

Mesenchymal stem cells remodeling of adsorbed type I collagen – effect of collagen oxidation

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Abstract: The effect of collagen type 1 (Col I) oxidation on Adipose Tissue Derived Mesenchymal Stem Cells (ADMSCs) remodeling is described as a model for acute oxidative stress. Morphologically, remodelling was presented by a mechanical rearrangement of adsorbed FITC-Col I and a trend for its organization in fibril-like pattern – a process strongly abrogated in oxidized samples, but without visible changes in cell morphology. The cellular proteolytic activity was quantified in multiple samples utilizing fluorescence de-quenching (FRET effect). In the presence of ADMSCs a significant increase of native FITC-Col I fluorescence was observed, almost absent in the oxidized samples. Parallel studies in cell-free system confirmed the enzymatic de-quenching of native FITC-Col I by Clostridial collagenase, again showing significant inhibition in oxidized samples. The structural changes in the oxidized Col I was further studied by Differential Scanning Calorimetry: an additional endotherm at 33.6°C along with the typical for native Col I at 40.5°C with sustained enthalpy (ΔH) was observed in oxidized samples. Collectively, it has been evidenced that remodeling of Col I by ADMSCs is altered upon oxidation due to the intrinsic changes in the protein structure, thus presenting a novel mechanism for the control of stem cells behaviour toward collagen.

Keywords: Keywords: Mesenchymal stem cells, collagen type I, remodeling, oxidation.

1. Introduction

Recent studies show that mesenchymal stem cells (MSCs) are strongly involved in the process of extracellular matrix (ECM) remodeling apart from their principal role in the various regenerative routes [1-2]. MSCs, often referred to as adult stem cells reside in most tissues assuring their repair upon damage and ageing [3], thus attracting great interest for most cell-based therapies [4]. Particularly Adipose Tissue Derived MSCs (ADMSCs) draw notable

attention combining relatively easy availability, multi-potency, and immunomodulatory activity [5]. ECM remodeling, comprising a tightly controlled balance between matrix protein formation and degradation, is a highly dynamic process constantly involved in the tissue reconstruction during development, cellular growth and differentiation, strongly affecting the whole mechanism of homeostasis [6-8]. Abnormal ECM remodelling lead to connective tissue disorders such as fibrosis, cancer[9], and over 200 genetic and autoimmune complaints [9,10]. Particularly, collagen remodeling has been extensively studied, shown to be cell-driven process that is critical during development, wound healing and regeneration, but also involved in various pathological conditions, such as inflammation, scar formation, aging, and tumor progression [11]. Besides the extensive studies on cellular collagen type I (Col I) processing by cells in 3D gel environment [12,13], substantially less attention was drawn to the investigation of the planar (collagen-coated) substrata, e.g., in 2D system. Nevertheless, these studies revealed that remodeling of substratum associated collagen is presented by three distinct morphological events: (1) mechanical reorganization; (2) extracellular fibrils deposition, and (3) proteolytic degradation of adsorbed collagen [2,14,15].

Collagen has been widely used as a naturally occurring biomaterial in tissue engineering and regenerative medicine [16], as a substrate of excellent biocompatibility, negligible immunogenicity, biodegradability and specific interaction with growth factors and cell adhesion molecules. As of today, over 29 types of collagen have been identified and classified into several groups according to the structures they form [17]. As a major protein component of the ECM, collagens contain a triple-helical domain with a unique periodical structure $(\text{Gly-X-Y})_n$ X and Y being repeatedly proline and 4-hydroxyproline, respectively [17]. Fibrillar type I collagen (Col I) is the most abundant type of collagen, comprising roughly 80-90% of the total collagen mass in the human body. It provides most tissues and organs with shape, firmness, maturity, integrity and connectedness [16]. In addition, Col I is a ligand for specific cell receptors like integrins, discoidin domain receptors, glycoprotein VI, and the mannose receptor family, controlling various important cellular activities, including extracellular matrix (ECM) formation and turnover [18]. The biosynthesis of collagen is a complicated process involving the number of post-translational modifications (PTMs), chain

association, folding, secretion, self-assembly, and progressive cross-linking [19]. The major PTMs depends on the oxidation of lysine and proline residues, being critical factors for the structural and biomechanical functions of Col I fibrils, strictly regulated by several sequential processes inside and outside the cell. The oxidation of lysine and proline may occur upon distinct environmental changes [19], for example as a part of the physiological collagen processing like fibrillogenesis and osteocalcification. It might be also a component of the oxidative stress [20,21], that is dependent on the production of reactive oxygen species (ROS) including free radicals and peroxides [20]. The oxidation of collagen (also other ECM proteins) caused by ROS further modulates the qualities of the ECM by altering its production, turnover, and modifications, finally, having strong impact on the cell–ECM interaction [22]. Oxidative stress is suggested to be important in numerous pathological conditions such as neurodegenerative diseases [23], various cardiovascular complications [24], atherosclerosis [21], fibrosis [25] and ageing [26].

Despite the extensive investigations on the role of oxidative stress in collagen genes expression and collagen turnover related to various chronic diseases, the studies *in vitro* utilizing direct cellular models are rather sparse [27-29]. Though it is clear native collagen undergoes intensive remodeling by cells, and particularly by fibroblasts, the specific role of MSCs was rather poorly investigated [16]. The effects of oxidative stress caused by ROS and accompanying regeneration of the injured tissues are also sparsely studied [30].

In this study, we aimed to learn more about the effect of Col I oxidation (as an *in vitro* model for the acute oxidative stress) on its remodeling by stem cells. For that purpose, we visualized morphologically the adsorbed Col I with the adhering ADMSCs and further developed a system for the quantification of observed cellular proteolysis using specially designed fluorescent probes.

Results

In this study we aimed to compare the biological response of ADMSCs adhering onto native and pre-oxidized Col I attempting to mimic the conditions of acute (short term)

oxidative stress that occur *in vivo*, assuming that it might have a significant effect on the stem cells behavior. For that purpose native Col I was labelled with FITC to easily follow its processing. For the preparation of oxidized FITC-Col I (FITC-Col I OXI) we used a previously described protocol [31]. The remodeling of adsorbed FITC-Col I was investigated employing two approaches, morphological and quantitative. For the morphological approach, ADMSCs were cultured on glass coverslips pre-coated with either native FITC-Col I or FITC-Col I OXI for 24 h. Then the samples were stained with Rhodamine-phalloidin and Hoechst to view simultaneously the substratum (green), the actin cytoskeleton (red) and the intact nuclei (blue), using the respective channels of the microscope. The second, quantitative approach was based on the de-quenching of FITC-Col I caused by the cellular proteolytic activity giving a proportional rise of the fluorescent signal (FRED effect) [32]. To confirm the de-quenching effect and for the comparison of native and oxidized Col I susceptibility to proteolysis, a separate experiment with collagenase from *Clostridium Histolyticum* (CH) were conducted.

2.1 Overall design of the experiments

A basic scheme of the study is presented on Figure 1. Substrates were coated with FITC-Col I or FITC-Col I-OXI (A). Cell adhesion was the next step (B), performed for 2 h in serum-free medium (to assure the attachment of ADMSCs to Col I only). Afterwards, 10% serum was added to each sample and cells were further cultured up to 24 h (C).

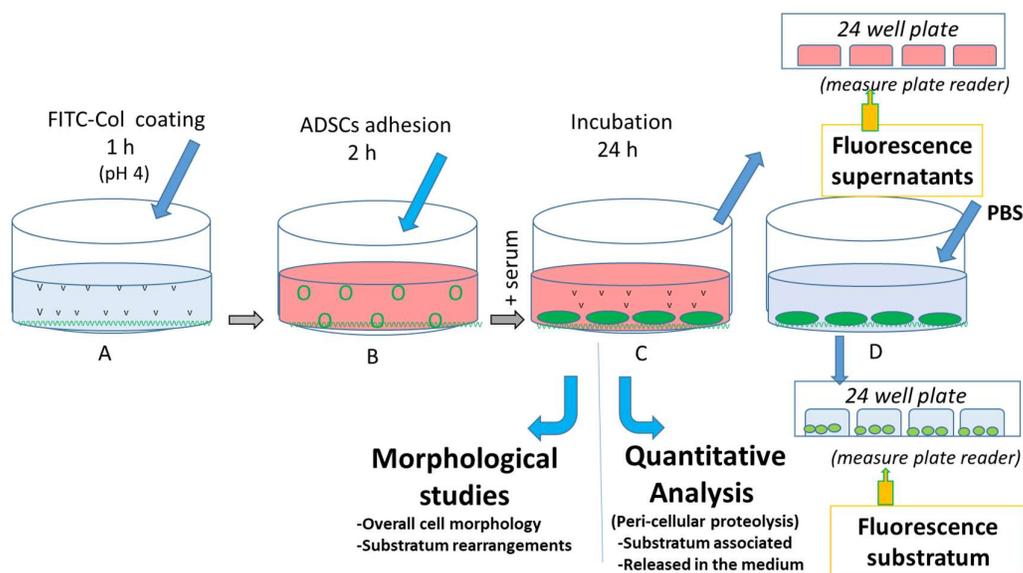


Figure 1. Overall design of the experiments of Col I remodeling by stem cells.

