JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med 2015; **9**: 1352–1362.
Published online 29 April 2013 in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/term.1747

RESEARCH ARTICLE

Carnitine and acetylcarnitine modulate mesenchymal differentiation of adult stem cells

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Abstract

Cellular metabolic activity, especially mitochondrial metabolism, plays a vital role in regulating cell proliferation and differentiation. Metabolism could therefore be an important factor to consider when using engineering technologies to stimulate tissue development and repair. The small metabolite carnitine and its derivative acetylcarnitine affect the activities of several pathways in mitochondrial metabolism, but their influence on cell differentiation has not yet been thoroughly studied. To elucidate the effects of these two small molecules on mesenchymal tissue engineering, we used adult stem cells as a platform in both monolayer and 3D hydrogel culture systems. We investigated the impact of these two small molecules on the differentiation of adult stem cells and analysed gene expression, cell proliferation and extracellular matrix deposition. We found that the molecules reduced adipogenesis but stimulated osteogenesis and chondrogenesis in both culture systems. Our results suggest that carnitine and acetylcarnitine could affect the differentiation rate of adult stem cells by regulating mitochondrial metabolism. The effects of these two small molecules give rise to the possibility of employing such metabolites in tissue-engineering systems to enhance cell differentiation and tissue development. Copyright © 2013 John Wiley & Sons, Ltd.

Received 1 August 2012; Revised 30 January 2013; Accepted 16 March 2013

Keywords **carnitine; acetylcarnitine; tissue engineering; adult stem cells; mesenchymal differentiation; mitochondrial metabolism**

1. Introduction

The general tissue-engineering paradigm uses cells and/or biomaterial scaffolds with biological signals to promote tissue growth and, eventually, to replace tissue lost from disease or trauma, or absent as a result of congenital abnormalities (Elisseeff *et al*., 2006). In this system, cellular metabolic activity may play an important role in stimulating cell survival and differentiation and regulation of tissue growth; however, the bioenergetics of engineered tissues have not undergone thorough consideration (Zhang *et al*., 2011). This role of cellular metabolism in the mesenchymal system has recently garnered significant attention, although the specific mechanism is not yet well understood. Initial studies have also reported that changes in the levels of unsaturated metabolites regulated the metabolic status of embryonic stem cells during differentiation (Yanes *et al*., 2010). Many vital metabolic activities take place in the mitochondrion, which is the main source of cellular energy. The link between mitochondrial bioenergetics and stem cell differentiation was recently pointed out in a stem cell osteogenesis study (Chen *et al*., 2008).

In stem cell biology and therapy, small molecules are frequently employed in activating stem cell differentiation and stimulating tissue production. They have several advantages over growth factors, including increased permeability through the plasma membrane, improved stability and delivery and finely tuned, reversible effects (Ding *et al*., 2003; Xu *et al*., 2008). Carnitine is a trimethylated amino acid (Figure 1a) and an indispensible part of cellular energy production through fatty acid oxidation. In the cytosol, fatty acids are activated into acyl-CoAs, which are later broken down by carnitine acyltransferase, with the acyl portion transferred to carnitine, forming

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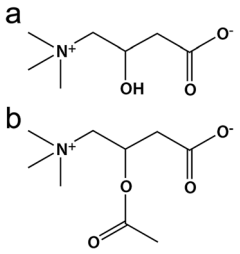

Figure 1. Schematics of the small molecules (a) carnitine and (b) acetylcarnitine

acylcarnitine on the mitochondrial outer membrane. Subsequently, the acylcarnitine is transported into the mitochondrial matrix, where it is reconverted to acyl-CoA to undergo β-oxidation (Demarquoy, 2011). Acetylcarnitine is an acetylated carnitine derivative (Figure 1b) and is converted from carnitine and acetyl-CoA in the mitochondrial matrix. Acetylcarnitine metabolism regenerates free CoA, which is important in fatty acid oxidation and the tricarboxylic acid (TCA) cycle (Rosca *et al*., 2009).

Knowledge of carnitine's function in fat metabolism and mitochondrial energy production extends back to the 1950s (Bremer, 1983). Its role in other cell functions has been recognized in recent years. *In vitro* studies have shown that carnitine could stimulate lipolytic gene expression and downregulate adipogenesis gene expression in 3T3-L1 adipocytes (Lee *et al*., 2006) and enhance extracellular matrix (ECM) synthesis in human primary chondrocytes (Stoppoloni *et al*., 2012). Carnitine and its derivative could also protect osteoblastic cells from apoptosis, besides promoting osteogenesis and bone regeneration (Colucci *et al*., 2005; Xie *et al*., 2008). The derivative acetylcarnitine has similar cellular functions to those of carnitine. Although acetylcarnitine is involved in reducing age-related oxidative stress and cell apoptosis (Calò *et al*., 2006; Rosca *et al*., 2009), the relationship between this small molecule and cell differentiation remains unclear.

In our study, we aimed to investigate the utilization of the small molecules carnitine and acetylcarnitine in mesenchymal tissue engineering. To elucidate their effects on adult stem cell differentiation and tissue production, we cultured bone marrow- or adipose-derived mesenchymal stem cells in differentiation media (adipogenesis, osteogenesis or chondrogenesis) containing carnitine or acetylcarnitine. Differentiation was monitored in both monolayer and three-dimensional (3D) poly(ethylene glycol) (PEG) hydrogel environments to determine the role of dimensionality in carnitine and acetylcarnitine-mediated differentiation, since, according to previous studies, 3D environments more closely mimic natural tissue and provide better conditions for stem cells to differentiate into target tissues (Williams *et al*., 2003; Yang *et al*., 2005). The effects were evaluated on gene expression, cell proliferation and ECM accumulation. We hypothesized that both carnitine and acetylcarnitine have an impact on mesenchymal differentiation of adult stem cells, and they could be employed to influence tissue production.

2. Materials and methods

2.1. Experimental design

Human adipose-derived stem cells (ASCs) and goat bone marrow-derived mesenchymal stem cells (MSCs) were used in this study. Adipogenesis, osteogenesis and chondrogenesis were conducted in an either two-dimensional (2D) or 3D environment with different concentrations of carnitine or acetylcarnitine and maintained for a suitable length of time *in vitro* (Table 1). Differentiation outcomes were evaluated by histological staining, gene analysis and biochemical assays. In all differentiation studies, cells cultured in normal differentiation medium without any carnitine or acetylcarnitine were used as controls. All experiments were performed in triplicate.

