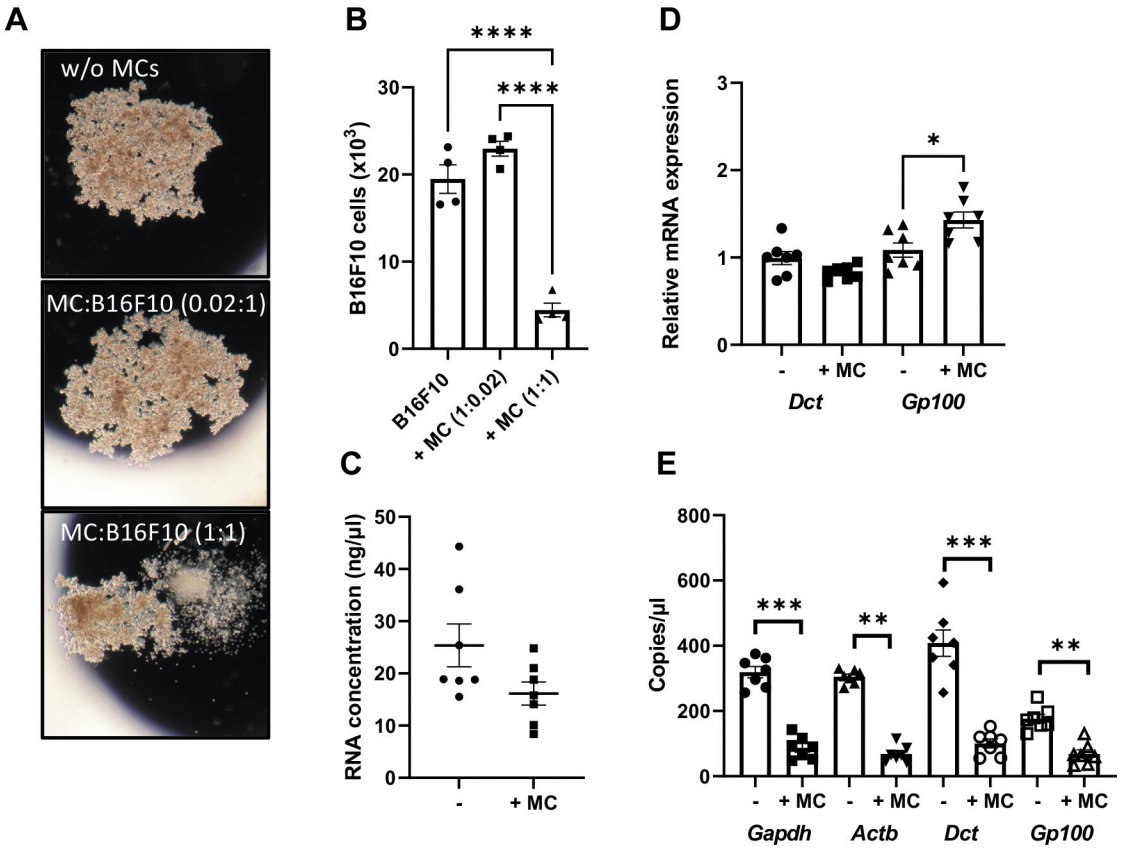

Figure 1. *Mast cells (MCs) inhibit melanoma spheroid growth. Melanoma spheroids were grown for 5 days in the absence or presence of MCs. A: Images of melanoma spheroids formed in hanging drops were taken with an inverted phase-contrast microscope. B: Melanoma cell numbers determined after trypsinization of spheroids, staining with trypan blue and counting the cells using a hemocytometer. Data are representative of two independent experiments and are given as means±standard error of the mean (SEM) (n=4). Differences were determined using one-way analysis of variance, and Tukey's multiple comparisons test. C: Melanoma spheroids, nontreated or treated with MCs (1:1 ratio of B16F10 and MCs), were harvested and total RNA was isolated, and the total RNA concentration was determined. Data shown represent mean values±SEM of two independent experiments (n=7). D: Quantitative polymerase chain reaction analysis for the expression of melanoma-specific genes: L-dopachrome tautomerase (Dct) and glycoprotein (Gp100). Expression of genes was evaluated relative to that of glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and normalized to non-treated spheroids. Results are presented as mean values ± SEM (n=7) of two independent experiments and were analyzed by Mann–Whitney test. E: Expression levels of housekeeping genes [Gapdh and β-actin (Actb)] and melanoma-specific genes (Dct and Gp100) were measured by droplet digital polymerase chain reaction. Results are presented as the mean±SEM (n=7) of two independent experiments and were analyzed by Mann–Whitney test. Significantly different at: *p≤0.05, **p<0.01, ***p<0.001 and ****p<0.0001.*

impact of MCs on melanoma growth was associated with a reduction in the expression of the melanoma-specific genes *Dct* and *Gp100*. However, the expression of *Dct* and *Gp100* normalized to the expression of *Gapdh* as housekeeping gene (and related to the expression in nontreated spheroids) was not affected by the presence of MCs (Figure 1D). When assessing absolute gene expression using ddPCR, we found that MCs caused a substantial reduction in the expression of both *Dct* and *Gp100*. However, the expression of house-keeping genes (*Gapdh* and *Actb*) was suppressed to a similar extent. These data indicate that MCs have the capacity to reduce melanoma spheroid growth and to cause an overall reduction in gene expression in melanoma cells.