The subsequent steps were different for the morphological estimation and for the quantitative evaluations of ADMSCs proteolytic activity, as detailed below.

2.2 Morphology of ADMSCs adhering on native or oxidized FITC collagen

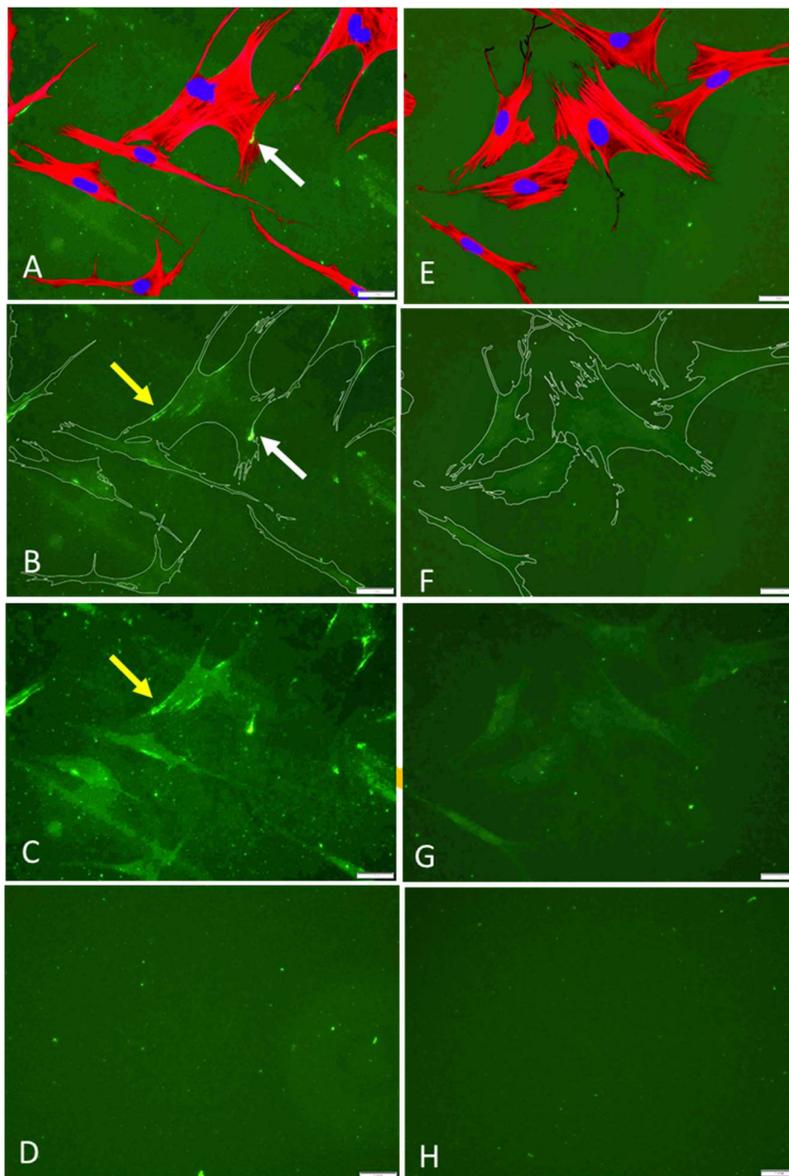


Figure 2. Morphology of ADMSCs adhering on native (A-C) and oxidized (E-G) FITC-Col I viewed together with the underlining substratum (green). A and E present the overall morphology of cells adhering on native and oxidized collagen, respectively. B and D present the underlining substrates only of the same samples with artificially superimposed cell

contours, while on C and G cell shapes are omitted. D and H show the plane substrates of both native and oxidized collagen, respectively, where no cell were added. The yellow arrows point the typical fibrillary collagen arrangements beneath the cells. The white arrows show the arrangements outside the cells along the cell's periphery. Bar 20 μm .

Figure 2 presents typical images of native (left row) and oxidized (right row) FITC-Col I substrata to which ADMSCs have adhered. The cells spread equally well on both substrata showing similar polarized morphology and spreading (compare 2A and E). However, as shown on the left panel (A - C) when adhering to regular Col I, ADMSCs tend to rearrange mechanically the underlining fluorescent layer forming typical fibrillary assemblies located mostly beneath the cells (yellow arrows) and sparsely at the cell's periphery outside the cells (white arrows). Note, on the oxidized samples such structures are missing (2E-G). For clarity on the lower panels (B, C) for the native and (F, G) for the oxidized samples are viewed the underlining substrates only, with artificially superimposed cell contours (B and F) or without them (C, G). Relatively homogenous accumulation of the labeled protein beneath the cells was observed on both native and oxidized samples. D and H represent the typical views of the plane substrata, native or oxidized respectively, where no cells were added. No significant difference in the morphology of the adsorbed proteins was found in this magnification. In the presence of cells, however, some discrete patterning of the adsorbed protein layer was observed, particularly stronger on native FITC Col I samples (C vs. G), suggesting a distinct proteolytic activity. On oxidized Col I samples only a homogenous accumulation of the protein beneath the cells was a typical find suggesting a rather missing proteolytic activity (C, D). Collectively these morphological data suggest that oxidized Col I substrates are barely remodelled by ADMSCs.

2.3 Quantitative measurement of Col I degradation

Col I was labeled with FITC according to Doyle ³³ with some modifications (see Methods section), basically directed to strictly adjusting the pH of samples to pH 9 assuring maximal FITC binding. In these conditions part of the fluorophore become day-quenched (possessing lowered fluorescence because of the FRET effect) [32] and putatively de-quench (increasing the fluorescence) upon proteolytic degradation of the carrying protein.

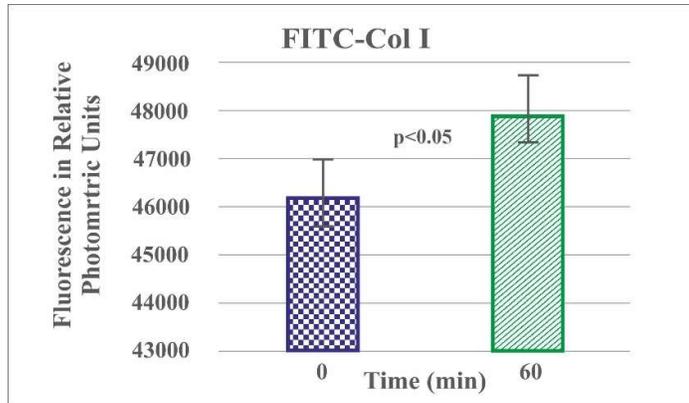


Figure 3. De-quenching of FITC-Col I in the presence of CH collagenase for 1h at 37°C.

Indeed, as we found in a preliminary study (Fig 3) our FITC-collagen was de-quenched, increased its fluorescence in the presence of collagenase CH, thus providing opportunity for the quantitative studies on collagen proteolysis. It has to be noted here that upon adsorption both FITC-Col I and FITC-Col I-OXI presented a sufficiently bright fluorescent background supporting the morphological investigations shown above.

On the other hand, in a parallel experiment aiming to compare the degradation profiles of native and oxidized FITC-collagen using the same collagenase CH degradation system, we found that while the native FITC-Col I significantly de-quenches upon proteolytic digestion for 60 and 120 min (compared to time 0 min) in collagenase CH solution (Figure 3). As shown on the next Figure 4, comparing Δ RPU for native (blue line) and oxidized samples (green line) at 60 and 120 min, this effect was much less pronounced for the oxidized FITC-Col I probe.