2.2. Cell isolation and expansion

ASCs were obtained from liposuction aspirates from three donors in accordance with the standards of the Johns Hopkins Institutional Review Board. Adipose tissue was washed with phosphate-buffered saline (PBS) to remove most of the blood. Afterwards the tissue was minced and digested with 0.1% collagenase type I (Worthington Biochemical, Lakewood, NJ, USA) at 37°C for 1.5 h. After centrifugation, floating fat and adipocytes were removed and the cell pellet was filtered through a 20 μm cell strainer and then incubated with erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA, pH 7.3) for 5 min to eliminate red blood cells. The ASCs were cultured at 37°C in 5% CO_2 in growth medium containing Dulbecco's modified Eagle's medium (DMEM)/F12 (Life Technologies, Carlsbad, CA, USA), 10% fetal bovine serum (FBS; HyClone, Thermo Scientific, Logan, UT, USA), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml

Table 1. Experimental design

Differentiation	Culture system	Cells	Time in culture (weeks)	Metabolite concentrations (mM)	
				Carnitine	Acetylcarnitine
Adipogenesis	Monolayer	ASCs	3	10	10
Osteogenesis	Monolayer	ASCs	3	10, 100	1, 10, 100
	Hydrogel	MSCs	4	10, 100	1, 10, 100
Chondrogenesis	Hydrogel	MSCs	3	10	10

DOI: 10.1002/term

streptomycin (Life Technologies) at a density of ca. 100 000 cells/cm^2. MSCs were isolated from adult caprine bone marrow aspirates (Thomas D. Morris Inc., Reisterstown, MD, USA), as previously described (Williams *et al.*, 2003). Briefly, marrow samples were washed twice with high-glucose DMEM (Life Technologies) and plated at a density of 120 000 mononuclear cells/cm^2. The cells were cultured in 5% CO_2 at 37°C, using growth medium containing high-glucose DMEM, 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Passage 4 cells were trypsinized, centrifuged and replated in monolayer culture or resuspended in polymer solution, as described below.

2.3. Differentiation media and monolayer cell culture

Adipogenic medium was composed of regular expansion medium supplemented with 1 μM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 100 μM indomethacin, 500 μM 3-isobutyl-1-methylxanthine and 10 μg/ml insulin (Sigma-Aldrich). Osteogenic medium contained 100 nM dexamethasone, 50 μM ascorbic acid-2-phosphate (Sigma-Aldrich) and 10 mM β-glycerophosphate (Sigma-Aldrich), in addition to high-glucose DMEM and 10% FBS. For chondrogenic differentiation, cells were induced in chondrogenic medium consisting of high-glucose DMEM, 1% ITS premix (BD Bioscience, San Jose, CA, USA), 100 nM dexamethasone, 40 μg/ml L-proline (Sigma-Aldrich), 50 μM ascorbic acid-2-phosphate and 100 μM sodium pyruvate (Life Technologies). In all cell cultures (monolayer and 3D), the media were changed three times/week until the end of the experiment, and different concentrations of carnitine or acetylcarnitine (both L-isomers; gifts from Sigma-Tau, Italy; acetylcarnitine was in the hydrochloride form) were added at every media change.

In monolayer cell culture studies (adipogenesis and osteogenesis), MSCs or ASCs were plated at 5000 cells/cm^2 initially and cultured in growth medium for approximately 5 days until they were confluent, before being exposed to differentiation media.

2.4. Hydrogel encapsulation and 3D culture

Formation of PEG gels for the chondrogenesis study was completed as described previously (Williams *et al.*, 2003). In short, UV-sterilized poly(ethylene glycol)–diacrylate (PEGDA; SunBio, Anyang City, South Korea) was dissolved in sterile PBS to give a 10% w/v solution. The photoinitiator Irgacure® 2959 (Ciba Specialty Chemicals, Tarrytown, NY, USA) was dissolved in 70% ethanol to give a 100 mg/ml stock solution and then added to the PEGDA polymer solution, with a final concentration of 0.05% v/v. Before polymerization, MSCs were suspended homogeneously in the polymer solution at 20 million cells/ml and aliquoted into 6 mm cylindrical moulds (100 μl/each) and photopolymerized under ultraviolet (UV) light (365 nm wavelength, 3 mW/cm^2 for 5 min). The hydrogels were then carefully removed from the moulds and incubated in 24-well tissue culture plates in chondrogenic medium for 3 weeks at 37°C with 5% CO_2.

The osteogenesis 3D study was conducted in PEG hydrogels functionalized with RGD (arginine–glycine–aspartate) peptide and the material was synthesized as previously described (Yang *et al.*, 2005). Polymer solution was prepared by mixing 2.2% w/v acryl–PEG–YRGDS and 12.8% w/v PEGDA in sterile PBS to yield a 2.5 mM concentration of the peptide, with 0.05% v/v photoinitiator. Goat MSCs were suspended in the polymer solution at 20 million cells/ml, and then the solution was aliquoted into 6 mm cylindrical moulds (100 μl/each) and photopolymerized under UV light. The hydrogels were carefully removed from the moulds and cultured in 24-well tissue culture plates in osteogenic medium for 4 weeks at 37°C with 5% CO_2.

2.5. Staining and histology

By the end of the first week, viability of MSCs undergoing chondrogenesis and encapsulated in PEG was determined by the Live/Dead Viability/Cytotoxicity Kit for mammalian cells (Life Technologies), which contains calcein-AM and ethidium homodimer-1. Generally, thin slices of samples from the hydrogels were stained with live/dead solution (4 μM calcein-AM and 4 μM ethidium homodimer-1) for 30 min at 37°C. They were then washed three times with PBS and imaged using a fluorescent microscope equipped with a 485 ± 10 nm optical filter for calcein AM (live cells) and a 530 ± 12.5 nm optical filter for ethidium homodimer-1 (dead cells).

In the adipogenesis study, monolayer cells were fixed in 10% formalin and lipid droplets were stained with 30 mg/ml oil red O (Sigma-Aldrich) in 60% isopropanol. In the osteogenesis study, calcium deposition was detected by 40 mM alizarin red S, pH 4.1 (Sigma-Aldrich) and alkaline phosphatase (ALP), stained according to the manufacturer's instructions (Sigma-Aldrich). Monolayer cells were rinsed with Tyrode's balanced salt solution and fixed for 30 s with citrate-buffered acetone. After washing with distilled water, the cell cultures were incubated in a fresh mixture of naphthol AS-MX phosphate and fast violet B salt solution in the dark for 45 min.

