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Tumor risk by tissue engineering: cartilaginous differentiation of mesenchymal stem cells reduces tumor growth

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Summary

Objective: Implantation of autologous chondrocytes (AC) is a promising option for the treatment of cartilage defects, but problems with cell harvesting, dedifferentiation, or the donor age limit the clinical outcome. Mesenchymal stem cells (MSC) gain much interest because of their simple isolation and multipotential differentiation capacity along with their immunosuppressive properties. The latter might introduce tumor manifestation. The influence of undifferentiated and chondrogenically differentiated MSC or AC on tumor growth and metastasis formation was investigated in a murine melanoma model.

Methods: Allogeneic melanoma cells and either syngeneic MSC (C3H10T1/2, transduced with enhanced green fluorescent protein gene) or AC were co-injected at a distance of 3 cm into the contra lateral groins of five mice/group, and evaluated macroscopically and histologically after 4 weeks.

Results: Undifferentiated MSC migrated to the tumor site and induced strong tumor growth and metastasis formation. Even avital MSC promoted tumor growth and spreading, but insignificantly without detectable MSC at the tumor site. Chondrogenically differentiated MSC did not migrate and had a significantly lower impact on tumor growth and spreading; AC had no measurable influence on melanoma cells.

Conclusions: Our data suggest that differentiation of MSC reduces MSC-dependent promotion of latent tumors and that native AC do not introduce any increased risk of tumor growth. The question of how far MSC should be differentiated prior to clinical application should be addressed in further studies.

Key words: Tissue engineering, MSC, Cartilage, ACI, Tumor growth, Cell labeling.

Introduction

Articular cartilage is frequently injured as a result of sports related trauma, but it has very limited capacity for repair due to its avascular nature. Therefore, treatment of focal cartilage defects is very challenging. There are several approaches to manage this problem as it causes pain and functional disability. At present, autologous chondrocyte implantation (ACI) is one of the most favored treatment options[1,2]. Autologous chondrocytes (AC) are harvested from the patients by a cartilage biopsy, expanded *ex vivo* and are reimplanted into the cartilage lesion. This procedure could be shortened and the complication rate might me minimized, if artificial cartilage engineered by mesenchymal stem cells (MSC) is used. A preoperative cartilage biopsy would be no longer necessary. This potential usage of MSC for cartilage repair is intensively investigated in several animal models[3–5].

There are different sources described for MSC, e.g., bone marrow, umbilical cord tissue, peripheral blood and placenta[6–10]. These cells can be separated from other tissues by their adherence characteristics and surface markers, and expanded more than 10^4-fold without loss of their multipotential differentiation capacity[11,12]. MSC are identified by the absence of CD34 and CD45 hematopoietic cell markers. They stain positive for CD90, endoglin/CD105 and vascular cell adhesion molecule-1 (VCAM-1/CD106) and SH3[9,10]. MSC express the major histocompatibility complex (MHC) class I but do not express MHC class II, B7-1/2, CD40 or CD40L molecules. A number of cytokines and regulatory molecules that play important roles in the proliferation and maturation of hematopoietic stem cells is also produced[13,14]. The C3H10T1/2 cell line applied for our experiments is bone marrow derived and has already been characterized regarding its mesenchymal stem cell quality[15].

MSC isolated from bone marrow aspirates have got multipotential differentiation capabilities and are the excellent candidates in tissue engineering (TE), but these cells also exhibit powerful immunosuppressive effects, about 200-fold compared to immunosuppression achieved by common immunosuppressants (e.g., cyclosporine A)[16]. For this reason, MSC are already used for clinical application in treatment of graft versus host disease (GVHD) after bone marrow transplantation with success[17,18]. Primary MSC are capable of homing to the bone marrow and survive in the long term[19,20]. Unfortunately, these systemic immunosuppressive effects could also result in a high risk of tumor manifestation that is found in patients receiving immunosuppressants[21–24]. Some studies could already show a consecutive tumor growth promotion after implantation of undifferentiated MSC in animal models, whereas tumor induction has only been described for embryonic stem cells (ESC)[25–28].

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Therefore, the aim of this study was to clarify whether bone marrow derived MSC (C3H10T1/2 cell line) with or without further cartilaginous differentiation, as well as mature AC utilized for ACI could display side effects favoring tumor growth in a murine melanoma model. This animal model allows to estimate the influence of MSC and other cells on B16 melanoma cell proliferation within the period of 4 weeks, when these cells are injected simultaneously into the contra lateral groins.

