Title

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DYSLIPIDEMIA IS A MAJOR FACTOR IN STEM CELL DAMAGE INDUCED BY UNCONTROLLED LONG-TERM TYPE 2 DIABETES AND OBESITY IN THE RAT, AS SUGGESTED BY THE EFFECTS ON STEM CELL CULTURE

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Abstract

Background.—Previous work showed that muscle derived stem cells **(MDSC)** exposed long-term to the milieu of uncontrolled type 2 diabetes **(UC-T2D)** in male obese Zucker **(OZ)** rats, were unable to correct the associated erectile dysfunction and the underlying histopathology when implanted into the corpora cavernosa, and were also imprinted with a noxious gene global transcriptional signature **(gene-GTS)**, suggesting that this may interfere with their use as autografts in stem cell therapy.

Aim.—To ascertain the respective contributions of dyslipidemia and hyperglycemia to this MDSC damage, clarify its mechanism, and design a bioassay to identify the damaged stem cells.

Methods.—Early diabetes **(ED)**-MDSC and late diabetes **(LD)**-MDSC were respectively isolated from nearly normal young OZ rats, and moderately hyperglycemic and severely dyslipidemic/obese aged rats with erectile dysfunction. Monolayer cultures of ED-MDSC were incubated 4 days in DMEM/10% fetal calf serum + or - aged OZ or LZ serum from non-diabetic lean Zucker rats (0.5–5%) or with soluble palmitic acid **(PA)** (0.5–2 mM), cholesterol **(CHOL)** (50–400 mg/dl), or glucose (10–25 mM).

Outcomes.—Fat infiltration was estimated by Oil red O, apoptosis by TUNEL, protein expression by western blots, and gene-GTS and microRNA **(miR)**-GTS were determined in these stem cells RNA.

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Results.—Aged OZ serum caused fat infiltration, apoptosis, myostatin overexpression, and impaired differentiation. Some of these changes, and also proliferation decrease occurred with PA and CHOL. The gene-GTS changes by OZ serum did not resemble the in vivo changes, but some occurred with PA and CHOL. The miR-GTS changes by OZ serum, PA, and CHOL resembled most of the in vivo changes. Hyperglycemia did not replicate most alterations.

Clinical Implications.—MDSC may be damaged in long-term UC-T2D/obese patients and be ineffective in autologous human stem cell therapy, that may be prevented by excluding the damaged MDSC.

Strengths and limitations.—The in vitro test of MDSC is innovative and fast to define dyslipidemic factors inducing stem cell damage, its mechanism, prevention and counteraction. Confirmation is required in other T2D/obesity rat models and stem cells (including human), as well as miR-GTS biomarker validation as stem cell damage biomarker.

Conclusion.—Serum from long-term UC-T2D/obese rats or dyslipidemic factors induce a noxious phenotype and miR-GTS on normal MDSC, that may lead in vivo to the repair inefficacy of LD-MDSC. This suggests that autograft therapy with MDSC in long-term UT-T2D obese patients may be ineffective, albeit this may be predictable by prior stem cell miR-GTS tests.

Keywords

cholesterol; erectile dysfunction; fat infiltration; microRNA; miR; muscle derived stem cells; myostatin; palmitic acid; Peyronie’s disease; free fatty acids; diabetes control; apoptosis

INTRODUCTION

Stem cell implantation into various types of damaged tissues has emerged as a promising therapy for several human urological disorders based on solid experimental evidence in animal models, and specifically in sexual medicine for erectile dysfunction (1,2) and Peyronie’s disease (3,4). These studies have translated to therapy the extensive biological research showing the ability of stem cells to proliferate indefinitely and differentiate into the desired cell lines intended to replace the lost or damaged cells, or alternatively assuming that they may favorably change the damaged tissue composition through paracrine or juxtacrine effects. The main premise for their efficacy and safety is the belief, or rather the lack of contrary evidence, that the “immortal” stem cells are essentially resistant in their tissues of origin, and later in their sites of implantation, to the chronic noxious conditions and endogenous factors in many diseases, and even some physiological processes that damage the differentiated cells. This assumption extends to the tissue repair elicited by endogenous (rather than implanted) local or recruited stem cells, such as in the penile corpora cavernosa (5,6) or tunica albuginea (7,8), where the potential damage to stem cells by the tissue milieu affected by the disease has not been studied.

Sixteen human clinical trials using implanted stem cells are registered for the treatment of erectile dysfunction in www.clinicaltrials.gov, but only 3 of them are in the USA with none in academic research institutions or hospitals, and two trials are listed as ongoing for Peyronie’s disease, but only one in the USA in a non-academic research institution. Proof of efficacy evaluated by the U.S. Federal Government is still pending. In addition, multiple

direct therapeutic applications of stem cell implantation in humans for these and other conditions are offered, as well as ongoing small research studies not registered in the clinicaltrials.gov site, generally with little or no information on previous supporting research or adequacy of study design. The common denominator in clinical applications or studies is the use of autograft (autologous) implants, from the same patients that are being treated, mainly because of allogenic or syngenic tissue rejection concerns when using stem cell banks. However, little is known regarding the efficacy and long-term safety of these procedures.

A main issue for all experimental stem cell therapy research in animal models driving the successive human use is rarely, if ever, discussed. This is that most or all studies are based on syngenic, not on autologous, implantation, thus ignoring the various noxious systemic milieus that may affect cells in situ prior to their use in humans as autografts. In other words, the stem cells are obtained mostly from healthy young animals, i.e, not having experienced the impact of chronic conditions such as aging, cancer, vascular disease, heavy smoking, severe obesity, diabetes, and others, and implanted as a sort of cell bank into the host animals. So far, the main novel related research on stem cell damage is purely on biological and autocrine, rather than exocrine, processes: the stem cell senescence (9,10), without understanding more precisely the host aging factors that trigger or accelerate it and present potential risks for their use in therapy.

Our previous study (11) defined the in situ impairment by a systemic or local noxious milieu in the tissue of origin, particularly long-term uncontrolled-type 2 diabetes **(UC-T2D)** and obesity in a rat model, and showed that in addition to their phenotype imprinting, these stem cells had lost the capacity to exert tissue and functional repair when implanted into another tissue damaged by the same environment in either syngenic or autologous hosts. There are some reports describing deleterious effects of the T2D milieu and its associated oxidative stress on the microenvironment impairing stem cells, inducing apoptosis, interfering with wound healing capacity, and other effects (e.g., 12–15), as well as obesity per se or associated with diabetes on the number of stem cells and progenitor cells, their lifespan, and other effects (e.g., 16–18). The impairment of stem cells was ascribed in some cases to dyslipidemia (e.g., 19, 20). Two very recent reviews referred to these recent papers and warned on potential effects on the therapeutic use of stem cells by the systemic and tissue milieu of metabolic syndrome and T2D (21) and obesity (22), even if the discussed approaches and mechanisms are rather different from the current study.

Specifically, we had shown (11) that muscle derived stem cells **(MDSC)** isolated from the skeletal muscle of aged male obese Zucker **(OZ)** rats with long-term UC-T2D and which we refer to as Late Diabetic MDSC **(LD-MDSC)** from animals suffering morbid obesity and corporal veno-occlusive dysfunction **(CVOD)**, when implanted as allografts into the penile corpora cavernosa of these same type of rats, failed to repair either CVOD or the underlying corporal histopathology. Moreover, the LD-MDSC had an abnormal pro-fibrotic/ inflammatory phenotype evidenced by their gene expression global transcriptional signature **(gene-GTS)**. In contrast, MDSC obtained from very young rats with early UC-T2D which we refer to as Early Diabetes MDSC **(ED-MDSC)**, were able as expected to repair both penile corporal tissue and erectile function (11). This was similar to what we found with

MDSC obtained from a non-diabetic rat strain for the therapy of erectile dysfunction in other animal models for risk factors, (23,24), and also with MDSC from normal mice but implanted into a T2D mouse model of limb ischemia (25).

In the current study we aimed to: 1) identify whether is dyslipidemia or hyperglycemia the main factor in the in vivo UC-T2D/obesity milieu that may cause stem cell damage, and get an insight into possible mechanisms mainly by subjecting in vitro the rat ED-MDSC to short incubations with the hyperglycemic and dyslipidemic serum of aged OZ rats, some specific dyslipidemic factors, and/or high glucose; 2) determine whether these agents affect fat infiltration, apoptosis, cell proliferation, protein expression, and the transcriptional imprinting of gene-GTS; c) verify whether microRNA **(miR)**-GTSs, are more sensitive than gene-GTSs in differentiating in vivo damaged (LD-MDSC) from normal (ED-MDSC) stem cells, and in identifying the noxious factors and providing an initial approach to their mechanism of action.

miRs are of high relevance, because they regulate protein translation, and transcription indirectly, and are widely accepted novel regulators and potential early biomarkers for various processes. This includes alterations of stem cell differentiation, cell cycle, apoptosis, and other types of damage (e.g.,26–29), diabetes, obesity and dyslipidemia (e.g., 30–32), and inflammation, and fibrosis (e.g, 33, 34), to name just a few. There are some recent publications on miRs in erectile dysfunction, (e.g., 35, 36), but none on Peyronie's disease.

MATERIALS AND METHODS

Rat model, MDSC and serum isolation.

Obese Zucker **(OZ)** rats (Crl:ZUC-Lepr^{fa}, catalog 185), and their Lean **(LZ)** counterparts (+/?, catalog 186) were obtained from Charles River (Wilmington, MA), treated according to the 'Principles of laboratory animal care' (The National Institutes of Health) with an Institutional Animal Care and Use Committee-approved protocol, and used for MDSC and serum isolation. The OZ rats are a model of metabolic syndrome starting to evolve at around 3–4 months into frank T2D, reaching a moderate hyperglycemia and morbid obesity which do not manifest in the LZ rats, and that is associated with erectile dysfunction, diabetic nephropathy and arteriosclerosis (11, 50–52).

The MDSC were initially described by J. Huard's group and extensively characterized by them (see e..g, 37–40), as well as by us (11, 23–25, 41–43). The ED-MDSC were obtained from the gastrocnemius of 2–3 male **OZ** rats at 12 weeks of age, when they are only mildly hyperglycemic and overweight, as compared to their lean non-diabetic counterpart **(LZ)** rats (11). In turn, LD-MDSC were from aged male OZ rats at 32 weeks of age, already severely obese and dyslipidemic but with only moderate hyperglycemia (see results), and having T2D/obesity-related CVOD. Sca-1 selected MDSC were isolated as described previously (11, 23–25, 41–43). The latter is the cell population containing MDSC. Cells were replicated on regular culture flasks (no coating) and used in the 10th-15th passage, since the mouse counterparts have been maintained in our laboratory for at least 40 passages with the same, or even increasing, growth rate (25, 41). Flow cytometry was performed to show that these cultures were Sca 1+/CD34+/CD44+ cells and expressed the key stem cell gene Oct 4 (11,

41). Low (5.6 mM) or high (22.2 mM) glucose media were used respectively for ED-MDSC and LD MDSC culture maintenance. Blood was withdrawn from the 32 week old OZ rats and from the age-matched LZ rats, and the sera (OZ serum and LZ serum, respectively) were obtained.

MDSC culture incubations.

