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RADIO ELECTRIC ASYMMETRIC CONVEYED FIELDS AND HUMAN ADIPOSE-DERIVED STEM CELLS OBTAINED WITH A NON-ENZYMATIC METHOD AND DEVICE: A NOVEL APPROACH TO MULTIPOTENCY

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ABSTRACT

Human adipose derived stem cells (hASCs) have been recently proposed as a suitable tool for regenerative therapies for their simple isolation procedure, and high proliferative capability in culture. Although hASCs can be committed into different lineages *in vitro*, the differentiation is a low-yield and often incomplete process. We have recently developed a novel non-enzymatic method and device, named Lipogems, to obtain a fat tissue derivative highly enriched in pericytes/mesenchymal stem cells by mild mechanical forces from human lipoaspirates. When compared to enzymatically dissociated cells, Lipogems-derived hASCs exhibited enhanced transcription of vasculogenic genes in response to pro-vasculogenic molecules, suggesting that these cells may be amenable for further optimization of their multipotency.

Here, we exposed Lipogems-derived hASCs to a Radio Electric Asymmetric Conveyer (REAC), an innovative device asymmetrically conveying radio electric fields, affording both enhanced differentiating profiles in mouse embryonic stem cells, and efficient direct multi-lineage reprogramming in human skin fibroblasts. We show that specific REAC exposure remarkably enhanced the transcription of prodynorphin, GATA-4, Nkx-2.5, VEGF, HGF, vWF, neurogenin-1 and myoD, indicating the commitment towards cardiac, vascular, neuronal and skeletal muscle lineages, as inferred by the overexpression of a program of targeted marker proteins. REAC exposure also finely tuned the expression of stemness related genes, including Nanog, Sox-2, and Oct-4. Noteworthy, the REAC induced responses were fashioned at a significantly higher extent in Lipogems-derived than in enzymatically-dissociated hASCs.

Therefore, REAC-mediated interplay between radio electric asymmetrically conveyed fields and Lipogems-derived hASCs appears to involve the generation of an ideal “milieau” to

optimize multipotency expression from human adult stem cells in view of potential improvement of future cell therapy efforts.

INTRODUCTION

The human body harbors multipotent stem cells within different "niches", including the bone marrow, dental pulp, fetal organs and adipose tissue (2,7,23). Human adult mesenchymal stem cells (hMSCs) may contribute to the normal homeostasis virtually in all tissues by replacing their own degenerated cells (9,15,20). The commitment and the ultimate fate of hMSCs are regulated by instructive signals, which comprise many biological molecules and biophysical factors. Bone marrow constitutes a common source of multipotent mesenchymal stem cells, but this population is rare (0.001% - 0.01%) (20). Recently, the human adipose tissue has been identified as a convenient alternative source of stem cells, generically referred to as "adipose-derived stem cells (hASCs) (35). These cells have been shown to be amenable for large-scale production of adipose tissue suitable for regenerative medicine and tissue banking (6), and can also be cryopreserved, setting the basis for long-term cell banking approaches (8). hASCs can differentiate *in vitro* after specific induction into different tissues for clinical application (16,35), but the differentiation process is often incomplete and occurs with a poor yield (11).

Recently, we have successfully isolated hASCs from a fat tissue product obtained with a novel non-enzymatic method and device, named Lipogems (5). The Lipogems-derived unexpanded tissue product encompassed a remarkably preserved stromal vascular fraction (SVF) consisting of a high yield of cells with pericyte identity, and mesenchymal stem cells. This stem cell population could be easily expanded in culture by simply transferring the

unexpanded Lipogems product into tissue culture without any manipulation, while in the same setting the enzymatic processing, and related washing of blood and oil contaminants from a lipoaspirate, would require considerably longer periods and additional manipulation (usually 40-50 min per sample), prior to placing the released cells into culture. Differently from the unprocessed lipoaspirate, the unexpanded Lipogems tissue product can be cryopreserved after being harvested from living or cadaveric donors, retaining superimposable phenotypic features, as well as the ability to release viable cells, after placing the product itself into the culture medium (5). These findings indicate that the Lipogems system can potentially pave the way to novel strategies and paradigms in the rescue of diseased tissues, due to its ability to provide a minimally manipulated derivative, the chance of immediate transfer into a clinical setting, and the possibility to be subjected to off-the-shelf strategies of cell culture and expansion *ex vivo*. Such a perspective is reinforced by the observation that a set of vasculogenic genes could be induced to a greater extent in Lipogems-derived than in enzymatically dissociated hASCs that had been exposed to vasculogenic molecules (5).

In the current study, we attempted at identifying a strategy to optimize multipotency expression from Lipogems derived-hASCs, avoiding the use of cumbersome viral vector-mediated gene delivery, or the development of synthetic molecules to manipulate the target cells. For this purpose, Lipogems-derived hASCs were exposed to a Radio Electric Asymmetric Conveyer (REAC) apparatus (24,25), an innovative device delivering radio electric asymmetrically conveyed fields (REACF) of 2.4 GHz with its conveyer electrodes immersed into the culture medium (18,19). The REAC technology was originally designed to convey asymmetrically the radio electric currents resulting from the interaction between the weak electromagnetic field produced by the instrument (24,25), with a radiated power of about 2 mW, and the electromagnetic field generated by the human body, with a radiated

power of about 54 mW (29). Recently, we provided evidence that REAC exposure afforded both optimization of the differentiating potential in mouse embryonic stem cells (19), and efficient direct multi-lineage reprogramming in human dermal skin fibroblasts (18).

In the present study, we comparatively investigated the effects produced by REAC exposure on the expression of stemness associated genes in Lipogems-derived and enzymatically dissociated hASCs. We also assessed whether REACF may affect the commitment towards complex lineages, including the myocardial, vascular, neuronal and skeletal muscle fates, and whether, in the affirmative, the degree of multilineage commitment may be differentially expressed among the two populations of exposed hASCs.

