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Basement membrane collagen type IV expression by human mesenchymal stem cells during adipogenic differentiation

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Abstract

During adipogenic differentiation human mesenchymal stem cells (hMSC) produce collagen type IV. In immunofluorescence staining differentiating hMSCs started to express collagen type IV when Oil Red O-positive fat droplets appeared intracellularly. Quantitative real time-polymerase chain reaction confirmed progressive increase of collagen type IV α1 and α2 mRNA levels over time, 18.6- and 12.2-fold by day 28, respectively, whereas the copy numbers of α3–α6 mRNAs remained rather stable and low. Type IV collagen was in confocal laser scanning microscopy seen around adipocytes, where also laminins and nidogen were found, suggesting pericellular deposition of all key components of the fully developed basement membrane. Immunofluorescence staining of matrix metalloproteinase-2 (MMP-2, 72 kD type IV collagenase, gelatinase A) and MMP-9 (92 kD type IV collagenase, gelatinase B) disclosed only faint staining of MSCs, but MMP-9 was strongly induced during adipogenesis, whereas MSC supernatants disclosed in zymography pro-MMP-2 and faint pro-MMP-9 bands, which increased over time, with partial conversion of pro-MMP-2 to its active 62 kD form. Differentiation was associated with increasing membrane type 1-MMP/MMP-14 and tissue inhibitor of metalloproteinase-2 (TIMP-2) staining, which may enable participation of type IV collagenases in basement membrane remodelling *via* ternary MT1-MMP/TIMP-2/MMP-2 or -9 complexes, focalizing the fully active enzyme to the cell surface. MMP-9, which increased more in immunofluorescence staining, was perhaps preferentially bound to cell surface and/or remodelling adipocyte basement membrane. These results suggest that upon MSC-adipocyte differentiation collagen type IV synthesis and remodelling become necessary when intracellular accumulation of fat necessitates a dynamically supporting and instructive, partly denatured adipogenic pericellular type IV collagen scaffold.

Keywords: adult stem cells • mesenchymal stem cells • cell differentiation • extracellular matrix • collagen type IV

Introduction

Human bone marrow mesenchymal stem cells are multipotent plastic adherent fibroblast-like cells [1–3]. During adipogenic differentiation, these cells would have to reform and/or remodel the interstitial matrix surrounding them [4, 5]. In particular, they have to generate a supporting basement membrane-like structure that surrounds adipocytes, but is missing around the MSCs. Therefore, MSC-to-adipocyte differentiation represents an interesting and dynamic model of the formation of human adipocyte basement membrane.

In experiments, where bovine intramuscular pre-adipose cells were cultured in adipogenic conditions, the expression of types I–VI collagens was increased during differentiation [6]. Murine stromal vascular cells differentiating to adipocytes showed in scanning electron microscopy a collagen network composed of types I,

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III, IV, V and VI collagen connecting these cells to fat cell clusters. Scanning electron microscopy showed that collagen IV and laminins were confined to close proximity of these cells and located in the basement membrane-like structures surrounding these cells [5]. This fits well with the dogma that basement membrane consists of two fused sheet-like networks, one of which is composed of collagen type IV and the other one of laminins, to which the adipocytes are integrated *via* their integrin and non-integrin matrix receptors [7,8]. Although often simply referred to as collagen type IV, human collagen IV genes actually encode six different α chains [α1(IV)–α6(IV)], which assemble into heterotrimers, most commonly $\alpha1\alpha1\alpha2$, $\alpha3\alpha4\alpha5$ and $\alpha5\alpha5\alpha6$ [9–11]. They are linked head-to-head to a two-dimensional (2D) basement membrane network, which gives basement membrane its tensile strength. Adipocyte basement membrane may be structurally important due to the support it provides for the large-size mature adipocytes containing lipids, which at the body temperature assume a semi-liquid state. In addition, it has been shown recently that collagen type IV is not only a product of differentiation, but by itself also influences the adipogenic differentiation processes [12, 13]. This would suggest that the role of collagen type IV may not be only restricted to providing structural support for the developing adipocyte, but collagen type IV α chains may also act as the key constituents in cell environment needed for certain stages of adipogenesis. Immunohistochemical studies of mature tissue fat cells have shown the presence of collagen type IV α1 and α2 chains, but also the presence of α5 and α6 chains at lower levels [14]. The presence of several different α chains may provide regulatory flexibility and suggests that the adipocyte basement membrane and its formation and maturation may require the presence of different trimer combinations. Composition of the basement membrane collagen type IV α chains changes during development in several tissues [15], which was also the hypothesis tested in our work. Indeed, it is remarkable that there is to our knowledge no previous publication analysing simultaneously the expression of all six possible α chains in the mature human adipocyte basement membrane and how this expressional profile develops during adipocyte formation from human adult tissue stem cells.

In addition, the forming basement membrane probably has to undergo remodelling depending on the developmental stage or the nutritional status of later stage mature adipocytes. Therefore, it was hypothesized that adipogenesis requires not only regulated synthesis of the specific α chain constituents into the 3D spherically organized and multilocular collagen type IV network, but also the proteolytic processing of already existing collage type IV matrix by matrix metalloproteinases (MMPs). Such processing may be needed not only for architectural purposes, but also for revealing important binding sites for the forming adipocyte [13]. Further, many growth factors are stored in extracellular matrix and undergo site and time-specific local release upon matrix/basement membrane degradation [16]. It has been reported that human adipocytes in primary culture and differentiating murine pre-adipocytes of the 3T3F442A and 3T3-L1 lines can express MMP-2 and MMP-9 and that MMP inhibition leads to inhibition of adipogenesis [17,18]. In contrast, if the differentiating human bone marrow MSCs were cultured on denaturated type I or type IV collagen matrix, they were induced to express MMP-8 and MMP-13, while the expression of TIMP-1, -2 and -3 was reduced [19]. Membrane anchored membrane type 1-MMP (MT1-MMP, MMP-14) has been more recently shown to be important in a mouse model of fat tissue development, especially in adipocyte–extracellular matrix interactions [20]. In this study differentiation of bone marrow–derived MSCs to adipocytes was for the first time used as a human cellular adipogenesis model system to study the expression of all different basement membrane collagen type IV α chains over time in relation to basement membrane formation and remodelling by type IV collagenases. Exact knowledge of basement membrane formation – one of the key events occurring during adipocyte differentiation from progenitor cells-helps to understand the bidirectional extracellular matrix – adipocyte related regulatory mechanisms guiding adipocyte formation, division and metabolism and, therefore, to develop better diagnostic, treatment and treatment monitoring methods for metabolic syndrome, diabetes and obesity in future.