Reduced growth of mouse and human melanoma spheroids in the presence of MC-conditioned media. The data above suggest that MCs can have a negative impact on melanoma spheroid growth, and this may potentially require MC-melanoma cell–cell contact or be independent on direct contact between the two cell populations. To approach this issue, we assessed whether factors present in MC-conditioned medium can influence melanoma spheroid growth. Indeed, when melanoma spheroids were developed in the presence of normal culture medium with the addition of MC-conditioned medium (10-50%), melanoma growth was reduced (Figure 2A). To address whether such a dampening effect of MC-conditioned medium on melanoma

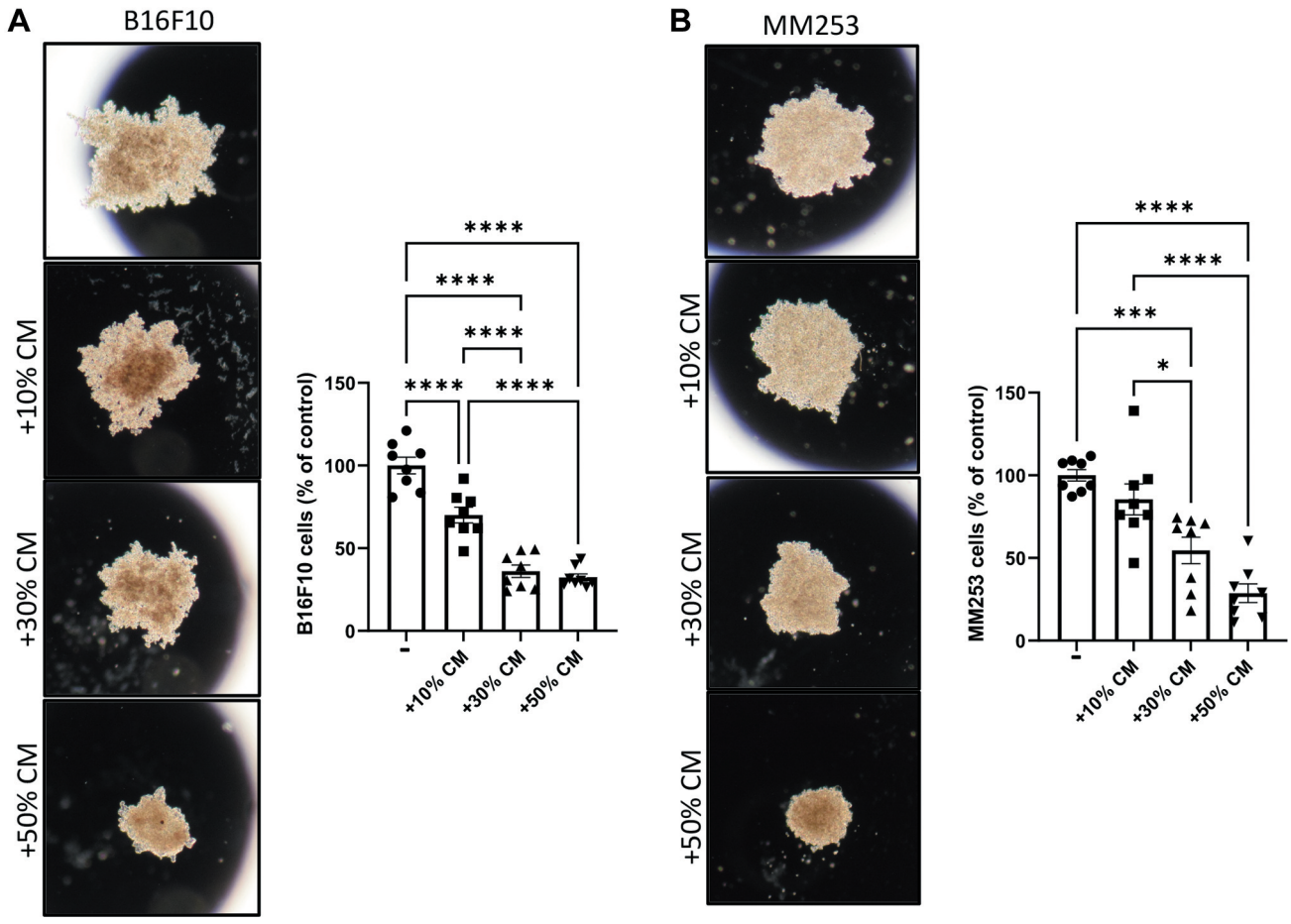

Figure 2. *Reduced growth of mouse and human melanoma spheroids in the presence of mast cell (MC)-conditioned medium. Mouse (A) and human (B) melanoma spheroids were developed for 5 days in the absence or presence of different amounts (10-50%) of conditioned medium (CM) from bone marrow-derived MCs or human skin MCs. Melanoma cell numbers were determined after trypsinization of the spheroids and staining with trypan blue. Data shown represent mean values±standard error of the mean of two independent experiments. One-way analysis of variance, Tukey's multiple comparisons test. Significantly different at: *p<0.05, ***p<0.001 and ****p<0.0001.*

growth can also be recapitulated in a human system, we cultured human melanoma cells (MM253) in the presence of conditioned medium from primary human skin MCs. As can be seen in Figure 2B, growth of human melanoma spheroids was also reduced in the presence of MC-conditioned medium. Altogether, these findings suggest that MCs secrete compounds that have the ability to interfere with the growth of melanoma spheroids.