MATERIALS AND METHODS

Description of Radio Electric Asymmetric Conveyer (REAC)

The REAC apparatus, was placed into a CO_2 incubator, was set with the "tissue optimization-regenerative protocol (TO-RGN)" at a frequency of 2.4 GHz and its conveyer asymmetric electrodes were immersed, as previously described (18,19), into the culture medium of Lipogems-derived, or enzymatically-dissociated hASCs. The distance between the emitter at 2.4 GHz and the culture medium was approximately 35 cm. The electromagnetic quantities have been measured with the spectrum analyzer Tektronix model 2754p, orienting the receiving antenna for maximum signal. With duration of single radiofrequency burst of 250 ms and an off interval of 2.5 seconds, we have obtained the following results: Radiated power is about 2 mW, Electric field E=0.4 V/m, Magnetic field 1mA/m, Specific Absorption Rate - SAR 0.128μW/g; determinate σ=1 A/V.m and ρ=1000Kg/m^3, the density of radio electric current flowing in the culture medium during the REAC single radiofrequency burst is **J**=30μA/cm^2. The electromagnetic field around the device is, of course, very irregular for the presence of metal walls of the incubator. However, were easily detectable points of the

highest level in the incubator. At a distance of 35 cm from the emitter, and in a very limited area around the receiving antenna we measured values of specific power around 400 μW/m². The REAC device that we used is registered under the trademark "B.E.N.E.- Bio enhancer - Neuro enhancer", and is produced by ASMED Srl, Italy.

Isolation and culture of hASCs

According to the policies approved by the Institutional Review Boards for Human Studies local ethical committees, all tissue samples were obtained after informed consent. Human subcutaneous adipose tissue samples were obtained from lipoaspiration/liposuction procedures, according to a recently described procedure (5). In this study, we processed a total number of 20 lipoaspirates, obtained from 9 male and 11 female donors, with age ranging between 32 and 55 years.

Each original lipoaspirate, with a volume ranging between 100 and 150 ml, was divided into two aliquots: between 40 and 100 ml of the sample were processed through the Lipogems device, as previously described (5); the rest was digested in collagenase A type I solution (Sigma-Aldrich) at a final concentration of 0.05%, under gentle agitation for 1 hour at 37°C, and centrifuged at 2000 rpm for 10 min to separate the stromal vascular fraction (SVF) from adipocytes. If necessary, the SVF fraction was treated with red blood cell lysis buffer for 5 min at 37°C, then centrifuged again. The supernatant was discarded and the cell pellet was resuspended and seeded in culture flasks in α-MEM supplemented with 20% heat-inactivated FBS, antibiotics (200 units/ml penicillin, 100 μg/ml streptomycin), L-Glutamine (1%), and incubated at 37°C in a humidified atmosphere with 5% CO_2. To obtain Lipogems-derived hASCs, the Lipogems product was simply transferred without any manipulation into tissue culture, according to a previously established procedure (5). hASCs slipped out from the

tissue cluster product, starting after day 2-3, attached to the tissue culture plastic, and reached 70-80% confluence in 7-12 days. Medium was changed every 4 days, but the non-adherent fraction of Lipogems product was removed from the culture only after two weeks. At confluence, cells were detached by treatment with trypsin-EDTA (Sigma-Aldrich), characterized by flow cytometry and subcultured. Cell differentiation was obtained culturing hASCs in tissue culture plates, at the concentration of 10^6 cells/well, under REAC exposure for a period of 24, 48 and 72 hours. Cells exposed to REAC for 72 hours were also cultured for additional 7 days (10-day total culture).

Assessment of Cell Viability and Apoptosis

Cell viability was determined by the trypan blue dye exclusion test (Life Technologies). Both attached and floating cells were harvested and counted by using countess automated cell counter. To assess apoptosis, Caspase 3 activity was detected using a commercial kit (Fluorimetric Caspase 3 Assay Kit, Sigma-Aldrich Corp., St. Louis, MO, USA) according to manufacturer's instruction. Briefly, cell pellet was dissolved in 35µl of Lysis Buffer, and cell lysates were added with the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC). Release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety was assessed as a measure of substrate hydrolysis by Caspase 3. Lactate dehydrogenase (LDH) release by dead cells was detected with LDH cytotoxicity assay kit II (MBL international corporation).

Flow cytometry analysis

Flow cytometry analysis was exploited to assess the percentage hASCs expressing specific cell phenotype markers. After a fixation/permeabilization step, cells were incubated with a primary antibody directed against β-3-tubulin (BD Biosciences), myoD (Santa Cruz), or α-

sarcomeric actinin (Sigma) (1 µg/10^6 cells) for 1 hour at 4 °C, and with 1 µg of fluorescein isothiocyanate-conjugated secondary antibody for 1 hour at 4 °C in the dark. After washing, cells were analyzed on a flow cytometer (FACSAria, BD Biosciences, San Jose, CA) by collecting 10,000 events, and the data were analyzed using the FACS Diva software (BD Biosciences).

Gene expression

Total RNA was isolated using Trizol reagent according to the manufacturer's instruction (Invitrogen). Total RNA was dissolved in RNAase-free water and, for RT-PCR, cDNA was synthesized in a 50-µl reaction volume with 1µg of total RNA and MMLV reverse transcriptase (RT) according to the manufacturer's instruction (Invitrogen). Quantitative real-time PCR was performed using an iCycler Thermal Cycler (Bio-Rad). Two µl cDNA were amplified in 50-µl reactions using Platinum Supermix UDG (Invitrogen), 200 nM of each primer, 10 nM fluorescein (BioRad), and Sybr Green. After an initial denaturation step at 94 °C for 10 min, temperature cycling was initiated. Each cycle consisted of 94 °C for 15 s, 55-59 °C for 30 s and 60 °C for 30 s, the fluorescence being read at the end of this step. Specific primers used in this study were from Invitrogen, and are reported in Table 1. To evaluate the quality of product of real-time PCR assays, melting curve analysis was performed after each assay. Relative expression was determined using the "delta-CT method" with hypoxanthine phosphoribosyltransferase 1 (HPRT1) as reference gene (30).