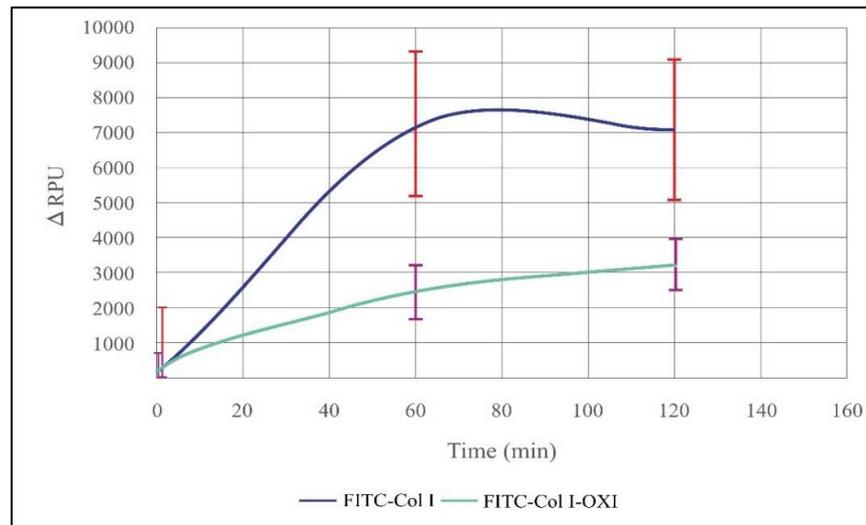


Figure 4. Relative changes in the fluorescence intensity presented as Δ RPU upon addition of FITC-Col (blue) or oxidized FITC-Col I-OXI (green) to a solution of Collagenase CH as substrates for proteolytic digestion at time 0, 60 and 120 min at 37°C. (B) Friedman test, presenting a statistically significant change of fluorescence in the native FITC-Col samples, but not in FITC-Col I OXI one.

Table 1. Chi-Square and significance of the relative changes in the fluorescence intensity (Δ RPU) upon collagenolytic action of Collagenase CH on FITC-Col and oxidized FITC-Col I-OXI calculated by Friedman Test Statistics.

Samples	FITC-Col I	FITC-Col I-OXI
Chi-Square	12.000	4.000
Sig. (p)	0.002	0.135

Applied Friedman test (Table 1.) to further confirm the significant increase of the fluorescence in the group of native FITC-Col I samples, but not in the oxidized ones, meaning that FITC-Col I-OXI is less susceptible to collagenases treatment. These data approve that our FITC-Col I preparations might be used for the quantification of ADMSCs proteolytic activity.

2.4 De-quenching of FITC-Col 1 by ADMSCs – effect of oxidation

According to the initial diagram (Fig 1), multiple samples (quadruplicates) of native FITC-Col I and oxidized FITC-Col I substrata (applied as coatings on the glass bottomed 24 well TC plates) were cultured for 24 h in the presence or absence of ADMSCs. The changes in the fluorescent signal measured for the substratum associated (adsorbed) and spontaneously

released FITC-Col I were further analysed. Indeed, given as rough data, the native FITC-Col I samples (-cells) show that distinct amounts of protein desorb spontaneously from the substratum after 24 h of incubation giving rise to a signal of about $(41858 \pm 3368 \text{ RPU})$. In the presence of cells (+ cells) the fluorescence increases to $44496 \pm 13685 \text{ RPU}$ (i.e. with about 7 %) basically confirming the de-quenching effect of ADMSCs. The same trend was observed for the substratum associated FITC-Col I: it increases from $11682 \pm 508 \text{ RPU}$ for controls (- cells) to $13253 \pm 5312 \text{ RPU}$ for the samples (+ cells), again confirming the proteolytic de-quenching of ADMSCs (in approximately 91%) but with values that are in the range of the scattering of signal obtained for the supernatants. The same situation was valid also for the oxidized Col I samples.

Figure 5A presents the ΔRPU for substratum associated FITC-Col I (measured from the bottom of the wells) and Panel B – those released in the medium. The left columns on both panels show the data for native FITC-Col I while the right columns – for the oxidized samples. A significant trend for increase of ΔRPU was observed for the substratum associated native FITC-Col ($p < 0.05$) vs. almost absent de-quenching for the oxidized samples. The same effect was detected for the released collagen (Figure 5B) showing again a significant de-quenching ($p < 0.05$) for the native FITC-Col I samples compared to a relatively small and nonsignificant increase ($p > 0.05$) in the oxidized ones.

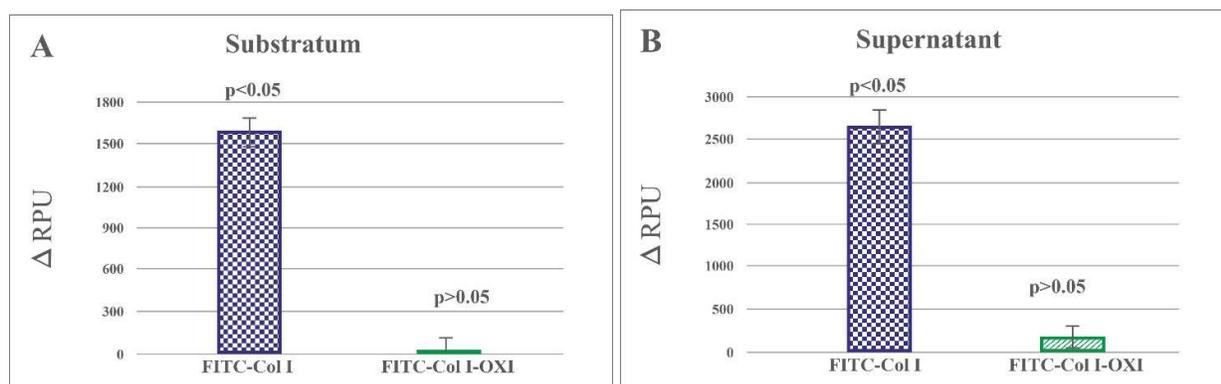


Figure 5. Relative changes in the fluorescence values of substratum associated (A) and spontaneously released (B) FITC-Col I after 24 h of incubation of samples with cells versus without cells. The data are presented as ΔRPU characterizing the

proteolytic de-quenching caused by the cells. The dramatic effect of oxidation is evident comparing the ΔRPU for native FITC-Col I (left columns blue) and oxidized FITC-Col I (right columns, green).

2.5 DSC analysis of FITC-Col 1 – effect of oxidation

To evaluate the level of structural changes in the oxidized Col I molecules we investigated the effects of oxidation on the thermal stability of FITC-Col I by DSC analysis. DSC measures the heat capacity of samples as a function of temperature and provides information about the thermal stability and putative structural changes in the molecule. DSC curves were used also to calculate the thermodynamic parameters, such as melting temperature (T_m), the total transition enthalpy (ΔH_{total}) and half-widths of transition ($\Delta T_{1/2}$).

As expected, the maximum of the heat absorption of native FITC-Col I was observed at 40.5°C (T_{M-main}) (Figure 6, Table 2). As a result of the oxidation, however, the thermogram results into splitting of two well-resolved transitions with melting temperatures at 33.6°C (T_{M-pre}) and 40.1°C (T_{M-main}), respectively (Table 2), which confirms our previous investigation on calf skin collagen Type I suggesting certain changes in collagen structure upon oxidation [31].

To further analyse this observation, we compared the enthalpy and the half width of transition of native and oxidized samples (Table 2). It should be noted that the value of total enthalpy (ΔH_{total}) after oxidation was very close to that of native collagen sample indicating only a discrete structural change in the collagen molecule upon oxidation. This was further confirmed by slightly higher half-width of transitions in oxidised sample as compared to native one.