ED-MDSC were incubated at an initial 20% confluence for 4 days on 6-well or 12-well collagen-coated plates, or on 8-well-removable compartment collagen-coated plates, in DMEM/10% fetal calf serum/5.8 mM glucose. For testing the effects of dyslipidemia in duplicate wells, either aged OZ or LZ serum was added to 0.5–5%, or soluble forms of palmitic acid, Na salt, conjugated to albumin **(PA)** or cholesterol-B-methylcyclodextrin **(CHOL)**, both from Sigma-Aldrich (St. Louis, MO), were added to a concentration of 0.5 and 1 mM or 50 and 100 mg/dl, respectively. Albumin and methyl-cyclodextrin were not used as vehicle controls because when they are added, they do not act as inert vehicles but are active agents in binding endogenous PA and cholesterol. Control wells contained no lipid additives. For testing the effects of hyperglycemia, glucose was added to a final concentration of 10–25 mM. Upon completion of treatment, the medium was discarded and cells were washed with PBS and subjected to fixation for histochemistry, or extracted fresh for protein for western blots, or for RNA for gene/miR-GTS, respectively.

Quantitative histochemistry.

Cells on the 12 well plates were subjected to Oil Red O staining for detecting fat droplets (11, 23, 34), and cells on the 8-well removable-partition plates were used for TUNEL determinations for establishing the apoptotic index (36). Quantitative image analysis (**QIA**) (11, 23–25, 34) was performed by computerized densitometry on 100X or 200X magnification pictures, using multiple pictures in order to cover the wells with as many fields as necessary, followed by QIA of all fields.

Western blots (11, 23–25, 36)

Protein extracts were subjected to western blot immuno-detection. The primary antibodies used were: a) calponin 1, mouse monoclonal (Santa Cruz Biotechnology, Inc. Santa Cruz, CA); b) α-smooth muscle actin **(ASMA)**, mouse monoclonal (Sigma/Aldrich St Louis, MO); c) myostatin **(GDF8)**, our mouse monoclonal against the myostatin carboxy-terminal 113 amino acids (44); d) proliferating cell nuclear antigen **(PCNA)** mouse monoclonal (Millipore, Billerica, MA, USA), e) caspase 3, mouse monoclonal (Cell Signalling Technology, Danvers, MA); f) glyceraldehyde 3-phosphate dehydrogenase **(GAPDH)**, mouse monoclonal (Millipore, Billerica, MA, USA), as a reference housekeeping protein. and g) beta-actin, mouse monoclonal, (Santa Cruz Biotechnology) also as a housekeeping protein. Membranes were incubated with secondary anti-mouse IgG, horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology, Danvers, MA) or anti-rabbit IgG linked to HRP (Amersham GE, Pittsburgh, PA). Bands were visualized using luminol (SuperSignal West Pico; Chemiluminescent, Pierce, Rockford, IL). For negative controls, the primary antibody was omitted. Densitometric analysis was performed in certain cases as stated, correcting by the housekeeping proteins.

Global transcriptional signatures (GTS).

A) Gene-GTS (11, 36, 45–47).—The alterations of mRNA levels on RNA isolated using the Qiagen RNeasy Micro Plus kit, and whose quality was determined by the Agilent 2100 Bioanalyzer, were estimated by DNA microarrays performed by the UCLA DNA microarray core, by the Affymetrix Rat Gene array for over 32,000 sequences, where various housekeeping genes allow the normalization between different samples. Only genes that were up- or downregulated by at least 2-fold were considered unless specifically detailed. The impact of the UC-T2D/obesity milieu on the LDMDSC expressed as the ratios between the LD-MDSC values for each selected gene and the respective control ED-MDSC values previously reported (11), but here selected, reorganized in descending order, and used to tabulate all other treatment ratios in the in vitro experiments referred to the newly determined control ED-MDSC values.

B. miR-GTS (36, 45, 46).—RNA was isolated from cells using the mirVana™ miRNA isolation kit (Ambion), and analysis was performed by Norgen Biotek Corporation (Thorold, ON, Canada) by next-generation sequencing for all miR transcripts listed in the Sanger miRBase Release 18.0. Treatment ratios, including the newly determined miR-GTS for ED-MDSC and LD-MDSC, were obtained as for gene-GTS, against the respective value for the ED-MDSC, and selected and tabulated as for gene-GTS (11). In this case, normalization was done by expressing per 10^7 total raw reads, correcting for various levels of amplification. Also, in one case (Table 1 Supplement) individual miR values were calculated for each specimen as per thousand of the total miRs in that specimen, which makes the comparison irrespective of the total raw reads, and ratios to the ED-MDSC were calculated as in the other cases. Both the gene-GTS and miR-GTS complete results are deposited in the GEO library.

Statistical analysis.

When applicable, values are expressed as the mean±SEM. The normality distribution of the data was established using the Wilk–Shapiro test. Multiple comparisons were analyzed by single factor ANOVA, followed by *post hoc* comparisons with the Bonferroni multiple comparison test.

RESULTS

The age progression of hyperglycemia and dyslipidemia in the OZ and LZ rats shows that the LD-MDSC were exposed in vivo to a noxious UC-T2D milieu.

The EDMDSC were isolated from 12 week old OZ rats, which when non-fasted had about 250 mg/dl glucose in the blood, whereas the age-matched LZ rats had 130 mg/dl (Fig. 1), showing that these stem cells from OZ rats were initially subjected to an early mild type 2 diabetes **(UC-T2D)**, rather than just to insulin resistance, but for a relatively short period. In contrast, the LD-MDSC were isolated from OZ rats that had experienced a non-fasting hyperglycemia peak at 24 weeks of 460 mg/dl, decreasing to 338 mg/dl at 28 weeks, versus 120 mg/dl at the latter age in the LZ rats. At 32 weeks of age, when the rats were obtained, although glycemia had lowered to 202+/−8 mg/dl in the non fasted OZ rats versus 104+/−4

in the LZ rats (not shown), this moderate or higher hyperglycemia had continued to exist in the LD-MDSC for at least 24 weeks.

Remarkably, these changes were accompanied by a steady increase of blood cholesterol from 150 mg/dl at 12 weeks of age to 420 mg/dl at 28 weeks in the non-fasted OZ rats, coupled to a consistent rise from 800 to 1,620 mg/dl in triglycerides, which in both cases presumably continued until 32 weeks of age (not measured). Therefore, the ED-MDSC were exposed short term to mild dyslipidemia, whereas the LD-MDSC were exposed long-term to very severe dyslipidemia.

Short-term in vitro exposure of ED-MDSC to dyslipidemia but not hyperglycemia induced stem cell damage, as evidenced by severe fat infiltration and apoptosis.

The highly dyslipidemic conditions of the UC-T2D/obesity milieu were represented at 32 weeks by the aged OZ serum used for the experiments described below. In contrast, the LZ serum from the 32 week old rats was comparatively (but not strictly) normolipidemic, with 140 mg/dl for cholesterol and 160 mg/dl of triglycerides in the non fasted LZ rats at this peak. These OZ dyslipidemic changes were in agreement with severe obesity that reached at 32 weeks body weights of 745 +/−26 g, vs only 494+/−15 g in the LZ rats (11).

We hypothesized that the aged OZ serum contains factors that would mimic the noxious in vivo milieu responsible for impairing the penile corporal tissue repair capacity of MDSC and their ability to restore erectile function and normal corporal histology (11). To test this, EDMDSC, previously reported as “normal” based on their ability to provoke adequate tissue repair, were first incubated in monolayer cell culture for 4 days, with OZ serum added to 0.5%, 2.5%, and 5% final concentration in standard DMEM medium (containing 10% fetal calf serum), and compared with the LZ serum or with no addition. The DMEM-0.1% glucose (5.6 mM) medium was used in this and all subsequent experiments (unless stated) to mimic a normoglycemic milieu, thus excluding hyperglycemia as a confounding factor for these experiments.

Fig. 1 B Supplement shows that even incubation with a very low 0.5% aged OZ serum for 4 days caused some fat infiltration in the ED-MDSC that would suggest stem cell damage, as denoted by Oil Red-O staining, and which was intensified at 2.5% **(D)**, whereas this effect occurred to a much lower extent with the age-matched LZ serum at both concentrations **(A and C)**. When aged OZ serum was increased to 5% (Fig. 2) the fat infiltration was very pronounced **(C)**, but was essentially not observed following 5% LZ serum **(B)** and was not observed in the absence of rat serum addition **(A)**. QIA confirmed the several-fold increase in stem cell fat infiltration for OZ serum versus LZ serum, expressed per stained nucleus (bar graphs; **D**, notice logarithmic Y axis).

In order to determine whether key dyslipidemic factors result in the same fat infiltration in ED-MDSC as for the aged OZ serum, similar incubations were performed adding either a representative of free saturated fatty acids, i.e., solubilized palmitic acid **(PA)**, at 0.5 (Fig. 3B) and 1 mM, (not shown) or cholesterol, at 50 **(C)** and 100 (not shown) mg/dl, final concentrations, in comparison to no addition **(A)**. The Oil Red O staining shows that there

was lower fat infiltration induced by either PA or cholesterol, measured by QIA **(D)**, as compared with the aged OZ serum effects shown in Fig. 2.

The aged OZ serum not only caused fat infiltration but also MDSC death, indicated by trypan blue staining **(not shown)**. Despite the fact that all the aged OZ rats used for serum donation had very milky sera denoting high lipid concentrations, there were differences in the proportion of dead cells caused by each specific aged rat OZ serum added at 5%, except for 3 of them. One of these sera was selected for just a relatively mild cell death effect and was used for the experiments throughout the study. Dilution of the other deleterious sera to 1% showed a concentration dependence of trypan blue uptake, and thus a corresponding dependence of the cell-killing effect.

To determine whether the variable rates of stem cell death were at least in part caused by apoptosis, the effects of the selected aged OZ serum at 5% on the ED-MDSC (lower number of trypan blue positive cells) were assessed by the TUNEL reaction. Fig. 4C shows that the 5% aged OZ serum used in this study caused MDSC apoptosis, but virtually none with LZ serum **(B)**, and none in the absence of added serum **(A)**. This was confirmed by QIA (bar graphs (**D)**, shown in non-logarithmic scale. The apoptotic effects were apparently lower than the unspecific cell killing detected by the trypan blue staining, implying that there was in part some nonapoptotic cell death.

A much higher apoptosis induction, within a considerable general effect on cell death that reduced MDSC number, occurred with PA at 0.5 (Fig. 5 B) and 1 mM (not shown), and lower with cholesterol at 50 **(C)** and 100 mg/dl (not shown), with the expected absence of apoptosis without addition **(A)**. QIA bar graphs **(D)** indicate this clearly in non-logarithmic scale for Y.

The fat infiltration and apoptosis was accompanied by reduced cell proliferation/ differentiation, and myostatin over-expression.

We then explored whether the aged OZ serum was able to induce some changes in protein expression affecting MDSC properties, as detected by western blot. Fig. 6 shows that ED-MDSC replication, represented by PCNA expression, was moderately reduced with aged OZ serum in comparison with its counterpart LZ serum, but only at 5%. Caspase 3, an indicator of mitochondrial apoptosis, was not increased contrary to what was expected, and was even mildly inhibited at 5% OZ serum, suggesting that the observed apoptosis resulted from other non-mitochondrial apoptotic pathways. ASMA, a smooth muscle cell and myofibroblast marker, was mildly reduced but not affected by higher concentrations. The spontaneous in vitro conversion of ED-MDSC into smooth muscle cells, a cell lineage that would be functionally desirable in penile corporal tissue repair, was more affected and severely reduced at 5%, as shown with calponin 1. The resulting increase in the ASMA/calponin 1 expression ratio would suggest a potentially deleterious spontaneous conversion into myofibroblasts.

In turn Incubation with PA at 1 mM reduced PCNA, ASMA, and calponin 1 levels, and at 2 mM blocked considerably PCNA and calponin 1 expression, and presumably induced some palmitoylation evidenced by an increase in the larger beta-actin band (Fig. 2 Supplement).