Akin to previous observations (18,19), separate time course analyses revealed that, based on the investigated genes, the optimal transcriptional response to REACF occurred within the first 24-72 hours of exposure, and that upon subsequent treatment withdrawal the mRNA expression proceeded for the following 4-7 days with a pattern superimposable to that obtained with a continuous 10-day exposure (not shown). For these reasons, gene and protein

expression were investigated throughout a 72-hour REAC exposure followed by a 4-7-day period of cell culturing in the absence of REACF.

Immunostaining

Cells were cultured for 3 days with or without REAC TO-RGN treatment and then for additional 4 days without REAC exposure. After 7 days, cells were treated with trypsin, and the resulting suspension was cultured at low density to permit visualization of individual cells. The cultures were fixed with 4% paraformaldehyde. Cells were exposed for 1 hour at 37 °C to mouse monoclonal antibodies against α-sarcomeric actinin (Sigma), β-3-tubulin (BD Biosciences), or myoD (Santa Cruz), or with rabbit polyclonal antibodies against myosin heavy chain (MHC) and von Willebrand Factor (vWF) (AbCAM), and stained at 37 °C for 1 hour with fluorescein-conjugated goat IgG.

All microscopy was performed with a Leica confocal microscope (LEICA TCSSP5). DNA was visualized with 1μg/ml 4',6-diamidino-2-phenylindole (DAPI).

Immunoblotting Analysis

Total cell lysates were electrophoresed on 10% Novex Tris-glycine polyacrylamide gels (Invitrogen, CA), in MOPS SDS Running Buffer, using the XCell SureLockTM Mini-Cell, according to the instruction provided by the manufacturer. After protein transfer to polyvinylidene difluoride (PVDF) membranes (Invitrogen, CA), membrane saturation and washing, the immunoreaction was carried out for 1 hour at room temperature in the presence of the primary antibody (antisera against GATA-4, β-3-tubulin, myoD, vWF, Sox-2, and Nanog) (AbCAM) diluted 1:1000. After additional washing, membranes were incubated with anti-rabbit (vWF, Sox-2, Nanog) or anti-mouse (GATA-4, myoD, β-3-tubulin) horseradish

peroxidase (HRP) conjugated secondary antibody (AbCAM). Targeted protein expression was assessed by a chemioluminescence detection system (ECL Western blotting detection reagents were from Amersham Biosciences). Data from REAC exposed cells have been reported relative to the expression of control untreated cells, and normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (rabbit polyclonal antibody against GAPDH, Santa Cruz).

Data Analysis

The statistical analysis of the data was performed by using the Statistical Package for Social Science (SPSS), version 13. For this study, were used nonparametric statistical tests: Kruskal-Wallis and Wilcoxon Signed Rank test. The first to evaluate the distributions and homogeneity of variance of each group at different times of observation, the second was used to evaluate, in the same group, the differences (Delta CT) between the data collected over a period of observation and the reference value at baseline. Test, and all results $P<0.05$ have been considered statistically significant.

RESULTS

Effects of REAC exposure on cellular proliferation and apoptosis

REAC exposure had no toxic effect on hASCs. In fact, there was no significant difference in cell growth between exposed and unexposed cells (Fig. 1*A*). Moreover, REAC did not affect significantly the amount of apoptotic (Fig. 1*B*) or necrotic cells (Fig. 1*C*), as compared with untreated hASCs. Trypan blue exclusion also revealed no significant difference between exposed and unexposed cells (not shown).

REAC TO-RGN exposure primes multilineage stem cell commitment

The prodynorphin, GATA-4 and Nkx-2.5 genes have been previously shown to act as major conductors in cardiac lineage commitment (17,27,31,32). Recently their expression was also found to be increased by REAC exposure in mouse ES cells (19), as well as in human dermal skin fibroblasts (18). Here, we show that exposure to REACF significantly enhanced the expression of cardiogenic transcripts even in human adult stem cells, as those derived from the Lipogems product (Fig. 2), also inducing a program of vasculogenic genes, including Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF), and vWF (Fig. 2). The transcriptional increase peaked as early as 24 h, then exhibiting different timely patterns, according to the investigated gene. In particular, while prodynorphin and GATA-4 were stably overexpressed throughout a period of 10 or three days, respectively (Fig. 2), the transcription of Nkx-2.5, VEGF, HGF, and vWF progressively declined after 24 h of treatment, still resulting greater than in unexposed cells at later times (Fig. 2).

The exposure of Lipogems-derived hASCs to REACF was able to induce the transcription of genes involved in both neurogenic and skeletal myogenic commitment, including neurogenin-1, and myoD, respectively (Fig. 3). Similar to cardiogenic and vasculogenic genes, these

transcripts exhibited an early enhancement at 24 h, declining thereafter, while retaining a higher level of expression even at 10 days, as compared to unexposed cells.

REAC asymmetrically conveyed radio electric fields exert a biphasic effect on stemness related genes

Stem cell multipotency is finely tuned by the expression of a number of transcripts, including Nanog (3), Sox-2 (22,34) and Oct-4 (33). Exposure of Lipogems-derived hASCs to REACF induced an early increase in the expression of these stemeness associated genes during the first 4-12 hours, followed by a significant down-regulation of transcript levels below the control value after 24 hours of treatment (Fig. 4). The inhibitory pattern persisted after 72 hours of exposure, being still clearly evident even when cells were maintained in culture for additional 7 days in the absence of REAC TO-RGN treatment (Fig. 4). Comparative analysis in REAC-exposed cells, revealed that the early overexpression, as well as the late inhibition of stemness genes were significantly more pronounced in Lipogems-derived than in enzymatically dissociated hASCs (Fig. 4).

