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Adipose stromal cell targeting suppresses prostate cancer epithelial-mesenchymal transition and chemoresistance

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Abstract

Fat tissue, overgrowing in obesity, promotes the progression of various carcinomas. Clinical and animal model studies indicate that adipose stromal cells (ASC), the progenitors of adipocytes, are recruited by tumors and promote tumor growth as tumor stromal cells. Here, we investigated the role of ASC in cancer chemoresistance and invasiveness, the attributes of tumor aggressiveness. By using human cell co-culture models, we demonstrate that ASC induce epithelial-mesenchymal transition (EMT) in prostate cancer cells. Our results for the first time demonstrate that ASC interaction renders cancer cells more migratory and resistant to docetaxel, cabazitaxel, and cisplatin chemotherapy. To confirm these findings in vivo, we compared cancer aggressiveness in lean and obese mice grafted with prostate tumors. We show that obesity promotes EMT in cancer cells and tumor invasion into the surrounding fat tissue. A hunter-killer peptide D-CAN, previously developed for targeted ASC ablation, suppressed the obesity-associated EMT and cancer progression. Importantly, cisplatin combined with D-CAN was more effective than cisplatin alone in suppressing growth of mouse prostate cancer allografts and xenografts even in non-obese mice. Our data demonstrate that ASC promote tumor aggressiveness and identify them as a target of combination cancer therapy.

Introduction

Despite numerous therapies aimed at malignant and vascular cells, resistance of many types of cancer to treatment remains a challenge. Progression of many carcinomas is associated with obesity, a medical condition resulting from expansion of white adipose tissue (WAT) [1]. A growing body of evidence indicates that WAT overgrown in obesity, rather than the lifestyle responsible for obesity onset, is linked with cancer progression [2]. Studies in mouse models have shown that overgrowth of WAT, which becomes inflamed, fibrotic, and dysfunctional in obesity [3], is sufficient to enhance cancer progression irrespective of diet

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Compliance with ethical standards

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[4]. In patients, abdominal adiposity and in particular overgrowth of periprostatic WAT are associated with aggressiveness of prostate cancer (PCa). Indeed, tumor invasion into surrounding WAT, is a determinant of PCa recurrence after treatment [2].

Adipocytes, the lipid-storing cells of WAT, differentiate from mesenchymal progenitors termed adipose stromal cells (ASC). These fibroblastic mesenchymal stromal cells (MSC) contribute to tumor stroma along with other non-malignant cells [5]. We have demonstrated that in obesity ASC undergo mobilization from WAT and migrate to tumors [4, 6]. Accumulating evidence indicates that ASC promote cancer progression in animal models [2, 7]. Our recent report links ASC trafficking from WAT to tumors in obese PCa patients with poor survival [6]. Both adipocytes and ASC secrete factors collectively termed adipokines, some of which are tumor-trophic [8]. Paracrine angiogenic, immunosuppressive, anti-apoptotic, and mitogenic adipokine signaling from adipose cells play an important role in tumor growth [2]. We had previously designed hunter-killer peptides D-WAT and D-CAN, composed of ASC-binding domain and a pro-apoptotic domain, which have a dose-dependent and specific cytotoxicity toward ASC but not toward other cell types [9–11]. In the mouse model, adipose ASC depletion expectedly resulted in WAT growth suppression without causing side effects [11]. Both D-WAT and D-CAN, administered subcutaneously, suppressed tumor growth, with D-CAN showing higher efficacy [10].

The roles of ASC in subsequent steps of cancer progression, which involve dissemination and survival of metastatic cells, have not been well defined. One of the potentially relevant properties of MSC is their ability to induce epithelial-mesenchymal transition (EMT) of adenocarcinoma cells, hallmarked by loss of E-cadherin and induction of N-cadherin, as well as a cascade of transcriptional factors changing cell signaling [12]. Previous studies have linked EMT with increased invasiveness, chemoresistance, and metastasis [13]. The ability of ASC to induce the EMT and chemoresistance in PCa has not been explored. Here, we investigated the interaction of cancer cells with ASC in cell culture and animal models of PCa. Our results indicate that ASC promote EMT, invasiveness, and chemoresistance in human PCa cells. We show that depletion of ASC with D-CAN suppresses these attributes of cancer aggressiveness and improves the efficacy of chemotherapy.