Materials and methods

Cell culture

Human Poietics® bone marrow–derived MSCs (Lonza Walkersville Inc., Walkdersville, MD, USA) were cultured in low-glucose DMEM (Invitrogen, Paisley, UK) with 10% foetal calf serum (FCS; StemCell Technologies, Vancouver, Canada). For adipogenic differentiation, 20×10^3 per cm^2 passage four to five cells were plated to six-well plates containing cover slips. Before starting the adipogenic differentiation cells were grown to full confluence. Cells were grown for various times from 0 to 28 days in adipogenic medium consisting of basal medium, supplemented with 10 μg/ml insulin, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine and 60 μM indomethacin. The medium was changed every 3–4 days.

Oil Red O staining

Cells at day 0, 7, 14 and 28 were fixed on cover slips in 4% paraformaldehyde at room temperature for 20 min., washed in 10 mM phosphate buffered, 150 mM saline (PBS, pH 7.4) and distilled water. Oil Red O staining solution was prepared fresh using 1 mg/ml stock solution in isopropanol by diluting it in H_2O in 3:2. After 10 min. of staining cells were washed in H_2O and nuclei were counterstained for 5 min. in 5 μg/ml 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA).

Quantitative real time-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cells of three different patients at day 0, 1, 3, 7, 14 and 28 of adipogenic differentiation using TRIzol reagent (Invitrogen) and mRNA using magnetic Oligo$(dT)_{25}$ polystyrene beads (Dynal, Oslo,

Table 1 Primer sequences used in quantitative RT-PCR and corresponding amplicon lengths

Gene	Forward primer	Reverse primer	Length (bp)
COL4A1	TGGTGACAAAGGACAAGCAG	TAAGCCGTCAACACCTTTGG	268
COL4A2	TGGGATGGATGGTTTCCAAG	CCCTTGACTCCTTTGATGTG	336
COL4A3	CTTGTCGGTGTACCAGGATG	GGGTCCTGTTAACCCTTTCA	357
COL4A4	CCTTTGGAGATGATGGGCTA	CATCCCGGGAGTTCCTTTAT	317
COL4A5	TCGTCGCTTTAGTACCATGC	ACATTCGATGAAGGGAGCTG	367
COL4A6	TCCTGGACAAACACCAACTG	TGGAGCTCCTTCAAATCCTG	250
PPARγ	GTGAAGGATGCAAGGGTTTC	TCAGCGGGAAGGACTTTATG	303
LPL	CCGGTTTATCAACTGGATGG	AATCACGCGGATAGCTTCTC	349
β-Actin	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG	295

Norway). Messenger RNA concentrations were measured spectrophotometrically and complementary DNA (cDNA) was synthesized from 50 ng mRNA using oligo(dT)$_{12–18}$ primers and SuperScript enzyme, followed by RNase H treatment (SuperScript Preamplification System; Invitrogen).

Quantitative RT-PCR was run in a LightCycler PCR machine using LightCycler FastStart DNA Master SYBR Green I kit (Roche, Mannheim, Germany), twice with each sample. Primers were designed with Primer3 (SourceForge), the sequences were searched using the NCBI Entrez search system and sequence similarity search was performed with the NCBI Blastn program. Primer sequences used for adipocyte differentiation markers and collagen type IV α chains are shown in Table 1. For the qRT-PCR standard curve, the gene of interest was amplified using PCR, extracted from an agarose gel and cloned into the pCRII-TOPO vector (Invitrogen). After identification of the plasmid by restriction enzyme analysis and sequencing, the concentrations were determined spectrophotometrically and serial dilutions were prepared for qRT-PCR analysis. The copy numbers of mRNA were determined in duplicates and normalized against β-actin gene. The data were presented as mean ± S.E.M. and single factor ANOVA and *t*-test were used to establish statistically important differencies.

Immunofluoresence microscopy

Cells on cover slips were fixed in 4% paraformaldehyde at room temperature for 20 min. or in methanol for 5 min. at −20°C and preserved in PBS-azide at +4°C. Cover slips for staining of collagen type IV α5 chain were pre-treated in 6M urea, 0.1M glycine solution for 1 hr. Cover slips from all relevant time-points were washed in PBS, pH 7.4, containing Triton X, 3 × 10 min., followed by incubations in (1) 10% pertinent normal donkey (Jackson ImmunoResearch, West Grove, PA, USA) or goat (Vector Laboratories) serum in 0.1% bovine serum albumin (Dako, Glostrup, Denmark) in PBS for 60 min.; (2) 1:100 diluted polyclonal rabbit anti-human collagen IV serum (Euro-Diagnostica AB, Malmö, Sweden), 1:100 diluted monoclonal mouse anti-human type IV collagen α1 and α2 chain IgG1/κ (clone M3F7; Developmental Studies Hybridoma Bank, University of Iowa, IA, USA), 1:200 diluted polyclonal rabbit anti-human type IV collagen α5 chain IgG (gift from Professor Jeffrey Miner; Ref. 21), 5 μg/ml polyclonal rabbit anti-human laminin IgG (Abcam, Cambridge, UK), 11 μg/ml monoclonal rat anti-human nidogen IgG2a (Chemicon International Inc., Temecula, CA, USA), 4 μg/ml affinity purified goat anti-human MMP-2 IgG, 4 μg/ml polyclonal affinity purified goat anti-human MMP-9 IgG (R&D Systems, Minneapolis, MN, USA), 20 μg/ml rabbit anti-human MT1-MMP Ig (Chemicon International Inc.) or 10 μg/ml rabbit anti-human TIMP-2 IgG (Neomarkers, Freemont, CA, USA) for 60 min.; (3) 10 μg/ml Alexa Fluor 488- or 568-labelled donkey anti-goat, goat antimouse, goat anti-rat or goat anti-rabbit IgG as required (Invitrogen) for 60 min.