Our study is based on a melanoma model which has been described previously: we support findings showing the characteristic high malignancy of B16 melanoma cells by syngeneic subcutaneous inoculation in C57BL/6 mice[29]. Additionally, the baseline of tumor growth in the control group was determined after allogeneic melanoma cell inoculation in C3H/He mice (baseline $\leq$ 30 µl of tumor volume), and subsequently co-injection of allogeneic melanoma cells and undifferentiated MSC showed a strong MSC-dependent increase in melanoma growth and metastases formation, as it has been shown previously[26]. The MSC utilized for our experiments were transduced with enhanced green fluorescent protein (EGFP) gene carrying the retroviral vector pBabePuro[30]. Thus, it was possible to show any migration of MSC to the tumor site and their metastases. Our study shows that chondrogenic differentiation of MSC reduces MSC-dependent promotion of latent tumors, and that native AC (normally used for ACI) do not introduce any increased risk for tumor promotion.

Material and methods

The experiments done in this study were approved by the regional government of Schleswig-Holstein (Germany), department of farming, environment and country places.

ANIMALS

Two different strains of mice were used according to their MHC antigen disparity: C3H/He and C57BL/6. Mice were bred in the central animal husbandry of the University Hospital Schleswig-Holstein, Campus Kiel, Germany. They were housed in the facilities of the Victor-Hensen-Haus and cared for according to the Laboratory Animal Care Guidelines. Thirty two adult animals aged 8–12 weeks were used and distributed among seven experimental groups [five animals (or two)/group, see Table I]. Three mice (C3H/He) aged 2 weeks were used for isolation of AC.

CELL CULTURE

The murine C3H10T1/2 MSC line and the B16-BL6 melanoma cell line were kindly given by Mrs. Danièle Nöel (INSERM U475, Montpellier, France). These cells were each cultured in complete Dulbecco Modified Eagle Medium (DMEM, Sigma, Germany) supplemented with 10% fetal calf serum (FCS, Seromed, Germany), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml amphotericin (Seromed, Germany). Any *in vitro* contamination of the different cells lines was avoided.

EGFP-GENE TRANSDUCTION IN MSC

We successfully performed the stable fluorescent labeling of the MSC with a retroviral system (pBabePuro). The retroviral vector consists of an internal ribosome entry site (IRES) element, an EGFP gene and a puromycin resistant gene for later selection of effectively transduced MSC. MSC were best transduced after incubation with the retroviral particles for 2 days. Not transduced cells were killed with 300 µg/ml puromycin (Seromed, Germany) within 4 days. MSC cell clones with high EGFP production were selected after punctual trypsinization from the monolayer culture (>99% EGFP-positive cells) and expanded for the animal experiments [Fig. 1(A, B)].

CARTILAGINOUS DIFFERENTIATION OF MSC AND RT-PCR

For cartilaginous differentiation 750,000 EGFP-gene-transduced C3H10T1/2 MSC were cultured in three-dimensional high density pellets at the bottom of an Eppendorf cap (Sarstedt, Germany) as described by Kurz *et al.*[31]. The differentiation medium contained the chondrogenic differentiation supplements: 0.1 mM dexamethasone and 10 ng/ml murine transforming growth factor (TGF-ß1) (Sigma, Germany). These pellets were exposed to 5% oxygen over a period of 10 days under otherwise normal culture conditions. The morphogenic genotype of the cells was confirmed by RT-PCR for aggrecan and collagen type II. Expression of collagen type II was negative in undifferentiated MSC, while there was a clear signal in cartilaginous differentiated MSC as well as in AC [Fig. 1(C)]. The EGFP production by MSC was not changed after cartilaginous differentiation (not shown). Prior to injection the cells were isolated from the pellets by collagenase treatment and subsequent filtration through a nylon mesh; cell viability and number were evaluated as described below.

RNA was isolated from the cell cultures using the Qiagen RNeasy Mini-Kit according to the manufacturer's instructions. Lysed samples were homogenized using the QIAshredder spin columns (Quiagen). Isolated RNA was determined for quantity and quality spectrophotometrically (260 and 280 nm). cDNA was

Table I

Statistical analysis of the detected primary tumor volumes 4 weeks after treatment using the student's t *test. The group B16-ALLO/- is the reference group with allogeneic mice (ALLO: C3H/He) and B16-BL6 melanoma cells: tumors with a volume of up to 30 µl had been found in this group and therefore been defined as background with a tumor incidence of 0%. The promotion of tumor growth was highly significant in syngeneic animals (SYN: C57BL/6) and in allogeneic animals (ALLO) receiving syngenic undifferentiated MSC (isolated from C3H/He). Cartilaginous differentiation of MSC (MSC-C) decreased the promotion of tumor growth, and even more the injection of MSC-AV. The impact of differentiation was clearly observed when native autologous chondrocytes (AC: isolated from C3H/He) were co-injected into the murine melanoma model. EGFP: cells were transduced with EGFP gene. Non-transduced MSC that were labeled with the fluorescent cell-tracker CM-DiI also favored tumor growth and metastasis formation. SD = standard deviation*