Results

ASC promote EMT in prostate cancer cells

First, we tested if ASC, derived from periprostatic WAT of patients with PCa described previously [6], confer EMT properties to PCa cells in direct co-culture model. As models of human PCa cells, we used LNCaP and PC3 cell lines transduced with GFP, which enabled their identification in mixed culture. LNCaP cells cultured alone grew as adherent cobblestone cultures, displayed epithelial phenotype, and expressed an epithelial marker E-cadherin (*Cdh1*) on the cell membrane (Fig. 1a). Co-culture with ASC resulted in LNCaP cell switch to mesenchymal fibroblastic morphology and E-cadherin downregulation/disappearance from the cell membrane (Fig. 1a). In addition, ASC-exposed LNCaP cells expressed N-Cadherin (*cdh2*), a mesenchymal marker strongly expressed by ASC (Fig. 1a). Similar results were observed for PC3 cells (Fig. S1A). These observations suggested that ASC induced EMT in cancer cells, which was confirmed by RT-PCR on FACS-sorted GFP+

cells. The mRNA analysis confirmed a reduction in *Cdh1* expression (Fig. 1b). In addition, upon ASC co-culture, we also observed induction of *Cdh2*, *fibronectin*, *vimentin*, *Zeb2*, and *Slug1* mRNA, confirming the EMT (Fig. 1b). These data indicate that ASC induce EMT in carcinoma cells.

ASC promote motility of prostate cancer cells

It has been reported that the EMT is coupled with induction of cell motility [13]. We, therefore, tested if ASC co-culture promotes migration of cancer cells. We performed a scratch ‘wound-healing’ assay to quantify motility of cancer cells plated with or without ASC (Fig. 1c). Data quantification revealed a significantly faster gap closure by GFP-labeled LNCaP cells in the presence of ASC (Fig. 1c). Similar results were observed for PC3 cells (Fig. S1B). An effect of ASC on cancer cell migration was also observed in a transwell Boyden chamber assay performed with ASC and cancer cells growing without direct contact for both LNCaP (Fig. 1d) and PC3 (Fig. S1C) cells. This suggests that soluble factors are sufficient to mediate the ASC effects. Our results indicate that ASC secrete factors inducing motility in carcinoma cells.

ASC promote chemoresistance of prostate cancer cells

Next, we tested if ASC confer PCa cells chemoresistance, another reported attribute of EMT. We used three conventional cancer drugs: microtubule disruptors cabazitaxel and docetaxel, as well as a DNA cross-linking agent cisplatin. Both classes of chemotherapeutics caused a dose-dependent decrease in numbers of adherent LNCaP (Fig. 2a, b; S2A) and PC3 (Fig. S2B-C) cells. Propidium iodide staining confirmed death of PC3 (Fig. 2c) and LNCaP (Fig. S2D) cells. In the presence of ASC, the majority of LNCaP cells still survived at docetaxel and cisplatin concentration tenfold higher than IC_{50} concentration for LNCaP grown without ASC (Fig. 2b). Upon co-culture with ASC, IC_{50} were also significantly higher for PC3 cells (Fig. S2B). Chemotherapeutic treatment resulted in selective loss of cells expressing higher levels of E-Cadherin, indicating that cells with mesenchymal phenotype are less sensitive to chemotherapy than epithelial cells (Fig. 2a). Treatment with the ASC-targeting peptide D-CAN abrogated the ASC-mediated chemoprotection of LNCaP cells on cytotoxicity from cabazitaxel (Fig. 2a), as well as docetaxel and cisplatin (Fig. S2C). Similar results were observed for PC3 cells (Fig. S2C). Interestingly, re-expression of E-Cadherin was not observed upon D-CAN treatment. This could be due to incomplete ASC depletion or to the lack of interaction between the sparse surviving cells. Treatment with D-CAN also abrogated migration observed for surviving cisplatin-treated LNCaP cells in ASC co-culture (Fig. 2a). Combined, these data indicate that ASC protect carcinoma cells from chemotherapy and suggest that this chemoprotection is mediated by EMT. As expected, we detected an increased level of intracellular reactive oxygen species (ROS) in LNCaP cells upon cisplatin treatment (Fig. 2d). Interestingly, this increase in ROS was not observed upon ASC co-culture (Fig. 2d).