Nuclei were stained for 5 min. in 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Corresponding non-immune goat IgG (Jackson ImmunoResearch, West Grove, PA), mouse IgG1/κ (Abcam) or rabbit IgG (R&D Systems) were used at the same concentration as and instead of the primary antibodies as negative staining controls. To avoid fat droplets smearing, cover slips were not mounted, but kept in PBS and lifted on glass objective slide only for a short period for microscopy using motorized revolving AX 70 system microscope (Olympus, Tokyo, Japan) coupled with 12 bits digital image camera (Sensicam, PCO imaging, Kelheim, Germany).

Confocal microscopy

Cells on cover slips were prepared for confocal microscopy as above for immunofluorescence microscopy. Before incubations with antibodies, the cover slips were put into acetone for 30 sec. to dissolve the fat droplets. Diluted polyclonal rabbit anti-human collagen IV serum, diluted 1:100, was used as the primary antibody (Euro-Diagnostica AB) in indirect immunofluorescence staining. Cover slips were mounted in SlowFade Gold (Invitrogen, Carlsbad, CA, USA). Confocal microscopy was carried out using a Leica TCS SP2 AOBS system (Leica Microsystems AG, Wetzlar, Germany) with argon 488 nm excitation line and HCX PL APO CS 40×/1.25 NA objective. Image stacks were acquired through the specimens using a standardized 120 nm z-sampling density. Selected stacks were further subjected to deconvolution and restoration using theoretical point spread function and iterative maximum likelihood estimation algorithm of Huygens Professional software (Scientific Volume Imaging BV, Hilversum, The Netherlands).

Zymography

Cell culture media samples collected at day 0, 7, 14, 21 and 28 of adipogenic differentiation were analysed for gelatinolytic activity using a gelatin-containing sodium dodecyl sulfate polyacrylamide gel under non-reducing conditions. Gelatinase zymography standards containing 92 kD

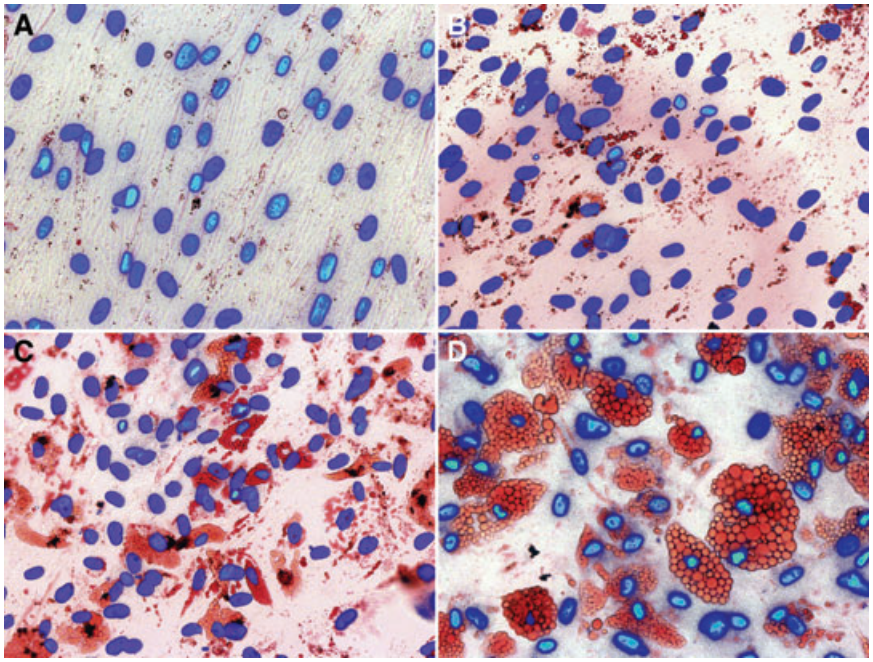

Fig. 1 Overlay figures of Oil Red O staining and 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstain of MSCs undergoing adipogenic differentiation: day 0 (**A**), day 7 (**B**), day 14 (**C**) and day 28 (**D**).

pro-MMP-9, 82 kD MMP-9, 72 kD pro-MMP-2 and 62 kD MMP-2 (Chemicon/Millipore, Billerica, MA, USA) were used. Twenty microlitres aliquots of cell culture medium were diluted with 80 μl SDS gel sample loading buffer (New England Biolabs, Ipswich, MA, USA) and incubated for 2 hrs at +22°C. Fifteen microlitres was loaded per well and run with 2 μl of 1:100 diluted Gelatinase zymography standard and low range pre-stained molecular weight markers (Bio-Rad, Hercules, CA, USA) on a 10% polyacrylamide gel (Bio-Rad) containing 1 mg/ml gelatine as substrate at 200 V for 1 hr. Gels were carefully removed from the plates and washed 3 × 10 min. in 0.05M Tris-HCl, 0.02% (w/v) NaN_3, pH 7.5 and 2.5% Tween 80, then 3 × 10 min. in the same buffer supplemented with 1 μM $ZnCl_2$ and 5 mM $CaCl_2$ at +22°C and finally incubated in this buffer for 48 hrs at +37°C. Gels were stained in 1.2 mM Coomassie brilliant blue (Serva, Heidelberg, Germany) for 1 hr and de-stained using several washes in 10% acetic acid and 20% methanol in distilled water. The gelatinase bands were visible as light bands against a dark blue background.