	Group name	Tumor volume [µl], $n = 5$ animals/group					Mean volume [µl]	SD [µl]	Incidence [%, based on tumor volume]		Student's *t* test ($\alpha < 0.05$)
		1	2	3	4	5			Tumor	Metastasis	*P* value
Injection of B16 melanoma cells alone	B16-ALLO/-	12	30	0	0	0	18	13	0	0	Control
	B16-SYN/-	112	178	96	150	148	137	33	100	100	<0.0001
Co-injection of B16 melanoma cells with MSC	B16-ALLO/ MSC-EGFP	105	250	200	186	60	160	76	100	80	<0.003
	B16-ALLO/ MSC-EGFP-AV	32	74	0	16	0	24	31	40	40	0.316
	B16-ALLO/ MSC-C-EGFP	60	105	36	48	30	56	30	80	0	0.012
	B16-ALLO/ MSC-CM-DiI	96	68				82	20	100		
Co-injection of B16 melanoma cells with AC	B16-ALLO/ AC	22	7	16	0	0	9	10	0	0	0.937

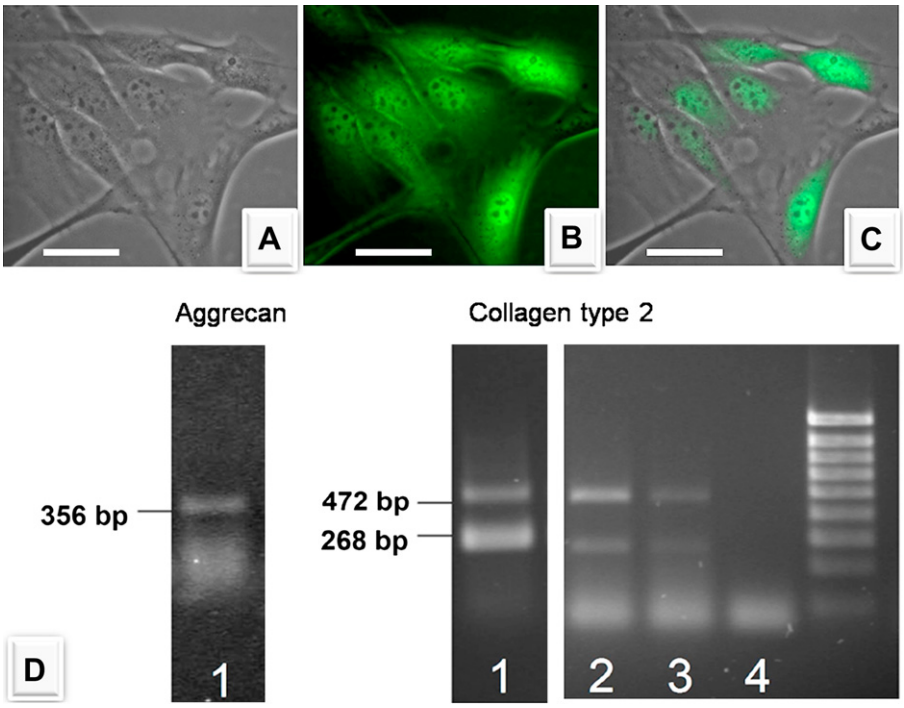

Fig. 1. A, B, and C: EGFP-gene-transduced MSC in monolayer culture after selection. A: phase contrast microscopy, B: fluorescence microscopy of the same cells, C: overlay of A + B. D: RT-PCR gel showing aggrecan and collagen type II-specific bands: characterization of native chondrocytes (1), cartilaginously differentiated MSC (2 and 3) and undifferentiated MSC (4) prior implantation into the animals for expression of collagen type II (268 bp and 472 bp) and aggrecan (shown for native chondrocytes, 356 bp).

generated using the QIAGEN OneStep RT-PCR Kit according to the manufacturer's instructions. For PCR the following bovine primers were used (0.5 g μM): collagen type II (Sense: GATCTGCACTGAATGGCTGA, Antisense: TCTGCCCAGTTCAGGTCTCT, product: 472 bp, annealing temperature: 62°C), aggrecan (Sense: AGGAGACCCAGACAGCAGAA, Antisense: ACAGTGACCCTGGAACTTGG, product: 356 bp, 64°C). RT-PCR reaction conditions were: reverse transcription 30 min, 50°C; initial activation step 15 min, 95°C; 3-step cycles: melting 30 s 94°C, annealing 60 s primer specific temp. (59–64°C, see above); a final extension step 10 min 72°C. Amplified products were separated on a 2% agarose gel, visualized fluorometrically and digitised.