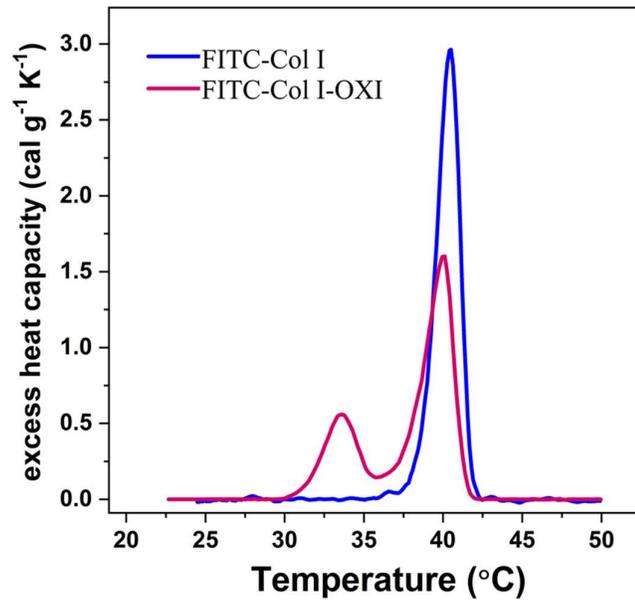


Figure 6. DSC thermograms of native FITC-Col I (blue line) and oxidized FITC-Col I-OXI (red line).

Table 2. Thermodynamic parameters: transition temperature (T_M), total calorimetric enthalpy (ΔH_{total}), transition half-widths ($\Delta T_{M-1/2}$) obtained from DSC profiles of FITC-Col I and FITC-Col I-OXI.

Collagen	T_{M-pre} (°C)	T_{M-main} (°C)	ΔH_{total} (cal/g)	$\Delta T_{M-pre-1/2}$ (°C)	$\Delta T_{M-main-1/2}$ (°C)
FITC-Col-I	-	40.5	5.58		1.72
FITC-Col I-OXI	33.6	40.1	5.50	2.58	2.09

* T_{M-main} – temperature of the main transition; T_{M-pre} – temperature of the additional pre-transition event; ΔH_{total} – total transition enthalpy; $\Delta T_{M-main-1/2}$ – half-width of the main transition; $\Delta T_{M-pre-1/2}$ – half-width of pre-transition.

3. Discussion

In vivo, cellular microenvironments are constructed of ECM proteins and proteoglycans where Col I play a major role, being under constant turnover to maintain tissue homeostasis [2, 9, 14-16]. In most forms of cellular activity, the cells tend to remodel their adjacent microenvironment via mechanical reorganization and proteolytic degradation [34, 35]. Here we anticipate that it reflects also the behavior of stem cells. Indeed, both morphological and

quantitative studies demonstrated that Col I undergo significant remodelling by stem cells. Morphologically it is presented by a mechanical translocation of the adsorbed protein and a tendency for its assembly in a fibril-like pattern. This phenomenon found to work for several cell types (endothelial cells, fibroblasts, hepatocytes, cancer cells, etc.) [14,35,36] is now demonstrated for ADMSCs. Likewise, other matrix proteins are also subjected to cellular remodelling, as shown for fibronectin, fibrinogen, vitronectin and type IV collagen, [14,15, 37-39] reflecting the constitutive property of cells to arrange their own ECM on the foreign material's interface [35,36]. Relatively little is known, however, on how stem cells behave in the acute oxidative environment known to affect the ECM turnover [40,41]. ROS have been long considered as pathological agents causing apoptosis under adverse conditions [23]. However, recent findings have challenged this dogma. Physiological levels of ROS are now considered as secondary messengers, mediating numerous cellular functions, including the behaviour of adult stem cells [42]. On the other hand, MSCs represent an important tool for tissue engineering, drug screening, and disease modeling [3,4,43], but their safe use, including clinical applications, still requires improvements to obtain functional stem cells with controlled behavior [43]. In this respect, the implication of native collagen matrices is a challenging approach as collagen is a natural ECM protein with easy tuneable properties [40,44]. However, *in vitro* stem cells easily lose their self-renewal and multi-lineage differentiation potential during cell doubling. The current strategies for maintaining MSCs stemness (self-renewal and differentiation) are largely focused on distinct ligand-receptor combinations, cell-cell adhesion (through N-cadherin) and adding soluble growth factors that stimulate differentiation [45-47]. However, the implication of additional measures that may support the maintenance of stem cells would increase the likelihood of success [48,49]. Our data unequivocally show that the oxidative environment alters the ADMSCs Col I remodelling, therefore we anticipate that one such measure is the modulation of the cell's oxidative environment. The potential of ROS for the regulation of physiological processes, for instance, chondrogenesis, is indicated by a chondrogenic cell line (ATDC5), which experienced an increase of ROS with time in culture [41,47]. Recent data suggest that the control of cellular redox status affecting their local microenvironment might be critical for MSCs survival,

expansion, and differentiation [41,42]. Nonetheless, ROS may lead to the generation of pathological environments triggering, for example, a perverse cycle of fibrosis involved in the pathogenesis of numerous diseases [42, 50]. Oxidative stress is generated when living cells are unable to neutralize excessive ROS, or are incapable to recycle the oxidized biomolecules. Particularly the (over) oxidation of proline (enzymatic and non-enzymatic) was shown to contribute to fibrotic processes in heart and amyloid formation [51-53].

Cell–substratum interaction is a complex process that is bi-directional and dynamic, mimicking to a certain extent the physiological interaction of cells with the ECM. Consequently, the adhering cells tend to rearrange adsorbed ECM components [15, 35-38]. To assure that cells attach exactly to collagen, as they may use also other adhesive proteins (like fibronectin, vitronectin, fibrinogen, etc.) [35,36-38], we used collagen pre-coating followed by 2 h cell adhesion in a serum-free medium (Figure 1) before the serum was added (step B). Thus, we avoided the competitive effect of other serum proteins. In addition, the collagen was fluorescently labelled that made easier its morphological visualization as detailed above and the subsequent quantifications of cellular proteolytic activity. Related to our experimental conditions ADMSCs visibly recognize the native and oxidized Col I equally well, as evidenced by the lack of difference in both adhesion and overall cell morphology (Figure 2A and E). However, a significant alteration in their ability to reorganize the oxidized FITC-Col I was observed (Figure 2 F and G), which is basically a novel observation presumably reflecting an intricate stem cell behaviour in an oxidizing environment. As the reorganization is known to require cellular proteolytic activity, [15,36,54] a similar effect might explain the restoring capacity of protease inhibitors on the extracellular collagen fibril deposition in human MSCs 2.

The ECM however undergoes also proteolytic remodelling, which is a mechanism for the removal of the excess ECM, a process often approximated with remodelling [15]. It has to be noted, however, that cell-dependent ECM remodeling includes likewise the process of ECM organization and fibrils formation, which is critical for their functionality and for the interaction with other cells [35,36]. It is generally agreed that oxidative stress *in vivo* is characterized by an impaired ratio between lowered collagen synthesis and accelerated degradation [54]. The direct data on the degradability of oxidized collagen, however, are

rather controversial. Chronic exposure to ROS causes an accumulation of damaged collagen and its fragmentation, which are more susceptible for proteolytic enzymes. Collagen cross-linking however, inhibits its degradation [55]. The disproportional collagen metabolism itself impairs cell-matrix interactions, which stimulates MMPs production [55- 56]. Therefore, considered in a broader sense, the capacity of cells to repair or to replace ECM proteins following acute oxidation is likely to be an important predictor of how well cells are able to respond to oxidative stressors [54].

The quantitative aspects of the in-vitro cellular proteolysis are still insufficiently settled. One approach to quantify such activity that we employed here was to measure the increasing fluorescent signal as a result of proteolytic de-quenching of an initially day-quenched protein (FRET effect) [32]. Originally the day-quenched substrates were developed as non-selective, broad-spectrum substrates for the analysis of protease activity in solution [57]. Consequently Jadzko et al [32] established a protocol for ECM proteins imaging in the presence of cells, adapted for confocal microscopy. Studies on the use of FRET effect for the quantification of cellular proteolysis in planar (2D) samples however are rather sparse and might be attributed mostly to a line of our previous investigations [15, 36-38].

An important observation concerning the proteolytic de-quenching of FITC-Col I is that it works well upon collagenase CH treatment giving significant rise of the total fluorescence. However, this de-quenching was substantially inhibited in oxidized FITC-Col I samples, meaning that oxidized collagen is more resistant to enzymatic degradation. We hypothesize that the effect of bacterial collagenase CH is connected with the specific binding in the active site and strong preference for glycine in P3 and P1', proline at P2 and P2' (according to proteases classification) in the cleavage site [58]. It is notable that the proline is particularly vulnerable to oxidation by metal ion generated ROS and can be disproportionally modified by oxidation [59-62]. Thus, the specific cleavage site could be lost.