Results

Adipogenesis

Adipogenic differentiation of MSCs was confirmed using Oil Red O staining (Fig. 1). Intracellular fat droplets were first seen at day 7 and their number and size had increased progressively at days 14 and 28 so that they practically filled the cellular cytoplasm of the newly developed adipocytes.

Quantitative RT-PCR showed increases in peroxisome proliferator-activated receptor gamma (PPARγ) and lipoprotein lipase (LPL) gene expression at day 14 when compared to day 0. PPARγ mRNA levels increased 9.5 ± 5.0-fold and LPL levels 813 ± 458-fold (mean ± S.E.M.) further confirming adipogenic differentiation.

Collagen IV

Collagen type IV expression was first studied using immunofluorescence staining of samples collected at culture day 0, 7, 14, 21 and 28. Collagen type IV had appeared at day 7, but its extent, intensity and pericellular organization increased towards the end of the observation period (Fig. 2A-E) by which time collagen type IV containing cellular basement membranes surrounded fat-filled adipocytes as disclosed by comparison of type IV collagen staining with micrographs taken from the same field but using bright light (Fig. 2E and F). Negative control staining confirmed the specificity of the staining (Fig. 2G).

Human collagen type IV may consist of six genetically different α chains, which were all separately studied using qRT-PCR at the mRNA level during adipogenic differentiation at day 0, 1, 3, 7, 14 and 28. These kinetic studies disclosed that mainly α1 and α2 mRNA levels increased over time, starting from day 3 and continuing over the whole observation period (Fig. 3). At day 0 and 28, the absolute mRNA copy numbers per 1000 β-actin mRNA copies were 0.45 and 8.33 (18.6-fold increase) for α1 chain, and 0.99 and 12.1 (12.2-fold increase) for α2 chain. Other collagen type IV α chain mRNA levels did practically not change much over time. To check, if the corresponding collagen type IV α1 and α2 chains were also expressed at protein levels,

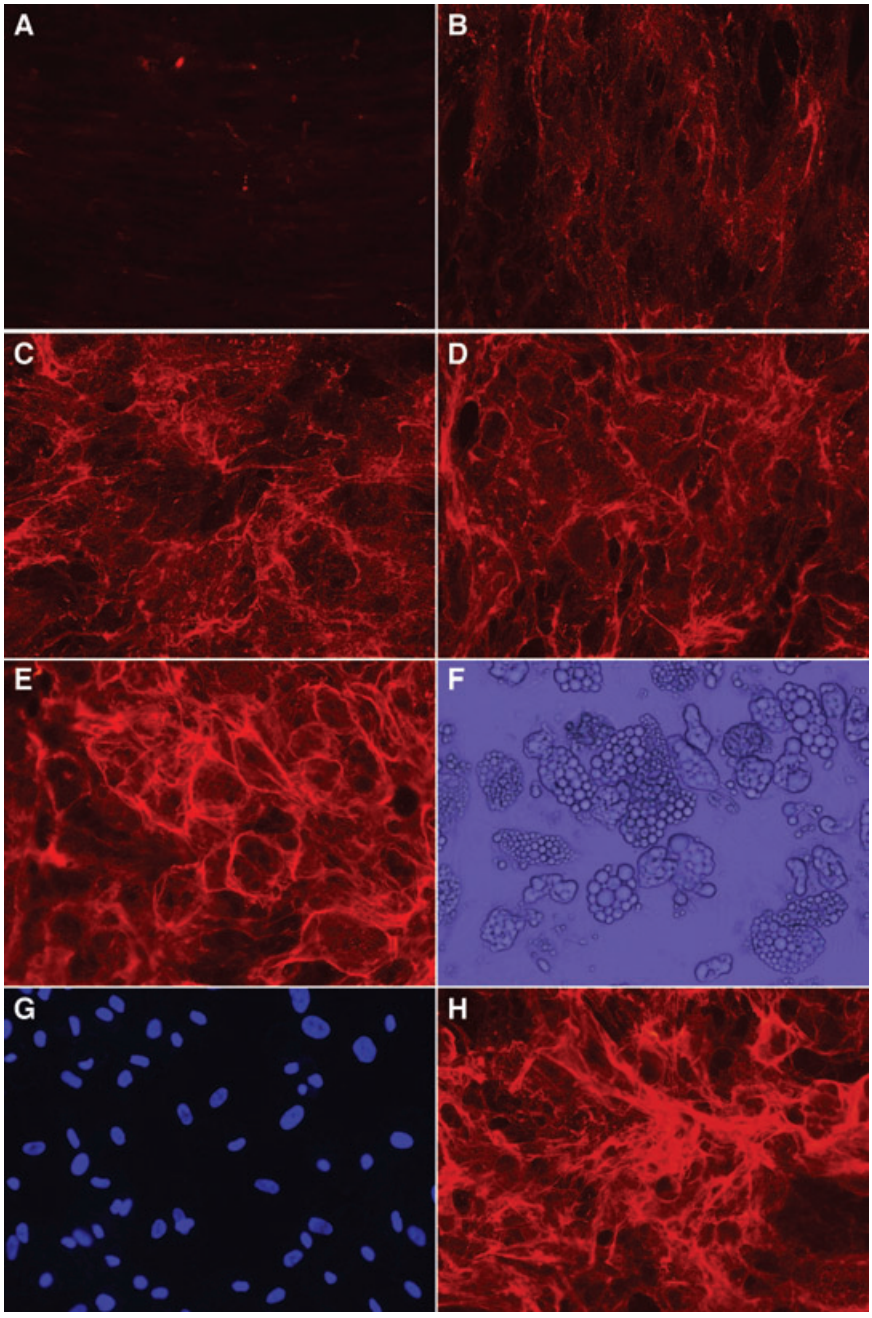

Fig. 2 General collagen IV staining at day 0 (**A**), day 7 (**B**), day 14 (**C**), day 21 (**D**) and day 28 (**E**) of adipogenic differentiation. Same field of view as in (**E**) is viewed with regular bright light showing the location of fat droplets containing cells (**F**). Overlay figure of negative control staining and 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstain (in blue colour) of day 28 cells (**G**). Day 28 cells stained with collagen IV α1/α2 chain specific antibody (**H**).

immunofluorescence staining was performed with collagen type IV α1/α2 chain specific antibody, which disclosed their presence in the adipocyte basement membranes (Fig. 2H). Immunofluorescence staining using collagen type IV α5 chain specific antibody was negative (Fig. 4B).