THE ISOLATION OF AUTOLOGOUS CHONDROCYTES (AC)

Samples of cartilage from the knee joints epiphysis of 2 weeks old mice (C3H/He) were submitted to a standard collagenase digestion process according to the protocol of Kurz *et al.*[32]. The cell yield of chondrocytes was about 12×10^4 per mouse. Isolated chondrocytes were seeded in a density of 100,000/cm^2 and cultured for up to 10 days in monolayer for expansion in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml amphotericin, and passaged one to two times in order to receive a substantial amount of cells. The differentiation of the cells as chondrocytes was verified using RT-PCR for extracellular matrix molecule mRNA of collagen type II and aggrecan. AC were washed with phosphate buffered saline (PBS), trypsinized, centrifugated by a speed of $300 \times g$ during 5 min and resuspended in PBS for cell counting and injection into mice.

CELL STAINING WITH CM-DIL

Stock solution of the fluorescent cell-tracer CM-Dil (Molecular Probes, Eugene, OR) was reconstituted at a concentration of 1 μg/μl in dimethylsulfoxide (DMSO). Cells were trypsinized, washed with PBS, and resuspended at the concentration of 10^7 cells/5 ml in 2 μg CM-Dil/ml PBS, prepared extemporarily. Cells were labeled by an incubation at 37°C for 5 min followed by 15 min at 4°C, in the dark. Unincorporated fluorescent dye was then removed by centrifugation at 300 g for 5 min and two washes in PBS. Cells were resuspended in PBS and maintained at 4°C until injection.

THE MURINE MELANOMA MODEL

B16 melanoma cells, MSC and AC were each prepared as a single-cell type suspension in separate tubes (5×10^5 cells in 100 μl PBS). The cell count and vitality (>90% vitality) was assessed by trypan blue exclusion with the help of the Neubauer chamber shortly before application. Subcutaneous application of the B16 melanoma cells was always performed separately into the right groin of the animals. The MSC or AC were given also subcutaneously as a co-injection at a distance of about 3 cm into the left groin. This means that melanoma cells on one side and MSC or AC on the other side where two distinct cell preparations injected in the same mouse, but not as a mixed preparation. Mice were examined two times a week and tumor incidence was evaluated by inspection and palpation. After 4 weeks animals were sacrificed and tumors and metastases were recovered. The length and diameter of melanoma tumors were measured in order to calculate tumor volumes prior to the histological analysis.

HISTOLOGY

Tumor samples and samples of some major organs (inguinal skin, brain, bone, lung, spleen, stomach, kidney, liver, peritoneum and gut) were fixed in 4% formaldehyde solution for several days and then processed for routine histology. Paraffin-embedded tissue sections (5 μm) were rehydrated and either stained with hematoxylin–eosin (HE) before examination or mounted in fluorescent mounting medium (Dako, Germany) for fluorescence visualization of EGFP-transduced cells.

STATISTICAL ANALYSIS

In this study, results are given as absolute values, mean values and standard deviation. For statistical analyses the Student's *t* test (two-tailed, unequal variance) was performed with the software program "XLSTAT 2008". A value of $P < 0.05$ was considered significant.

Results

THE B16 MELANOMA CELLS ARE VERY MALIGNANT IF INOCULATED IN SYNGENEIC ANIMALS

To demonstrate the vitality and malignancy of B16 melanoma cells 5×10^5 of these cells were injected into the right groin of the mice. The tumor incidence in syngeneic mice was 100% just after 2 weeks by palpation (Fig. 2). B16

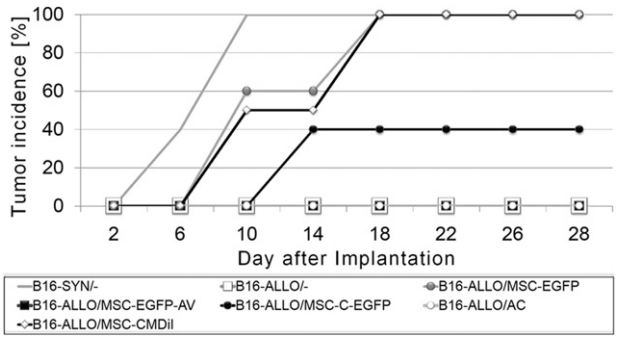

Fig. 2. Palpated tumor incidence. Five animals/group were treated according to the murine melanoma model with either one injection of 5×10^5 B16 melanoma cells into the right groin or an additional co-injection of 5×10^5 MSC or AC into the contra lateral groins. They were examined twice a week for palpable tumors. Tumor development was rapid in syngeneic animals (B16-SYN/-), where the animals had to be sacrificed already after 2 weeks because of suffering. Palpable tumors were also found in animals receiving co-injection of undifferentiated MSC (B16-ALLO/MSC-EGFP) after 10 days and in two cases receiving cartilaginously differentiated MSC (B16-ALLO/MSC-C-EGFP) after 14 days, whereas no tumor was palpable in the control group (B16-ALLO/-), or in case of co-injection of MSC-AV (B16-ALLO/MSC-EGFP-AV) or native chondrocytes (B16-ALLO/AC). EGFP: cells were transduced with EGFP gene. Non-transduced MSC that were labeled with the fluorescent cell-tracker CM-DiI also favored tumor growth and metastasis formation.