Unequivocally also sound the data for ADMSCs induced enzymatic remodelling of FITC-Col I, characterized by a significantly higher de-quenching of samples (+cells) compared to the samples (-cells). Interestingly, the fluorescence of native FITC-Col I increased with about 11 %, which is higher than the effect of CH collagenase (about 5%) suggesting an involvement

of more active MMPs secreted by ADMSCs. On the other hand, the ability of ADMSCs to de-quench the substratum associated FITC-Col I suggests that adsorbed collagen is sensitive to the peri-cellular proteolysis. The same tendency, however, was observed in the supernatants meaning that the proteolysis continued also in the medium, in fact targeting the spontaneously released collagen. It has to be noted, however, that the fluorescent signal in the medium is approx. 4 times higher than the signal measured from the substratum, meaning that only about 25 % of Col I remains there after 24 h of incubation, valid for both native and oxidized samples. The reason for this unexpectedly high spontaneous desorption of FITC-Col I might be attributed to the withdrawal of equilibrium between adsorbed and released Col I due to its initially low concentration in the medium (after washing) [63]. The competitive Vroman effect [64] of serum proteins also cannot be excluded assuming that 10% of serum was added to the system after ADMSCs adhesion (see Fig 1). The Vroman effect dictates that the protein of highest mobility adsorbs first on the substratum but later are replaced by less mobile proteins that have a higher affinity for the surface [64]. The later might come from the serum, but also secreted by the cells. The competitive effect of some small amino acids like glycine is also an acceptable mechanism [65]. Nevertheless, this result leads to the assumption that the “noise” from spontaneously released FITC Col I in the medium cannot screen the effect of ADMSCs induced FITC-Col I de-quenching confirming that the proteolysis continued in the supernatants and is driven by soluble proteases.

Only scant information is available on the mode by which matrix proteases degrade ECM substrates. The members of the Matrix metalloproteinases (MMPs) family of zinc- and calcium-dependent endopeptidases, cooperatively degrade variety of extracellular proteins of which collagen is being particularly resistant to other proteases due to its tightly packed structure [66]. The mammalian cells secrete MMPs that play pivotal roles in various physiological processes, and are among the key molecules that regulate the ECM development and remodelling [67]. ADMSCs express various MMPs, including the membrane-type (MT) MMPs (MMP-1, -2, -3, -7, -8, -9, -14, -15, -28) of which MMP-1, MMP-8 and MMP-14/ MT-1 degrade fibrillary collagens [68,69]. We haven't investigated which exactly proteases are involved in the observed de-quenching effect but considering that enzyme works on both the

substratum bound and released collagen suggests that possibly all three types are involved. We have to consider however that morphological studies show that the space under the cells is not significantly affected and even some accumulation of FITC-Col I beneath the cells was observed (Figure 2). Why FITC-Col I accumulate there, moreover in both native and oxidized samples, remains unclear. One possible explanation is that the higher amount of FITC-Col I in the medium activate its transcytosis across the adhering cells. Transcytosis, the vesicular transport of macromolecules from one side of a cell to the other, is a strategy used by multicellular organisms to selectively move material between two environments without altering the unique compositions of those environments [70]. We could not find however data for the transcytosis of collagen by MSCs, though the link between protein transcytosis and reactive oxygen species is demonstrated in other cell systems [71].

Nevertheless, our results raise the question: why oxidized collagen is less sensitive to remodeling, both mechanical and enzymatic? We anticipate that the process of collagenolysis depends on multiple interactions of mammalian collagenases with different exosites, serving to align the active site of collagenase which possesses a strong preference for the cleavage site perfectly matching the repetitive amino acid sequence of the native Col I molecules [72]. They are located near the peptide bond that will be cleaved, along with the local unwinding of the triple helix [72, 73]. Any changes in the structure of collagen, as a result of oxidation, could prevent the proper aligning of collagenase and subsequently the degradation of type I collagen. Moreover, it was shown that the proline and hydroxyproline abundance in certain positions impacts the conformation of collagen molecule even its affinity to integrin receptors [74]. It may be a hint to understand the morphological observations for absent ADMSCs collagen reorganization in oxidized samples known to require integrin activity^{15,18}. The missing difference in the overall cell morphology however suggests that such altered integrin activity is unlikely, which endorsed the view that some intrinsic structural changes in the collagen molecule upon oxidation are responsible for the effect.

In order to confirm the putative structural changes in Col I molecule, we performed DSC analysis comparing the native and oxidized samples (Figure 6). In fact, this was a follow up study from our previous investigation on calf skin collagen denaturation profile [75] and

the effect of oxidation [31]. Here we confirm that the native FITC collagen type I undergoes similar change in the thermal transition under heating, i.e. a single cooperative peak at 40.5°C. Thermal denaturation of oxidised collagen however resulted in a splitting of the main transition into two well-resolved transitions, i.e. along with the above typical collagen endotherm, a new transition at 33.6 °C appears. Interestingly, the enthalpy (ΔH), which reflects the energetically aspect of the transition, was found to be similar to those of native FITC-Col I. ΔH is basically dependent on the fraction of native protein in the solution. [76] Consequently, if this fraction is less in the total protein, ΔH would drop down correspondingly [77], which is obviously not the case as we did not find such a significant change. To be noted that the transition half-widths (Figure 6 B) were still close to the native sample, which is an indication that the observed pretransition is caused by a rather discrete kind of damage to the collagen molecule. This allows us to speculate that upon oxidation in acute conditions collagen molecule undergoes mostly intrinsic reorganization causing a lowered transitional temperature and does not go into the denatured state [75,76]. This again points to the possibility that the low digestibility of collagen in an oxidizing environment depends on the distinct changes in the collagen structure, but not because of its denaturation.

Collectively, all these data lead us to believe that we have encountered a novel mechanism for the control of MSCs behaviour via subtle changes in their oxidative environment, presumably valid also for other cell systems.

4. Material And Methods

4.1 Collagen preparation

Collagen type I (Col I) was isolated from rat tail by standard procedure combining acetic acid extraction and salting out with NaCl as described elsewhere [78-79]. Rat tails were obtained from control animals of other experiments performed in Medical University Pleven, tendons were gently removed from the tails, cleaned from tail debris, rinsed with distilled water and PBS. The fatty waste was removed by pouring the tails for 5 min in acetone and 70% isopropanol consequently. Col I was solubilized in 0.5 M acetic acid on magnetic stirrer at 4°C

for at least 48 h. After centrifugation at 4000 rpm at 4°C, the supernatant was dialyzed extensively against 0.05M acetic acid before collagen was salted out by 1/5 volume of 4.5M NaCl. After centrifugation at 8000 rpm at 4°C, the pellets were re-dissolved in 0.05 M acetic acid and dialyzed against 0.05M acetic acid overnight. All procedures were run at 4°C. Such collagen preparations are strongly enriched of type I collagen [78,79]. The collagen concentration in the solutions was measured by modified Lowry assay [80] and by the optical absorbance at 230 nm [81].

4.2 *Fluorescent labeling of collagen*

FITC labelled collagen was prepared according to the modified protocol of Doyle [33]. Briefly, 4 ml of Col I solution in 0.05M acetic acid (2.5 mg/ml) was titrated with 0.1M M borate buffer-pH (9.0) and mixed with 50µL FITC dissolved in DMSO at a concentration of 1 mg/ml, then incubated at room temperature for 90 min in dark. The reaction was stopped by 0.05M Tris buffer (pH 7.4) and the excess of FITC was removed by intensive dialysis versus 0.05M acetic acid. Aliquots of FITC labelled Col I was stored at +4°C for up to 3 months.

4.3 *Collagen oxidation procedure*

FITC-Col I oxidation was performed according to the previously described protocol by incubating collagen solution (2 mg/ml) in 0.05M acetic acid, pH 4.3, with freshly prepared 50µM FeCl₂ and 5mM H₂O₂ for 18 hours at room temperature [31]. The reaction was stopped with EDTA at a final concentration of 10mM. The excess of oxidants was removed by intensive dialysis against 0.05 M acetic acid.