The negative impact of MCs on melanoma spheroid growth is independent of MC-restricted proteases and serglycin. MCs are highly granulated cells, with various MC-restricted proteases and serglycin proteoglycans accounting for a major fraction of the total granule content (21). To assess whether any of these compounds influence melanoma growth, we developed melanoma spheroids in the presence of wild-type MCs or MCs with either multiple deficiency in MC-restricted proteases [chymase, tryptase, carboxypeptidase A3, (15)] or deficient in serglycin (14). However, the impact of MCs on melanoma growth was not affected by deficiency in these compounds, suggesting that the ability of MCs to impair melanoma spheroid growth is independent of MC-restricted proteases and serglycin proteoglycan (Figure 3).

MC-conditioned medium affects the proliferation of melanoma cells. To provide insight into the mechanism by which MCs affect growth of melanoma spheroids, we assessed the effect of MC-conditioned medium on cell-cycle regulation in melanoma cells recovered from spheroids. As seen in Figure 4A, a substantial fraction of the melanoma cells in non-treated spheroids were in the G_2 phase, *i.e.*, in a proliferating state. However, the fraction of cells in the G_2

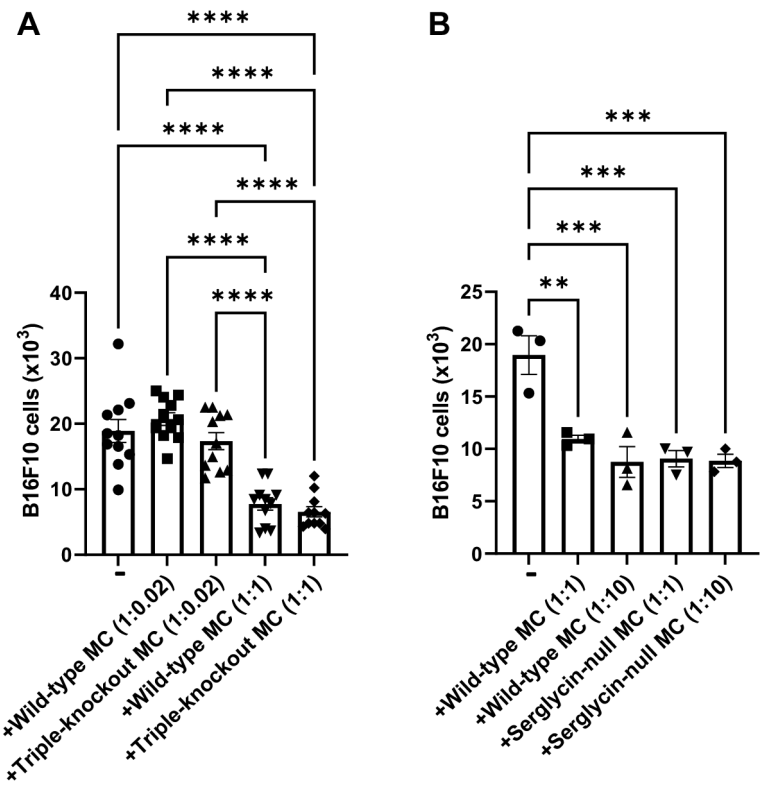

Figure 3. *The suppressing effects of mast cells (MCs) on melanoma spheroid growth is independent of serglycin and of major MC proteases. Melanoma spheroids were developed in the absence of MCs, or in the presence of either wild-type (WT), serglycin-null or triple knockout (mouse MC protease 4-null, mouse MC protease 6-null, carboxypeptidase A3-null) MCs. On day 5, melanoma spheroids were harvested, trypsinized, stained with trypan blue and counted with a hemocytometer. Data shown represent the means±standard error of the mean (n=11) of three independent experiments (A), or representative of two experiments (n=3) (B) and were analyzed by one-way analysis of variance and Tukey's multiple comparisons test. Significantly different at: **p<0.01, ***p<0.001 and ****p<0.0001.*

phase was significantly reduced in the presence of MC-conditioned medium, suggesting reduced proliferation (Figure 4A), and this was accompanied by a corresponding increase in the population of cells in the G_1/G_0 phase. In agreement with this, a reduction of the number of Ki67-positive cells was seen in melanoma spheroids that had been treated with MC-conditioned medium and then transferred to an extracellular matrix (ECM; Matrigel) to preserve spheroid integrity (Figure 4B). Potentially, an increased rate of apoptosis could contribute to the observed reduction of melanoma spheroid growth in the presence of MCs or MC-conditioned medium. To address this possibility, we additionally stained melanoma cells recovered from the spheroids with annexin V and Draq7. This revealed that MC-conditioned medium evoked a modest decrease in the fraction of viable cells, accompanied by a marginal increase in double annexin V/Draq7-positve cells (necrotic/late apoptotic) (Figure 4C), suggesting that the MC-conditioned medium caused minimal cell death of the melanoma cells.