ASC depletion suppresses obesity-induced EMT in mouse tumor model

To assess the relevance of our cell culture findings in vivo, we used HMVP2, a basal-epithelial cell line derived from the spontaneous Hi-Myc mouse PCa model [14]. We placed FVB male mice on high-fat (60 kcal%) diet (HFD) for 12 weeks to induce diet-induced

obesity (DIO), reflected as mean weight of 45 g; control age-matched males kept on control, low fat (10 kcal%) diet had a mean weight of 34 g (Fig. 3a). After HMVP2 spheroids were grafted subcutaneously as described [14], tumors grew significantly faster in obese mice (Fig. 3b). Upon tumor recovery, histological analysis revealed a higher degree of tumor invasion into the surrounding WAT (Fig. 3c). Tumors in obese mice displayed higher lymphocyte infiltration (Fig. S3A) and a higher frequency of cell proliferation (Fig. S3B). These results, consistent with our previous findings in Hi-Myc mice [15], indicated that HMVP2 grafts present an adequate model to study the effect of obesity on PCa. IF for E-Cadherin and N-cadherin revealed that the tumors were composed of both epithelial and mesenchymal cells. Tumors in obese mice tended to contain fewer epithelial E-Cadherin+ and more mesenchymal N-cadherin+ cells, indicating that obesity promotes the EMT in this PCa model (Fig. 3d; Fig. S3C).

To test the role of ASC in the obesity-associated EMT promotion, we injected 14.5 mg/kg D-CAN into mice as described previously [10] during HMVP2 growth; control mice were injected with PBS. We did not detect an effect of peptide treatment on body weight, confirming previous observation [11] that ASC depletion does not cause acute WAT loss (Fig. 3a). There was, however, a significant suppressive effect of D-CAN on tumor growth in both obese and lean mice (Fig. 3b). Hematoxylin/eosin (H&E) staining of resected tumors revealed that invasion into the surrounding WAT observed in obese mice was notably suppressed in DIO mice treated with D-CAN (Fig. 3c). Obesity-associated increase in lymphocyte infiltration and cell proliferation was also reduced in tumors of DIO mice treated with D-CAN (Fig. S3A-B). E-cadherin expression was increased in tumors of mice treated with D-CAN, while N-cadherin expression was reduced to undetectable levels (Fig. 3d; Fig. S3C). Combined, these results indicate that obesity-associated tumor EMT is promoted by ASC, a process that can be prevented by D-CAN treatment.

ASC depletion improves efficacy of chemotherapy in mice

Finally, we tested if ASC inactivation could improve PCa chemotherapy outcome in vivo. FVB mice (raised on regular chow) subcutaneously grafted with HMVP2 spheroids were treated with cisplatin (0.5 or 2.4 mg/kg), D-CAN (0.5 mg/kg), or a combination of 0.5 mg/kg cisplatin and D-CAN. Compared with PBS sham-injected controls, cisplatin dose of 0.5 mg/kg had only a subtle effect on tumor growth (Fig. 4a–c). D-CAN (injected at 0.1× of its dose effective in Fig. 3) also did have a strong effect on tumor growth. Caliper measurements indicated that both 2.4 mg/kg cisplatin and 0.5 mg/kg cisplatin/D-CAN treatments resulted in tumor growth suppression (Fig. 4a; S4A). While mice injected with 2.4 mg/kg cisplatin became moribund and began dying around day 20, mice treated with 0.5 mg/kg cisplatin/D-CAN displayed no signs of toxicity. Individual tumor analysis upon necropsy revealed that tumors in ‘0.5 mg/kg cisplatin / D-CAN’ group were not only smaller and anemic (Fig. S4B) but also more necrotic than those in ‘cisplatin only’ and ‘D-CAN only’ groups (Fig. 4b). Based on tumor weights at endpoint, 0.5 mg/kg cisplatin/D-CAN therapy was four times more effective than 0.5 mg/kg cisplatin alone (Fig. 4c). We reproduced these results for human LNCaP xenografts in immunodeficient RAG1 mice with DIO pre-induced for 5 months. D-CAN/cisplatin combination treatment, initiated when tumors were 0.3 cm³ in size, halted tumor growth in this experiment (Fig. 4d). Combined,

our results show that the potentiating effects of ASC on tumor growth/EMT can be reversed by ASC depletion leading to enhanced efficacy of chemotherapy.