As expected, cholesterol at all concentrations did not exert palmitoylation, but did block calponin-1, and also reduced ASMA. PCNA was lowered at only 200 and 400 mg/dl. Fig. 7 represents the quantitative densitometric determinations of band intensities only in the duplicate experiments, agreeing with the visual inspections, except that the PCNA decrease by serum was not significant. In turn, increasing concentrations of glucose failed in 1-week incubations of EDMDSC to cause the changes induced by the aged OZ serum or PA (not shown).

We had reported that the repair-ineffective LD-MDSC, but not the repair-effective EDMDSC, implanted into the corpora cavernosa of 24-week old OZ rats with CVOD, increased myostatin expression in the tissue, either from the MDSC themselves or from their interaction with the tissue (11). Therefore, we tested the possibility that the aged OZ serum could induce in vitro a effect similar to the one observed in vivo in the presence of the T2D/obesity milieu on the ED-MDSC. Myostatin is a lipofibrotic inducer and inhibitor of skeletal muscle mass made in this tissue, and a modulator of muscle/adipocyte differentiation, that we recently showed is also expressed in the corporal smooth muscle in the rat, and in the tunica albuginea myofibroblasts, and is weakly expressed in MDSC (11, 25, 48, 53, 54).

Fig. 8 shows that this was the case since there was a concentration-dependent increase of myostatin induced by aged OZ serum, in this case in the ED-MDSC themselves, not just in the MDSC/tissue interaction seen in vivo, and this did not occur with control age-matched LZ serum. Hyperglycemia per se was unable to induce over-expression of myostatin in the EDMDSC when they were incubated for 1 week with increasing concentrations of glucose, either as such or after an azacytidine incubation to stimulate de-methylation and “stemness” (41). PA reduced myostatin expression (not shown).

The in vivo exposure of the LD-MDSC to the UC-T2D/obesity milieu induced a potential noxious imprint of their gene-GTS, which was partially reproduced by dyslipidemia in vitro, but not by hyperglycemia.

The induction of an abnormal gene GTS on ED-MDSC by 9 month exposure to the UC-T2D milieu is a landmark of stem cell damage and loss of tissue repair capacity, as previously reported (11). Therefore, we aimed to investigate here whether this could be replicated in vitro by short-term incubations under normal 0.1% (5.6 mM) glucose, with aged OZ serum, PA, or cholesterol, in comparison with 0.4% (22.4 mM) glucose alone. RNA was isolated and gene GTS were then obtained for the in vitro incubations. The respective basal data for the ED-MDSC controls and the LD-MDSC/ED-MDSC ratios in the in vivo series previously reported (11) are now presented in Tables 1 and 2 to allow for comparison with the new in vitro data. The respective ED-MDSC control values in the two series were used since the ED-MDSC were originated from different culture passages and the gene-GTS microarrays were done on different dates. In addition, values were standardized by similar RNA inputs, procedures, and internal housekeeping genes.

Table 1 shows a selective comparison of the effects of the in vivo exposure to the UCT2D milieu, with only some previously reported (11), with the results of the 4 day in vitro exposure to aged serum, PA, cholesterol, or high glucose shown in the subsequent columns. The in vivo ratios between the LD-MDSC and the ED-MDSC, for genes that may be related

to stem cell damage processes, are now sorted in decreasing order and restricted to the in vivo changes where mRNA levels were up-regulated in LD-MDSC versus ED-MDSC by >2.0 (represented by rounding up >1.95; highlighted in yellow). The respective in vitro changes that had the same direction (>1.95) are highlighted in yellow, or where opposite changes (<0.5); occurred, they are highlighted in green. Myostatin inclusion is the exception, since the LD/ED ratio was 1.5 in vivo, but is important in terms of its relevance to the miR-GTS changes presented in subsequent figures. The yellow gene IDs in the first column indicate that there was a similar direction (up-regulation) in any one of the in vitro changes and the few IDs without highlighting correspond to the absence of in vitro agreement with the in vivo values within the stated ranges. Of note for the discussion (not shown in the table), the LD-MDSC/ED-MDSC ratios for antioxidant enzymes in our gene-GTS were considerably elevated for glutathione peroxidase (Gpx3: 2.9) and superoxide dismutase (SOD3: 5.1), thus counteracting the only oxidant enzyme ratio that is elevated, the one for xanthine dehydrogenase (Xdh: 4.5), so that oxidative stress does not seem to act as a major factor in the in vivo MDSC damage.

Table 2 is structured similarly, but showing the down-regulation of the in vivo LD/ED ratios (i. e, <0.5), and compared to the respective <0.5 (yellow highlighting) or >2.0 (green) changes in the in vitro treatments.

Out of the >32,000 gene sequences (with gene ID) measured, only several hundred. were up-regulated and we selected from them only 33 based on relevance for potential affected noxious pathways of the in vivo upregulated mRNAs. The majority of them (23) were changed in the same direction by either PA or cholesterol, and 6 by both agents, whereas remarkably only 1 was changed by aged OZ serum and 4 by hyperglycemia. Only 6 did not have any agreement in the in vitro results with the in vivo upregulated genes. Of note, the myostatin mRNA was uniformly increased in vivo in the LD-MDSC vs ED-MDSC and in vitro by serum, but only by a 1.5 factor. Cholesterol in vitro considerably downregulated myostatin. In turn, of the several hundred sequences that were downregulated we presented in Table 2 only 31 of the in vivo downregulated mRNAs that were changed in vitro in a similar direction, 20 of them by either PA or cholesterol, and 8 by both agents. Again, remarkably, none was with aged serum or with hyperglycemia, and only 2 did not show any in vitro correspondence.

Collectively this indicates that the dyslipidemic factors exerted in vitro a substantial number of changes in the gene-GTS in a similar direction as the imprinting of the LD-MDSC gene-GTS induced by the diabetic milieu in vivo, but high glucose in vitro was virtually inactive as shown for myostatin and other protein expression. This suggested that dyslipidemia rather than hyperglycemia was the main in vivo and in vitro factor in damaging the MDSC. However, the diabetic aged OZ serum was unable to imprint in vitro the ED-MDSC with the noxious in vivo gene-GTS phenotype seen in LD-MDSC, possibly due to the in vitro exposure being too short as to affect their gene-GTS in comparison with the several months operating in vivo.

The in vivo and in vitro gene-GTS changes induced by dyslipidemia were accompanied by parallel considerable changes in miR-GTS.

Therefore, we focused next on changes on a more sensitive and earlier GTS, that could precede and predict the final gene-GTS, namely potential alterations in the miR-GTS transcription. The RNA was this time isolated separately with a procedure tailored to miR recovery and quantification by next-generation sequencing. We had detected (above) over-expression of myostatin both as protein and as mRNA, and both in vivo in LD-MDSC, and in vitro in ED-MDSC with OZ-MDSC. Therefore, we have separated the miR-GTS data into two sets, as myostatin-related (Table 3) or myostatin-unrelated (Table 4) miRs, including all the in vitro treatment ratios vs the control values without addition, for each listed miR irrespective of the magnitude of their changes. This was done because of the putative relevance of myostatin protein overexpression by UC-T2D in vivo or by the aged OZ serum. The results have been compiled as down (<0.5) or up (>2.0) regulation of miR expression in the LD-MDSC vs ED-MDSC ratios, with the same rounding up or down as for the gene-GTS ratios, and presented together with the in vitro values.

Table 3 shows that the in vivo T2D/obesity milieu: a) led to down-regulation of 12 miRs related to myostatin (with none upregulated), implying a subsequent up-regulation of myostatin mRNA/protein expression (as observed), since most miRs act as inhibitors of gene transcription/translation (the reference numbers **55–67** for the inter-relation of individual miRs with myostatin expression are indicated in the table); b) 6 of these miRs were also downregulated in vitro in the ED-MDSC by the aged OZ serum, and remarkably all by PA and 5 by cholesterol, 2 by all in vitro and in vivo (blue highlighting) and only in one case the result was opposite (upregulation) to the one in vivo; c) some of these changes were also seen with the aged LZ serum, but were less intensive; and d) the changes, in this case down-regulation, were more intense for the MDSC miR-GTS than for the MDSC gene-GTS. Based on the level of miR expression (red highlight), the observed down-regulation, its correlation with in vitro changes, and the relevance of each miR to myostatin and stem cell damage-related factors, miRs 21, 199a,b, 27a, 23a, and 181a, stand out.

Table 4 shows in turn, miRs unrelated to myostatin, 3 upregulated and 22 downregulated as a result of long-term in vivo exposure to the UC-T2D/obesity milieu, with all but 5 in the same direction by the aged OZ serum in vitro, all but 5 by PA and all but 12 by cholesterol. Again, the non-diabetic aged LZ serum exerted similar changes as the aged OZ serum, but much less intensive. This suggests that many of these miRs selected with the same criteria (except that they are unrelated to myostatin), may be relevant to the dyslipidemic effects but in this case through myostatin unrelated pathways, such as miR 99a, 152, 26a, 99b, 100, 148b, 10 a, etc.

Remarkably, when the changes listed in Tables 3 and 4 are expressed now in Table 1 Supplement as ratios between the % changes for each one of these miRs over the total expression for the overall listed miRs for each sample (instead of between normalized by raw values), most of the myostatin-related miRs (IDs highlighted in blue) selected from Table 3 preserve approximately the changes presented in Table 3. The same applies to all the other non-myostatin related miRs from Table 4 (non-highlighted IDs), placed there together.

The comparison between percents shows (highlighted in yellow) the considerable agreement between the direction of changes in vivo and in vitro, and the brown highlighting of IDs indicate that at least 4 in vitro ratios are in the same direction as in the in vivo situation. The only opposite directions are highlighted in green. This alternative normalization by % is independent of the total miR expression values for each sample and therefore is not subject to variability in initial RNA inputs or rounds of amplification during sequencing, thus indicating that the changes are specific for each miR and not resulting simply from a general down- or up-regulation of miRs in total.

The few differences between Tables 3 and 4 versus Table 1 Supplement may derive from the fact that Tables 3 and 4 give approximately the actual individual and total miR amounts. However, the latter is subject to variability in initial RNA inputs or rounds of amplification during sequencing.

DISCUSSION

Our previous in vivo data (11) had pioneered the demonstration that in the OZ rat the in vivo long-term exposure of stem cells from an early diabetic stage, our ED-MDSC, to an uncontrolled T2D/obesity milieu induced their failure to repair the tissue by replacing their lost corporal smooth muscle cells, the functional CVOD and its underlying corporal histopathology, and this damage was associated with the LD-MDSC imprinting with a noxious gene-GTS, and, upon their corporal tissue implantation, with the overproduction of myostatin, a pro-lipofibrotic protein that affects stem cell lineage, inhibits the generation of skeletal muscle mass, and is also present in the smooth muscle (48).

In the current work we have now extended these findings by pioneering an in vitro approach to reproduce some of these alterations and study their mechanism specifically by showing in short-term incubations of ED-MDSC: 1) the severe fat infiltration, apoptosis and nonapoptotic cell death, proliferation reduction (in the case of PA and CHOL), differentiation inhibition, and the overproduction of myostatin, induced specifically by the dyslipidemic serum, but none of these elicited by hyperglycemia; 2) the milder fat infiltration with the added dyslipidemic factors, water-soluble palmitate and cholesterol, but more intense apoptosis, particularly with PA, confirming the noxious role of dyslipidemia; 3) their association with changes in the gene-GTS, only some resembling the ones observed in vivo under the T2D/obesity milieu, but with changes in the miR-GTS that are representative of the in vivo observed miR-GTS; and 4) the significance of the miR-GTS for their potential use as a diagnostic tool to detect the stem cells damaged by their original in vivo exposure to the T2D/obesity milieu, in this case the LD-MDSC.