MC-conditioned medium affects the transcriptome of melanoma spheroids. To provide an increased understanding of how MCs can affect melanoma, we next performed a transcriptome analysis to address the effects of MC-conditioned medium on relative gene expression in the melanoma spheroids. For this, melanoma spheroids were grown either in the absence or presence of MC-conditioned medium for periods of up to 5 days, followed by Ampliseq transcriptome analysis. As seen in a multidimensional scaling plot analysis, the transcriptomes of control spheroids and spheroids treated with MC-conditioned medium clustered closely together after 24 h of incubation, indicating minimal effects of MCs on the gene-expression patterns at this time point. In contrast, control and treated melanoma spheroids showed a clear separation after a prolonged culture period (5 days), indicating profound effects of the MC-conditioned medium on gene-expression patterns (Figure 5A). These findings were substantiated by a volcano plot analysis, which confirmed both the minimal effects of MC-conditioned

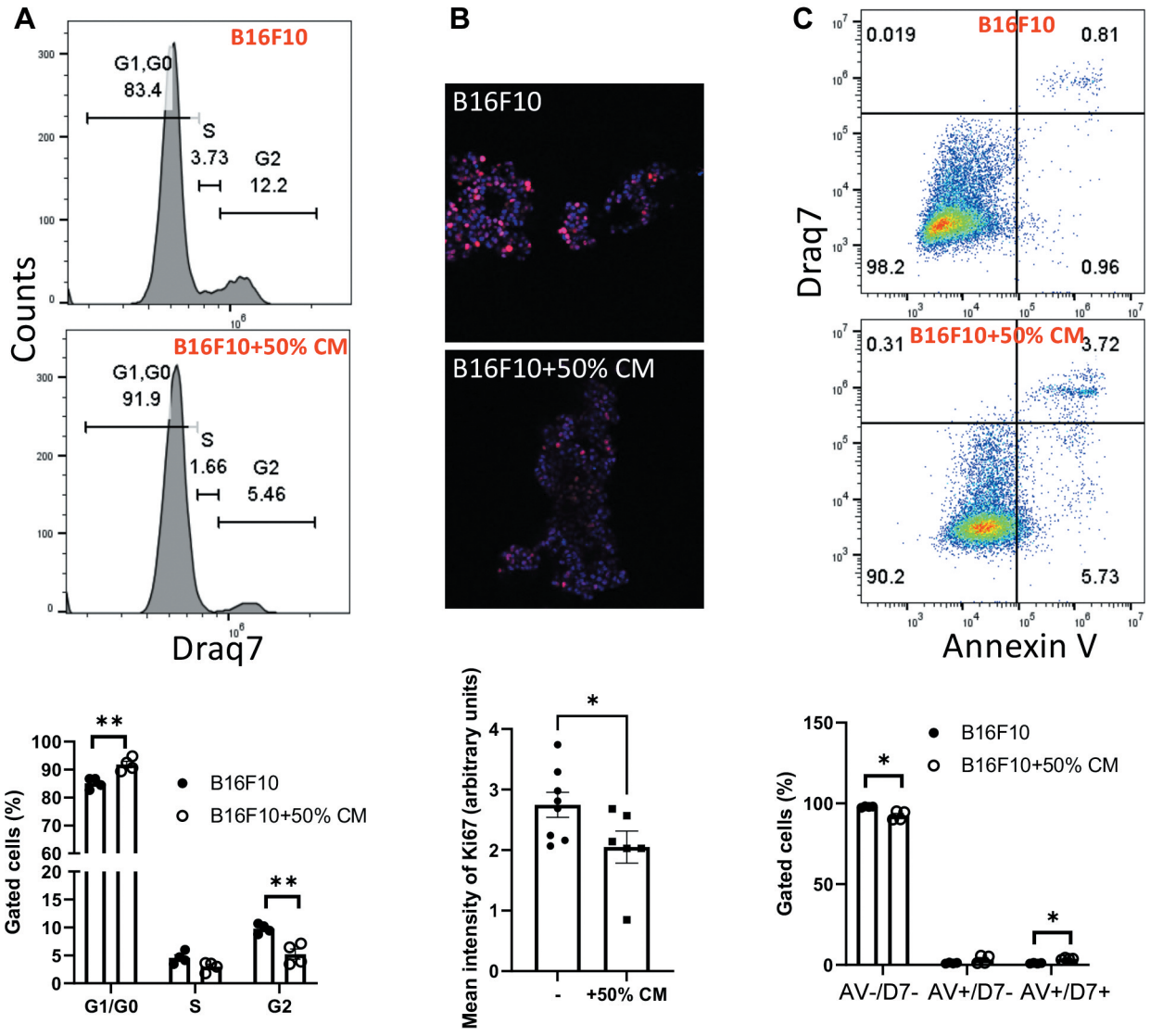

Figure 4. *Mast cell (MC)-conditioned medium affects the proliferation of melanoma cells. A: Melanoma spheroids were developed in the absence or presence of 50% of MC-conditioned media (CM). On day 3, melanoma spheroids were harvested and transferred to Matrigel prior immunostaining for Ki67 and analysis with a confocal microscope (B); trypsinized to a single-cell suspension and either permeabilized, stained with Draq7 and analyzed with a flow cytometer (C) or stained with annexin V and Draq7 (D). Data shown are representative of two independent experiments and are given as means±standard error of the mean (n=4-7). Mann–Whitney test, significantly different at: *p≤0.05 and **p<0.01.*