4.4 *Cells*

Human ADMSCs of passage 1 were obtained from Tissue Bank BulGen using healthy volunteers undergoing liposuction with written consent. The cells were maintained in DMEM/F12 medium containing 1% GlutaMAX™, 1% Antibiotic-Antimycotic solution and 10% Fetal Bovine Serum (FBS) all purchased from (Thermo Fisher Scientific, USA), The medium was replaced each 2nd day until the cells reach approximately 90% confluence to be used for the experiments up to 4th passage.

4.5 Morphological studies

For the morphological studies, standard (22x22 mm) glass coverslips (ISOLAB Laborgerate GmbH) were coated with FITC-Col I, either native or oxidized, dissolved in 0.05M acetic acid (100 µg/ml, 60 min, 37°C). The glass samples were placed on 6-well TC plates (Nunc, Denmark) and seeded with 5×10^4 ADMSCs at final volume of 2 mL in serum-free medium (to assure the attachment to Col I only). To control the initial cell attachment at the 2nd hour of incubation the samples were monitored under phase contrast using inverted microscope (Leica DM 2900). At the end of the second hour 10% serum was added to each sample and the cells were further cultivated up to 24 h, then fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 before fluorescence staining. To visualize actin cytoskeleton, red fluorescent Rhodamine-Phalloidin (Invitrogen) was used (dilution 1:100 in PBS), while cell nuclei were stained by Hoechst 33342 (Sigma-Aldrich) in dilution 1:2000 from a 10 mg/ml stock solution. Finally, the samples were mounted upside down on standard glass slides with Mowiol (Sigma-Aldrich) and viewed using the green (FITC collagen), red (actin cytoskeleton) and blue (nuclei) channels of an upright fluorescent microscope (Olympus BX53) with objectives UPlan FLN at low (10x/0.50) and high (40x/0.50) magnification. The different colors were merged by Adobe Photoshop image processing software. At least three representative images were acquired for each sample.

4.6 Assay of collagen degradation *in vitro*

FITC-Collagen was labeled (see above) under conditions assuring a maximum of lysine ε-amino group exposure resulting in partial quenching of the fluorophore. In a preliminary study we found that the FITC-Col I sample was sensitive to proteolytic degradation: upon bacterial collagenase treatment, the sample de-quenches, resulting in an increase of the fluorescent signal emission due to the Resonance Energy Transfer (FRET effect) [32]. To standardize the de-quenching effect and evaluate the proteolytic degradation in the native and oxidized samples, a basic solution of Collagenase type I from *Clostridium histolyticum* (Genaxxon bioscience) at a concentration of 3.7 mg/ml in TC medium was used to which natural FITC-Col I or oxidized (FITC-Col I-OXI) were added (10 µg/ml) followed by 1.0- and

2.0- hours incubation (as indicated) at 37°C. Fluorescence of the quadruplicated samples was measured in 24 well glass bottomed plate (Greiner, Sensoplate black, Germany) using Multimode Microplate Reader (Mithras² LB 943, Berthold Technologies GmbH & Co. KG, Germany) set to adsorption/emission rate of 495/530 nm. Fluorescence intensity is presented either directly as Relative Photometric Units (RPU) or as a calculated difference in the fluorescent signal between samples with collagenase versus controls (TC medium only), reflecting better the proteolytic de-quenching of FITC-Col I.

4.7 Assay of collagen degradation by living ADMSC

To measure the cell-dependent proteolytic activity the same approach of FITC-Col I de-quenching was used. Glass bottomed 24 well TC plates (see above) were pre-coated with 100 µg/ml native or oxidized FITC-Col I in 0.05 M acetic acid then washed 3 times with PBS before ADMSCs (1x10⁴ per well) were added in a final volume of 1 ml serum-free medium. After 2 h of incubation in serum-free medium (assuring single protein adhesion of cells to Col I), 10% serum was added to each sample and the cells were further cultured up to 24 h in a humidified CO₂ incubator. Then the supernatants were collected for the fluorescence measurement of released FITC-Col, while the adsorbed (substratum associated) FITC-Col I was measured directly from the bottom of the plate (in 1ml PBS) using Microplate Reader (see above) set at 495/530 nm. Symmetric samples without cells (-cells) were processed in the same way. All experiments were quadruplicated. The measured fluorescence intensity is presented directly in RPU or ΔRPU (calculating the difference between samples (+cells) versus (-cells)), thus characterising quantitatively the ADMSCs dependent proteolytic de-quenching of FITVC-Col I.

4.8 DSC measurements

DSC measurements were performed using DASM4 (Privalov, BioPribor) built-in high-sensitive calorimeter with a cell volume of 0.47 ml. The samples were diluted in 0.05 M acetic acid prior to DSC running. Protein concentration was adjusted to 2 mg/ml. To prevent possible degassing of the solution under study, constant pressure of 2 atm was applied in the cells. The samples were heated at a scanning rate of 1.0 °C/min from 20°C to 65°C and were preceded by

a baseline run with buffer-filled cells. Each collagen solution was reheated after the cooling from the first scan to evaluate the reversibility of the thermally induced transitions. The calorimetric curve corresponding to the second (reheating) scan was used as an instrumental baseline and was subtracted from the first scans, as the collagen thermal denaturation is irreversible. The obtained excess heat capacity profiles were normalized to the protein concentration. The calorimetric data were analyzed using Origin Pro 2018 software package assuring the calculation of the total enthalpy (ΔH) of the transitions in the DSC scan among other thermodynamic parameters.

4.9 Statistical analysis

Data was analyzed by using SPSS Statistics for Windows, Version 23.0 (Armonk, NY: IBM Corp). All quantitative results were obtained from at least four samples for analyses. Descriptive data was compared using Chi-Square and Mann-Whitney U tests. Comparison of differences between groups was performed by non-parametric test of Friedman; pair wise comparison was performed using post-hoc analyses by Dunn-Bonferroni. Data were expressed as mean \pm standard deviation (SD). Difference with $p < 0.05$ was considered to be statistically significant.

Conclusions

Both morphological and quantitative approaches demonstrate that the native Col I undergo significant remodelling by stem cells. A completely novel observation, however, is that the oxidized collagen in acute conditions cannot be remodelled by ADMSCs, both mechanically and enzymatically, confirmed quantitatively by the absent proteolytic de-quenching of FITC-Col I. Parallel studies in the cell-free system show that the oxidative environment generally alters the enzymatic de-quenching of FITC-Col I. DSC analysis confirm that all these effects depend rather on the intrinsic structural changes of the oxidized Col I molecules than on the altered functionality of ADMSCs.

Institutional Ethics Committee Statement:

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Committee of Medical University-Pleven (APPROVAL N 601-KENID 20/05/19).

Author Contributions: AG and KP-R – Conceptualization of the study ; KP-R, SG, BK, ST and AG – Methodology and Investigation; AG, KP-R, ST and KS – Analysis and interpretation; AG – Writing – Original Draft Preparation; AG and KP-R Writing – Review & Editing , KP-R –Project Administration, AG – Primary responsibility for final content. All authors have read and agreed to the published version of the manuscript.

Funding: This study was conducted with the financial support from Medical University – Pleven, Bulgaria within the internal projects (MU-Project 15/2019 and 16/2020) and the multidisciplinary project BG05M2OP001-1.002-0010 financed by the operative program “Science and education for intelligence growth”. The partial support of Tissue Bank BulGen - Sofia is also acknowledged.

Acknowledgements: We are in depth acknowledge for the technical help of I. Mihova from UMBL Lozenec-Sofia and V. Ivanova from MU-Pleven for technical assistance, as well as of G. Altankov Jr. for the processing of fluorescent image files.

Conflicts of Interest: The authors declare no competing financial interest.