Discussion

The biological connection between cancer and obesity is incompletely understood [2]. As the prevalence of obesity is rising, insights into the underlying mechanisms are urgently needed. Currently, lean and obese PCa patients are treated the same. Development of therapies tailored to obese patients may improve their survival. There is emerging evidence that WAT-derived cells contribute to the therapy resistance of carcinomas [16, 17]. The ability of WAT-derived cells to promote metastatic dissemination has been reported for breast cancer [18, 19]. There is building evidence that these effects are due to the EMT induced by the cells of WAT. While the role of EMT in metastatic dissemination is controversial, increased migratory capacity and resistance to therapy is undoubtedly a hallmark of EMT [13]. Here, we show that ASC promote both EMT, invasiveness, and chemoresistance in PCa cells.

Recently, CXCL12 (also known as stromal-derived factor alpha, SDF1a), was found to account for obesity-associated STAT3 signaling and prostate carcinoma progression in Hi-Myc mice [15]. Because STAT3 signaling underlies EMT and CXCL12 is highly expressed by ASC, this chemokine may be the EMT-inducing factor responsible for the observed ASC effects. Interestingly, adipocytes, that differentiate from ASC, induced EMT in LNCaP cells to a similar extent as ASC (Fig. S5A). The effect of adipocyte co-culture on PCa cell chemoresistance was also comparable to that of ASC (Fig. S5B). This suggests that both ASC and adipocytes may support cancer progression. At this point, it is unclear if mechanisms through which these cell populations act are distinct. It is possible that targeting both of these cell populations will be synergistic.

Conclusion

Our results reinforce previous reports indicating that ASC promote cancer progression [4, 6] and resistance of cancer cells to chemotherapeutics in cell culture settings [16, 20]. We demonstrate that an experimental drug D-CAN, which reduces the numbers of ASC in tumors [10], suppresses the EMT-associated attributes of cancer aggressiveness in cell culture and in mice. The effect of ASC ablation on metastatic dissemination remains to be tested. Previous studies have shown that EMT enables carcinoma aggressiveness via reducing ROS levels [21]. Therefore, the lack of ROS increase in ASC-co-cultured PCa cells treated with cisplatin suggests a possible mechanism of chemoprotection, which is consistent with the reported effects of fibroblasts on oxidative stress in cancer cells [22]. Recently, we reported that D-CAN targets human ASC [9]. Here, we further establish D-CAN as an experimental therapeutic that can be used to interrogate the role of ASC in cancer progression and aggressiveness. Our data, demonstrating that ASC depletion potentiates chemotherapy at a non-toxic dose in mouse models, may pave the way to combination therapies with lower side effects and an improved anti-cancer efficacy.

Materials and methods

Cell culture

Human LNCaP and PC3 cell lines were purchased from ATCC. Murine HMVP2 cell line characterized previously [14, 15], PCa cell lines and co-culture cells were grown in RPMI 1640 / 10% FBS. (Life Technologies). Human primary ASC cells from periprostatic adipose tissue described previously [6] were grown in EBM2 medium (Lonza). For co-culture experiments, non-fluorescent human ASC (0.5×10^3) were seeded first; after they adhered, GFP+ cancer cells (3×10^3) were seeded on top per well of a 96-well plate. Analysis was performed after 24 h of co-culture. Cancer cell numbers in culture were determined using Cytation5 (Biotek) / Gen5™ Software by total nucleated (DAPI channel) GFP+ cell (GFP channel) counts. Propidium iodide (1 µg/ml) staining was performed for 30 min.

Quantitative real-time PCR

GFP+ cancer cells were sorted with BD FACS Aria II from co-culture. mRNA extraction, complementary DNA, and PCR were processed as described [6, 11]. Expression of human genes was normalized to human *18S* RNA. The SYBR green primers are listed in Table S1.