Hydrogel samples were cut into equal halves and fixed overnight in 10% formalin, following a standard histological dehydration procedure, and embedded in paraffin. Tissue sections were prepared at a thickness of 5 μm. For the chondrogenesis study, samples were stained with haematoxylin and eosin (H&E) for cell morphology and safranin O for basophilic glycosaminoglycans (GAG), as described previously (Hwang *et al.*, 2006). Osteogenesis sections were also stained with H&E and alizarin red S to visualize calcium deposition. Excess stain was removed by thoroughly washing with deionized water. All histological staining images were taken through a Nikon 2000E microscope in brightfield or phase-contrast mode.

2.6. Biochemical analyses

Hydrogel samples were lyophilized, mechanically homogenized by pestle and then digested in papainase solution (Worthington Biochemical) for 16 h at 60°C, followed by centrifugation to obtain the supernatant. DNA content was determined using the Hoechst Dye 33258 assay (Sigma-Aldrich), and GAG content was determined using a dimethylmethylene blue (DMMB) dye assay, as described previously (Hwang *et al*., 2006). Osteogenic samples used for calcium quantification were harvested in 0.5 M HCl; monolayer cells were removed from the tissue-culture plates mechanically by cell scrapers following sonication; hydrogel samples were homogenized by pestle and then vortexed for 16 h at 4°C. Calcium in the supernatant was quantified using a calcium assay kit (Point Scientific, Canton, MI, USA), according to the manufacturer's protocol. For osteogenesis monolayer samples, ALP activity was measured by incubation with *p*-nitrophenol phosphate substrate (Sigma-Aldrich) in glycine buffer, pH 10.4, at 37°C. After 15 min the reaction was quenched by adding 1 M NaOH solution. The absorbance at 405 nm was measured on a microplate reader (Biotek, Synergy 2, Winooski, VT, USA).

2.7. Reverse transcription and real-time quantitative polymerase chain reaction (qRT–PCR) analysis

Total RNA was isolated from cells grown on tissue culture plates or in hydrogels by TRIzol® (Life Technologies) and cDNA was synthesized using a SuperScript First-Strand Synthesis System (Life Technologies). The real-time qPCR was performed on the StepOnePlus™ Real-Time PCR System (Life Technologies) with SYBR® Green PCR Master Mix (Life Technologies). Each PCR was carried out in triplicate. The relative expression of each target was calculated using the $\Delta\Delta C_T$ method and *β-actin* was the endogenous reference, as described previously (Livak and Schmittgen, 2001). All expression levels of samples were normalized to controls. Primer sequences and product sizes are listed in Table 2.

2.8. Cell proliferation assay

Cell proliferations of ASCs undergoing adipogenic differentiation with different carnitine or acetylcarnitine concentrations were assessed by the water-soluble tetrazolium salts (WST-1) assay. Cells were seeded on a 96-well plate at a density of 10^4 cells/well in 200 μl growth medium and incubated at 37°C overnight. Afterwards, the medium was changed to differentiation medium, with or without carnitine or acetylcarnitine. Cells were incubated for 24 h at 37°C and then 100 μl WST-1 reagent (Cayman Chemical, Ann Arbor, MI, USA) was added to each well after the medium had been aspirated. The plate was incubated for 40 min at 37°C and then the absorbance of each well at 460 nm wavelength was measured using a microplate reader (Biotek, Synergy 2).

2.9. Statistical analysis

All the quantitative results were reported as means of three measurements ± standard deviation (SD). Statistical significance compared to the control condition was determined by one-way ANOVA and the results were marked as $p < 0.05$ (significant) or $p < 0.001$ (highly significant).

Table 2. Primer sequences and product sizes for qRT–PCR

Gene		Sequence	Product size (bp)
β-actin	Forward	5′-TGGCACCACACCTTCTACAATGAGC-3′	396
	Reverse	5′-GCACAGCTTCTCCTTAATGTCACGC-3′	
αP2	Forward	5′-GTACCTGGAAACTTGTCTCC-3′	418
	Reverse	5′-GTTCAATGCGAACTTCAGTCC-3′	
GLUT4	Forward	5′-AGCAGCTCTCTGGCATCAAT-3′	275
	Reverse	5′-CAATGGAGACGTAGCACATG-3′	
LPL	Forward	5′-GAGATTTCTCTGTATGGCACC-3′	276
	Reverse	5′-CTGCAAATGAGACACTTTCTC-3′	
Leptin	Forward	5′-GGCTTTGGCCCTATCTTTTC-3′	325
	Reverse	5′-GCTCTTAGAGAAGGCCAGCA-3′	
PPARγ1	Forward	5′-GCTCTAGAATGACCATGGTTGAC-3′	250
	Reverse	5′-ATAAGGTGGAGATGCAGGCTC-3′	
Type I collagen	Forward	5′-GCCAAGAGGAAGGCCAAGTC-3′	108
	Reverse	5′-AGGGCTCGGGTTTCCACAC-3′	
Type II collagen	Forward	5′-GTGGAGCAGCAAGAGCAAGGA-3′	334
	Reverse	5′-CTTGCCCCACTTACCAGTGTG-3′	
Sox9	Forward	5′-TTCATGAAGATGACCGACGA-3′	326
	Reverse	5′-CACACCATGAAGGCGTTCAT-3′	
Aggrecan	Forward	5′-CACGATGCCTTTCACCACGAC-3′	182
	Reverse	5′-TGCGGGTCAACAGTGCCTATC-3′	
cbfa1	Forward	5′-CCACCCGGCCGAACTGGTCC-3′	258
	Reverse	5′-CCTCGTCCGCTCCGGCCCACA-3′	

DOI: 10.1002/term

3. Results

3.1. Adipogenesis in monolayer culture

Carnitine and acetylcarnitine supplementation resulted in decreased adipogenesis. At the microscopic level, cells treated with either small molecule exhibited decreases in lipid droplet accumulation, which were visualized by oil red O staining. The acetylcarnitine-treated group had even less lipid than the carnitine-treated group (Figure 2a–c). This inhibitory effect was also reflected in mRNA levels. Based on qPCR results, several adipogenic genes were downregulated after exposure to carnitine or acetylcarnitine (Figure 2d–g). There was a five-fold decrease in *GLUT4* expression in the group treated with 10 mM acetylcarnitine. Expressions of *PPARγ1* and *leptin* also decreased significantly (two- to three-fold) in the same group. A similar trend for *GLUT4* expression was observed in the carnitine-treated group. Exposure to carnitine did not result in significant changes in the expression of either *leptin* or *PPARγ1*. There was no significant change in the expression level of *αP2* or *LPL* (Figure 2g). WST-1 assay demonstrated increased cell proliferation rates with carnitine or acetylcarnitine exposure (Figure 2h).