melanoma cells rapidly developed tumors and metastases in syngeneic C57BL/6 mice, because they are poorly immunogenic. The animals had to be sacrificed after 2.5 weeks since they seemed to suffer much from the melanoma tumor and its metastases. There were many large metastases formations as described for lymphatic and cavernous tumor cell spread, although the mean tumor volume was only 137 μl at that time point (Table I and Fig. 3). The incidence of metastases formation was 100%. There were also 1−2 mm large subcutaneous satellite metastases.

THE B16 MELANOMA CELLS ARE REJECTED BY ALLOGENEIC MICE

Allogeneic animals were able to reject B16 melanoma cells (5×10^5) injected into the right groin. There were no metastases in tissue samples of these animals and subcutaneous primary tumors had a maximum tumor volume of $\leq$30 μl after 4 weeks. Therefore, the baseline for tumor growth was set to >30 μl and the tumor incidence was defined as 0%, if the primary tumors were not bigger than the baseline volume (Table I and Fig. 3).

THE B16 MELANOMA CELLS ARE NOT REJECTED BY ALLOGENEIC MICE WHEN CO-INJECTED WITH UNDIFFERENTIATED MSC

In the presence of undifferentiated MSC co-injected into the contra lateral groin at a distance of about 3 cm the tumor volume increased significantly ($P < 0.003$ for ALLO/MSC-EGFP; Table I). The incidence of tumor growth or metastases formation was 100%. Even in two mice which were treated with un-transduced but CM-DiI-labeled MSC the MSC promoted tumor growth, indicating that tumor progression was not an unspecific result of cell transduction (Table I). The EGFP-gene-transduced or CM-DiI stained MSC were detectable at the tumor site and within its metastases under the fluorescence microscope indicating migration of the MSC (Fig. 4). All primary tumors and all metastases in these experimental groups demonstrated a strong infiltration by labeled MSC, although the MSC were primarily located at the edges of the tumors. We did not see any migration of these cells into other tissue samples lacking metastases.

We also investigated whether avital (AV) lyophilized MSC exhibit the same effects. After destruction of the migration ability (there were no MSC visible at the tumor site, Fig. 4), the mean tumor volume declined to 24 μl (Table I). However, in two cases tumor growth exceeded the baseline of 30 μl tumor volume and metastases formation was detectable. Therefore the incidence of tumor growth and metastases formation was 40%, although promotion of tumor growth by avital MSC (MSC-AV) was insignificant ($P = 0.316$). The little but measurable positive effect on tumor growth and metastasis formation could probably be attributed to soluble factors of MSC.

THERE IS NO SIGNIFICANT INCREASE IN B16 MELANOMA TUMOR GROWTH AFTER CO-INJECTION OF CARTILAGINOUSLY DIFFERENTIATED MSC

In this set of experiment we co-injected 5×10^5 melanoma cells and 5×10^5 cartilaginously differentiated MSC subcutaneously into the contra lateral groins. At the end of the experiment, the incidence of tumor growth was still 80% (due to the definition that every primary tumor which exceeds the volume of 30 μl is defined as tumor formation), but for metastases 0% (Table I). However, the mean tumor volume was 56 μl only, and compared to the group of animals receiving undifferentiated MSC (mean tumor volume 160 μl) the tumor volume was significantly diminished ($P = 0.022$). Additionally, no cartilaginously differentiated MSC were visible at the tumor site, indicating loss of migration properties (Fig. 4).

THERE IS NO SIGNIFICANT PROMOTION OF B16 MELANOMA TUMOR GROWTH WHEN CO-INJECTED WITH AC

With this set of experiments we investigated whether AC have an influence on tumor growth or metastasis formation as it is demonstrated for undifferentiated MSC in this study. AC were successfully isolated from femur epiphyses of 2-week-aged mice. The cartilage characteristics were shown by RT-PCR. In contrast to undifferentiated MSC the cells were positive for collagen type II [Fig. 1(C)] and aggrecan mRNA. The mice received a co-injection of 5×10^5 B16 melanoma cells with 5×10^5 AC into the contra lateral groins. At the end of the experiment, the incidence of both, tumor growth and metastases, was 0%. The mean tumor volume was 26 μl and similar to that of the control group with only allogeneic B16 melanoma cell inoculation and therefore below the baseline of the promotion of tumor growth (Table I and Fig. 4).