Our current experimental results agree with, and confirm, some of the assumptions from a recent review (21) condensed on its statement that “the environment (so-called niche) from which mesenchymal stem cells **(MSC)** are isolated may determine their usefulness”, particularly in relation to the review citations on the role of apoptosis and the reduction of proliferation in their deterioration induced by metabolic syndrome and T2D. However, our results do not seem to support the review’s proposed main role of oxidative stress and reactive oxygen species **(ROS)** in stem cell damage, at least in or case in the LD-MDSC.

Moreover, our evidence on dyslipidemia as a major factor in the stem cell damage is different from the stem cell senescence or autophagy concepts proposed there. In turn, our work has not focused on the putative (21) membrane derived vesicles role via AMK/mTOR on stem cell multipotency, but we do agree that the T2D/obesity induced release of cytokines (mainly interleukins and chemokines) may be worth to study.

We had previously discussed the implications of the gene-GTS alterations caused by a 9 month experience of the UC-T2D/obesity milieu in vivo (11), so it was now interesting to find that PA and to a lesser extent cholesterol, induced in vitro a much faster imprinting on many of those genes in just 4 days in the treated ED-MDSC. Again, hyperglycemia was ineffective in modifying gene-GTS, although based on its well known effects through pathways such as AGE (advanced glycation end products) production we cannot discard that it may contribute to the dyslipidemia damage. However, although the added aged OZ serum induced some changes, they were not the expected ones that would mimic the in vivo modifications. We believe that the complex nature of the aged OZ serum composition, its dilution in vitro (added to only 5%) that reduces its dyslipidemic factor impact, and above all an insufficient time of contact, were potential reasons for blunting the effects of serum on the gene-GTS, in contrast to the UTT2D/obesity milieu.

The discrepancy between some of the effects on the stem cells exerted by the T2D/obesity exposure in vivo and the aged OZ serum exposure in vitro, was compensated by the finding of a considerable and distinctive alteration of the miR-GTS found in the in vivo exposed LD-MDSC that was partially reproduced by the miR-GTS changes induced in vitro by the aged OZ serum. This makes sense if one considers that miRs are early inhibitors of gene transcription and translation, with miR-GTS changes necessarily preceding the resulting gene-GTS mRNA changes. Therefore, the miR-GTS is a more sensitive marker for the initial phenotype changes induced by a noxious factor than the gene-GTS. The fact that PA and to a much lesser extent, cholesterol, induced a much faster imprinting than the aged OZ serum of the gene-GTS in the ED-MDSC, and substantial changes in the miR-GTS, suggest that these factors are at least partially responsible for the dyslipidemic serum effects.

Of particular relevance, considering the observed in vivo UC-T2D/obesity-induced, and the in vitro aged OZ serum-induced upregulation of myostatin protein in MDSC and the lesser up-regulation of myostatin mRNA, is the in vivo and in vitro induced alterations of miR-GTS in both conditions for individual miRs related to myostatin. In fact, the selection of the miRs downregulated in vivo in the MDSC by the UC-T2D/obesity milieu in terms of their relationship to myostatin is validated in the bibliographic assessment presented in Table 2 Supplement, by showing how each of these miRs are also related to potential stem cell damage processes. The observed down-regulation of miRs is in general associated with up-regulation of the respective gene, thus agreeing with the observed higher myostatin protein. Some of the selected miRs 21, 199a,b, 27a, 23a, and 181a, act either as inhibitors of myostatin expression or of downstream pathways (55–67), thus also acting as modulators of stem cell replication, and of damaging processes such as apoptosis, inflammation, fibrosis, etc, or are involved in dyslipidemia or UCT2D pathways. The relevance of the in vivo and in vitro elicited changes in miR-GTS extend to processes affected by non-myostatin related miRs. (Table 3 Supplement). Although their discussion exceeds the paper scope and length,

miRs 99a, 100, Let 7a and 11a may be selected for further bibliographic analysis to explore their significance for stem cell damage through pathways other than myostatin.

We believe that the current work not only expands our previous observations (11), but it also pioneers an initial mechanistic approach by emphasizing dyslipidemia as the main noxious factor of MDSC damage, over hyperglycemia, and of an in vitro approach for a fast procedure to study these effects. The role of myostatin in this process is still unknown, but if its observed upregulation on the ED-MDSC by aged OZ serum is relevant to their fat infiltration and other damage, this would fit the well known pro-adipogenic/obesogenic role of myostatin (68–70), and compound the stem cell damage with other noxious systemic changes.

The limitations of this work stem essentially from it being a very innovative approach to an important conceptual issue, that still needs to be confirmed in future work in other animal models of UC-T2D/obesity, and subsequently in the human in order to establish clinical significance. This opens up questions both in vivo and in vitro, such as: a) can lower concentrations of serum or lipid factors during a longer but still short period be a more effective paradigm to mimic the in vivo exposure, and show in the case of serum more inhibition of proliferation?; b) can a causal effect of the overproduction of myostatin on the MDSC damage be found by inhibiting myostatin expression? c) how generally these noxious phenotypes and epigenetic changes may occur in other stem cells relevant for the therapy of erectile dysfunction or Peyronie’s disease, e.g., adipose-derived, bone marrow, endothelial stem cells, etc, and if so, how long and intensive the in vivo exposure needs to be to damage the stem cells?; d) is fat infiltration the trigger of the apoptosis and genomic changes, or are both related but independent?; e) what role mediators other than dyslipidemia, such as oxidative stress, and even AGEs produced by hyperglycemia (21) may play in the stem cell impairment, and what are the biochemical pathways involved in the process?; f) what is the time course for affecting “stemnesss”, i.e, stem cell proliferation and death, cell lineage commitment, etc, and can these changes not just affect tissue repair, but reach situations where even the host health is compromised by for instance aberrant cell lineage commitment?; g) may chronic conditions in animal models other than UC-T2D/obesity, like hypertension, vasculopathies, heavy smoking, cancer, very old age, affect the stem cell milieu similarly to UC-T2D/obesity; h) do these processes affect not just implanted but also endogenous stem cells and their recruitment to injured tissues?; i) perhaps the most important, can this stem cell damage be prevented and/or corrected by in vivo diabetes control, or ancillary pharmacological treatment like what we showed with pioglitazone in the OZ rat for CVOD (58), or ex vivo in the laboratory with simple biological/ chemical procedures prior to implantation as proposed (21)?

Until some of the questions are experimentally tested it may be prudent to exert considerable caution regarding the clinical implantation as autografts of stem cells from long-term UC-T2D/obesity patients, and possibly from other serious chronic conditions, to avoid unpredictable repair efficacy impairment, undesirable cell lineage commitment, or even general biological toxicity. However, based on our proof of concept, one important action may be taken soon, namely, to conduct validation research on miR-GTS as potential biomarkers to detect the degree of damage on autologous stem cells as a pre-requisite to

approve their efficacy and safety prior to their in vivo implantation into the target affected organs of patients with chronic serious co-morbidities. This requires two research stages for at least T2D/obesity patients: a) to establish the effects of the duration of MDSC exposure and the intensity of the dyslipidemia in the in vivo milieu specifically on miR-GTS, and comparing with other types of autologous stem cells, using several animal models and target organs; b) transferring and validating these results to in vitro work with the counterpart human stem cells, and if possible isolating them from patients with severe T2D/obesity. This will allow to compare the “normal” miR-GTSs of the stem cells isolated from young and healthy individuals, with the “abnormal” ones in the same type stem cells from the patients who would be both donors and potential recipients of the autologous implants. Finally, if the T2D/obesity damage of stem cells is validated in the human, it may be speculated that obtaining normal stem cells from young healthy individuals and cryopreserving them, may be useful for future autograft reimplantation when needed if the subjects have developed T2D/obesity complications. However, this would require thorough research.

In summary, this study expands and confirms our previous demonstration that long-term exposure of MDSC to the T2D2/obesity environment damages their tissue repair capacity and induces a noxious gene-GTS, by now showing that also the miR-GTS is considerably disturbed in vivo, particularly for miRs affecting myostatin expression, and that these alterations can be partially reproduced in vitro by short-term incubation of MDSC with highly dyslipidemic serum and dyslipidemic factors, which induce some similar miR-GTS changes, severe fat infiltration, apoptosis, overproduction of myostatin, and other noxious effects not seen with hyperglycemia alone. This allows in vitro mechanistic studies faster and easier to perform than in vivo. This implies, if supported by future animal and human studies, that both the therapeutic effects of implanted autologous MDSC in highly dyslipidemic T2D/obesity patients, their endogenous recruited MDSC cell repair, and even of other stem cells, may be compromised. The miR-GTS should be further studied and validated as a potential biomarker for screening stem cell damage prior to implantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations:

CHOL	water soluble cholesterol-methyl cyclodextrin
ED-MDSC	MDSC from male 12 weeks old OZ rats, with early T2D, incipient mild hyperglycemia/dyslipidemia and moderate overweight
gene-GTS	gene global gene transcriptional signature
LD-MDSC	MDSC from 32 weeks old male OZ rats, with late T2D, moderate hyperglycemia, high dyslipidemia and morbid obesity
LZ	Lean Zucker
LZ serum	serum from 32 weeks old, non-T2D LZ rats

MDSC	muscle derived stem cells
MSC	mesenchymal stem cells
miR	microRNA
miR-GTS	global miR transcriptional signature
PA	water soluble palmitic acid Na salt/albumin
OZ	Obese Zucker
OZ serum	serum from 32 weeks old, late T2D OZ rats
ROS	reactive oxygen species
QIA	Quantitative image analysis
UC-T2D	uncontrolled T2D
T2D	type 2 diabetes mellitus

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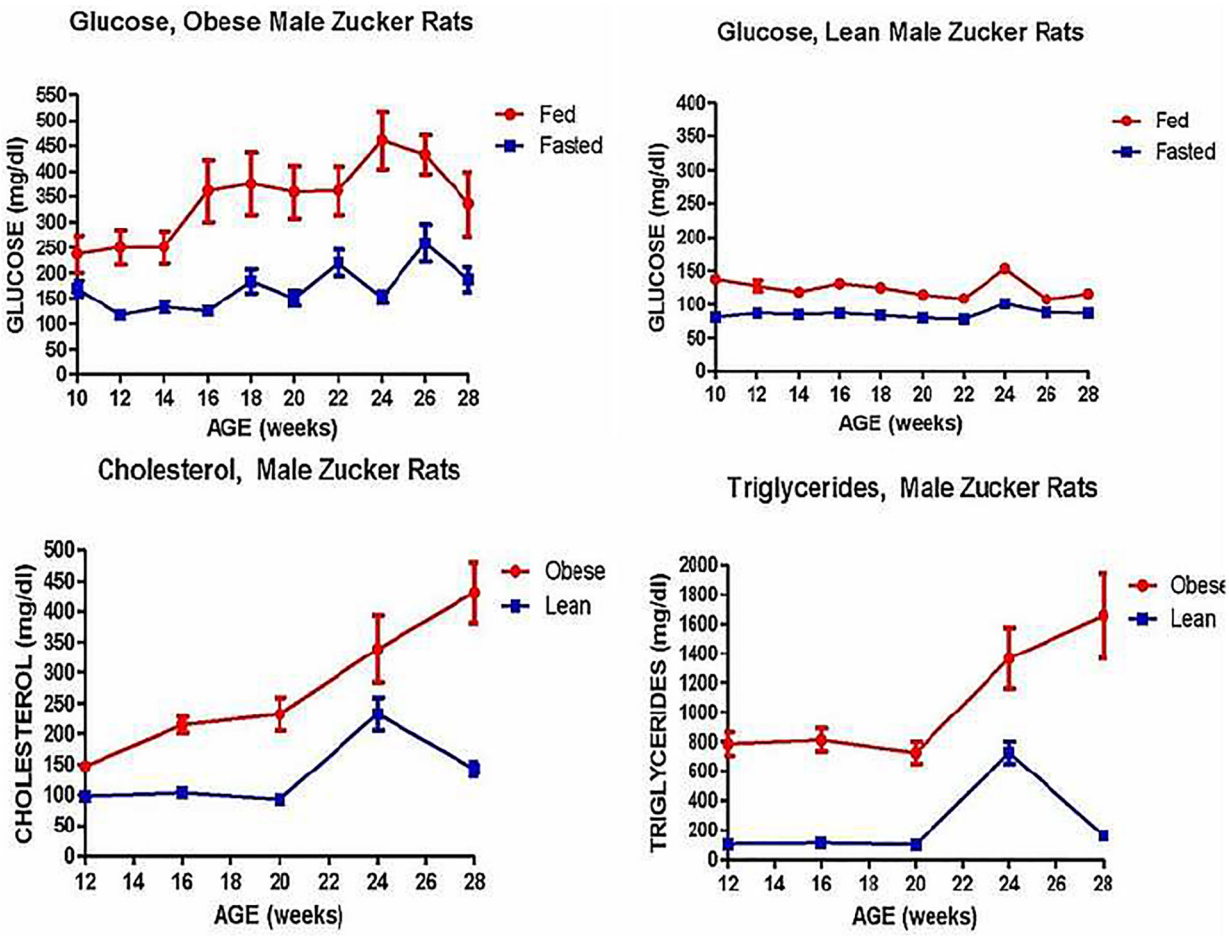

Figure 1. A moderate hyperglycemia but intense dyslipidemia develops with age in male diabetic obese Zucker (OZ) rats in contrast to the related strain non-diabetic lean Zucker (LZ) rats.
Animals were subjected to weekly evaluation of glucose, cholesterol, triglycerides, and insulin (not shown) in the serum from 12 to 28 weeks of age.