medium on gene-expression patterns after short-term culture, and the extensive effects seen after longer periods of treatment. As visualized in the volcano plot (Figure 5C), MC-conditioned medium strongly down-regulated the relative expression of numerous genes, but also profoundly up-regulated the expression of others. In total, 401 genes were significantly down-regulated, and 533 genes were significantly up-regulated in response to the MC-conditioned medium. Effects on gene expression patterns were also visualized in a heat map analysis (Figure 5B). Kyoto Encyclopedia of Genes and Genomes pathway analysis of the gene-expression patterns revealed that MC-conditioned medium not only induced up-regulation of cancer-related genes, but also of genes with a role in virus (measles) defense. Up-regulation of genes related to virus defense was also supported by GO Biological Processes analysis, whereas GO Molecular Function pathway analysis revealed an up-regulation of genes involved in protein binding and transferase activity. Among the pathways that were down-regulated by MC-conditioned medium, we noted a preponderance of those related to protein and amino acid metabolism, based on Kyoto Encyclopedia of Genes and

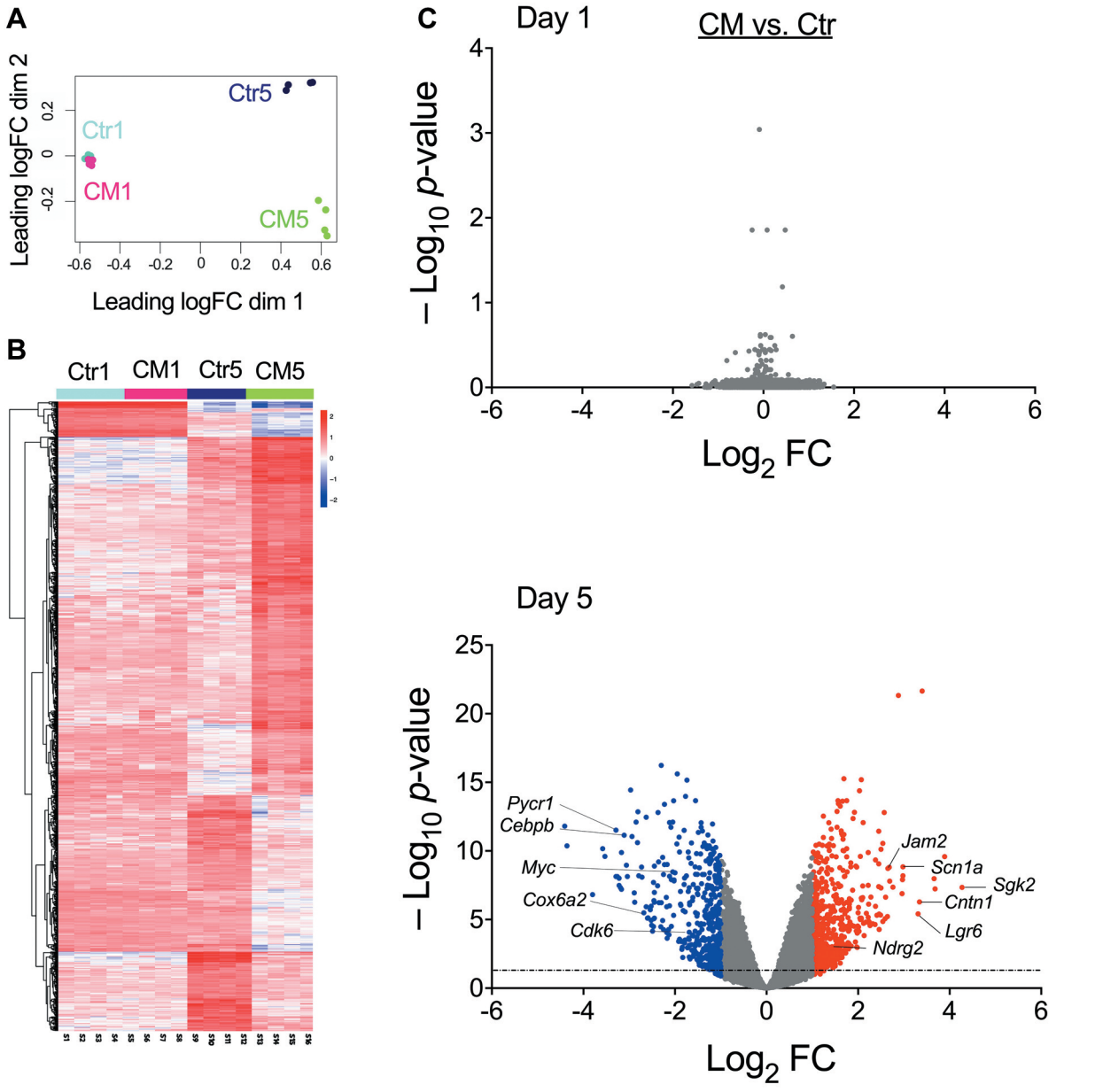

Figure 5. *Continued*

Genomes, GO Biological Process and GO Molecular Function analyses (Figure 5D).