To avoid blurred visualization in immunofluorescently labelled collagen type IV, type IV collagen staining was also visualized in thin cross-sections produced using optical sectioning of stained specimens in confocal laser scanning microscopy. This clearly disclosed a thin basement membrane-like deposition of type IV collagen around the adipocyte (Fig. 4A).

Basement membrane laminins (Fig. 4C) and nidogen (also known as entactin; Fig. 4D) were also localized to adipocyte surface suggesting that all key components of the

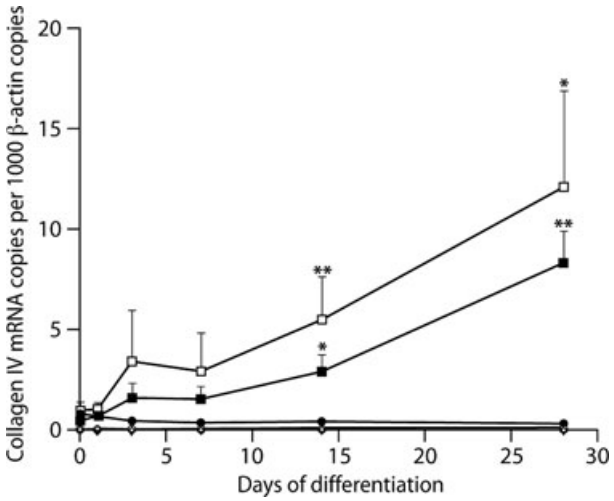

Fig. 3 Quantitative RT-PCR analysis of human mesenchymal stem cells during adipogenic differentiation. Messenger RNA copy numbers normalized to 1000 β-actin mRNA copies coding for type IV collagen α1 (filled squares), α2 (empty squares), α3 (filled triangles), α4 (empty triangles), α5 (filled diamonds) and α6 (empty diamonds). Values are the mean and S.E.M. ($n = 3$; for clarity, S.E.M. is shown only for type IV collagen α1 and α2). Statistically, important differences in expression levels in comparison to day 0 levels are marked with $*P < 0.05$ and $**P < 0.01$.

basement membrane are deposited around the developing adipocyte. Negative control staining confirmed the specificity of the staining (not shown).

Metalloproteinases

Metalloproteinases able to degrade basement membrane type IV collagen and some related molecules were first studied using immunofluorescence staining. Metalloproteinase-2 was constitutively expressed at both day 0 and day 28 and did not seem to increase nor accumulate in the forming adipocytes in immunocytological staining (Fig. 5A and B). In contrast, MMP-9 was not observed in MSCs at day 0, but was clearly seen in adipocytes at day 28 (Fig. 5C and D). Tissue inhibitor of metalloproteinases TIMP-2 was similarly induced upon adipogenesis (Fig. 5E and F). Membrane type 1-MMP (MT1-MMP or MMP-14) was not seen in MSCs, but stained faintly in adipocytes at day 28 (Fig. 5G and H). Negative staining controls using normal goat (Fig. 5I) or rabbit (Fig. 5J) IgG confirmed the specificity of the staining.

To supplement staining of cell associated MMP-2 and MMP-9, zymography was used to study gelatinolytic enzymes released into the cell culture supernatants. In accordance with the above-mentioned constitutive MMP-2 staining, bands corresponding to the 72 kD pro-MMP-2 and 62 kD MMP-2 were seen at day 0, 7, 14, 21 and 28, with a slight and somewhat variable increase over time of the active 62 kD band (Fig. 6, lanes 2–6). In spite of induction of cell-associated MMP-9 seen in immunostaining during adipogenesis and its clear cut presence in adipocytes at day 28, the 92 kD pro-MMP-9 band and in particular the 82 kD MMP-9 band were very faint or absent in the cell culture supernatants and the increase over time of the 92 kD pro-MMP-9 band in the supernatant samples was minimal (Fig. 6, lanes 2–6).

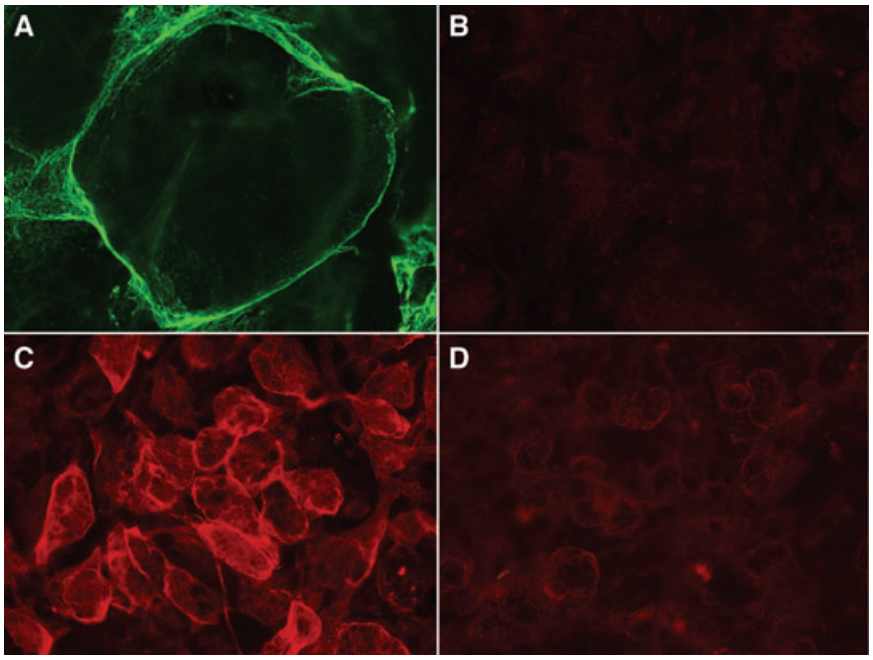

Fig. 4 Immunofluorescence staining of general type IV collagen at day 28 visualized in confocal laser scanning microscopy demonstrates the sharp outline of type IV collagen network around the developing adipocyte (**A**). Type IV collagen α5 chain is not detected in forming adipocytes (**B**), while other basement membrane key components laminin (**C**) and nidogen (entactin; **D**) were expressed pericellularly around adipocytes.