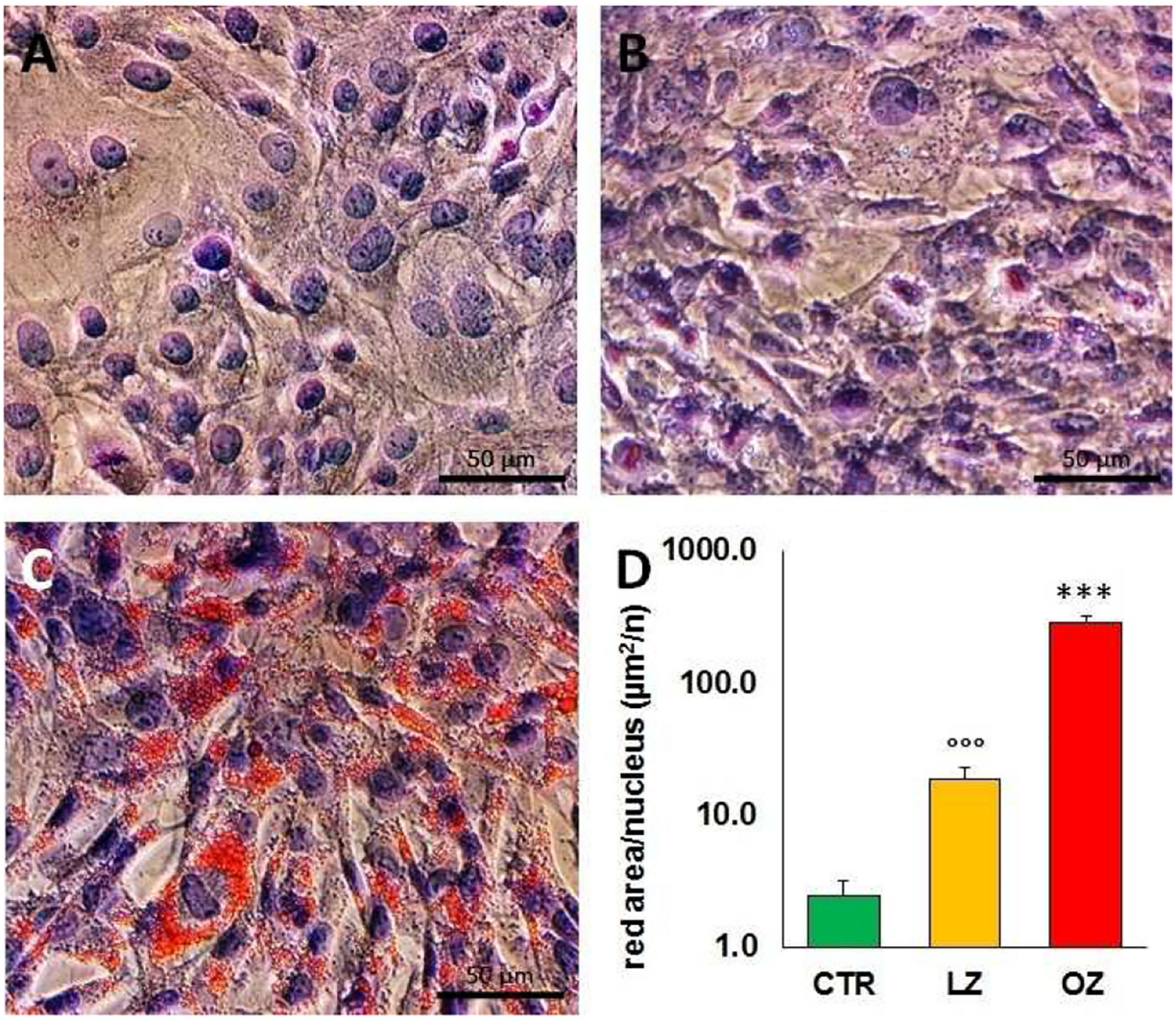

Figure 2. Incubation of ED-MDSC with a higher level of aged OZ serum induced a considerable intracellular infiltration by fat globules.

ED-MDSC were incubated with no addition **(A)**, or with added 5% LZ serum **(B)** or 5% OZ serum **(C)** as in Fig. 2 Supplement, and stained with Oil Red O. Pictures were taken at 200X, but QIA was applied to multiple fields at 100X **(D)** Graph of red area (fat) per cell. ***p≤0.005 (CTR vs OZ); $^{\circ\circ\circ}$p≤0.005 (LZ vs OZ)

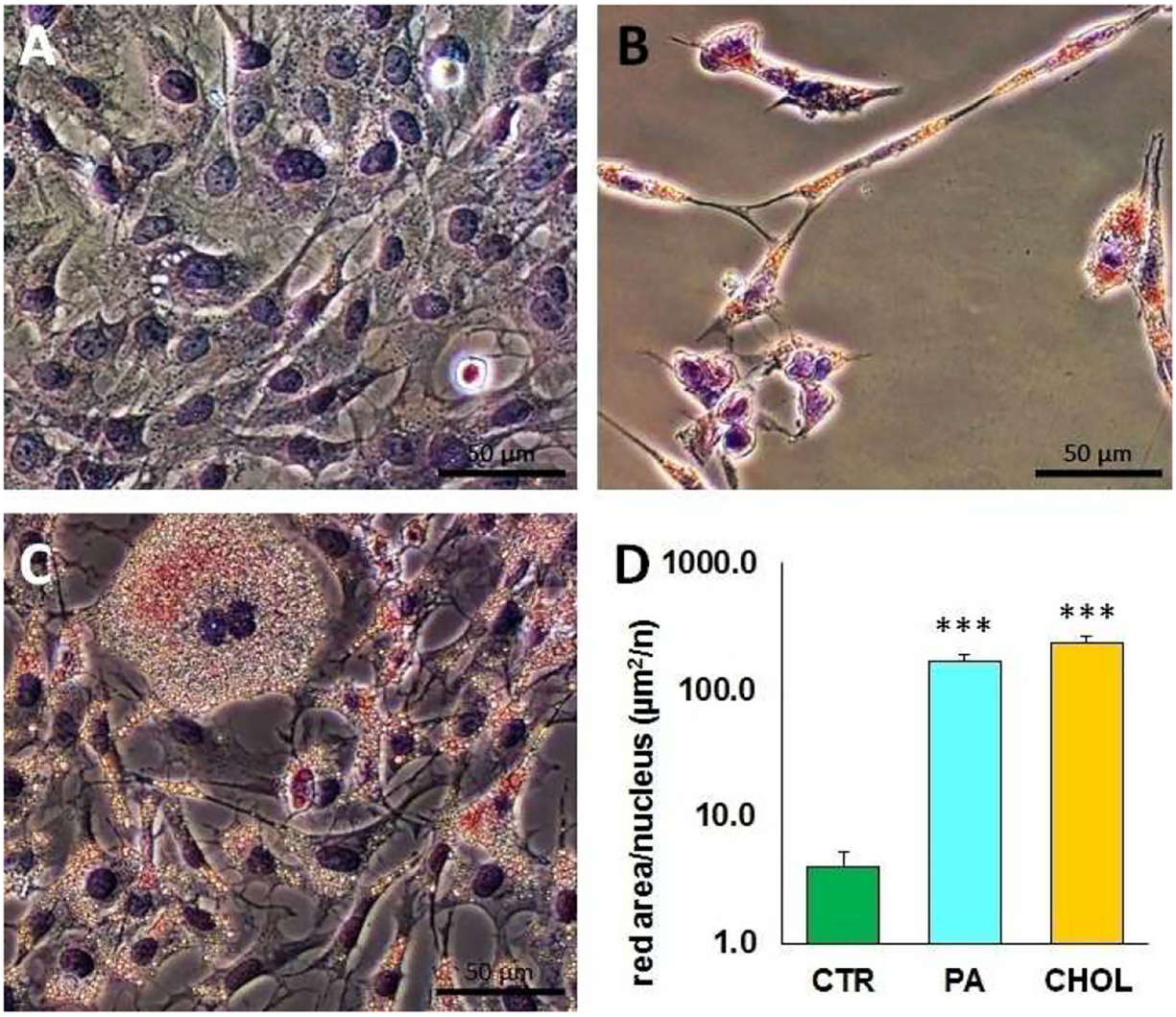

Figure 3. Incubation of ED-MDSC with water-soluble preparations of the sodium salt of palmitic acid (PA) as representative of saturated free fatty acids, or cholesterol (CHOL), induced a milder infiltration of fat globules.