Discussion

In our study we explored the potential promotion of tumor growth and metastasis formation from latent melanoma cells introduced by MSC or AC in a murine melanoma model. Both cell types are already utilized in regenerative medicine: the latter for repair of cartilage defects as used in ACI[1,2] and undifferentiated MSC for the treatment of GVHD, transplant rejection and autoimmune diseases because of their potent immunosuppressive effects[16–18,33–36]. Unfortunately, these side effects of MSC could display the promotion of latent malignant tumors under certain

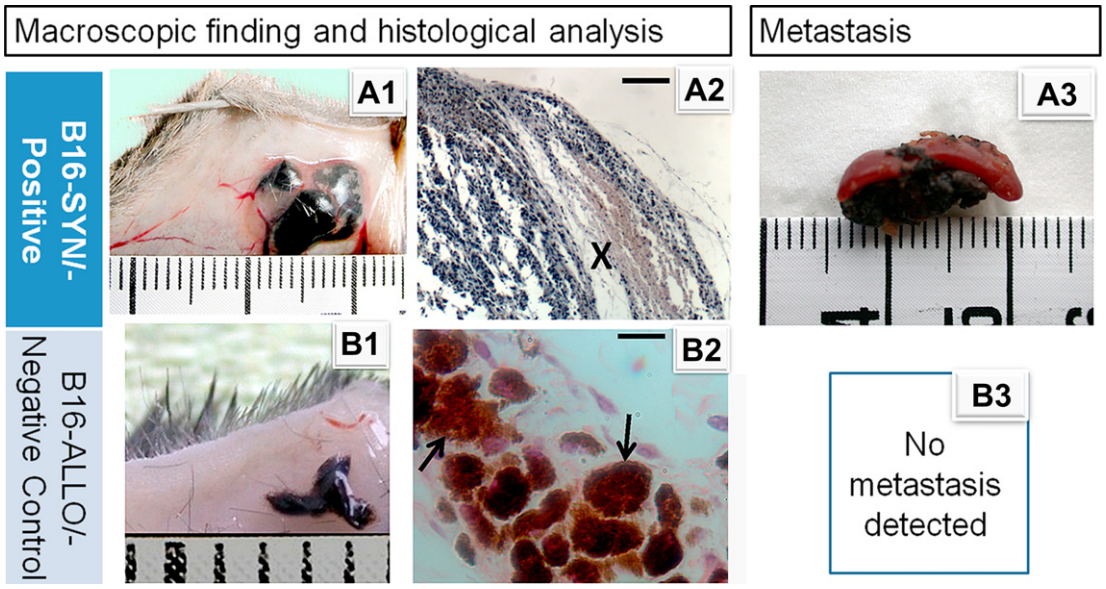

Fig. 3. Macroscopic and histological findings in the control groups. A1, B1, and A3 show scales for mm. (A1–A3) Large tumors and metastases were recovered in animals of the positive control (B16-SYN/-) that was carried out to explore the high malignancy of B16 melanoma cells in syngeneic mice. These animals were treated with subcutaneous injection of 5×10^5 B16 melanoma cells into the right groin. (A1) A large primary tumor is visible in the subcutaneous tissue. (A2, bar = 160 μm) The histological analysis shows part of an isolated round tumor with necrotic zones (X) which indicate high expansion of the tumor cells. (A3) A spleen metastasis is shown exemplarily. (B1–B3) In the negative control (B16-ALLO/-) allogeneic animals were treated with subcutaneous injection of B16 melanoma cells. (B1) A small subcutaneous melanoma cell cumulus is visible. (B2, bar = 20 μm) Cells in the tumor are plump and filled with large amount of melanin pigments (arrows). (B3) No metastasis was detectable in the negative group.

circumstances and accelerate their manifestation in MSC-transplanted patients as noticed after administration of immunosuppressants. The tumor risk for immunocompromised patients is set 90-fold for lymphoma, 37-fold for malignant melanoma and 14-fold for cervical cancer[21–24]. One should also recapitulate case reports that showed spontaneous tumor regression in immunocompetent patients with prior diagnosis of bronchial carcinoma, gut tumors, malignant melanoma or breast cancer[37–41]. For review see Blattman and Greenberg[42].

Tumor growth promotion by undifferentiated MSC has been demonstrated in different animal models[21,22]. The goal of this study was to estimate the influence of cartilaginously differentiated MSC as well as the influence of differentiated AC on B16 melanoma tumor growth *in vivo*. Prior to administration in the animal experiments, the MSC were labeled with EGFP-gene transduction *via* the retroviral vector pBabepuro or labeled with CM-Dil (Chloromethylbenzamido derivative, Invitrogen) as described in prior studies[12]. We did not see any morphological or proliferative changes of these cells after transduction or CM-Dil staining. EGFP expression was stable *in vitro* as well as *in vivo*. We preferred EGFP cell labeling because of its stability, good detectability in histological tissue samples and no adverse effects at the side of injection. In comparison, animals receiving CM-Dil-labeled MSC displayed a dermatitis at the injection site. However, it cured over a period of 2 weeks without any treatment. The detection of CM-Dil-labeled MSC was very difficult because of autofluorescence and weak CM-Dil-fluorescence. It can be therefore concluded that EGFP-gene transduction of MSC improves the conditions for both cell application and subsequent detection of the labeled cells.