Western blot experiments conducted in Lipogems-derived hASCs revealed that GATA-4, vWF, β-3- tubulin and myoD were significantly induced by REAC exposure (Fig. 5). The protein expression of Sox-2 and Nanog also mirrored the transcriptional responses elicited by REAC exposure, being significantly downregulated below the levels observed in control unexposed cells after 24 hours of TO-RGN treatment (Fig. 5).

Confocal microscopy analysis shown the appearance of tissue specific markers for cardiogenic (α-sarcomeric actinin, MHC), neurogenic (β-3-tubulin), skeletal muscle (myoD), and endothelial (vWF) commitment in REAC-exposed, Lipogems-derived hASCs, indicating that the induction of a tissue restricted program of gene and protein expression by REACF delivery converged to the regulation of lineage specification at the intact cell level (Fig. 6).

Lipogems-derived hASCs are more sensitive to the REAC TO-RGN treatment than enzymatically dissociated cells

We have recently shown (5) that Lipogems derived hASCs, exhibited expression patterns of pericyte identity, including CD146+/90+/34-, and CD146+/34+, a pattern detected in a pericyte subset that may be transitional between pericytes and supra-adventitial adipose stromal cells, and/or a set of endothelial (progenitor) cells, as well as the hMSC pattern CD90+/CD29+/CD34-. Moreover, the cell percentage with pericyte and hMSC profile was significantly higher in Lipogems-derived than in enzymatically dissociated hASCs (5). Both cell populations were negative for the expression of the hematopoietic markers CD14, CD34, and CD45.

Here, comparative flow cytometry analysis of the expression of β-3-tubulin, myoD and α-sarcomeric actinin, highlighting a neural, skeletal myogenic and cardiogenic commitment, respectively, was performed in Lipogems-derived and enzymatically dissociated hASCs cultured in the absence or presence of REAC-REACF for 72 hours, and then left untreated for additional 4 days. Results confirmed that in both cell populations the expression of these tissue restricted markers was significantly higher in exposed than in unexposed cells (Fig. 7). Moreover, among REAC-treated cells, the percentage of each lineage commitment from Lipogems-derived hASCs significantly exceeded the percentage detected from enzymatically dissociated hASCs (Fig. 7). These results were confirmed even when, following a previous 72-hour REAC exposure, the cells were left untreated for additional 7 days (not shown).

DISCUSSION

The current study indicates that the REAC technology was able to afford an efficient commitment of hASCs towards multiple lineages, including the cardiovascular, as well as the skeletal muscle and neuronal fates. So far, the multilineage potential of hASCs has been limited by their low differentiation efficiency (16,35). To this end, a significant increase in the myogenic commitment of hASCs could be obtained by composite approaches including their transduction with engineered MyoD protein (28), cell growing onto nanostructured scaffolds with tailored fiber orientation (4), or the cellular fusion with mouse C2C12 myoblasts (10). Noteworthy, hASC exposure to REAC-REACF acted at both transcriptional and protein expression level to afford a high-throughput of commitment towards complex lineages without the needs of viral/protein transduction or nanopatterned manipulation of environmental cues. The REAC induced increase in the expression of the investigated lineage restricted genes and proteins was an early event, already reaching a maximum within the first 24 h of exposure, then exhibiting a progressive decline over time, although the transcripts remained detectable above the control level even in the later phase of the observational time course. Such transcriptional profiles are in agreement with previous findings, showing the early and transient nature of the timely pattern of gene and protein expression of most of the tissue restricted transcription factors involved in stem cell commitment to cardiovascular, neural and skeletal muscle lineages (13,17,21,26,27). So far, a remarkable complexity has been encountered in the expression profiles of these transcription factors, with unexpected multidirectional actions, as shown by the ability of Nkx-2.5 itself to drive non-cardiogenic decisions, including the commitment to neuronal differentiation in both skeletal muscle and ES cells (23). Deciphering the molecular mechanisms that interconnect the transcriptional units intervening in REAC-mediated commitment of hASCs may provide a glimpse into the cellular circuitry that specifies the attainment of multiple fates from these cells.

The ability of REAC treatment to trigger an early expression of stemness related genes, including Sox-2, Oct-4 and Nanog, concomitant with the expression of lineage restricted genes, suggests that the REAC action may have been exploited through an optimization of stem cell multipotency. Such a view is further supported by the high yield of cells expressing markers of terminal commitment at the intact cell level, as shown by confocal microscopy experiments. To this end, the downregulation in stemness gene and protein expression observed after 12 h of REAC exposure may be worthy of consideration, since it is now evident that after their induction Sox-2, Nanog and Oct-4 need to be downregulated to allow cell progression towards a differentiated state (1,12,14,22).

Here, we provided a comparative analysis of the transcriptional/signaling and differentiating outcomes of REAC-REACF in hASCs isolated by enzymatic digestion of the lipoaspirate or after its processing with Lipogems, an innovative non-enzymatic method simply using mild mechanical forces to yield a tissue product harboring a preserved SVF highly enriched in pericytes and mesenchymal stem cells. Following exposure to a mixture of hyaluronan, butyric and retinoic acids, Lipogems-derived hASCs have been shown to respond with higher transcription of essential vasculogenic genes, including VEGF, KDR, encoding a major VEGF receptor, and HGF, as compared with hASCs obtained with enzymatic dissociation (5). The present findings show for the first time that, even in the presence of a “physical milieu”, as that provided by REAC asymmetrically conveyed radio electric fields, Lipogems-derived hASCs exhibited significantly greater responses in the expression of stemness genes than enzymatically obtained cells. Consistent with this observation, flow cytometry analysis of selected differentiating markers revealed that the magnitude of commitment along myocardial, neuronal and skeletal muscle lineages in Lipogems-derived hASCs significantly exceeded the extent observed in enzymatically dissociated cells.

We have previously hypothesized that the enhanced response to chemical stimuli afforded in Lipogems- compared to enzymatically-derived hASCs may be attributable to the fact that lipoaspirate processing through the Lipogems device, avoiding the use of collagenase and other enzymes, may have preserved the cell surface environment and glycocalyx composition better than other methods based on enzymatic dissociation. The mechanisms accounting for the improved response to REAC-REACF yielded by Lipogems-derived hASCS still remain to be elucidated, but we cannot exclude that a better preserved cellular environment, as that afforded by the Lypogems method, may also represent a prerequisite for enhanced cellular responsiveness even to physical energy.