ED-MDSC were incubated with no addition **(A)**, or with added PA at 0.5 **(B)** or 1 mM (not shown), or added CHOL at 50 mg/dl **(C)**, or 100 mg/dl (not shown) and stained with Oil Red O. Pictures were taken at 200X, but QIA was applied to multiple fields at 100X **(D)** Graph of red area (fat) per cell. ***p≤0.005 (CTR vs OZ)

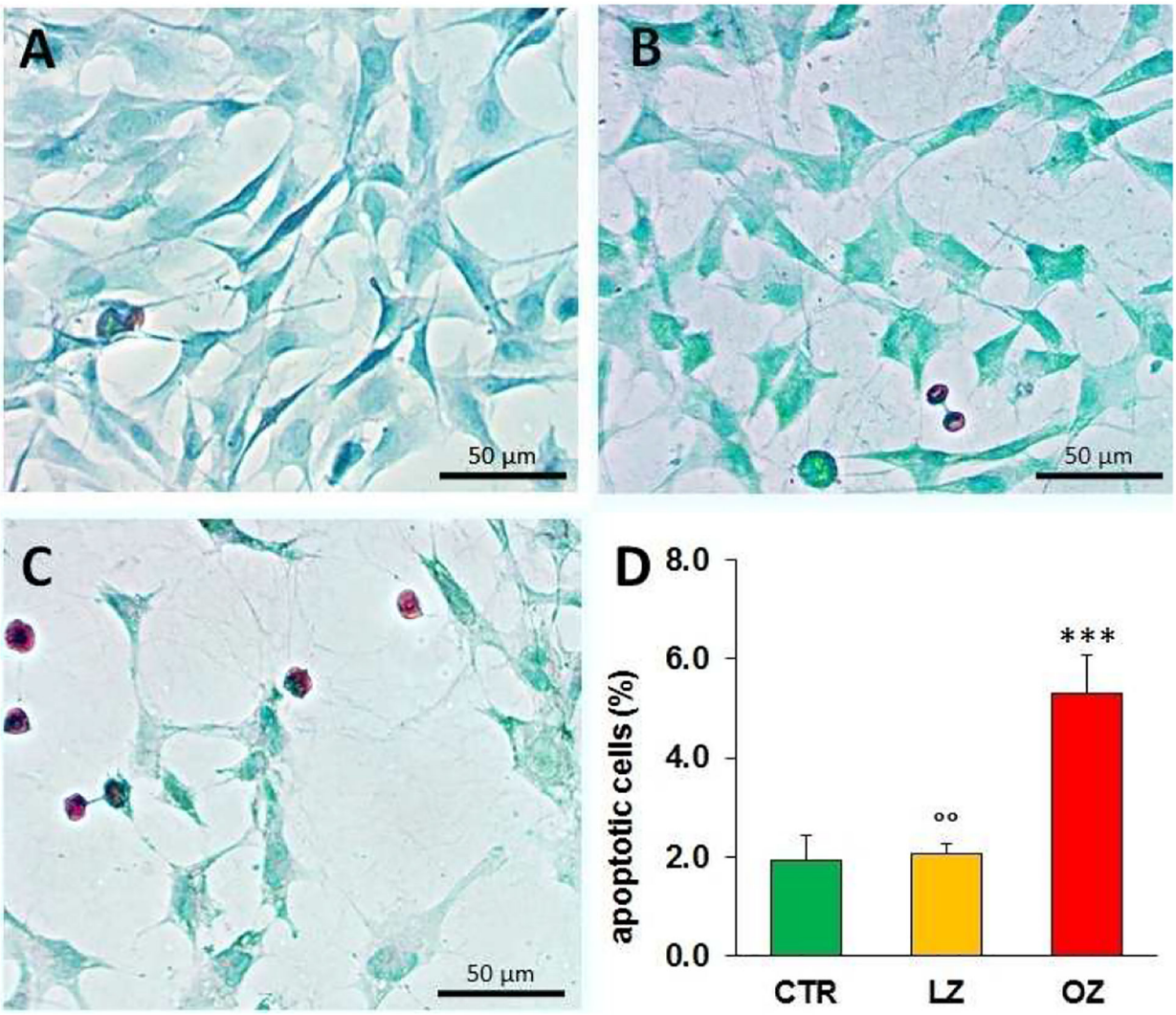

Figure 4. Incubation of ED-MDSC with 5% aged OZ serum induced moderate apoptosis.

ED-MDSC were incubated with no addition **(A)**, or with added 5% serum from aged LZ rats **(B)** or 5% serum from aged OZ rats **(C)** as in Fig. 2 and stained with the Tunel reaction. Pictures were taken at 200X, but QIA was applied to multiple fields at 100X **(D)**. Graph of apoptotic index. ***$p \leq 0.005$ (CTR vs OZ); $^{\circ\circ}p \leq 0.01$ (LZ vs OZ) The morphology of the control cells appears slightly different than in the previous figures stained for Oil Red O, but leaving aside the typical stem cell coexistence of morphological variants, is the different background color in both types of reactions what mostly creates this impression.

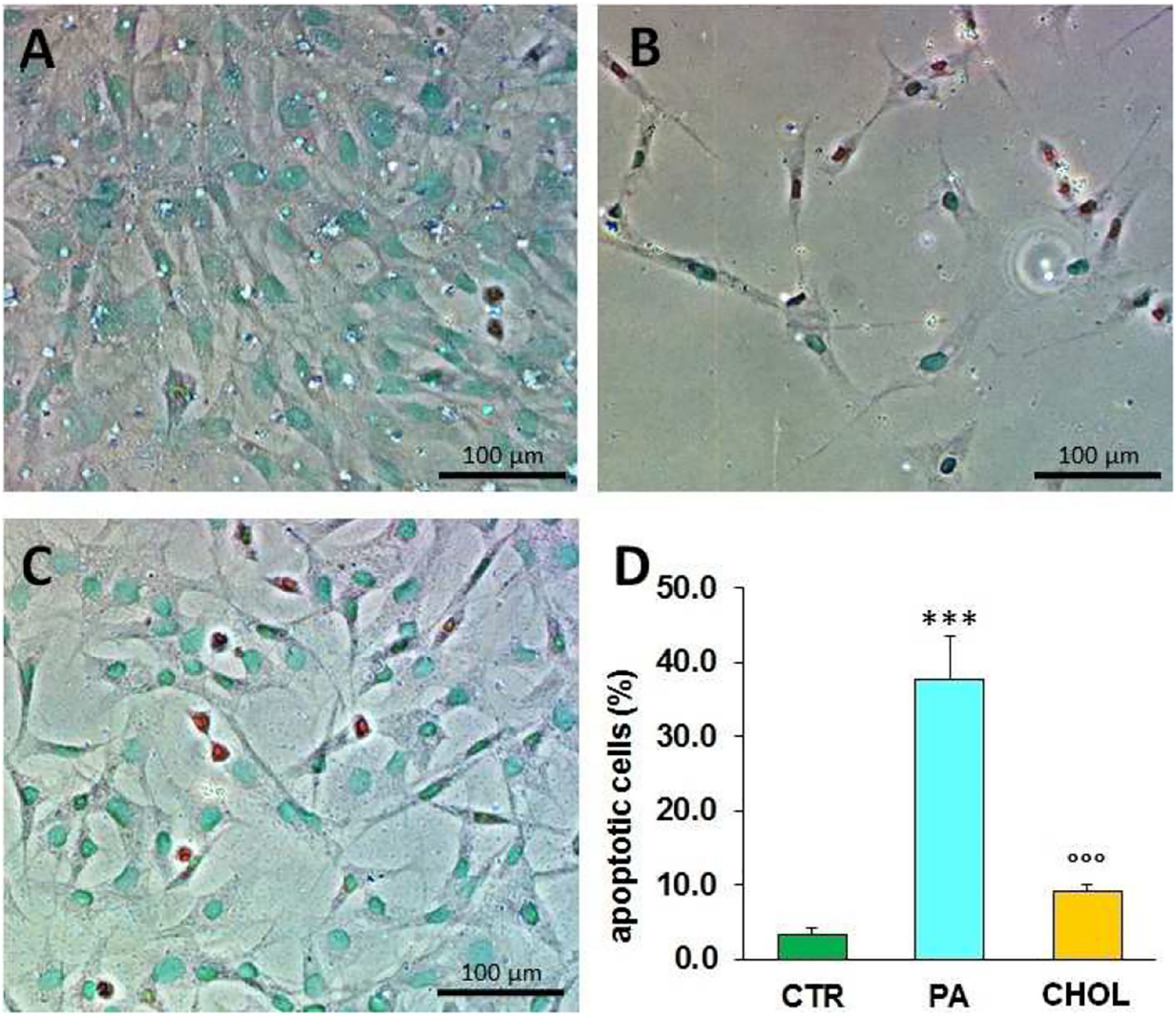

Figure 5. Incubation of ED-MDSC with preparations of water-soluble palmitic acid (PA), or cholesterol (CHOL), induced considerable apoptosis.

ED-MDSC were incubated with no addition **(A)**, or with added PA at 0.5 **(B)**, or added CHOL at 50 mg/dl (B) or 100 **(C)** and stained with the Tunel reaction. Pictures were taken at 200X, but QIA was applied to multiple fields at 100X **(D)** Graph of apoptotic index. ***p≤0.005 (CTR vs OZ); $^{○○○}$p≤0.005 (LZ vs OZ)

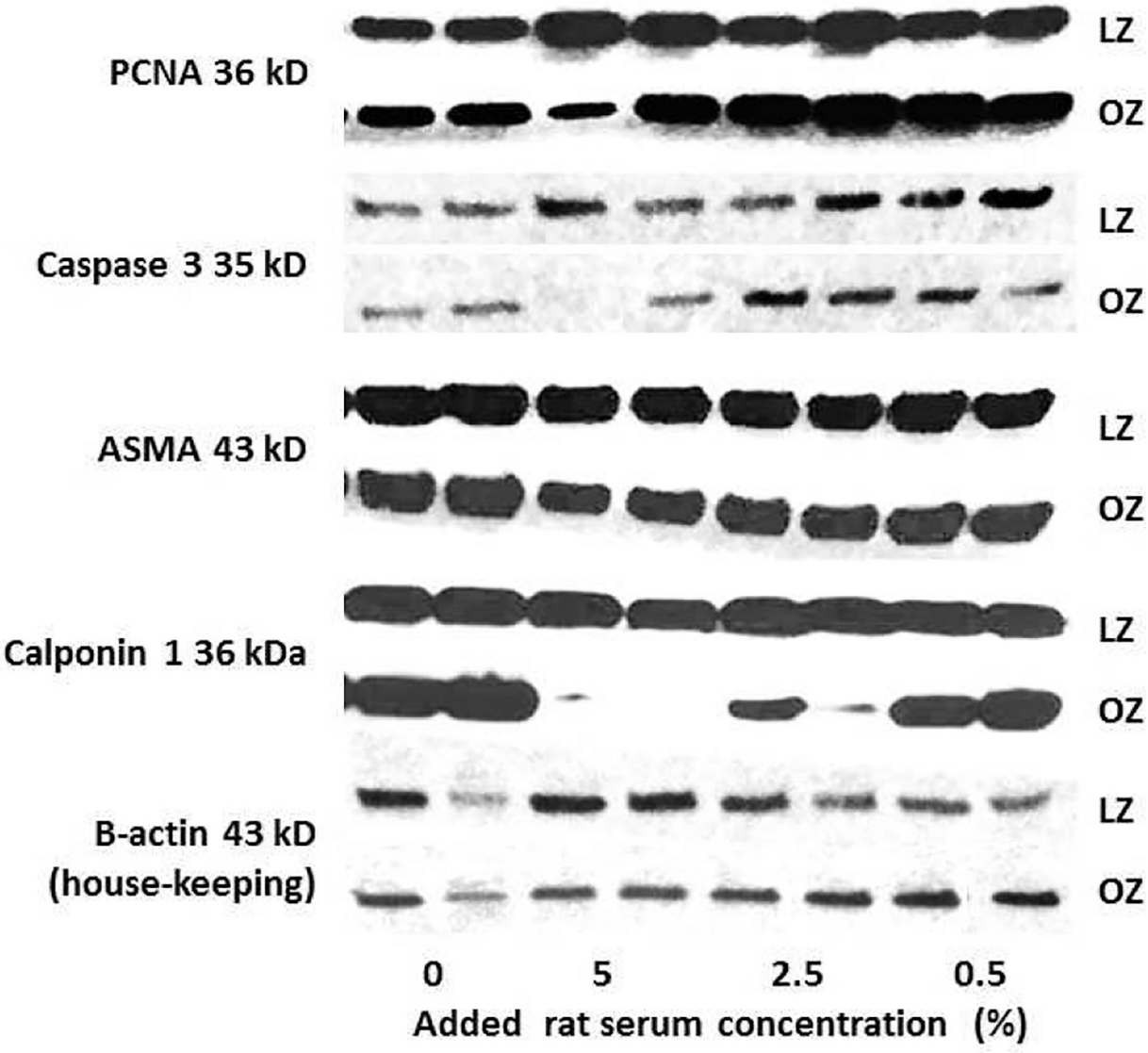

Figure 6. Incubation of ED-MDSC with OZ serum caused a moderate decrease in cell replication and smooth muscle differentiation.