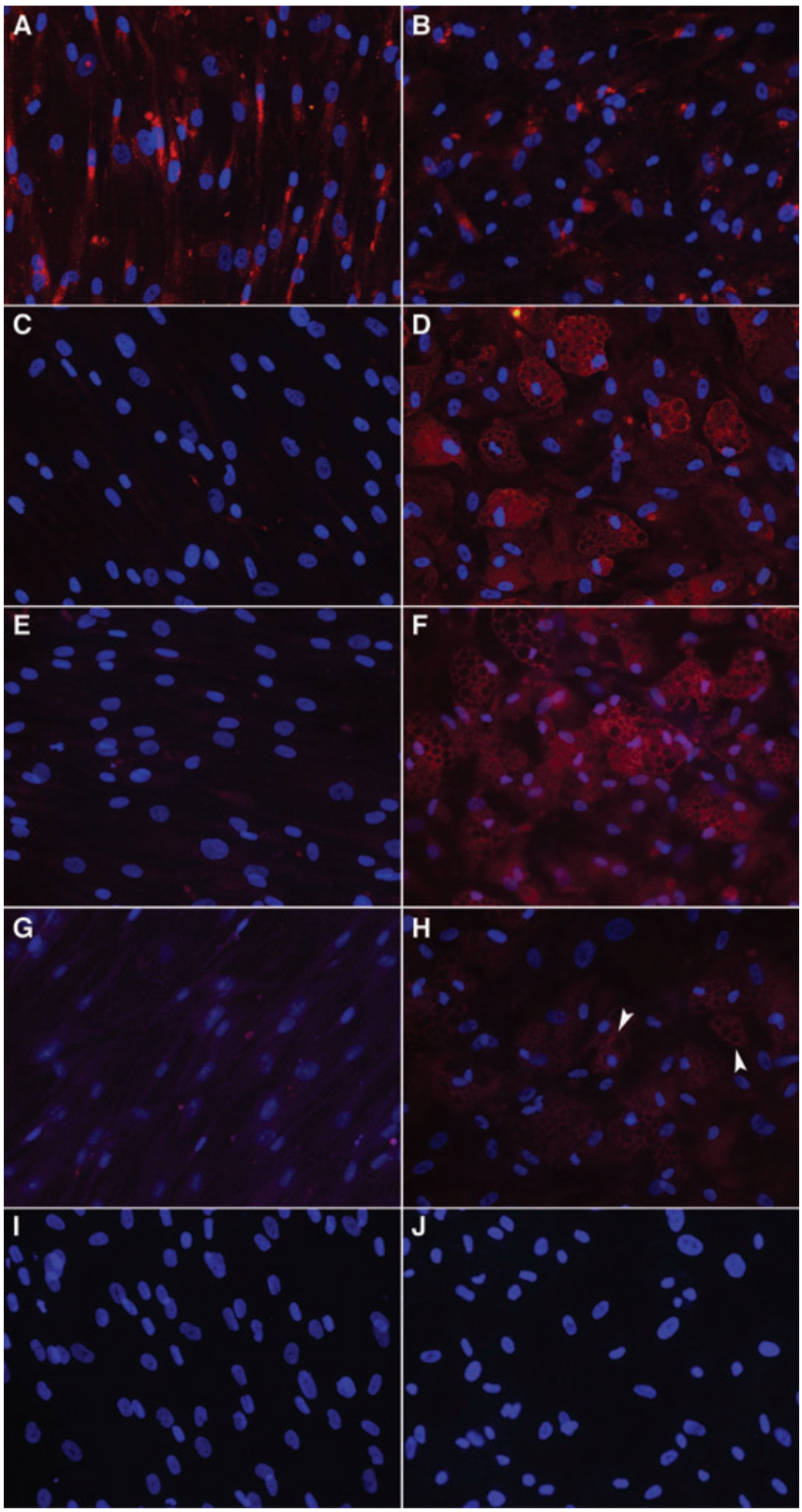

Fig. 5 Overlay figures of immunofluorescence staining of MMP-2 (**A**, **B**), MMP-9 (**C**, **D**), TIMP-2 (**E**, **F**) or MT1-MMP (**G**, **H**) (red colour) and 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstain (in blue colour). (**A**, **C**, **E**, **G**) Undifferentiated mesenchymal stem cells; (**B**, **D**, **F**, **H**) cells after 28 days of adipogenic differentiation. Arrowheads on H show faintly positive staining of MT1-MMP in forming adipocytes. Negative control staining of day 28 differentiated cells with non-immune goat (**I**) and rabbit IgG (**J**).

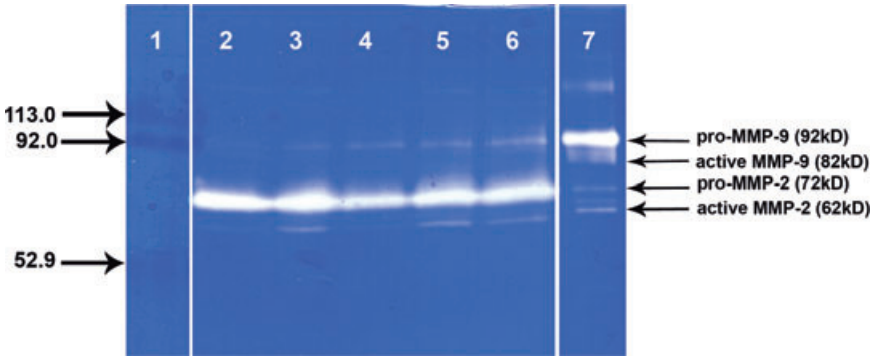

Fig. 6 Gelatin zymography of matrix metalloproteinase 9 (MMP-9) and MMP-2 in cell culture media samples of day 0 (lane 2), day 7 (lane 3), day 14 (lane 4), day 21 (lane 5) and day 28 (lane 6) of adipogenic differentiation. Gelatinase zymography standards are run on lane 7 and molecular weight markers on lane 1.