In conclusion, the synergistic interplay between REAC asymmetrically conveyed radio electric fields and Lipogems-derived hASCs has been shown to generate an ideal "milieau" to optimize stem cell multipotency. These outcomes were achieved after extremely brief exposure pulses, showing long-lasting persistence of cell commitment upon the cessation of REAC treatment. Studies are on the way to dissect the electrophysiological and functional properties of REAC-committed, Lipogems-derived hASCs, and to assess whether improved tissue healing may result from their transplantation in defined animal models of heart failure, neurodegeneration and skeletal muscle dystrophy. In the affirmative, these present observations may represent the underpinning for future cell therapy approaches.

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CONFLICT OF INTEREST

Salvatore Rinaldi and Vania Fontani have invented and patented the REAC technology. Carlo Tremolada has invented and patented the Lipogems device. The other Authors report no conflicts of interest.

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FIGURE LEGENDS

Figure 1. *Effect of REAC on cell viability, apoptosis and necrosis.*

After plating (2×10^5/well), cells were left untreated (white bars) or treated (black bars) with REAC for the indicated times, and counted to estimate cell growth (A), or processed to assess the amount of apoptosis (B), or necrosis (C) (mean ± S.E.; n=6). In B, release of the AMC moiety (pmol/□l) after cleavage of the fluorogenic Ac-DEVD-AMC caspase substrate by cell lysates was calculated from a standard curve determined with defined AMC solutions. In C, cell supernatants and pellets were collected, and total proteins extracted from both fractions. LDH activity in each supernatant was normalized to total LDH activity of their own pellet + supernatant.

No significant difference was observed between exposed and unexposed groups (two-tailed, unpaired Student's *t* test, or one-way analysis of variance with subsequent Bonferroni test).

Figure 2. *Effect of REAC TO-RGN exposure on the expression of genes orchestrating Lipogems-hASC commitment toward a cardiovascular lineage.*

Cells were exposed for 24, 48 hours or 72 hours in the absence or presence of REAC, or were treated with REAC for 72 hours and then left unexposed for additional 7 days (day 10 from time zero). The amounts of prodynorphin, Nkx-2.5, GATA-4, VEGF, HGF, and vWF mRNA from REAC-treated or untreated cells were normalized to HPRT1, and the mRNA expression of REAC-exposed cells was plotted at each time point as fold change relative to the expression in control untreated cells, defined as 1 (mean ± S.E.; n=6). All the REAC TO-RGN-treated cells at each time point were significantly different from each control untreated cells (mean ± S.E.; n=6; $P<0.05$).

Figure 3. *Effect of REAC TO-RGN exposure on the expression of genes orchestrating Lipogems-hASC commitment toward neurogenic and skeletal muscle lineages.*

Cells were exposed for 24, 48 hours or 72 hours in the absence or presence of REAC, or were treated with REAC for 72 hours and then left unexposed for additional 7 days (day 10 from time zero). The amounts of neurogenin-1, and myoD mRNA from REAC-treated or untreated cells were normalized to HPRT1, and the mRNA expression of REAC-exposed cells was plotted at each time point as fold change relative to the expression in control untreated cells, defined as 1 (mean ± S.E.; n=6). All the REAC-treated cells at each time point were significantly different from each control untreated cells (mean ± S.E.; n=6; $P<0.05$).

Figure 4. *REAC TO-RGN treatment affords a biphasic effect on the transcription of stemness associated genes.*

Enzymatically dissociated hASCs (white bars) and Lipogems-derived hASCs (black bars) were exposed for 4, 8, 12, 24, 48 or 72 hours in the absence or presence of REAC, or were treated with REAC for 72 hours and then left unexposed for additional 7 days (day 10 from time zero). The amount of Sox-2, Oct-4, and Nanog mRNA from REAC-treated or untreated cells were normalized to HPRT1, and the mRNA expression of REAC-exposed cells was plotted at each time point as fold change relative to the expression in control untreated cells, defined as 1 (mean ± S.E.; n=6). All the REAC-treated cells at each time point were significantly different from each control untreated cells (mean ± S.E.; n=6; $P<0.05$). In REAC-treated, Lipogems-derived hASCs, the expression level of each mRNA was significantly different from that detected in enzymatically dissociated hASCs (mean ± S.E.; n=6; $P<0.05$), except for the gene expression of Oct-4, at 24h-10d, and the gene expression of Nanog, at 24 and 48 h.

Figure 5. *Lipogems-hASC exposure to REAC modulates the expression of selected, tissue-restricted, and stemness-related proteins.*

Total lysates were obtained from Lipogems-derived hASCs exposed for 24, 48 hours or 72 hours in the absence (white bars) or presence (black bars) of REAC, or treated with REAC for 72 hours and then left unexposed for additional 7 days (day 10 from time zero). Samples were subjected to Western blot analysis, using polyclonal antisera raised against GATA-4, vWF, β-3-tubulin (Tubulin), myoD, Sox-2, and Nanog. Sizes of the bands were determined with prestained marker proteins. Densitometric analysis was performed using Quantity one (BioRad). Data are reported relative to the expression of control untreated cells and normalized to the expression level of GAPDH (mean ± S.E.; n=6). All the REAC-treated cells at each time point were significantly different from each control untreated cells (mean ± S.E.; n=6; $P<0.05$), except for the expression of GATA-4 at 72h.

Figure 6. *REAC-mediated Lipogems-hASC differentiation.*

Expression of α-sarcomeric actinin (α-Actinin), myosin heavy chain (MHC), β-3-tubulin (Tubulin), myoD, and von Willebrand Factor (vWF) was assessed in cells cultured in the absence or presence of REAC, for 72 hours, and cultured for additional 4 days without REAC treatment in tissue chamber slides suitable for immunofluorescence staining. Nuclei are labeled with DAPI (*blue*). Scale bars are 40 μm. Representative of five separate experiments. For each differentiation marker, fields with the highest yield of positively stained cells are shown.