3.2. Osteogenesis in monolayer culture

Osteogenesis in monolayer culture was promoted by both carnitine and acetylcarnitine. After 1 week, ASCs showed detectable ALP activity on the cell membrane, confirmed by both the ALP assay (Figure 3a) and ALP staining (Figure 3d–i). The representative ALP images correlated well with quantitative analysis. At week 1 there were increases in ALP activity in the 10 and 100 mM carnitine-treated groups (Figure 3e, f); however, in the acetylcarnitine-treated groups, only 1 mM acetylcarnitine (Figure 3g) enhanced ALP activity, while the higher acetylcarnitine concentration groups showed detrimental effects on ALP activity (Figure 3h, i).

Increased osteogenesis was also reflected in DNA content (Figure 3b) and ECM deposition (calcium content, Figure 3c; alizarin red staining, Figure 3j–o) in some

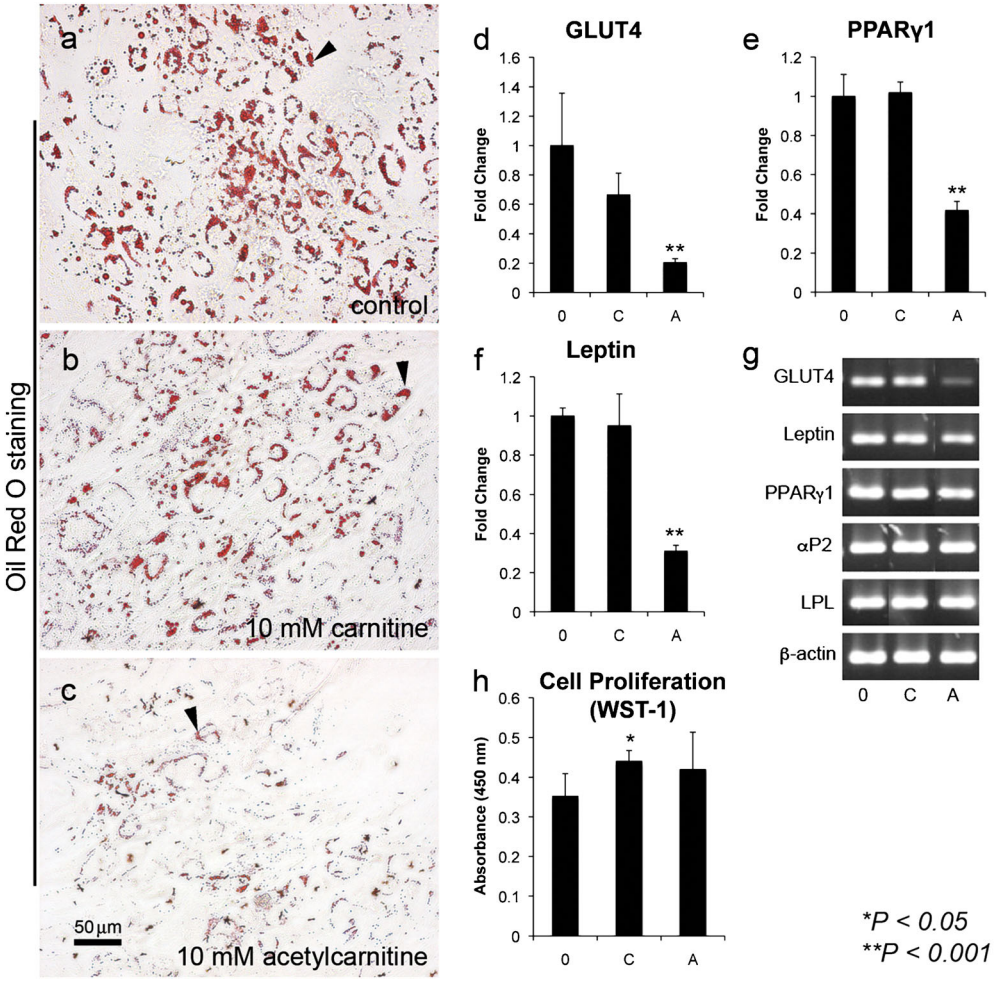

Figure 2. The effects of carnitine and acetylcarnitine on the adipogenesis of ASCs in monolayer culture. ASCs were cultured in adipogenic medium supplemented with 10 mM carnitine or acetylcarnitine. (a–c) Oil red O staining with decreased lipid-aggregation (arrowhead) for both supplementations; (d–g) qRT–PCR results with reduced expression of adipogenic markers *GLUT4*, *PPARγ1* and *leptin*; (h) WST-1 assays exhibited an increasing trend of cell proliferation with supplemented carnitine or acetylcarnitine. 0, control; C, 10 mM carnitine; A, 10 mM acetylcarnitine