Discussion

Adipocytes can be produced from bone marrow–derived mesencymal stromal cells [22], although most committed adipocyte progenitors may actually reside as mural cells in the stromal-vascular compartment of the vasculature of the adipous tissue [23]. In this work, studying adipocyte basement membrane formation, we used differentiating bone marrow mesenchymal stromal cells as a useful human *in vitro* model for adipocyte and basement membrane formation. Upon adipogenic differentiation hMSCs pass determined and committed stages to finally terminally differentiate to mature adipocytes [24]. In this work conventional adipogenic stimuli were used to induce MSCs into the adipogenic lineage, the success of which was verified by Oil Red O staining and by demonstration of conventional adipocyte biomarkers peroxisome proliferator-activated receptor-γ and lipoprotein lipase. Analysis of the synthesis of various collagen type IV α chains disclosed three interesting results. Firstly, in the sensitive qRT-PCR hMSCs were shown to contain low collagen type IV α chain mRNA copy numbers, but staining of the corresponding translated protein was negative, when an antibody, which recognizes epitopes shared by all collagen type IV α chains, was used in immunofluorescence staining. This suggests that the collagen type IV α-chain mRNA molecules are not translated to the corresponding proteins in MSCs or that this occurs to such a low extent that the concentrations of the final protein products are below the detection limit of the immunofluorescence staining.

Secondly, day-28 adipocytes contained 18.6- and 12.2-fold increased concentrations of collagen type IV α1 and α2 chain mRNA copies and, accordingly, staining of these cells using a collagen type IV α1/α2 specific antibody confirmed that also the corresponding collagen type IV α chain proteins are present. Thirdly, in contrast, all the other collagen type IV α chains, α3–α6, continued to be expressed at low levels or declined even further during adipogenesis. These findings suggest that the forming collagen network of adipocyte basement membrane is composed of $\alpha1\alpha1\alpha2$ heterotrimers and not of $\alpha3\alpha4\alpha5$ or $\alpha5\alpha5\alpha6$ heterotrimers. Interestingly, although the known combinations suggest that the expected type IV collagen trimer is composed of two α1 and α2 chains and in spite of the fact that during adipogenesis α1 chain copy numbers increased more than the α2 chain copy numbers, the absolute mRNA copy numbers of type IV collagen α2 chains were actually slightly higher than those of the type IV collagen α1 chains.

Interestingly, mature human subcutaneous fat adipocytes have been stained, in addition to α1 and α2 chains, also quite weakly for α5 and α6 chains [14]. It is now described that during the MSC-adipocyte differentiation process the mRNA levels of α3 to α6 chains were significantly lower compared to α1 and α2 chains, and that the expression of α5 actually decreased during the process (2.6-fold by day 28). This was further proven at protein level by negative staining using collagen type IV α5 specific antibody, which basically excludes the presence of α5 chain-containing $\alpha3\alpha4\alpha5$ and $\alpha5\alpha5\alpha6$ type IV collagen trimers. These results show that forming/differentiating adipocytes do not need and do not produce at any stage of their early differentiation any type IV collagen chains than α1 and α2. This would suggest that it is possibly $\alpha1\alpha1\alpha2$ trimer that is needed and sufficient for developmental process *per se* and not the other possible combinations, although these may be present in mature fat containing and locally adapted tissues. Indeed, the actual reduction of α5 chain expression may not be accidental, but being the key component of some of the other possible trimers, it may be the way how the forming adipocyte further regulates its surroundings by excluding the possibility of the formation of other trimers. Our model is a pure adipogenesis and basement membrane formation model and is not affected by interactions with other cells or by stimuli occurring in living organism. It may be that tissue and location specific post-differentiation signals (*e.g.* mechanical stimuli, *etc.*) induce adipocytes to express also low levels of α5 and α6 chains. This would be compatible with the observations suggesting that fat cells and their extracellular matrix are affected by their local tissue environment, such as mechanical stimuli [25].

Apart from the studies of the various type IV collagen α chains in developing adipocyte basement membrane, kinetic studies disclosed that the mRNA copy numbers started to increase at culture day 3 simultaneously with other basement membrane components (nidogen and laminins, data not shown) and then continued to do so over the whole study period in an almost linear fashion to show statistically important differences by day 14 and 28.

Immunofluorescence staining disclosed collagen type IV already at day 7, but collagen type IV staining was then still quite weak and patchy and it did not yet seem to fully surround individual fat cell bodies in generation. First at day 14, when cytoplasmic fat droplet accumulations were clearly already seen in Oil red O staining, did the adipocyte basement membrane seem to uniformly surround the adipocyte. During later phases of differentiation, immunofluorescence became stronger and more distinct suggesting consolidation of the pericellular basement membrane. This type of collagen IV network occurred in association with pericellular laminin network and nidogen (entactin), which is supposed to link these two basement membrane networks to each other. This suggests that all the molecular key components of a fully established basement membrane are produced by the differentiating cells. This timing of the basement membrane formation seems logical, because its appearance thus coincided with the appearance and accumulation of Oil Red O-positive intracellular fat droplets suggesting that the synthesis and organization of the pericellular collagen type IV basement membrane scaffold occurs first when the need for structural support becomes imminent. However, adipocyte basement membrane participates also in outside-in signalling, which can guide adipogenesis, in part *via* denaturation.