In the murine melanoma model, we first investigated whether undifferentiated MSC from passages three to five promote tumor growth if co-injected subcutaneously with melanoma cells at a distance of about 3 cm into the contra lateral groins of the animals. At the end of the experiment, we noticed that undifferentiated MSC favored the tumor growth significantly ($P < 0.003$) and were detectable at the tumor site and even inside the metastases. Our results are in concordance with prior studies that explored the tumor risk for MSC in diverse animal models[25,26]. Additionally, we explored whether maturation of MSC could influence the promotion of tumor growth. After cartilaginous differentiation of the MSC tumor growth favoring was significantly diminished ($P = 0.022$), and there were no EGFP-gene-transduced MSC detectable inside or close to the subcutaneous tumors. This suggests that differentiation of MSC reduces the ability of the cells to migrate and to promote tumor growth. These results, especially a significant promotion of tumor growth in the case of non-EGFP-transduced, CM-Dil-labeled MSC indicate that any immunomodulation by EGFP-gene transduction reported in further studies is non relevant in this tumor model[43,44].

However, it has to be mentioned that differentiation of the cells might have influenced the cell stability or viability (even though the latter had been evaluated prior to injection). The reduced influence of differentiated MSC in this study could therefore be not only the result of differentiation but also reduced cell activity or viability.

We therefore used AV lyophilized undifferentiated MSC to find out whether vitality and the ability to migrate are essential for promotion of the tumor growth. To our knowledge, we are the first group using MSC-AV in a murine melanoma model. We could show that even MSC-AV seem to promote tumor growth and metastases formation, even though insignificantly ($P = 0.316$). The residual positive effect could be referred to soluble factors within the lyophilisate administered subcutaneously to the animals. One should consider that the immunosuppressive effects of