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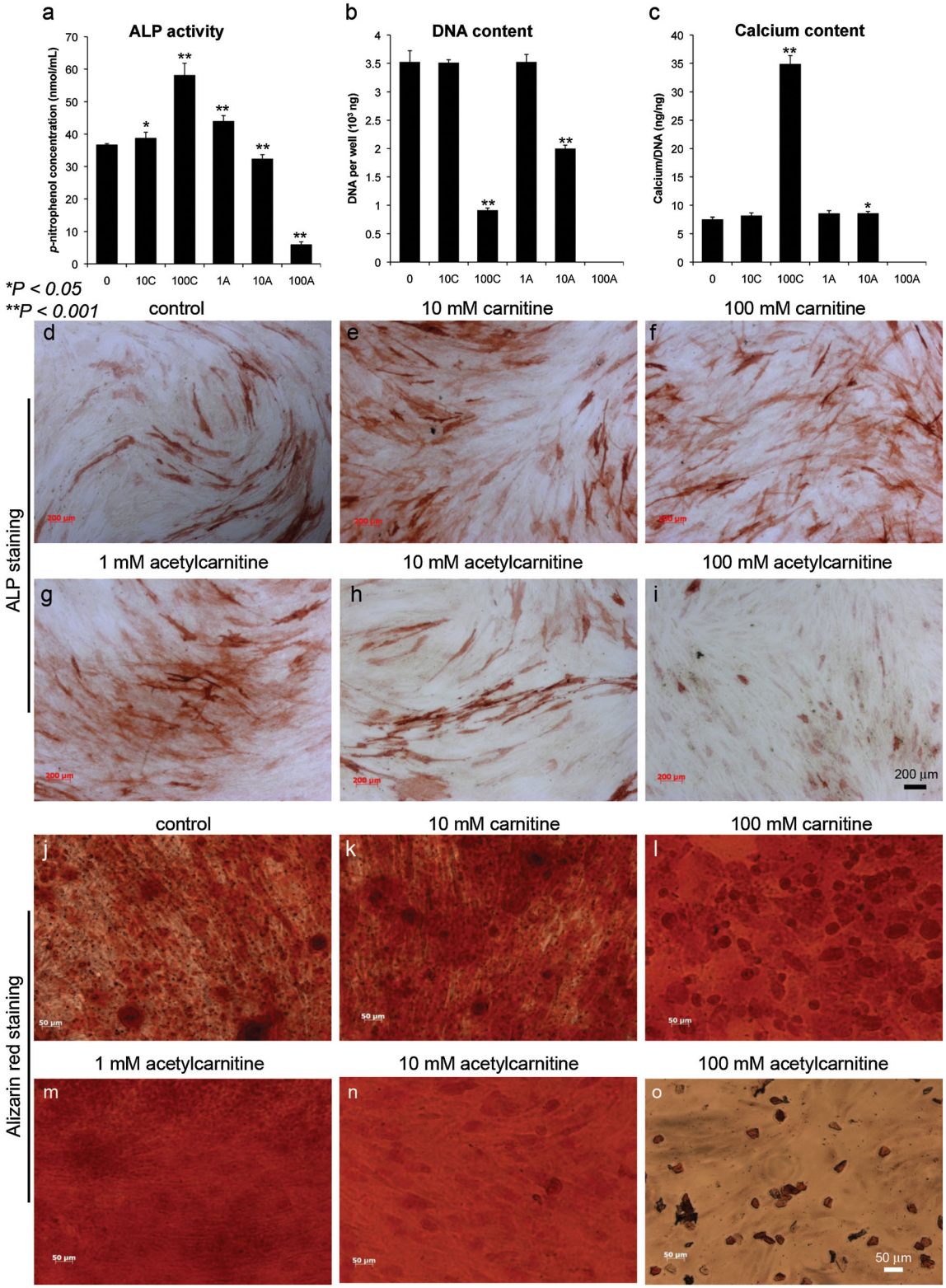

Figure 3. The effects of carnitine and acetylcarnitine on the osteogenesis of ASCs in monolayer culture. (a) At day 7, the ALP activity increased at 10 mM carnitine, 100 mM carnitine and 1 mM acetylcarnitine, while it decreased at 10 and 100 mM acetylcarnitine. (b) The DNA content at day 14 of differentiation was decreased at 100 mM carnitine, 10 mM and 100 mM acetylcarnitine. (c) At day 14, the calcium production increased in the 100 mM carnitine-treated group. Staining: (d–i) ALP; (j–o) alizarin red. 0, control; C, carnitine; A, acetylcarnitine; numbers (1, 10 or 100) indicate the concentrations (mM)

treated groups. The 10 mM carnitine group (Figure 3k) was comparable to the control (Figure 3j) in DNA content and calcium deposition, while the 100 mM carnitine group (Figure 3l) decreased the total amount of DNA but produced more calcified matrix. In the 1 mM acetylcarnitine group (Figure 3m) there was no effect on DNA but calcium

DOI: 10.1002/term

deposition was promoted. However, 10 mM acetylcarnitine (Figure 3n) significantly decreased DNA content and calcium deposition was slightly increased. The DNA and calcium contents in the 100 mM acetylcarnitine group (Figure 3o) were too low to be determined.

3.3. Osteogenesis in a 3D hydrogel environment

Similar to what occurred in monolayer culture, carnitine and acetylcarnitine were also beneficial to osteogenesis in a 3D hydrogel system. Supplementation with either carnitine or acetylcarnitine to the osteogenic medium significantly promoted cell proliferation in the hydrogels (Figure 4a). In the 10 mM and 100 mM carnitine groups, DNA contents were noticeably higher than in control samples. Similarly, all three concentrations of acetylcarnitine produced more DNA than the control group, while the effects were greater in the 10 and 100 mM groups than in the 1 mM group.

In histological analysis (alizarin red staining) of the constructs, we noticed higher cell densities in the treated groups vs the control (Figure 4e–j). According to the quantitative calcium assay (Figure 4b), both carnitine and acetylcarnitine had positive effects on mineral deposition, and we observed similar effects with respect to DNA content. Alizarin red staining for calcium deposition confirmed the quantitative data, that there was an increase in mineralization in carnitine- and acetylcarnitine-supplemented groups compared to the control. A significant upregulation of the early osteogenesis marker transcriptional factor *cbfa1* (Figure 4c) was observed in the 1 mM and 100 mM acetylcarnitine groups, while in the carnitine-treated groups this was not significantly different from that in the control group, and there was a similar trend in the expression of type I collagen (Figure 4d).