It was hypothesized that during MSC-to-adipocyte differentiation, the formation of pericellular adipocyte basement membrane does not only require regulated synthesis of its constituent collagen monomers, but also remodelling of the restrictive and probably unelastic pericellular basement membrane. Because type IV collagenases, constitutively expressed MMP-2 (72 kD type IV collagenase, gelatinase A) and inducible or regulated MMP-9 (92 kD type collagenase, gelatinase B) [26–29], are supposed to play a major role here, their eventual presence in MSCs and induction during adipogenesis were studied. MMP-2 was produced already by MSCs and located mostly in the perinuclear area, perhaps in the Golgi complex, while MMP-9 seemed to be absent. Although in this work overconfluent cultures were used for adipogenic differentiation, this MMP-2 and MMP-9 expression pattern in undifferentiated hMSCs is in line with earlier published results, where slightly different culture conditions were used [30]. A somewhat similar staining pattern of MMP-2 was seen in cells cultured in the adipogenic medium for 28 days. Because these cells, cultured for 28 days, contain a mixture of MSCs and already quite well differentiated adipocytes, it is possible that most of the MMP-2 positive cells in such cultures represent non-differentiating MSCs. In contrast, according to immunocytological staining MMP-9 was clearly induced in differentiating adipocytes, although it did not much increase in cell culture supernatant. The most plausible explanation for this apparent discrepancy is that the induced MMP-9 is produced to satisfy cellular needs for basement membrane remodelling and is, therefore, engaged in enzyme–substrate interactions at the intimate cell surface–basement membrane interface. Indeed, it has been described that type IV collagenases can be focalized to the pericellular area *via* MT-MMP and TIMP-2 in ternary complexes, where TIMP-2 forms a bridge between these two MMPs [31–33]. Inhibitory TIMP usually blocks both the active site and the C-terminal domain of its MMP target. In contrast, when it forms a bridge between MT-MMP and another MMP, it blocks the active site of the MT-MMP (which is inhibited) and binds to the C-terminal domain of the other MMP, so that its catalytic site remains in a fully active state. Indeed, active MMP-9 has recently been reported to bind to cell surface by an as yet unresolved mechanism, which contributes significantly to its proteolytic activity against type IV collagen. In contrast to soluble MMP-9, such membrane-bound MMP-9 was 21- and 68-fold more resistant to inhibition by TIMP-1 and TIMP-2 [34]. This is totally in concert with the current findings that adipogenesis, in addition to MMP-9, also induced MT1-MMP and TIMP-2 and although MT1-MMP itself cannot degrade basement membrane collagen type IV [35], it may act as a critical localizing component in the cell surface-bound ternary proteolytic complex.

Interestingly, very recent experiments have shown that it is the de-naturated, not the native, collagen type IV network that promotes the adipogenic differentiation capabilities of human MSCs [13]. Native collagen type IV displays two adipogenic $\alpha_2\beta_1$ integrin binding sites and only one $\alpha_v\beta_3$ integrin binding site located in the non-collagenous domain of the monomer; in contrast, in its denatured state the same monomer displays 15 adipogenic $\alpha_v\beta_3$ integrin binding sites [13].

Therefore, the role of MMPs in differentiating adipocytes may not only be related to mechanical/structural physical remodelling of the basement membrane, but also to their ability to convert native collagen locally to denatured collagen and, thus, to change the regulatory properties of collagen type IV network in cellular differentiation processes. Indeed, native collagen IV matrix is unsupportive of adipogenic differentiation and very little adipogenesis occurs on such matrix even in the presence of adipogenic stimuli [13]. From the point of view of treating obesity, it is interesting that collagenases can be inhibited, even *in vivo*, with, for example doxycyclines (a non–anti-microbial effect) and bisphosphonates [36–39]. Because basement membrane type IV collagen is one of the most prominent substrates of MMP-2 and MMP-9, MMP-2 is often called '72 kD type IV collagenase' and MMP-9 '92 kD type IV collagenase'. However, they have also many other substrates [16]. Therefore, they degrade collagen type IV but they can participate in the degradation/remodelling of some other components of extracellular matrix as well. In conclusion, these results suggest that upon MSC differentiation and adipocyte formation cells synthesize type IV collagen α1 and α2 chain containing basement membrane and develop enzymatic capability to remodel it using MMP-2 (72 kD type IV collagenase) and MMP-9 (92 kD type IV collagenase) and a potential MMP-2/9 regulating capacity *via* production of MMP-2/9-focalizing MT1-MMP and regulatory TIMP-2.

In practical terms, the results of this study are of interest also from adipose tissue engineering and regenerative medicine point of view. Although, many studies have been conducted using different type IV collagen containing matrices as scaffolds to support adipogenesis, the results have been varying and the optimal scaffold composition and properties remain so far unclear [40–43]. Knowing the exact profile of type IV collagen α chains produced by differentiating adipocytes themselves, enables possibly the optimization of the search for an ideal scaffold and provides an endogenous reference for compositional evaluation

and comparison of matrices obtained from different sources and by different purification methods.

When intracellular fat starts to accumulate, production of a dynamically supporting and instructive, possibly partly denatured and adipogenic pericellular type IV collagen scaffold becomes necessary. In our work, we studied the initial stages of adipocyte and basement membrane formation in cell cultures. The extent and properties of final extracellular matrix in endogenous 3D fat tissue is known, however, to vary in different locations. Sbarbati *et al.* described three different types of white adipose tissues (WAT), namely deposit WAT in abdominal area, structural WAT in limbs and hips and fibrous WAT tissue in areas subjected to severe mechanical stress. These tissues differ as to their fat cell content, collagen structure, vascularity, and possibly also in the number of the progenitor niches, which may affect their suitability in various regenerative procedures [25]. Such fat tissue types probably reflect site-specific modulation of the basic adipogenic process by different tissue microenvironments.

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Conflict of interest

The authors confirm that there are no conflict of interest.

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