Figure 7. *Cell lineage marker expression in Lipogems- or enzymatically-derived hASCs treated in the absence or presence of REAC.*

Lipogems-derived hASCs, or enzymatically dissociated hASCs (Enz hASCs) were exposed to REAC for 72 hours and then left untreated for additional 4 days. Flow cytometry analysis was performed in cells stained with primary antibodies specific for β-3-tubulin (Tubulin), MyoD, or α-sarcomeric actinin (α-Actinin), and FITC-conjugated secondary antibodies, when necessary. *, significantly different from untreated (mean ± S.E.; n=6; $P<0.05$).

GENE	FORWARD PRIMER	REVERSE PRIMER
GATA-4	TGGCCTGTCATCTCACTACG	TAGCCTTGTGGGGAGAGCTT
Nkx-2.5	CAAGTGTGCGTCTGCCTTT	GCGCACAGCTCTTTCTTTTC
Prodynorphin	TGGCCAAGCTCTCTGGGTCA	TCATGGCCCATGCTATCCCC
VEGF	AGAAGGAGGAGGGCAGAATC	ACACAGGATGGCTTGAAGATG
HGF	ATTTGGCCATGAATTTGACCT	ACTCCAGGGCTGACATTTGAT
vWF	CAACACCTGCATTTGCCGAA	ATGCGGAGGTCACCTTTCAG
HPRT1	AGCCCTGGCGTCGTGATTA	TGGCCTCCCATCTCCTTCA
Sox2	CACATGAACGGCTGGAGCA	TGCTGCGAGTAGGACATGCTG
OCT4	CTCACCCTGGGGGTTCTAT	CTCCAGGTTGCCTCTCACTC
NANOG	CATGAGTGTGGATCCAGCT	CCTGAATAAGCAGATCCAT
MyoD	GGCATGATGGACTACAGCG	GGAGATGCGCTCCACGATGCT
Neurogenin1	TTCCTCACCGACGAGGAAGACTGT	TCAAGTTGTGCATGCGGTTGCGCT