References

1. Song, Y. H.; Shon, S. H.; Shan, M. R.; Stroock, A. D.; Fischbach, C., Adipose-derived stem cells increase angiogenesis through matrix metalloproteinase-dependent collagen remodeling. *Integrative Biology* **2016**, *8* (2), 205-215. DOI: 10.1039/c5ib00277j
2. Han, S., Li, Y.Y. & Chan, B.P. Protease inhibitors enhance extracellular collagen fibril deposition in human mesenchymal stem cells. *Stem Cell Res Ther* *6*, 197 (2015). <https://doi.org/10.1186/s13287-015-0191-1>

3. Zuk, P. A.; Zhu, M.; Mizuno, H.; Huang, J.; Futrell, J. W.; Katz, A. J.; Benhaim, P.; Lorenz, H. P.; Hedrick, M. H., Multilineage cells from human adipose tissue: Implications for cell-based therapies. *Tissue Eng.* **2001**, *7* (2), 211-228. DOI: 10.1089/107632701300062859
4. Wade, R. J.; Burdick, J. A., Engineering ECM signals into biomaterials. *Mater. Today* **2012**, *15* (10), 454-459. DOI: 10.1016/s1369-7021(12)70197-9
5. Frese, L.; Dijkman, P. E.; Hoerstrup, S. P., Adipose Tissue-Derived Stem Cells in Regenerative Medicine. *Transfusion Medicine and Hemotherapy* **2016**, *43* (4), 268-274. DOI: 10.1159/000448180
6. Karsdal, M. A.; Genovese, F.; Madsen, E. A.; Manon-Jensen, T.; Schuppan, D., Collagen and tissue turnover as a function of age: Implications for fibrosis. *J. Hepatol.* **2016**, *64* (1), 103-109. DOI: 10.1016/j.jhep.2015.08.014
7. Frantz, C.; Stewart, K. M.; Weaver, V. M., The extracellular matrix at a glance. *J. Cell Sci.* **2010**, *123* (24), 4195-4200. DOI: 10.1242/jcs.023820
8. Bosman, F. T.; Stamenkovic, I., Functional structure and composition of the extracellular matrix. *J. Pathol.* **2003**, *200* (4), 423-428. DOI: 10.1002/path.1437
9. Karsdal, M. A.; Nielsen, M. J.; Sand, J. M.; Henriksen, K.; Genovese, F.; Bay-Jensen, A. C.; Smith, V.; Adamkewicz, J. I.; Christiansen, C.; Leeming, D. J., Extracellular Matrix Remodeling: The Common Denominator in Connective Tissue Diseases Possibilities for Evaluation and Current Understanding of the Matrix as More Than a Passive Architecture, but a Key Player in Tissue Failure. *Assay Drug Dev. Technol.* **2013**, *11* (2), 70-92. DOI: 10.1089/adt.2012.474
10. Grutters, J. C.; Lammers, J.-W. J., Connective tissue diseases. In *Clinical Respiratory Medicine*, Spiro, S. G.; Silvestri, G. A.; Agusti, A., Eds. Saunders: Philadelphia, PA, 2012; pp 653-666.
11. Cipak, A.; Mrakovcic, L.; Ciz, M.; Lojek, A.; Mihaylova, B.; Goshev, I.; Jaganjac, M.; Cindric, M.; Sitic, S.; Margaritoni, M.; Waeg, G.; Balic, M.; Zarkovic, N., Growth suppression of human breast carcinoma stem cells by lipid peroxidation product 4-hydroxy-2-nonenal and hydroxyl radical-modified collagen. *Acta Biochim. Pol.* **2010**, *57* (2), 165-171.
12. Krahn, K. N.; Bouten, C. V. C.; van Tuijl, S.; van Zandvoort, M.; Merckx, M. Fluorescently labeled collagen binding proteins allow specific visualization of collagen in tissues and live cell culture. *Anal. Biochem.* **2006**, *350* (2), 177-185. DOI: 10.1016/j.ab.2006.01.013
13. Langhans Sigrid A. Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning. *Frontiers in Pharmacology*, **2018**, (9) ISSN=1663-9812, DOI=10.3389/fphar.2018.00006
14. Maneva-Radicheva, L.; Ebert, U.; Dimoudis, N.; Altankov, G. Fibroblast remodeling of adsorbed collagen type IV is altered in contact with cancer cells. *Histol. Histopathol.* **2008**, *23* (7), 833-842.
15. Coelho, N. M.; Llopis-Hernandez, V.; Salmeron-Sanchez, M.; Altankov, G., Dynamic Reorganization and Enzymatic Remodeling of Type IV Collagen at Cell-Biomaterial Interface. In *Insights into Enzyme Mechanisms and Functions from Experimental and Computational Methods*, Christov, C. Z., Ed. **2016**; Vol. 105, pp 81-104.
16. Lee, C. H.; Singla, A.; Lee, Y., Biomedical applications of collagen. *Int. J. Pharm.* **2001**, *221* (1-2), 1-22. DOI: 10.1016/s0378-5173(01)00691-3

17. Sorushanova A, Delgado LM, Wu Z, et al. The Collagen Suprafamily: From Biosynthesis to Advanced Biomaterial Development. *Advanced Materials (Deerfield Beach, Fla.)*. **2019** Jan;31(1):e1801651. DOI: 10.1002/adma.201801651. PMID: 30126066.
18. Leitinger, B., Transmembrane Collagen Receptors. In *Annual Review of Cell and Developmental Biology, Vol 27*, Schekman, R.; Goldstein, L.; Lehmann, R., Eds. **2011**; Vol. 27, pp 265-290.
19. Yamauchi, M.; Sricholpech, M., Lysine post-translational modifications of collagen. In *Lysine-Based Post-Translational Modification of Proteins*, Scott, I., Ed. **2012**; Vol. 52, pp 113-133.
20. Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T. D.; Mazur, M.; Telser, J., Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **2007**, 39 (1), 44-84. DOI: 10.1016/j.biocel.2006.07.001
21. Bonomini, F.; Tengattini, S.; Fabiano, A.; Bianchi, R.; Rezzani, R., Atherosclerosis and oxidative stress. *Histol. Histopathol.* **2008**, 23, 3, 381-390.
22. Watson, W. H.; Ritzenthaler, J. D.; Roman, J., Lung extracellular matrix and redox regulation. *Redox Biology* **2016**, 8, 305-315. DOI: 10.1016/j.redox.2016.02.005
23. Ramalingam, M.; Kim, S. J., Reactive oxygen/nitrogen species and their functional correlations in neurodegenerative diseases. *J. Neural Transm.* **2012**, 119 (8), 891-910. DOI: 10.1007/s00702-011-0758-7
24. Saremi, A.; Arora, R., Vitamin E and Cardiovascular Disease. *Am. J. Ther.* **2010**, 17 (3), E56-E65. DOI: 10.1097/MJT.0b013e31819cdc9a
25. Daniil, Z. D.; Papageorgiou, E.; Koutsokera, A.; Kostikas, K.; Kiropoulos, T.; Papaioannou, A. I.; Gourgoulialis, K. I., Serum levels of oxidative stress as a marker of disease severity in idiopathic pulmonary fibrosis. *Pulm. Pharmacol. Ther.* **2008**, 21 (1), 26-31. DOI: 10.1016/j.pupt.2006.10.005
26. Reeg, S.; Grune, T., Protein Oxidation in Aging: Does It Play a Role in Aging Progression? *Antioxidants & Redox Signaling* **2015**, 23 (3), 239-255. DOI: 10.1089/ars.2014.6062
27. Dichi, I., Bregano, J. W., Simao, A. N. C., Cecchini, R. Role of Oxidative Stress in Chronic Diseases. 1st ed.; CRC Press: **2014**. ImprintCRC Press . DOI: <https://doi.org/10.1201/b16653>
28. Liu, C.; Yang, Q.; Fang, G.; Li, B. S.; Wu, D. B.; Guo, W. J.; Hong, S. S.; Hong, L., Collagen metabolic disorder induced by oxidative stress in human uterosacral ligament-derived fibroblasts: A possible pathophysiological mechanism in pelvic organ prolapse. *Mol. Med. Report.* **2016**, 13 (4), 2999-3008. DOI: 10.3892/mmr.2016.4919
29. Serras F. The benefits of oxidative stress for tissue repair and regeneration. *Fly (Austin)*. **2016**;10(3):128-133. doi:10.1080/19336934.2016.1188232
30. Gonçalves, R. V. E.; Costa, A. M. A. E.; Grzeskowiak, L. E., Oxidative Stress and Tissue Repair: Mechanism, Biomarkers, and Therapeutics (Special Issue in: Oxidative Medicine and Cellular Longevity). Hindawi: **2019**.
31. Komsa-Penkova, R.; Koynova, R.; Kostov, G.; Tenchov, B., Discrete reduction of type I collagen thermal stability upon oxidation. *Biophys. Chem.* **2000**, 83 (3), 185-195. DOI: 10.1016/S0301-4622(99)00135-0