3.4. Chondrogenesis in 3D PEG hydrogel culture

Overall, there was an increase in chondrogenesis in both the carnitine- and acetylcarnitine-treated groups. qPCR results indicated downregulation of the expression for

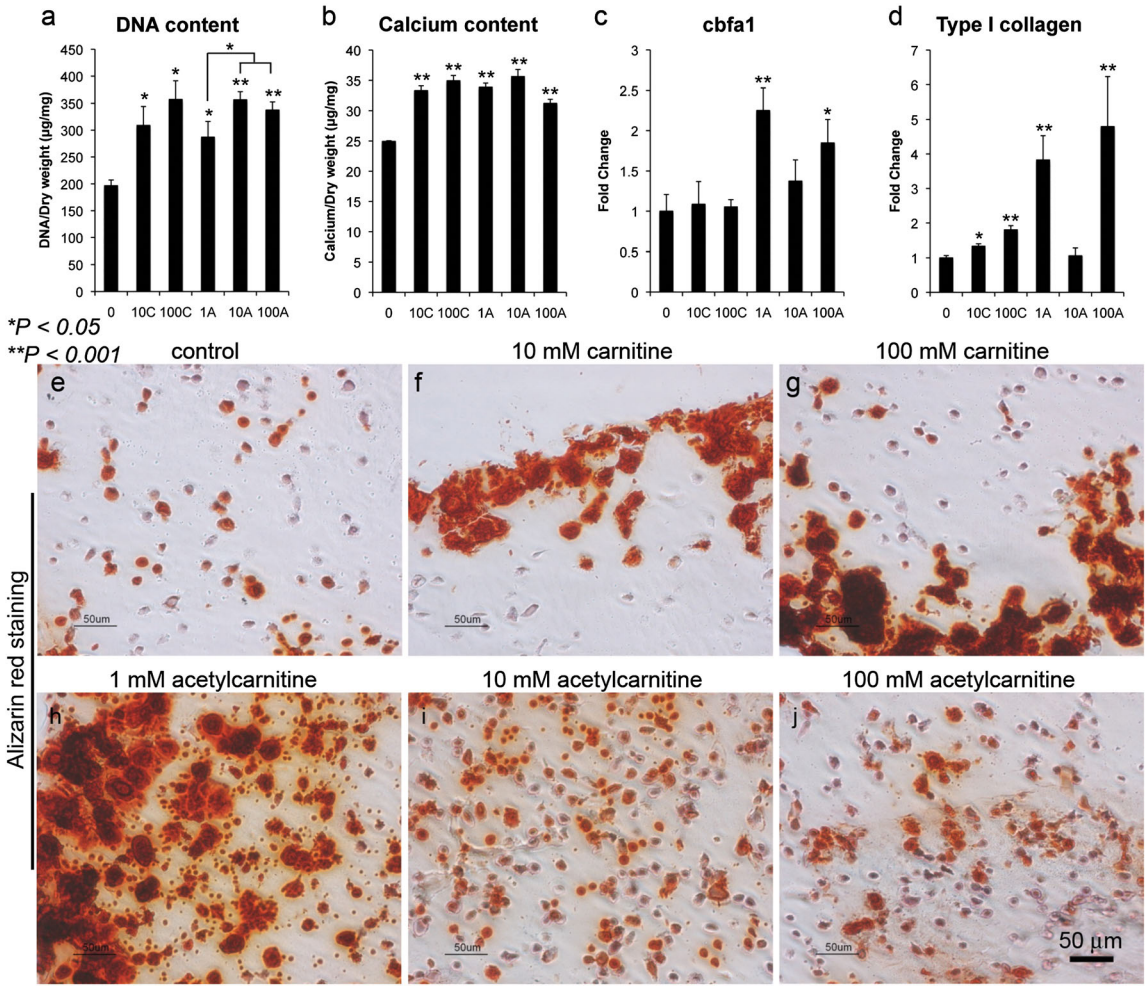

Figure 4. The effects of carnitine and acetylcarnitine on the osteogenesis of MSCs encapsulated in PEG–RGD hydrogels. (a) The DNA content showed significant increases in cell proliferation in all treated groups. (b) Calcium production in all conditions was also significantly higher than in the control group. (c, d) qPCR results: expression of the early osteogenesis marker transcriptional factor *cbfa1* was upregulated in acetylcarnitine-treated groups; similar trends were observed in the expression of type I collagen. (e–j) Alizarin red S staining revealed an increase in calcium deposition with acetylcarnitine and carnitine treatment. 0, control; C, carnitine; A, acetylcarnitine; numbers (1, 10 or 100) indicate the concentrations (mM)

DOI: 10.1002/term

the osteogenic markers *cbfa1* and *type I collagen*, but upregulation of the expression of the chondrogenic markers *type II collagen*, *sox9* and *aggrecan* (Figure 5a–e). In the carnitine-treated groups, *sox9* expression increased more than eight-fold (Figure 5b). Figure 5g is the live/dead staining image 1 week after encapsulation, showing that almost all cells were alive. Carnitine also had positive effects on the amount of ECM produced. The GAG content in the hydrogel constructs supplemented with 10 mM carnitine was significantly higher than in the unsupplemented control (Figure 5f), while the GAG content in the acetylcarnitine-supplemented group remained similar to that of the control. The GAG content result was consistent with the histological staining (Figure 5h–m). H&E and safranin O stainings displayed more cell aggregation in the carnitine- and acetylcarnitine-treated groups than in the control group.

4. Discussion

The primary metabolic function of carnitine is to facilitate the transport of long-chain fatty acids across the inner mitochondrial membrane for β-oxidation (Bremer, 1983). Mitochondrial metabolism plays a very important role in