TABLE 1

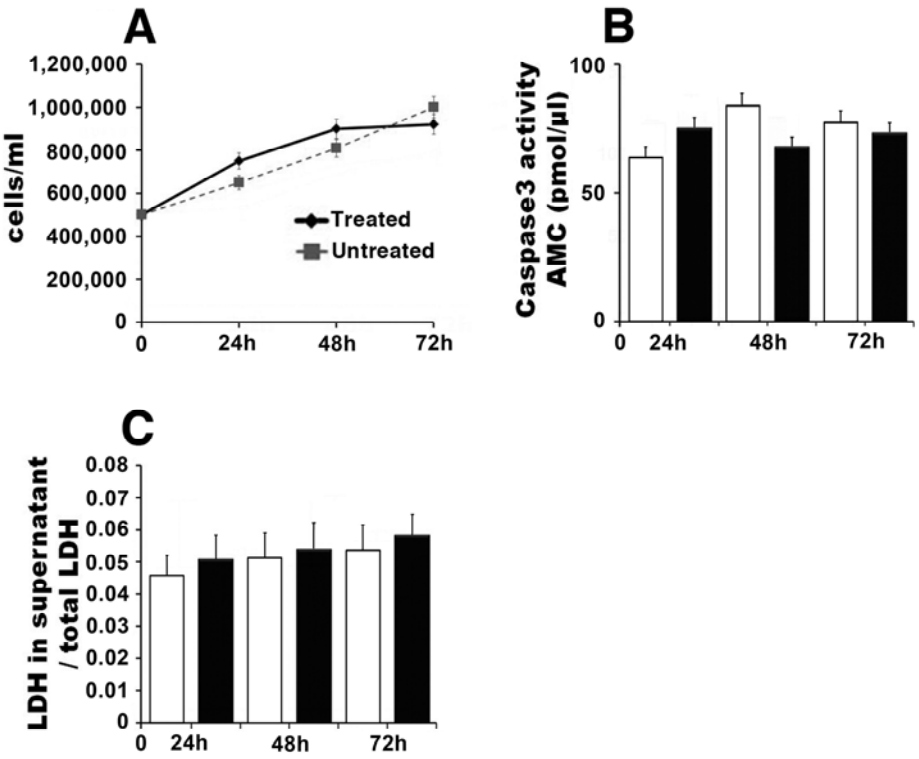


FIGURE 1

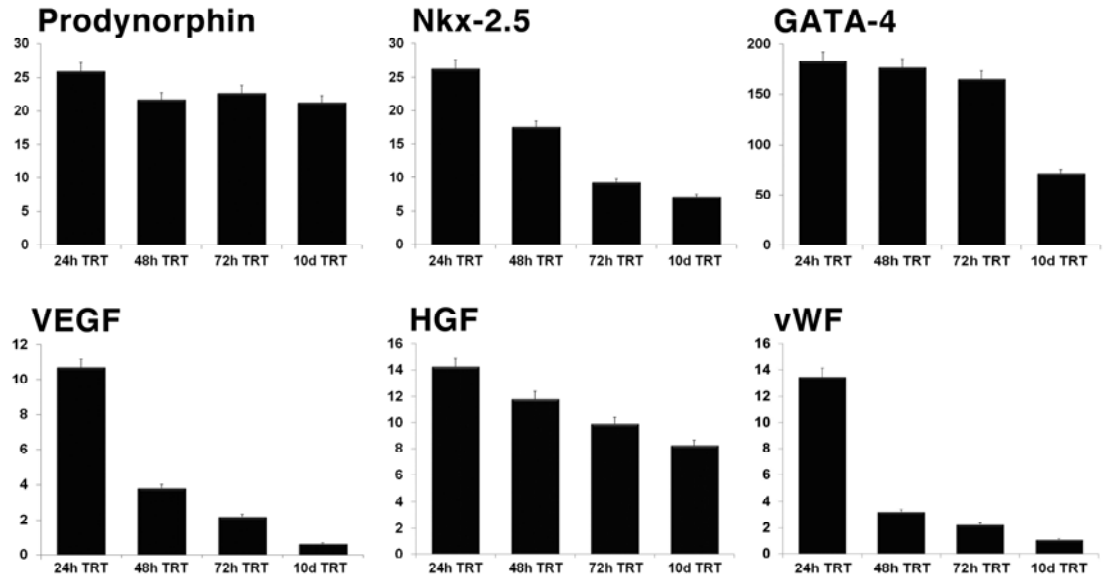


FIGURE 2

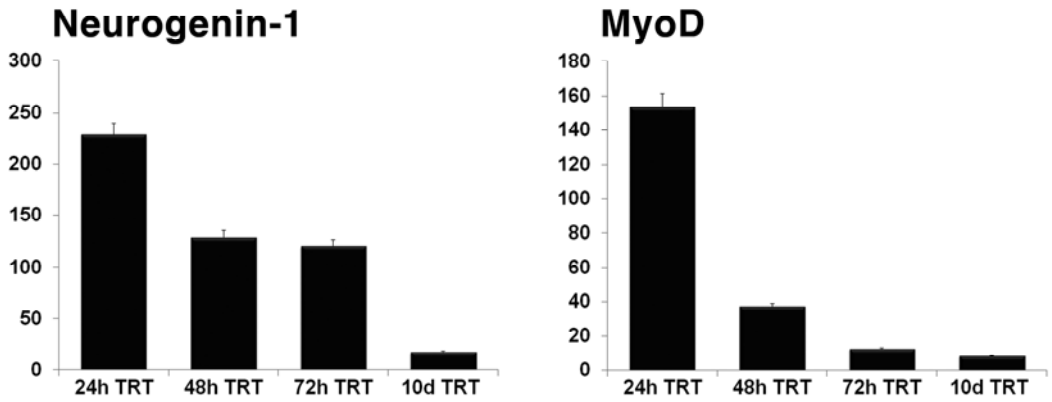


FIGURE 3

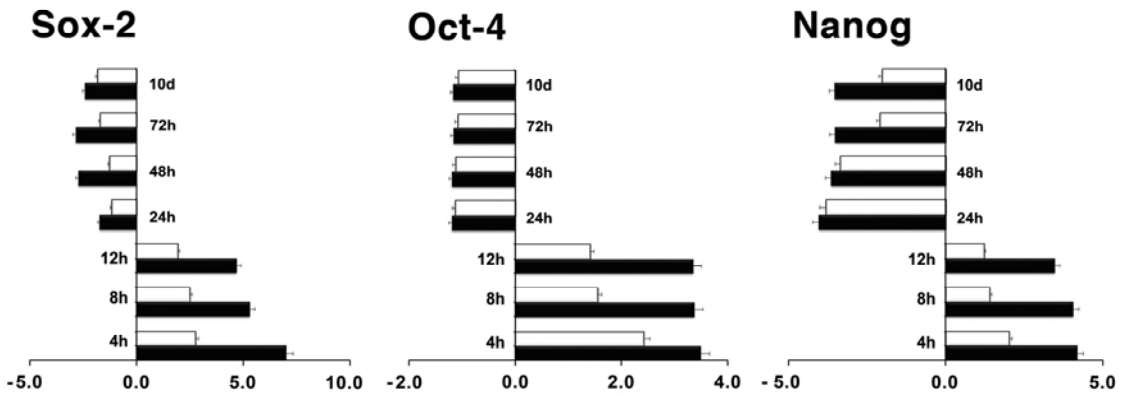
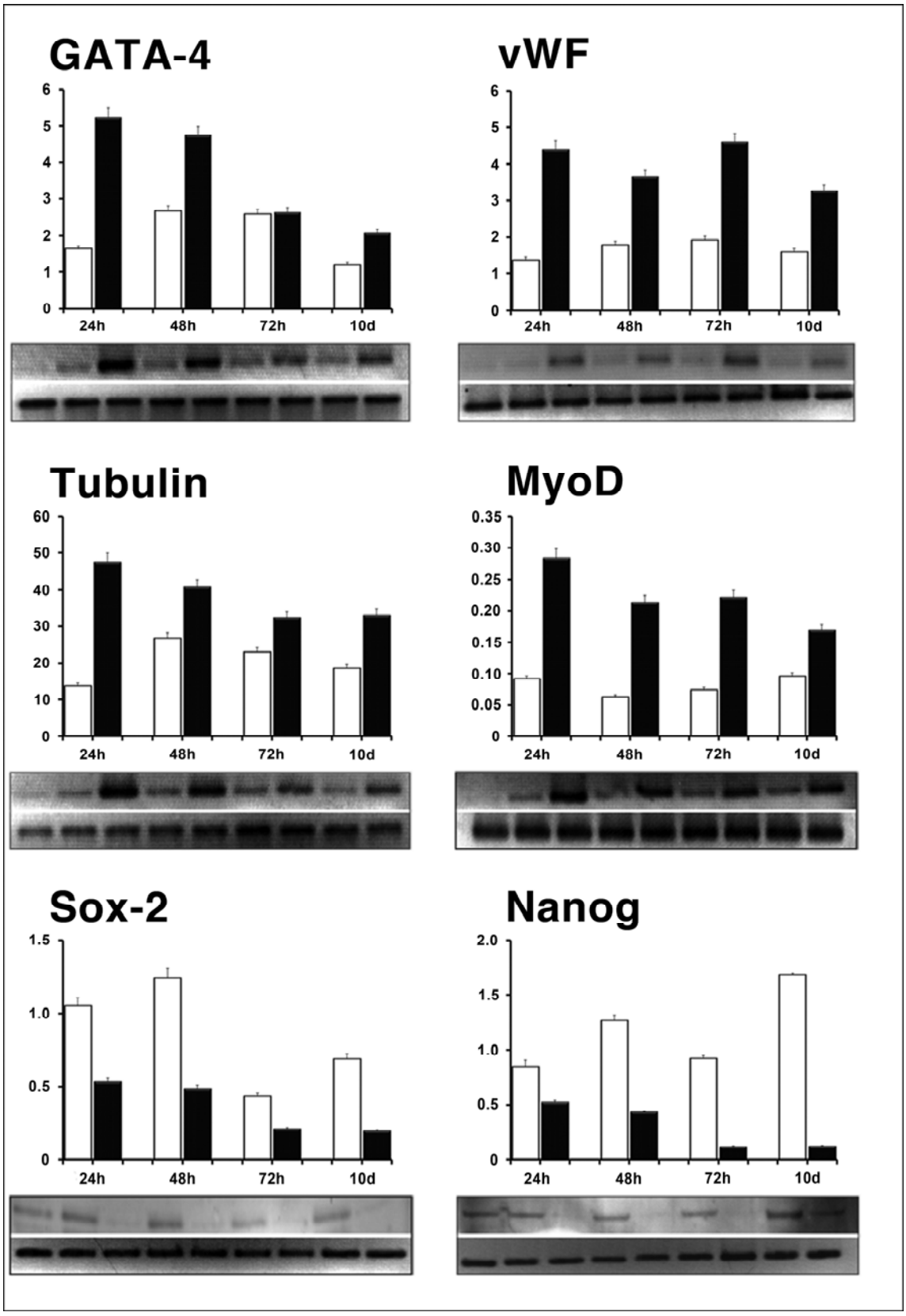