ED-MDSC were incubated in duplicate with no addition, with added 0.5%, 2.5%, or 5% aged OZ or LZ serum, and subjected to western blot as indicated, with beta-actin as a housekeeping gene. See Fig. 7 for quantitative analysis.

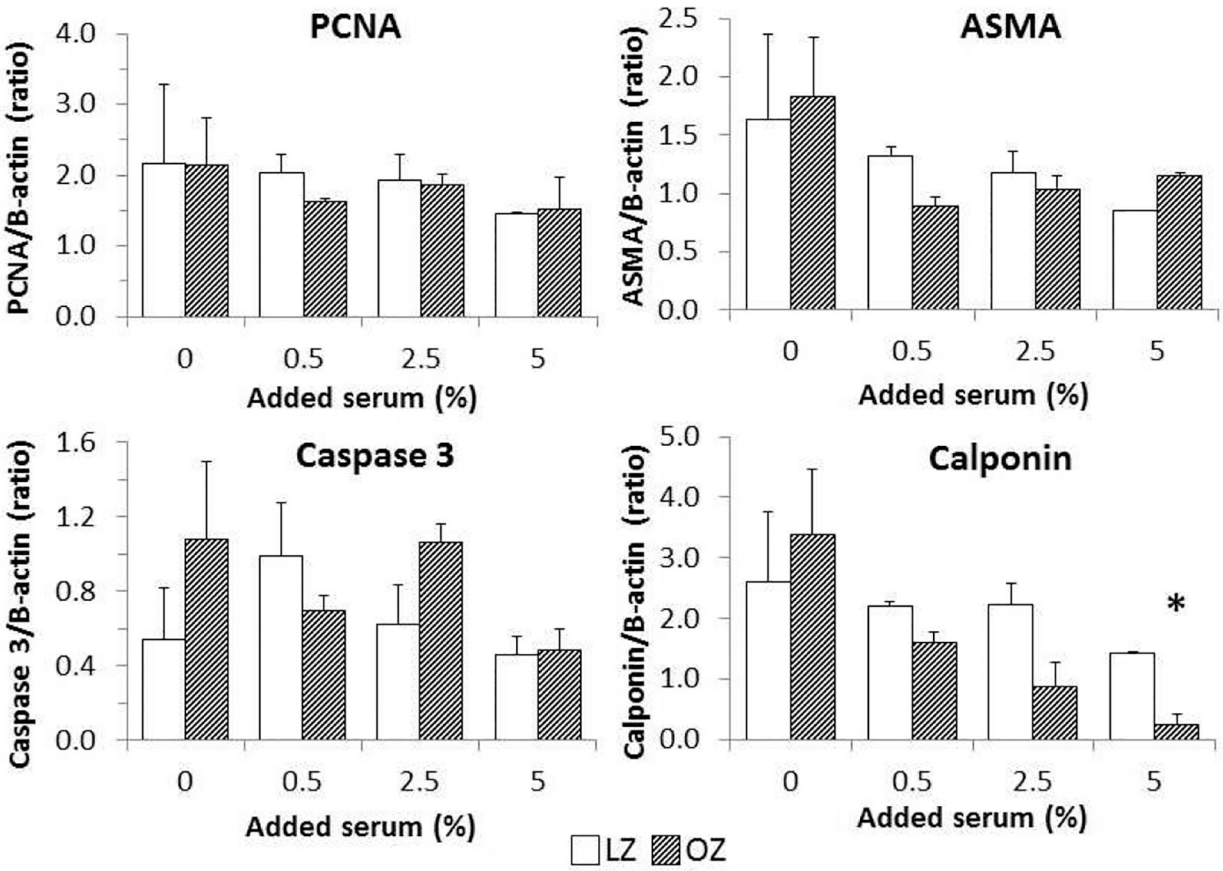

Figure 7. The quantitative image analysis (QIA) of the protein band densitometries in the incubations of ED-MDSC with OZ serum and palmitic acid sodium salt confirmed the visual inspection of the western blot images.

The selected bands of the duplicate experiments seen in Fig. 6 and in Fig. 2 Supplement (single experiment with cholesterol excluded) were subjected to QIA and the means+/−SEM were statistically compared for variance in each type of incubations. *p≤0.05 compared to control

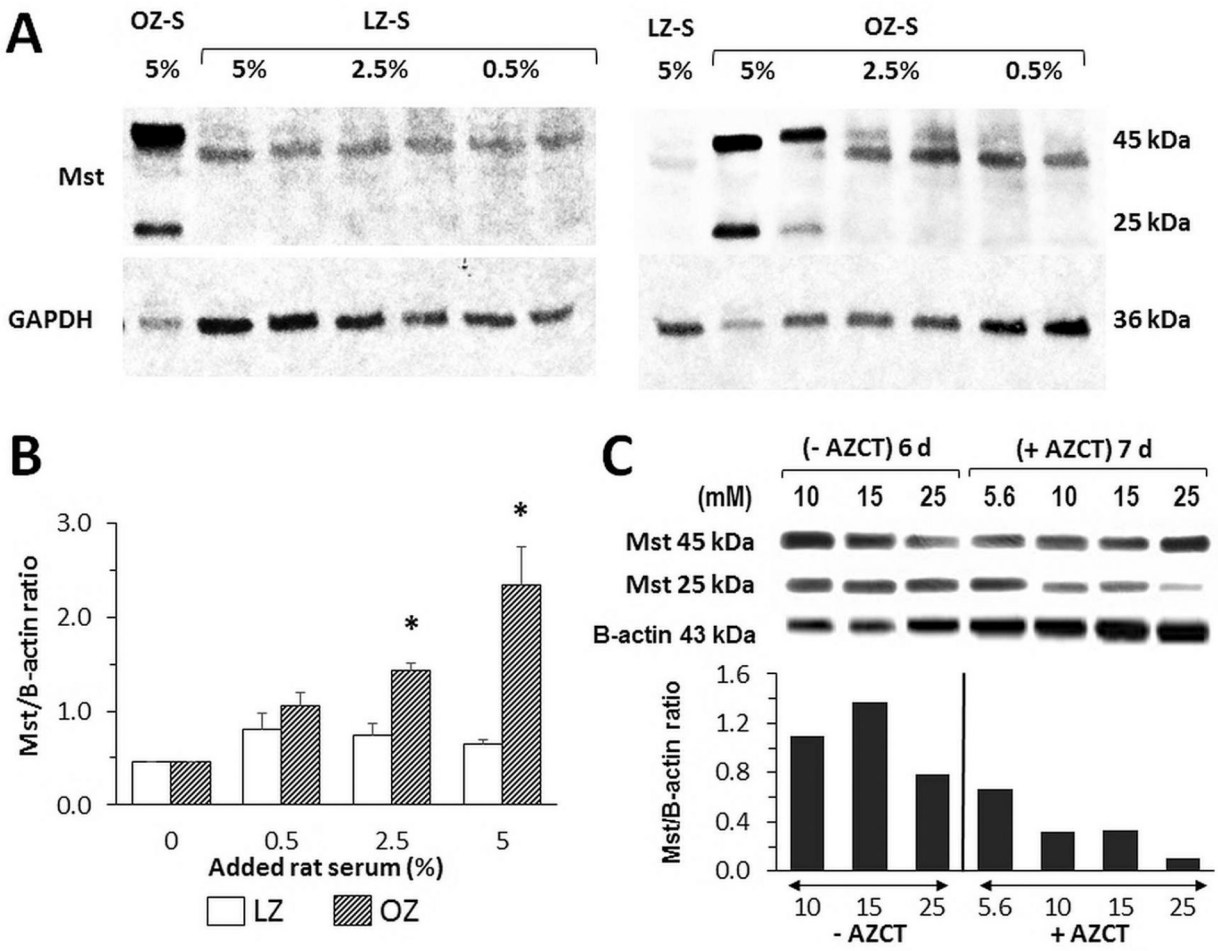

Figure 8. Incubation of ED-MDSC with increasing aged OZ serum caused a concentration dependent expression of myostatin protein, a pro-lipofibrotic and muscle mass inhibitor, but the incubation with increasing glucose exerted an opposite effect.

Incubations were performed on 6 well plates with no addition, or in duplicate with increasing concentrations of OZ or LZ serum **(A)**, or of glucose either before or after 2 mM azacytidine for 2 days to stimulate stemness **(C)**. Cell homogenates were subjected to western blot as indicated, with beta-actin as housekeeping gene, and densitometry was applied for the A samples **(B)**. Other biomarkers for fibrosis, apoptosis, replication, and SMC differentiation were assayed in C, but no changes were observed (not shown). *p≤0.05 compared to control

Table 1.

The long-term in vivo exposure of MDSC to T2D in aged OZ rats (LDMDSC) caused the transcriptional upregulation of many genes in comparison with the unexposed ED-MDSC, affecting inflammation, fibrosis, chemokines, and other noxious pathways, and most of these changes were replicated by the short term in vitro incubation with dyslipidemic factors, but not by hyperglycemia or aged OZ serum.

The cell culture basal values for each gene in the global transcriptional signatures **(GTS)** for EDMDSC were obtained separately for: a) the comparison with the GTS of LD-MDSC, and b) with all the in vitro incubation GTS comparisons, in order to compensate for different passage numbers and separate DNA microarray assays. The ratios for the in vivo exposure LDMDSC/ED-MDSC, and for the different in vitro exposures of ED-MDSC vs the in vitro control ED-MDSC were obtained and highlighted in yellow when >2.0 or <0.5. The many up-regulated LD/ED genes are selected in this table for their relevance to stem cell damage related processes, and their IDs are highlighted in yellow only when one or more of the in vitro changes were in the same direction as the in vivo ones. Green highlighting indicates occasional opposite ratios.

In vivo LD/ED MDSC up-regulated genes (mRNAs)		In vivo T2D effects		In vitro effects of serum (S), lipidemic factors (PA: palmitic acid; CHOL: cholesterol) and glucose (G) (HG: highG; LG: low G)				
Gene ID	**Gene Description**	**Basal ED**	**LD/ ED**	**Basal ED-2**	**OZS/ ED-2**	**CHOL /ED-2**	**PA/ ED-2**	**HG/ LG**
II1a	interleukin 1 alpha	56	67.7	99	0.7	0.7	3.0	0.7
Fgf7	fibroblast growth factor 7	309	24.1	194	0.8	3.4	3.3	1.1
Mt2A	metallothionein 2A	758	20.8	251	0.5	3.3	1.2	0.6
CxcI1	chemokine (C-X-C motif) ligand 1	476	17.2	577	0.6	2.6	6.4	0.9
Mmp9	matrix metallopeptidase 9	176	15.0	173	0.7	1.9	0.9	0.9
Angpt4	angiopoietin 4	189	11.1	715	0.7	2.0	1.1	0.7
Bdkrb1	bradykinin receptor B1	88	9.8	74	0.8	2.7	1.6	1.2
Ccr1	chemokine (C-C motif) receptor 1	64	7.9	23	1.1	6.7	14.7	1.2
Itga2	integrin, alpha 2	148	6.8	154	0.9	2.5	1.2	0.9
Cxcl5	chemokine (C-X-C motif) ligand 5	90	5.4	50	1.0	2.1	1.4	1.3
II6	interleukin 6	370	4.9	195	1.7	1.5	0.6	2.2
Xdh	xanthine dehydrogenase	400	4.5	619	1.0	2.4	0.9	1.3
Wnt4	wingless-type MMTV, member 4	306	4.5	842	0.7	1.5	0.3	0.8
Smad6	SMAD family member 6	290	4.4	736	0.8	0.9	0.5	0.9
Thbs2	thrombospondin 2	746	4.1	5360	1.1	0.8	0.3	1.4
Angpt1	angiopoietin 1	70	4.0	180	1.7	0.7	1.0	2.1
Cpt1a	carnitine palmitoyltransf 1a, liver	412	3.5	472	1.7	1.7	2.0	1.0
Col15a1	collagen, type XV, alpha 1	278	3.3	438	0.6	0.4	0.5	0.6
Mmp3	matrix metallopeptidase 3	638	3.2	2279	0.9	1.1	3.1	1.0
Cxcl16	chemokine (C-X-C motif) ligand 16	1407	3.1	2477	1.6	2.2	2.6	2.0