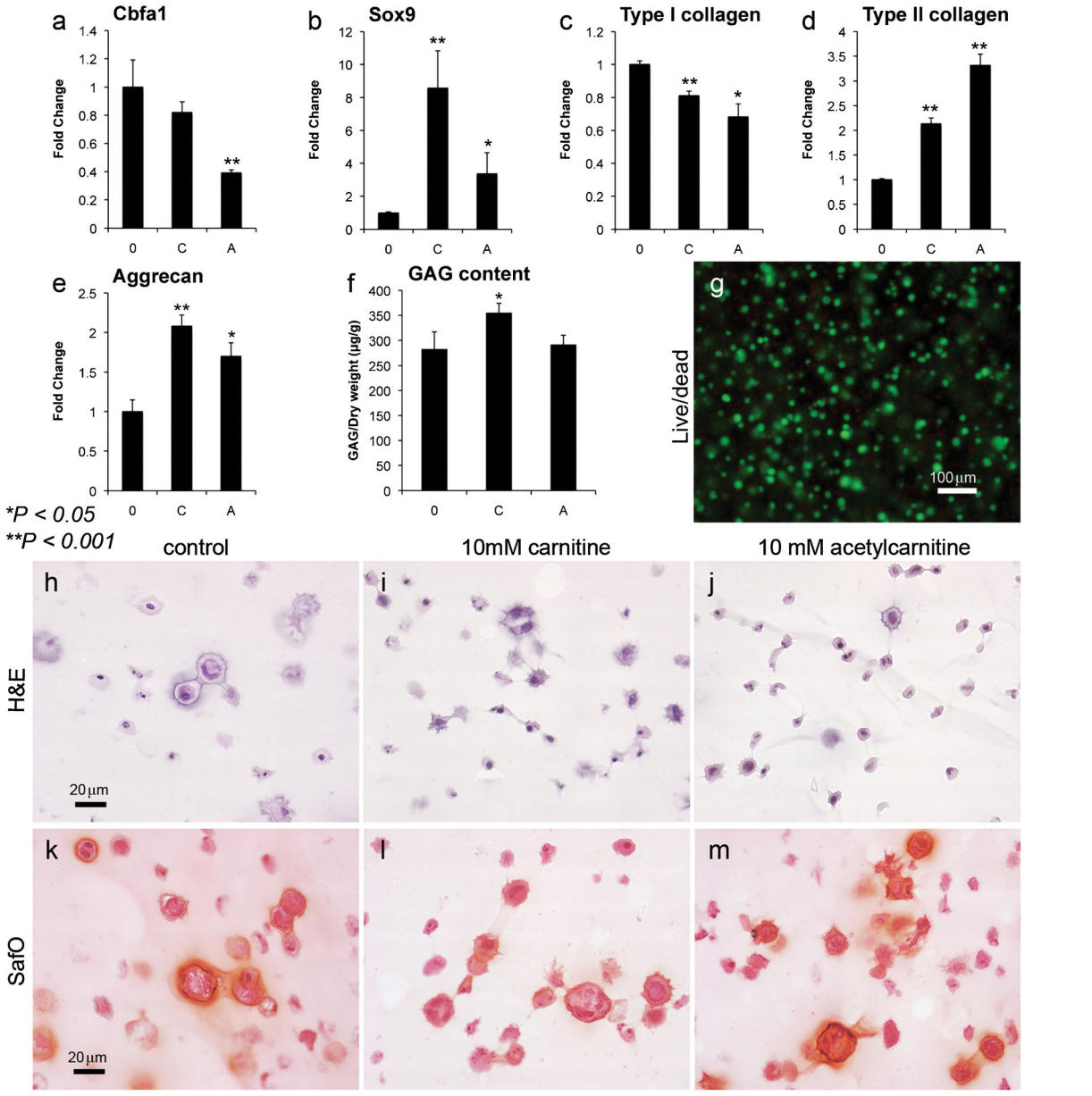

Figure 5. The effects of carnitine and acetylcarnitine on chondrogenesis of MSCs encapsulated in PEG hydrogels. (a–e) qPCR results: gene expression exhibited downregulation of early bone transcription factors *cbfa1* and *type I collagen* and significant upregulation of *type II collagen*, *sox9* and *aggrecan*. (f) GAG content assay. (g) Live/dead staining 1 week after encapsulation. Histological staining: (h–j) H&E; (k–m) safranin O (SafO) for GAG. The staining results at 3 weeks were consistent with the quantification data that both carnitine and acetylcarnitine (each 10 mM) promoted cell aggregation and GAG deposition. 0, control; C, 10 mM carnitine; A, 10 mM acetylcarnitine

DOI: 10.1002/term

early cell differentiation (Tormos *et al*., 2011), which rationalizes the use of carnitine or its derivative acetylcarnitine to influence stem cell differentiation and, eventually, tissue development. Our results indicated that carnitine and acetylcarnitine each had a role in the regeneration of different tissues. In either monolayer or the 3D PEG system, they demonstrated multiple effects on stem cell mesenchymal differentiation. In particular, we observed that both molecules inhibited adipogenesis, while osteogenesis and chondrogenesis were stimulated.

Carnitine has been used experimentally to enhance fat metabolism (Bernard *et al*., 2008), which explains why both carnitine and acetylcarnitine diminish adipogenic differentiation in monolayer ASCs culture, as shown by the reduced accumulation of lipid droplets (Figure 2a–c). We also observed decreases in adipocyte-specific gene expression (Figure 2d–g). PPAR (peroxisome proliferator-activated receptors) is a family of transcription factors that regulate the differentiation of preadipocytes into adipocytes (Wu *et al*., 1998). The activation of *PPARγ* is one of the earliest events during *in vitro* adipogenesis and leads to subsequent expression of most adipocyte-specific markers (Rosen *et al*., 2000).

GLUT4 is the major insulin-dependent glucose transporter, expressed exclusively in adipose tissue, cardiac and skeletal muscles (Hou and Pessin, 2007). In our study, acetylcarnitine downregulated the expression of *PPARγ*, which consequently led to the decreased expression of *GLUT4*. Without this adipose tissue-specific glucose transporter, the response of ASCs to insulin was attenuated, therefore less adipogenic differentiation was observed. Leptin is a hormone secreted by adipocytes, and in storage tissue such as white adipose tissue, lipid storage is increased in response to it (McClelland *et al*., 2004). The mRNA level of *leptin* was also decreased, and this is consistent with the decreased accumulation of lipid droplets observed in the acetylcarnitine-treated group (Figure 2c, f). In conclusion, acetylcarnitine decreased the expression of *PPARγ1*, *GLUT4* and *leptin*, and this shows that the influence of acetylcarnitine on the adipogenesis of adult stem cells starts from the primitive commitment stage and continues to the terminally differentiated adipocytes stage (Janderova *et al*., 2003).

The inhibitory effect of carnitine on adipocyte or preadipocyte differentiation was previously observed and reported in the literature (Lee *et al*., 2006; Luo *et al*., 2008). Our findings indicate, for the first time, a negative feedback on stem cell adipogenesis exposed to carnitine or acetylcarnitine. The WST-1 assay is a colorimetric assay based on the cleavage of WST-1 tetrazolium salt by mitochondrial dehydrogenases (part of the respiratory chain) and is a measurement of cellular respiration, metabolic rate and proliferation. Therefore, the elevated cell proliferation levels confirm that the effect of carnitine and acetylcarnitine on differentiation was through the manipulation of mitochondrial metabolism (Figure 2h). However, the carnitine-treated group only showed downregulated *GLUT4* expression and the decrease in lipid accumulation was smaller than the acetylcarnitine-treated group. Excess acetylcarnitine could generate more acetyl-CoA, leading to a higher acetyl-CoA:CoA ratio, which inhibited pyruvate dehydrogenase and consequently reduced the activity of the TCA cycle (Rosca *et al*., 2009). There was a correlation between decreased TCA flow and increased fatty acid oxidation in 3T3-L1 adipocytes (Wiczer and Bernlohr, 2009), and this is a possible mechanism explaining the better effect of acetylcarnitine than carnitine in reducing adipogenesis in ASCs.