Scratch wound healing assay

For the scratch (gap closure) assay [15], non-fluorescent ASC were (where indicated) seeded first; GFP+ cancer cells were seeded on top to form a monolayer. Cells were starved for 4 h and then scratched with a pipette tip to create a gap. For automated image capturing and analysis, the Cytation5 (Biotek)/Gen5™ Software was used to monitor wound closure in GFP channel. ImageJ calculated Metric of (Sum Area) obtained from each scratch assay along the traced edge of the green fluorescent area was used to quantify cell migration. Representative pictures of migration (gap closure) at 0, 24 or 36 h after the scratch were taken.

Transwell migration assay

The transwell assay was performed as previously described [6, 15]. ASC were seeded into the bottom chamber and PCa cells were seeded onto the upper chamber of a transwell filter with 8 µm pores (Costar) after serum starvation for 4 h. After 48 h, non-migrated PCa cells on top of filters were removed using cotton-tipped swab, and migrated PCa cells at the bottom of the filter were fixed and stained with 0.4% crystal violet in 10% ethanol. For each experiment, images were captured for three independent transwells and stained cells in nine random fields were counted using Cytation5 (Biotek)/Gen5™ Software.

Reactive oxygen species assay

Live cells were stained with CellROX® Deep Red (Molecular Probes®, C10422) (5 µM) in the dark for 25 min followed by DAPI stain. 30,000 GFP+ singlets were analyzed in the APC channel per sample with a BD FACS Aria II / FlowJo software.

Cell culture drug treatments

Docetaxel (Alfa Aesar, J60174), cabazitaxel (APExBIO, B2157, and cisplatin (Tocris, 2251) stocks were prepared according to manufacturers' protocols. D-CAN, composed of ASC-homing peptide WAT7 (CSWKYWFGE) linked via aminohexanoic acid with an amphipathic sequence KFAKFAKKFAKFAK, was described previously [10]. D-CAN was synthesized from D-amino acids, cysteine-cyclized, and acetate salt chromatographically purified to 99% and quality-controlled (mass spectroscopy) by Ambipharm. In cell culture experiments, 0.01 mM D-CAN, 50 ng/ml docetaxel, 50 ng/ml cabazitaxel, and 5 µg/ml cisplatin were used, except for Fig. 2c.

Mouse experiments

Studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of UTHealth. FVB/N and RAG1 male mice were purchased from Jackson Laboratories. DIO induction, body composition measurement, and grafting of HMVP2 spheroids or LNCaP cells were as described [14]. Drug treatment began when tumors became palpable. Tumors were harvested and processed after 4 weeks (twelve injections, 3 times weekly) of treatment, or when they exceeded 1.5 cm³ or became ulcerated. D-CAN was injected subcutaneously as described [10] in Fig. 3 or retro-orbitally in Fig. 4. Cisplatin was administered retro-orbitally. Mice were maintained on the DIO or control diets for the duration of the experiments.

Immunofluorescence

Tissues and tumors were fixed in 10% formalin and cells from culture were fixed in 4% paraformaldehyde and stained as described [6, 14, 15]. Primary antibodies were as follows: anti-E-cadherin R&D AF748 (1:100); anti-N-Cadherin Abcam ab98952 (1:100); anti-CD3 BD Pharmingen 550275 (1:100). The secondary antibodies were as follows: Donkey Alexa 488-conjugated IgG or Cy3-conjugated IgG. Nuclei were stained with DAPI or Hoechst 33258 (Invitrogen). Images were acquired with a confocal Leica TCS SP5 (Leica), Carl Zeiss upright Apotome Axio Imager Z1 or Cytation5 (Biotek).