Melanoma spheroids which developed in the presence of MC-conditioned medium displayed increased growth and increased adhesive properties after transfer to an ECM. Fibroblasts are the main producers of ECM, and since fibroblasts were not present in our spheroid system, our cultures had a relatively low ECM content [see also (22-24)]. Hence, in this sense, spheroid systems may mimic the milieu prevailing in the tumor parenchyma. In contrast, the tumor stroma has a substantial content of fibroblasts and is thereby rich in ECM (25, 26), and we next asked whether factors released by MCs can influence the properties of melanoma spheroids in an ECM-rich environment. To address this, we transferred control spheroids and spheroids that had developed in the presence of MC-conditioned medium to Matrigel. Potentially, this replicates a setting in which melanoma cells egress from the tumor parenchyma to the stroma, *e.g.* in the process of metastatic dissemination (26). As seen in Figure 6A, control

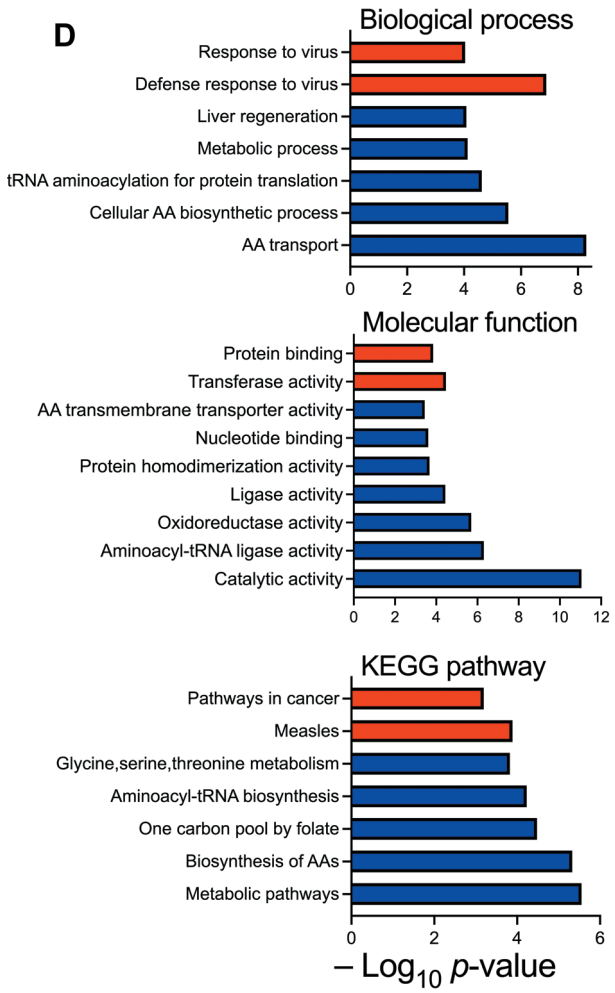

Figure 5. *Effects of mast cell (MC)-conditioned medium on transcriptome of melanoma spheroids. Melanoma spheroids were cultured for 1 or 5 days either alone (Ctr1, Ctr2) or in the presence of MC-conditioned medium (CM1, CM2), followed by Ampliseq transcriptome analysis. A: Multidimensional scaling plot displaying the clustering of samples. B: Heat map displaying hierarchical clustering of differentially expressed genes (DEGs); red represents up-regulation and blue down-regulation. Original data were scaled to have a mean of 0. C: Volcano plots of log2 fold-change (FC) of gene expression after treatment of melanoma spheroids with MC-conditioned medium. DEGs with p<0.05 and absolute FC ≥1 are indicated in red and those with p<0.05 and absolute FC ≤1 are indicated in blue. The horizontal dashed line denotes a cut-off of p<0.05. D: p-Values for Gene Ontology (GO; http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/) pathway enrichment analyses for DEGs in melanoma spheroids treated with MC-conditioned medium versus untreated spheroids. All p-values were adjusted using the Benjamini–Hochberg procedure (p<0.05). AA: Amino acids.*

spheroids were extensively dispersed after their transfer to the Matrigel. In contrast, spheroids that had developed in the presence of MC-conditioned medium clumped tightly together after transfer to Matrigel. To monitor the growth capacities of the control spheroids and spheroids developed in the presence of MC-conditioned medium, the expansion of individual melanoma spheroids was followed over time. As seen in Figure 6B, spheroids developed in the presence of MC-conditioned medium showed a substantially higher growth capacity in Matrigel *versus* spheroids that were developed in the absence of MC-conditioned medium. Moreover, even after prolonged culture in Matrigel, spheroids that originally developed in the presence of MC-conditioned medium clumped much more tightly together in comparison with spheroids developed in its absence, compatible with a higher expression of adhesion molecules.

The role of nutrient starvation effects in the regulation of melanoma spheroid growth in the presence of MC-conditioned medium. Our findings suggest that MC-conditioned medium affects the growth of melanoma spheroids, implying that MCs secrete factors that affect melanoma cells. However, although such effects were seen even at low concentrations of MC-conditioned medium (down to 10% of the growth medium; see Figure 2B), we were unable to completely exclude that our findings might be attributed to the nutrient consumption that might have occurred in the medium that had been conditioned by MCs. To assess this possibility, we replaced the MC-conditioned medium with equal amounts of nutrient-poor buffer (PBS). When assessing the effects of medium supplemented with PBS at various ratios on the melanoma growth, we found that the impairment of melanoma spheroid growth was similar to the effects of MC-conditioned medium (Figure 7).