In vivo LD/ED MDSC up-regulated genes (mRNAs)		In vivo T2D effects		In vitro effects of serum (S), lipidemic factors (PA: palmitic acid; CHOL: cholesterol) and glucose (G) (HG: highG; LG: low G)				
Gene ID	**Gene Description**	**Basal ED**	**LD/ ED**	**Basal ED-2**	**OZS/ ED-2**	**CHOL /ED-2**	**PA/ ED-2**	**HG/ LG**
Bmp2	bone morphogenetic protein 2	153	2.9	257	0.9	1.8	3.5	1.2
Mmp13	matrix metallopeptidase 13	63	2.9	145	0.5	0.7	1.7	0.8
Mmp23	matrix metallopeptidase 23	712	2.9	2815	1.0	0.8	0.2	1.0
Cd68	Cd68 molecule	109	2.6	109	1.1	3.0	1.4	1.0
Tgfbr3	transforming growth factor, beta rec, III	157	2.5	440	1.0	2.1	1.4	1.2
II7	interleukin 7	79	2.4	98	0.7	1.2	1.9	0.9
Cd274	CD274 molecule	291	2.3	69	1.2	1.8	2.2	1.4
Mmp10	matrix metallopeptidase 10	64	2.3	41	1.0	1.4	4.6	1.0
BCI2I11	BCL2-like 11 (apoptosis facilitator)	207	2.3	136	0.9	2.1	1.6	1.2
Myocd	myocardin	2047	2.2	519	1.0	0.4	0.5	0.7
Tnfrsf11b	tumor necrosis factor recept. family, 11b	2323	2.1	1723	2.8	2.8	2.3	2.3
PIa2g2a	phospholipase A2, group IIA	51	2.0	268	1.0	4.2	2.7	1.2
Mstn	myostatin	56	1.5	721	1.5	0.1	1.4	1.2

Table 2.

The gene-GTS alteration caused in vivo by T2D or in vitro by dyslipidemia occurred also in opposite direction in a set of downregulated genes selected by their in vivo relevance.

See Table 1 caption, but here only the LD-MDSC downregulated genes are selected with the same procedure as in Table 1

In vivo LD/ED MDSC down-regulated genes (mRNAs)		In vivo T2D effects		In vitro effects of serum (S), lipidemic factors (PA: palmitic acid; CHOL: cholesterol) and glucose (G) (HG: high G; LG: low G)				
Gene ID	**Gene Description**	**Basal ED**	**LD/ ED**	**Basal ED-2**	**OZ/ ED-2**	**CHOL/ ED-2**	**PA/ ED-2**	**HG/ LG**
Ednra	endothelin receptor type A	785	0.5	408	0.9	0.9	0.4	1.1
Col12a1	collagen, type XII, alpha 1	10193	0.5	10115	1.0	0.4	0.5	0.9
Cdh3	cadherin 3	2526	0.5	510	0.9	0.9	0.4	0.9
Col6a1	collagen, type VI, alpha 1	3359	0.5	3729	0.7	0.7	0.5	0.7
Bmp6	bone morphogenetic protein 6	544	0.5	809	1.2	0.5	0.2	1.1
Igfbp5	insulin-like growth factor binding protein 5	344	0.5	182	0.8	1.6	0.4	0.9
Col3a1	collagen, type III, alpha 1	3402	0.5	7135	0.7	0.6	0.4	0.8
Tgfb2	transforming growth factor, beta 2	5511	0.4	5305	1.0	0.5	0.4	0.9
Fzd2	frizzled family receptor 2	2888	0.4	2526	0.8	0.8	0.3	0.9
Myh10	myosin, heavy chain 10, non-muscle	4476	0.4	1868	1.0	0.8	0.5	0.8
Thbs4	thrombospondin 4	1766	0.4	543	1.0	0.9	0.5	0.9
Fads1	fatty acid desaturase 1	6074	0.4	3411	0.6	0.5	0.6	0.7
Cd200	Cd200 molecule	1692	0.4	356	2.1	0.9	1.2	2.8
Myh1	myosin, heavy polypeptide 1, sk muscle	5196	0.3	1868	1.0	0.8	0.5	0.8
Pltp	phospholipid transfer protein	1735	0.3	315	1.1	1.1	0.4	1.2
Cnnm2	cyclin M2	1611	0.3	2564	1.0	0.5	0.7	1.2
Casp12	caspase12	846	0.3	921	1.2	0.5	0.7	1.2
Col11a1	collagen, type XI, alpha 1	1658	0.3	2942	1.1	0.6	0.4	1.1
Fads2	fatty acid desaturase 2	4337	0.2	2839	0.6	0.3	0.4	0.7
Casp4	caspase 4, apoptosis-rel cysteine peptid	633	0.2	840	0.9	0.5	0.6	1.2
Fzd8	frizzled family receptor 8	2907	0.2	1713	0.9	0.5	0.4	1.1
Adamtsl3	ADAMTS-like 3	767	0.2	846	0.8	0.4	0.2	0.9
Cxcl10	chemokine (C-X-C motif) ligand 10	3152	0.2	370	1.1	0.9	3.7	0.9
Tnnc1	troponin C type 1 (slow)	1080	0.2	166	1.0	0.5	0.6	0.9
Fgf1	fibroblast growth factor 1	1828	0.2	2049	1.3	0.8	0.4	1.4
Cmklri	chemokine-like receptor 1	749	0.2	402	0.9	1.1	0.4	1.2
Itga11	integrin, alpha 11	7179	0.2	6784	0.9	0.7	0.4	0.9
Itga4	integrin, alpha 4	2243	0.1	843	1.7	0.5	0.5	0.9

In vivo LD/ED MDSC down-regulated genes (mRNAs)		In vivo T2D effects		In vitro effects of serum (S), lipidemic factors (PA: palmitic acid; CHOL: cholesterol) and glucose (G) (HG: high G; LG: low G)				
Gene ID	**Gene Description**	**Basal ED**	**LD/ ED**	**Basal ED-2**	**OZ/ ED-2**	**CHOL/ ED-2**	**PA/ ED-2**	**HG/ LG**
Fndc1	fibronectin type III domain containing 1	1772	0.1	1924	1.0	0.3	0.2	1.7
Omd	osteomodulin	477	0.1	116	1.3	0.5	0.2	2.6
ItgbI1	integrin, beta-like 1	2858	0.0	2129	1.5	0.9	0.4	1.7

Table 3.

The long-term in vivo exposure of MDSC to the T2D diabetic milieu in the LD-MDSC, induced also a considerable change in the miR-GTS, and for this table specific miRs are selected by their relevance to myostatin, accompanied by many similar direction changes caused by treatment of MDSC in vitro with dyslipidemic serum and factors.

See Tables 1 and 2 for captions, with individual, downregulated miRs selected here for their relevance to myostatin. The red highlighting indicates basal values >20,000 per 10^7 total raw reads so that every sample was normalized in this way. See Table 2 Supplement for the myostatin and stem cell related significance.

Myostatin-related miRs changed in amount by T2D in LD vs ED	In vivo T2D effects (OZ rat)		In vitro effects of serum (S) and lipidemic factors (PA: palmitic acid; CHOL: cholesterol)				Reference numbers for myostatin-related miRs
	Basal value	Ratio to ED-MDSC	Ratios to ED-MDSC				
	ED (per 10^7reads)	LD/ED	OZS/ ED	LZS/ ED	PA/ ED	CHOL/ ED	
23a-3p	18,140	0.50	1.51	1.62	0.30	0.46	55
30e-5p	6,244	0.42	0.71	0.77	0.54	0.58	56
27a-3p	11,885	0.41	0.82	1.35	0.33	0.42	55,58,59,61, 62,67,See 60
181a-5p	10,092	0.39	1.21	1.29	0.43	0.50	64
101a-3p	2,549	0.38	1.62	2.26	0.51	0.57	57
199a-5p	62,452	0.29	0.13	0.23	0.25	0.52	57, see 60
199a-3p	20,968	0.25	0.15	0.37	0.33	0.63	57, see 60
29a-3p	1,872	0.25	0.80	1.16	0.53	0.58	63 (as miR-29)
214-3p	4,056	0.22	0.09	0.15	0.25	0.67	64
21-5p	547,830	0.16	0.32	0.31	0.26	0.41	65
101b-3p	1,648	0.09	0.19	0.26	0.30	0.59	57
132-3p	790	0.02	0.02	0.06	0.21	0.59	60

Table 4.

The in vivo induced changes in individual myostatin-related miRs were also accompanied by changes in other individual miRs assumed to be unrelated to myostatin that were reflected as well on the in vitro dyslipidemic treatments.

See Table 3 for the caption, but these miRs are not so far associated with myostatin. See Table 3 Supplement for their stem cell related significance.

Other miRs changed in amount by T2D in LD vs ED	In vivo T2D effects (OZ rat)		In vitro effects of serum (S) and lipidemic factors (PA: palmitic acid; CHOL: cholesterol)			
	Basal value	Ratio to ED	Ratios to ED			
	ED (per 10^7 reads)	LD/ED	OZS/ ED	LZS/ ED	PA/ ED	CHOL/ ED
99a-5p	30,750	2.65	1.75	2.25	0.29	0.32
25-1 p	6,350	2.20	1.90	5.17	2.07	1.53
10b-5p	11,639	2.20	2.67	4.36	0.15	0.45
28-5p	1,234	0.36	0.30	0.40	0.22	0.29
152-3p	22,285	0.33	0.20	0.40	0.32	0.58
26a-5p	58,443	0.32	0.79	1.57	0.34	0.66
Let-7f-5p	64,915	0.32	0.71	1.21	0.38	0.45
31a-5p	2,868	0.29	0.17	0.35	9.49	0.65
196b-5p	881	0.27	0.17	0.44	3.34	0.53
342-3p	4,379	0.26	0.20	0.31	3.25	0.52
99b-5p	507,430	0.25	0.47	0.52	0.27	0.45
148a-3p	16,262	0.25	0.22	0.30	0.53	0.58
221-5p	14,946	0.24	0.57	0.33	0.32	0.34
let-7g-5p	15,492	0.22	0.89	1.17	3.38	0.41
100-5p	409,443	0.22	0.19	0.25	3.24	0.48
362-5p	852	0.20	0.35	0.64	0.52	0.40
92a-3p	12,454	0.17	1.26	0.91	0.83	0.47
148b-3p	21 215	0.16	0.52	0.64	0.27	0.30
146a-5p	14,087	0.14	0.04	0.02	0.86	0.49
196a-5p	1,584	0.14	0.36	0.62	0.44	0.50
Let-7i-5p	68,254	0.12	0.44	0.66	0.36	0.66
10a-5p	16,557	0.10	0.19	0.26	0.13	0.28
10b-3p	1,643	0.10	0.39	0.53	0.30	0.59

Other miRs changed in amount by T2D in LD vs ED	In vivo T2D effects (OZ rat)		In vitro effects of serum (S) and lipidemic factors (PA: palmitic acid; CHOL: cholesterol)				
	Basal value	Ratio to ED	Ratios to ED				
	ED (per 10^7 reads)	LD/ED	OZS/ ED	LZS/ ED	PA/ ED	CHOL/ ED
212-5p	1,026	0.08	0.02	0.07	0.35	0.76
224-5p	1,368	0.06	0.20	0.31	0.76	0.75