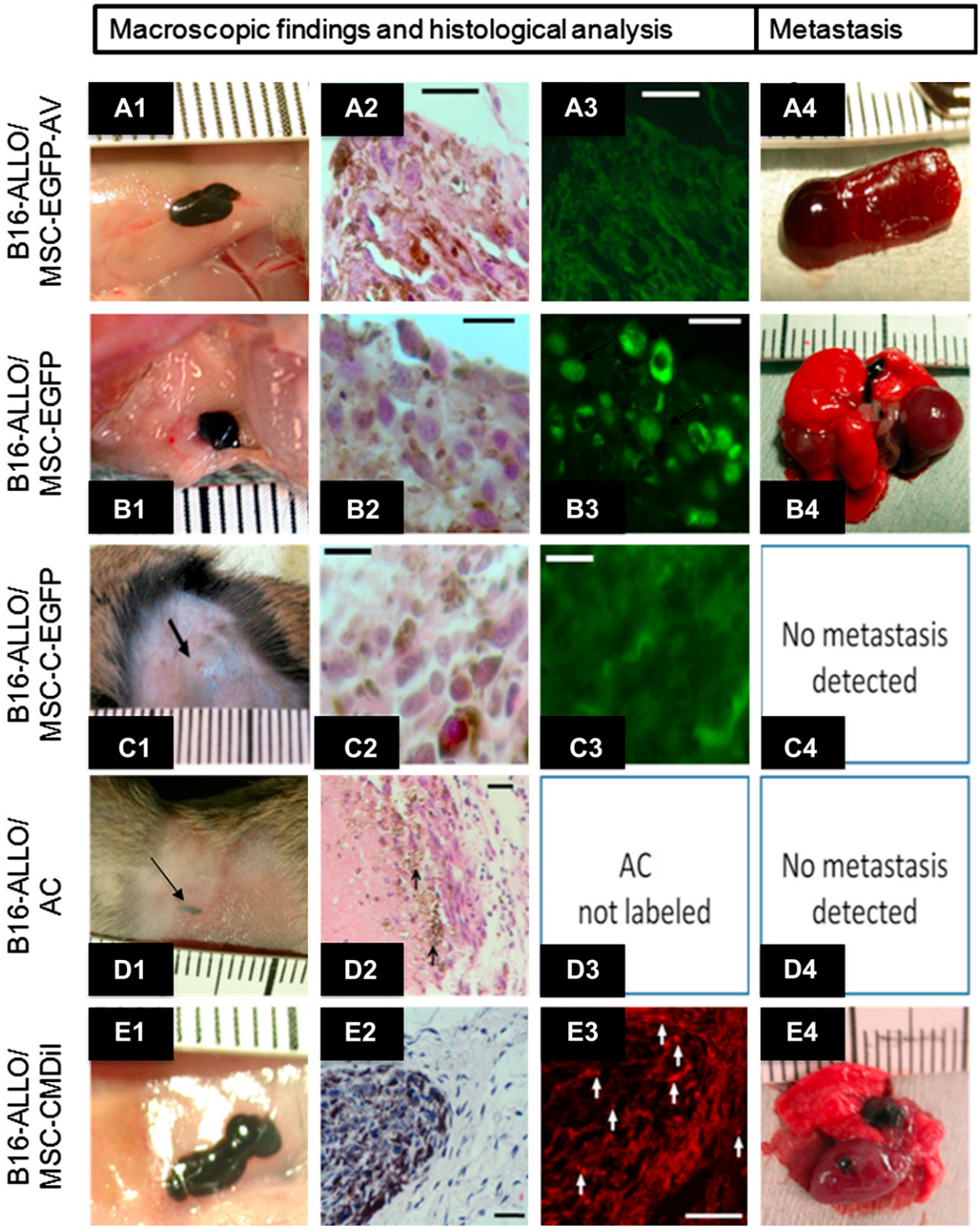

Fig. 4. Macroscopic and histological findings of tumor growth after 4 weeks of treatment in the allogeneic (ALLO) mouse melanoma model. A1−E1, A4, B4 and E4 show scales for mm. Pictures A−E1 show examples of primary tumors in the subcutaneous tissue, A−E2 and A−E3 show corresponding tissue areas of isolated tumors with HE staining (2) or fluorescence microscopy (3), pictures A−E4 show examples of metastases, if present. (A1−A4) Two of five animals receiving 5×10^5 MSC-AV together with 5×10^5 B16 melanoma cells developed tumors and metastases. (A2−A3, bars = 25 μm) No EGFP-gene-transduced MSC are visible at the tumor side. (A4) A spleen metastasis is shown in the circled area. Similar tumor growth promotion and migration behaviour was found for non-transduced CM-Dil-labeled undifferentiated MSC when co-injected with B16 melanoma cells (E1−E4, bars = 20 μm). (B1−B4) Animals treated with vital MSC and B16 melanoma cells show primary tumors in five of five cases and metastases in four of five cases. (B2−B3, bars = 20 μm) EGFP-positive MSC (arrows) are detectable in tissue samples of the primary tumor. (B4) A lung metastasis is demonstrated in the circled area. (C1−C4, bars = 20 μm) Animals receiving co-injection of cartilaginously differentiated MSC (MSC-C) showed small primary tumors in four of five animals without any indication of MSC migration to the tumor site or metastasis formation. (D1−D4) No metastases were recovered in animals treated with native autologous chondrocytes (AC) and melanoma cells in the presence of remarkable minimal primary tumors similar to that of the control group. (D2, bar = 20 μm) Arrows show melanin pigments.

MSC have been attributed to inhibition of T-cell proliferation and generation of $CD8^+$ regulatory cells by secretion of soluble factors in cocultures[31]. However, the exact mechanism of the immunosuppressive effects is still unknown. It should also be considered that vital undifferentiated MSC might proliferate in contrast to lyophilized MSC. Therefore, promotion of tumor growth by MSC-AV might be reduced because of a decreased amount of relevant factors.

In addition, we explored whether AC exhibit similar effects on tumor promotion when administered in the murine melanoma model as described above for MSC. In animals receiving AC, we did not notice any significant promotion of tumor growth compared to the control group treated with allogeneic B16 melanoma cells without co-injection ($P = 0.937$). Thus, we were able to show that AC, which were passaged one to two times do not promote tumor growth and that promotion of tumor growth is a phenomena observed for MSC, especially for undifferentiated MSC.

The results of this paper assume that there is a possible risk of promotion and manifestation of latent malignant tumors after administration of MSC, even locally. These effects are probably related to the low maturation grade or proliferation capacity of these cells. Therefore, the differentiation procedures of MSC and the mechanism of tumor growth promoted by MSC should be extensively explored as presumption for functional tissue produced by TE. Since C3H10T1/2 is a MSC line generated from mouse embryos, it is suggested that further studies should demonstrate the influence of adult MSC on tumor growth of latent tumors dependent on the status of differentiation. Passaged AC, on the other hand, did not show any promotion of tumor growth and do therefore not introduce any latent risk of tumor manifestation in transplanted patients. However, the cells used here still showed some collagen type II mRNA expression. AC, that are often used for treatment of cartilage defects (e.g., ACI), may sometimes be completely dedifferentiated, and the impact of this dedifferentiation should be investigated in further studies.

Conflict of interest

The authors have no conflicts of interest.

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