Discussion

There is a vast amount of documentation showing that MCs are present in a wide range of malignant settings. In particular, a large number of clinical studies have firmly established the presence of MCs in virtually all types of tumors, and there are also numerous studies where MC presence has been correlated to disease outcome (4-8). In addition, a role for MCs in cancer has been documented through studies made in animal models, based on mice that either lack MCs altogether or lack individual MC products (4, 5). However, although these studies collectively have linked MCs to malignant conditions, there is still limited knowledge of how MCs act under such settings. One obstacle for such investigations is the inherent challenge of performing detailed mechanistic approaches in an *in vivo* setting, and for this purpose it can therefore be valuable to develop *ex vivo*/*in vitro* settings that recapitulate prevailing *in vivo* tumor conditions. One strategy for this, which is rapidly gaining increased attention, is to study tumor cells in a 3D context such as spheroids (19, 20), and it has indeed been suggested that such approaches can provide valuable insight into tumor properties in an *in vivo*-like milieu (19, 20).

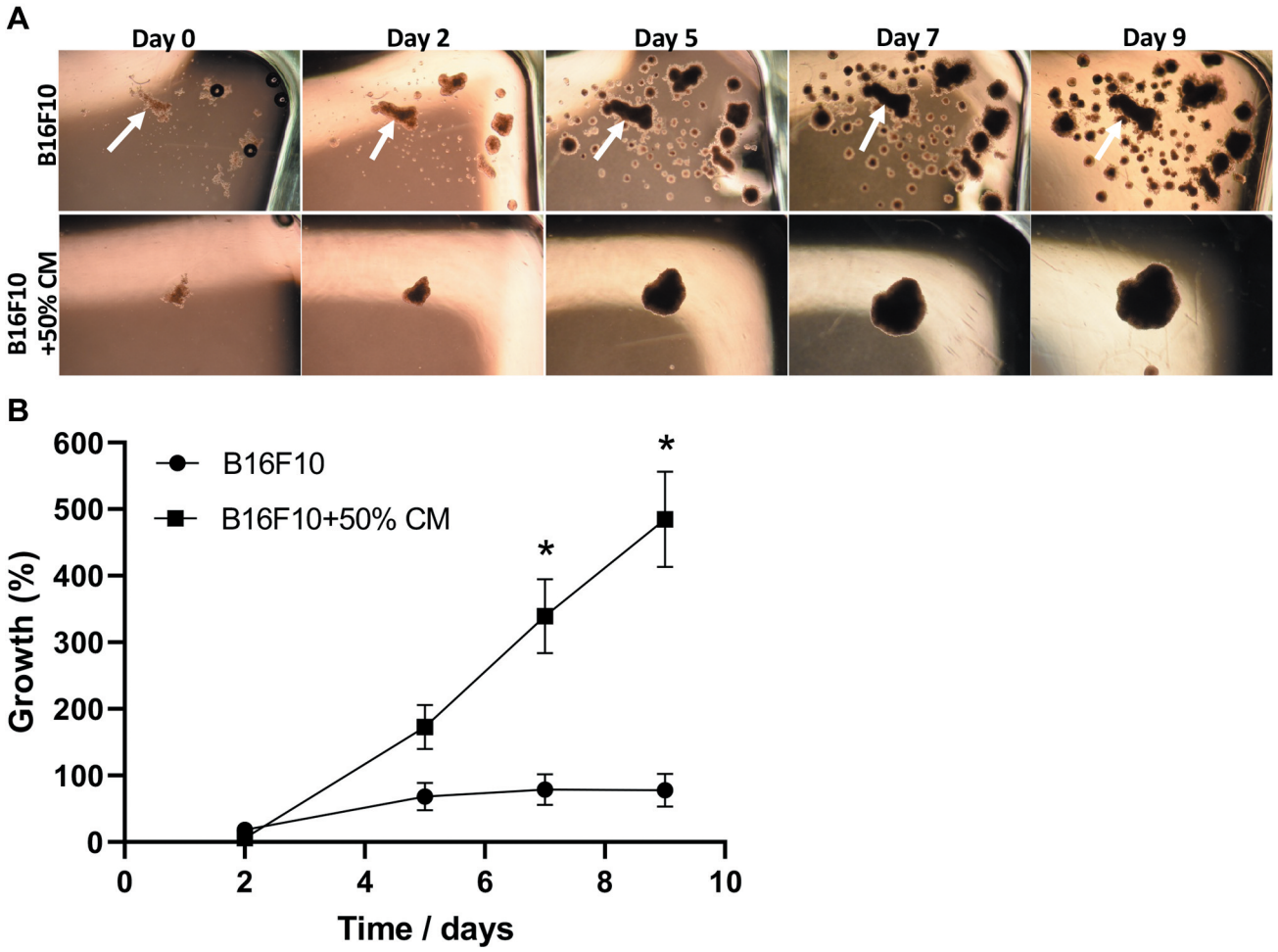

Figure 6. *Enhanced growth in Matrigel of melanoma spheroids developed with mast cell (MC)-conditioned medium. A: Melanoma spheroids were developed in the absence or presence of 50% of MC-conditioned media (CM). After 5 days, spheroids were transferred into Matrigel, covered with medium, and cultured further for 9 days. Growth of individual spheroids was followed over time. Images were taken at the indicated time points, and spheroid size was recorded. Representative spheroids were used for quantification of growth (arrows). B: Quantification of spheroid growth data. Data are representative of three independent experiments and are given as means±standard error of the mean (n=4). Multiple Mann–Whitney tests. Significantly different at: *p≤0.05.*