FIGURE 4



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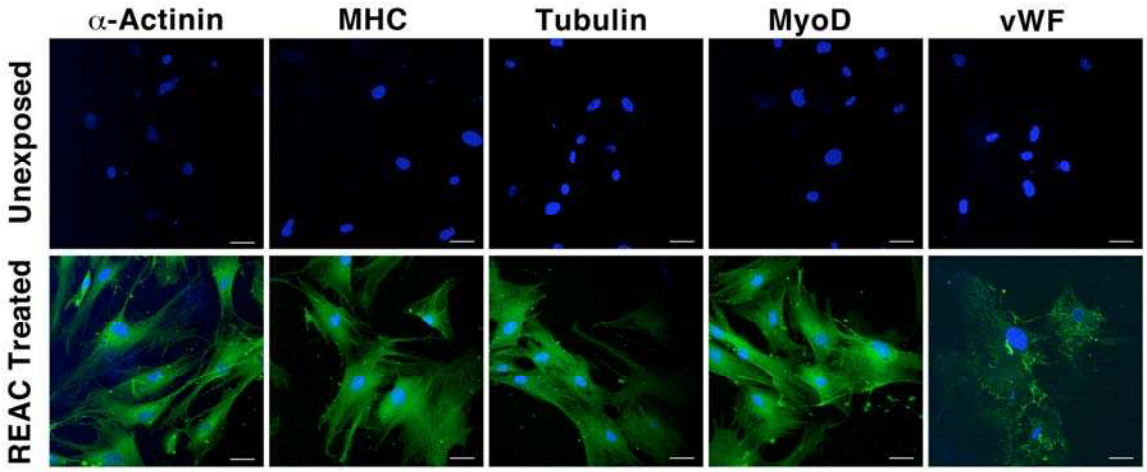

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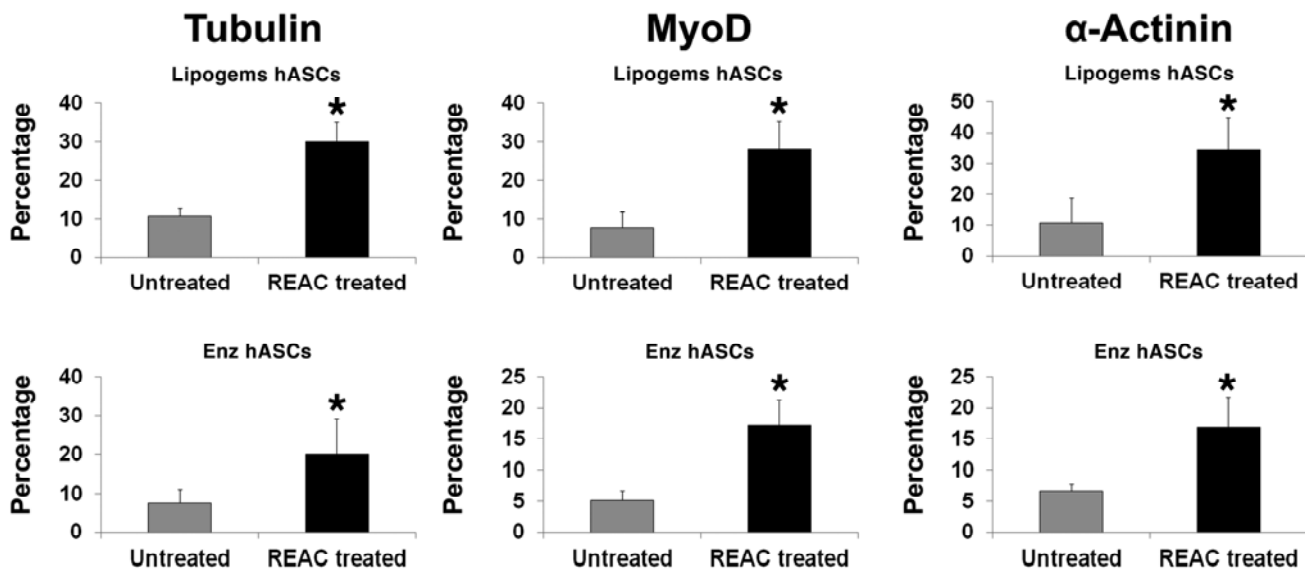

FIGURE 7