Statistical analysis

For cell quantification analyses, $N = 10$ individual fields of view. Statistical analyses were performed using GraphPad Prism was used to graph data as mean $\pm$ SEM and to calculate P -values with one-way ANOVA or two-tailed unpaired Student's t -test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

ASC promote EMT and motility in human prostate cancer cells. **a** Direct 24 h co-culture with human ASC induces mesenchymal morphology in adjacent GFP⁺ LNCaP cells (green). Concomitant loss of E-cadherin and expression of N-cadherin detected by IF on fixed cells indicates EMT (yellow) in PCa cells co-cultured with ASC (red). Nuclear staining (dim in ASC) is blue. **b** RT-PCR analysis of *E-cadherin*, *N-cadherin*, *fibronectin*, *Vimentin*, *slug1*, and *zeb2* mRNA expression, normalized to 18 S RNA, in FACS-sorted GFP⁺ LNCaP cells, confirms ASC-induced EMT. **c** Representative pictures of wound closure in GFP⁺ LNCaP

monolayer ($\pm$ co-cultured ASC) at 0 and 24 h after the scratch was made. Graph: quantification presented as mean surface area of the gap $\pm$ SEM of three independent experiments. **d** Representative pictures of migrated LNCaP cells, stained with crystal violet, on the outside of the transwell membrane after 48 h of co-culture. Graph: quantification of migrated cells presented as $\pm$ SEM of three independent experiments. * $P < 0.01$. Scale bar = 50 μm

Fig. 2.

ASC promote chemoresistance and ASC depletion potentiates chemotherapy. **a** Representative pictures of GFP⁺ LNCaP cells alone or with ASC (24 h) treated with cabazitaxel for 24 h and subjected to E-cadherin IF or wound closure assay for 36 h. Arrows: ASC nuclei. Red insets show only E-Cadherin / DNA signal for area in white above. Note that in ASC co-culture D-CAN restores cabazitaxel cytotoxicity and prevents gap closure upon cisplatin treatment. **b** GFP⁺ LNCaP cell survival upon treatment with increasing concentrations of docetaxel and cisplatin. Note increased survival in ASC co-culture. Relative numbers of viable GFP⁺ cells per well $\pm$ SEM of three independent experiments are plotted, * $P < 0.01$. **c** After GFP⁺ PC3 cells treated with 100 ng/ml cisplatin (CISP), 1 ng/ml docetaxel (DTX), or 1 ng/ml cabazitaxel (CBTX) for 16 h, nuclei were stained with propidium iodide (PI, arrow). Note that ASC decrease cell death, as quantified in the graph; * $P < 0.05$. **d** GFP⁺ LNCaP grown with (right) or without (left) ASC, treated with 2 μ g/ml cisplatin for 16 h (green) or untreated (red) were stained with CellROX® Deep Red and analyzed in APC channel by flow cytometry. Note that ASC suppress ROS generation upon chemotherapy, as quantified in the graph; * $P < 0.05$

Fig. 3.

Cancer progression in mice upon ASC depletion. Mice fed either a low-fat (10 kcal%) or high-fat (60 kcal%) diet and grafted with HMVP2 spheroids were analyzed after D-CAN (14.5 mg/kg) treatment. **a** Mouse weights demonstrate obesity in 60 kcal% groups and lack of D-CAN effect on body mass. **b** Tumor growth rate suppression by D-CAN treatment (initiated on day 21). *P < 0.05 compared to untreated mice on the same diet. **c** H&E-stained sections of HMVP2 grafts grown in lean and obese mice revealing decreased tumor/WAT invasion upon D-CAN treatment. a: adipocytes. **d** E-cadherin and N-cadherin IF in tumor sections without or with D-CAN treatment, indicates EMT suppression upon ASC ablation. Blue: DNA. Scale bar = 50 μ m. **c, d** graphs: mean $\pm$ SD; *P < 0.05

Fig. 4. ASC depletion improves outcome of cisplatin cancer treatment in mice. **a–c** HMVP2 spheroid grafts in FVB mice fed normal chow ($n = 8/\text{group}$). When tumors reached 0.1 cm (day 1), mice were injected with PBS; 1.6 mg/kg D-CAN; 0.5 mg/kg cisplatin (C 0.5); 2.4 mg/kg cisplatin (C 2.4); or 1.6 mg/kg D-CAN + 0.5 mg/kg cisplatin. **a** HMVP2 tumor volume at terminal time point. **b** Mean % of necrotic tumor area with representative H&E-stained sections of tumors shown below; n: necrosis. **c** Excised tumor weight at terminal time point for the two groups from **(a)**. **d** Individual tumor growth time-course for LNCaP xenografts ($n = 2/\text{group}$) in DIO RAG1 mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (one-way ANOVA)