In this study, we adopted a spheroid system with the aim of studying how MCs can influence melanomas. Our findings reveal that MCs and MC-conditioned media have an apparent dampening effect on melanoma spheroid expansion. Hence, these findings would be in agreement with previous studies where it has been suggested that MCs can have a protective impact in melanoma but are in apparent discrepancy with others where tumor-promoting effects of MCs in melanoma have been suggested (4-8). In an attempt to further dissect the underlying mechanisms, we also assessed for effects on mRNA expression in the melanoma cells. This analysis revealed extensive effects on gene expression patterns after prolonged culture (5 days). When examining gene-expression pathways, we found that numerous genes related to cancer progression were affected. For example, we observed suppression of expression of *Myc,* cyclin-dependent kinase 6 *(Cdk6),* N-myc down-regulated gene 2 (*Ndgr2)* and cystathionine gamma-lyase *(Cth),* all of which are implicated in tumor progression (27-29). Another striking finding was the increased expression of adhesion-related molecules in the melanoma cells. In particular, substantially enhanced expression of contactin (*Cnt1*) and junctional adhesion molecule B (*Jam2*) was seen. Intriguingly, both of these cell adhesion molecules as well as *Cth* have been implicated in metastasis (29-31). Together, these findings could thus raise the possibility that conditioned medium may prime melanoma cells to enhance their capacity for metastatic spreading. In line with this, we noted that melanoma spheroids that had

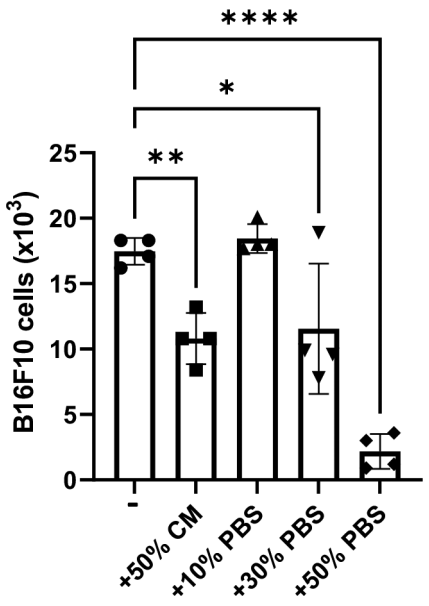

Figure 7. *Nutrient starvation has a similar suppressing effect on melanoma spheroid growth as mast cell (MC)-conditioned medium. Mouse melanoma spheroids were developed for 5 days in the absence or presence of 50% of MC-conditioned media (CM) or different amounts (10-50%) of phosphate buffer solution. Melanoma cell numbers were determined after trypsinization of the spheroids and staining with trypan blue. Data shown represent mean values±standard error of the mean from a representative experiment. One-way ANOVA and Dunnett's multiple comparisons test. Significantly different at: *p≤0.05, **p<0.01 and ****p<0.0001.*

been developed in the presence of conditioned medium had an increased potential for expansion and increased adhesive properties (possibly through their increased expression of adhesion molecules) after transfer to a milieu rich in ECM (Matrigel).

Altogether, one potential conclusion from these findings would be that MCs suppress the proliferation of melanoma cells in a tumor parenchyma-like milieu and that MCs have profound effects on the gene-expression profile of melanomas. However, we also checked the possibility that the observed effects might have been due to nutrient deprivation in medium that had been conditioned by the MCs, rather than to the effects of factors secreted by MCs. Indeed, when performing experiments to directly address this possibility, we saw similar effects on proliferation in melanoma cells that had been exposed to nutrient-poor medium as in cells exposed to MC-conditioned medium. Hence, we cannot exclude the possibility that the observed effects on melanoma spheroid growth were due to nutrient starvation in the conditioned media, rather than due to factors secreted by MCs. Furthermore, we cannot rule out that the effects of MC-conditioned medium on gene-expression patterns in the melanoma spheroids can also be explained by nutrient starvation effects rather than by effects mediated by MCs. The noted effects on melanoma phenotype may be attributed to starvation effects. Notably though, nutrient starvation is a common feature of solid tumors (32), and this study may thus provide insight into the phenotypic switch that may occur in melanomas under nutrient stress.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Authors' Contributions

MG conceived the study, designed, planned, and performed the experimental work, interpreted data, and contributed to the writing of the article; TN performed experimental work and interpreted data; TH contributed to the design of experiments; HW contributed to the design of experiments; ML performed experimental work; AP contributed to the design of the work, interpreted data and contributed to the writing of the article; GP conceived the study, planned and interpreted the experiments and wrote the article. All Authors read the article and provided input.

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