Our findings confirm that carnitine and acetylcarnitine supplementation consistently enhanced cell proliferation (DNA contents) and osteogenesis in hydrogel culture (Figure 4). Recently, it has become clear that balanced regulation of reactive oxygen species (ROS) is critical for cell function and viability, stem cell development and differentiation (Tormos *et al*., 2011). Chen *et al*. (2008) suggested that, during osteogenic induction in MSCs, oxidative phosphorylation would replace glycolysis as a means of producing cellular energy; during osteogenesis, aerobic mitochondrial metabolism was upregulated, while accumulation of intracellular ROS was prevented. This relationship between enhanced metabolic output and reduced glycolysis may provide a link between carnitine and cell differentiation. Carnitine was found to protect cardiocytes against apoptosis by inhibiting cytochrome *c* release in mitochondria (Oyanagi *et al*., 2011), leading to increased oxidative phosphorylation. It was also reported that carnitine supplementation resulted in reduced intracellular ROS production in a H_2O_2-challenged hepatocytes model (Li *et al*., 2012). The above evidence supports that carnitine is beneficial for osteogenesis by regulating mitochondrial bioenergetics and reducing oxidative stress.

Carnitine and its derivatives have been used orally to enhance bone volume in osteoporotic mice (Patano *et al*., 2008) and also demonstrated *in vitro* osteogenic potential in human osteoblasts (Colucci *et al*., 2005). In our monolayer osteogenic study, calcium and ALP activity increased with carnitine concentration, with 100 mM carnitine exhibiting the greatest effect on calcification and ALP activity (Figure 3a, c). Histological evaluation by alizarin red staining for calcium deposits was consistent with the calcium assay and ALP activity (Figure 3j–o). However, 100 mM acetylcarnitine exhibited detrimental effects on cell proliferation and ECM development (Figure 3b, c), which is probably due to the abrupt increase in osmolarity and acidity in the medium. A similar concentration gradient was employed in 3D hydrogel culture experiments. All treated groups had significantly increased cell proliferation and more ECM accumulation (Figure 4a, b). Cells in monolayer developed clusters of calcified matrix, whereas the encapsulated MSCs produced calcified matrix directly surrounding each cell (Figure 4e–j). Nonetheless, unlike the 100 mM acetylcarnitine group in monolayer culture, no obvious side-effects were observed in any group of 3D hydrogel culture. Compared to monolayer culture, the 3D environment provides the encapsulated cells with a microenvironment closer to that of native tissue. Due to the pore sizes and dimensions of the hydrogel, a fast equilibration of acetylcarnitine concentration could also

DOI: 10.1002/term

be established within the gel, whereas cells cultured in monolayer are exposed directly to doses of acetylcarnitine (Varghese *et al*., 2007). This implies the importance of using a 3D system to study the effects of metabolites on tissue engineering, as different results may be obtained from monolayer and 3D cultures, even in similar culture media.

Understanding the factors essential for chondrogenesis is an important step in the treatment of cartilaginous diseases such as osteoarthritis (OA). Mitochondrial dysfunction is involved in human OA development, resulting in cartilage degradation, matrix calcification and chondrocyte apoptosis (Blanco *et al*., 2011). ROS are also believed to be one of the driving factors in OA (Goldring and Otero, 2011). Therefore, carnitine and acetylcarnitine could be helpful for chondrogenesis through increasing mitochondrial activity. This is supported by our chondrogenesis study. In hydrogel culture, the bone markers *cbfa1* and *type I collagen* were significantly downregulated, while the upregulation of the chondrogenesis-specific genes *sox9*, *type II collagen* and *aggrecan* confirmed a positive effect on mRNA level (Figure 5a–e). One of the key factors modulating chondrogenesis is the cellular potential to produce GAG. MSCs undergoing chondrogenesis have enhanced GAG production when treated with 10 mM carnitine. However, unlike what occurs in adipogenesis, the positive effects of acetylcarnitine were more restricted to the mRNA level and are not reflected downstream on the ECM (Figure 5f).

In addition to facilitating fatty acid transportation, carnitine and acetylcarnitine have been associated with anti-apoptotic and anti-oxidant effects on different cell types. Abdelrazik *et al*. (2009) observed that certain carnitine concentrations had an anti-apoptotic effect in mouse embryos. Rump *et al*. (2010) hypothesized that acetylcarnitine administration protected the brains of alcohol-fed mice from alcohol-induced oxidative damage by protecting normal functioning of mitochondria in neurons. The protective effects of acetylcarnitine, which is used clinically in treating neurological diseases (Malaguarnera, 2012), may be advantageous to chondrogenesis, particularly in inhibiting the inflammatory effects typical of damaged cartilage. In the PEG hydrogel environment, carnitine- and acetylcarnitine-treated MSCs undergoing chondrogenesis showed better survival, with increased cell–cell contact and spreading than MSCs in the untreated groups (Figure 5h–m).

Our findings support the feasibility of modulating adult stem cell differentiation with carnitine and acetylcarnitine. However, carnitine palmitoyltransferase I is the rate-limiting factor in mitochondrial β-oxidation (Foster, 2004); once its capacity is reached, higher concentrations of carnitine or acetylcarnitine may not generate better effects. This may be the reason why we did not observe clear dose-dependent behaviour in osteogenesis studies: all concentrations gave rise to similar increases in DNA and calcium content in 3D PEG culture (Figure 4a, b).

5. Conclusion

In summary, we investigated the relationship between cell metabolic activity and tissue engineering, and the role of small metabolites in manipulating adult stem cell mesenchymal differentiation. Our results indicate that carnitine and acetylcarnitine are both beneficial to osteogenesis and chondrogenesis and detrimental to adipogenesis of adult stem cells. This finding suggests that the use of small molecule metabolites may have a promising future use in stem cell differentiation, as, through regulation of cellular activities and mitochondrial metabolism, we will be able to manipulate the rate of stem cell differentiation and, eventually, accelerate tissue development.

Acknowledgements

This study was supported by the Sigma-Tau Foundation, Italy, and dedicated its founder, Claudio Cavazza. We also thank Dr Tae Kyun Kim for valuable comments on the manuscript.

Conflict of interest

The authors have declared that there is no conflict of interest.

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