32. Jedeszko, C.; Sameni, M.; Olive, M. B.; Moin, K.; Sloane, B. F., Visualizing Protease Activity in Living Cells: From Two Dimensions to Four Dimensions. *Curr. Protoc. Cell Biol.* **2008**, 39 (1), 4.20.1-4.20.15. DOI: 10.1002/0471143030.cb0420s39
33. Doyle, A. D., Fluorescent Labeling of Rat-tail Collagen for 3D Fluorescence Imaging. *Bio-Protocol* **2018**, 8 (13). DOI: 10.21769/BioProtoc.2919
34. Friedl, P.; Wolf, K., Proteolytic and non-proteolytic migration of tumour cells and leucocytes. In *Proteases and the Regulation of Biological Processes*, Saklatvala, J.; Nagase, H.; Salvesen, G., Eds. **2003**; Vol. 70, pp 277-285.
35. Altankov, G.; Groth, T., Reorganization of substratum-bound fibronectin on hydrophilic and hydrophobic materials is related to biocompatibility. *Journal of Materials Science-Materials in Medicine* **1994**, 5 (9-10), 732-737. DOI: 10.1007/bf00120366
36. Llopis-Hernandez, V.; Rico, P.; Moratal, D.; Altankov, G.; Salmeron-Sanchez, M., Role of Material-Driven Fibronectin Fibrillogenesis in Protein Remodeling. *BioResearch Open Access* **2013**, 2 (5), 364-373. DOI: 10.1089/biores.2013.0017
37. Tzoneva, R.; Groth, T.; Altankov, G.; Paul, D., Remodeling of fibrinogen by endothelial cells in dependence on fibronectin matrix assembly. Effect of substratum wettability. *Journal of Materials Science-Materials in Medicine* **2002**, 13 (12), 1235-1244. DOI: 10.1023/a:1021131113711
38. Toromanov, G.; Gugutkov, D.; Gustaysson, J.; Planell, J.; Salmeron-Sanchez, M.; Altankov, G., Dynamic Behavior of Vitronectin at the Cell-Material Interface. *Acs Biomaterials Science & Engineering* **2015**, 1 (10), 927-934. DOI: 10.1021/acsbiomaterials.5b00147
39. Coelho, N. M.; Salmeron-Sanchez, M.; Altankov, G., Fibroblasts remodeling of type IV collagen at a biomaterials interface. *Biomaterials Science* **2013**, 1 (5), 494-502. DOI: 10.1039/c3bm00163f
40. Wei, X. W.; Liu, B. Y.; Liu, G.; Yang, F.; Cao, F.; Dou, X. J.; Yu, W. T.; Wang, B. J.; Zheng, G. S.; Cheng, L. L.; Ma, Z. J.; Zhang, Y.; Yang, J. H.; Wang, Z. H.; Li, J. L.; Cui, D. P.; Wang, W.; Xie, H.; Li, L.; Zhang, F.; Lineaweaver, W. C.; Zhao, D. W., Mesenchymal stem cell-loaded porous tantalum integrated with biomimetic 3D collagen-based scaffold to repair large osteochondral defects in goats. *Stem Cell. Res. Ther.* **2019**, 10. DOI: 10.1186/s13287-019-1176-2
41. Tangtrongsup, S.; Kisiday, J. D., Modulating the oxidative environment during mesenchymal stem cells chondrogenesis with serum increases collagen accumulation in agarose culture. *J. Orth. Res.* **2018**, 36 (1), 506-514. DOI: 10.1002/jor.23618
42. Sart, S.; Song, L. Q.; Li, Y., Controlling Redox Status for Stem Cell Survival, Expansion, and Differentiation. *Oxid. Med. Cell. Longev.* **2015**, 2015. DOI: 10.1155/2015/105135
43. Zhu, S. C.; Yuan, Q. J.; Yin, T.; You, J.; Gu, Z. P.; Xiong, S. B.; Hu, Y., Self-assembly of collagen-based biomaterials: preparation, characterizations and biomedical applications. *Journal of Materials Chemistry B* **2018**, 6 (18), 2650-2676. DOI: 10.1039/c7tb02999c
44. Ricard-Blum, S., The Collagen Family. *Cold Spring Harb. Perspect. Biol.* **2011**, 3 (1). DOI: 10.1101/cshperspect.a004978
45. N. Chosa and A. Ishisaki. Two novel mechanisms for maintenance of stemness in mesenchymal stem cells: SCRG1/BST1 axis and cell-cell adhesion through N-cadherin *Jpn Dent Sci Rev.* **2018** Feb; 54(1): 37-44.

- 46 Xu Y, Chen C, Hellwarth PB, Bao X. Biomaterials for stem cell engineering and biomanufacturing. *Bioact Mater.* 2019;4:366-379. Published 2019 Dec 2. doi:10.1016/j.bioactmat.2019.11.002
- (47) Kim, K. S.; Choi, H. W.; Yoon, H. E.; Kim, I. Y., Reactive Oxygen Species Generated by NADPH Oxidase 2 and 4 Are Required for Chondrogenic Differentiation. *J. Biol. Chem.* **2010**, 285 (51), 40294-40302. DOI: 10.1074/jbc.M110.126821
48. Somoza, R. A.; Welter, J. F.; Correa, D.; Caplan, A. I., Chondrogenic Differentiation of Mesenchymal Stem Cells: Challenges and Unfulfilled Expectations. *Tissue Engineering Part B-Reviews* **2014**, 20 (6), 596-608. DOI: 10.1089/ten.teb.2013.0771
49. Abraham, L.C; Zuena, E; Perez-Ramirez, B; Kaplan, D.L. Guide to collagen characterization for biomaterial studies. *J Biomed Mater Res B Appl Biomater.* **2008**; 87 (1):264-285. doi:10.1002/jbm.b.31078
50. Liu, R. M.; Desai, L. P., Reciprocal regulation of TGF-beta and reactive oxygen species: A perverse cycle for fibrosis. *Redox Biology* **2015**, 6, 565-577. DOI: 10.1016/j.redox.2015.09.009
51. Rosin, N. L.; Sopel, M. J.; Falkenham, A.; Lee, T. D. G.; Legare, J. F., Disruption of Collagen Homeostasis Can Reverse Established Age-Related Myocardial Fibrosis. *Am. J. Pathol.* **2015**, 185 (3), 631-642. DOI: 10.1016/j.ajpath.2014.11.009
52. Butterfield, D. A.; Boyd-Kimball, D., Oxidative Stress, Amyloid-beta Peptide, and Altered Key Molecular Pathways in the Pathogenesis and Progression of Alzheimer's Disease. *Journal of Alzheimers Disease* **2018**, 62 (3), 1345-1367. DOI: 10.3233/jad-170543
53. Rodriguez-Pascual, F.; Rosell-Garcia, T., Lysyl Oxidases: Functions and Disorders. *J. Glaucoma* **2018**, 27 Suppl 1, S15-s19. DOI: 10.1097/ijg.0000000000000910
54. Cecarini, V.; Gee, J.; Fioretti, E.; Amici, M.; Angeletti, M.; Eleuteri, A. M.; Keller, J. N., Protein oxidation and cellular homeostasis: Emphasis on metabolism. *Biochimica Et Biophysica Acta-Molecular Cell Research* **2007**, 1773 (2), 93-104. DOI: 10.1016/j.bbamcr.2006.08.039
55. Tu, Y.; Quan, T. Oxidative Stress and Human Skin Connective Tissue Aging. *Cosmetics*, **2016**, 3, 28.
- 56 Nowotny K, Grune T. Degradation of oxidized and glycoxidized collagen: role of collagen cross-linking. *Arch Biochem Biophys.* **2014**;542, 56-64. doi:10.1016/j.abb.2013.12.007
57. Menges, D. A.; Ternullo, D. L.; TanWilson, A. L.; Gal, S., Continuous assay of proteases using a microtiter plate fluorescence reader. *Anal. Biochem.* **1997**, 254 (1), 144-147. DOI: 10.1006/abio.1997.2408
58. Eckhard, U.; Huesgen, P. F.; Brandstetter, H.; Overall, C. M., Proteomic protease specificity profiling of clostridial collagenases reveals their intrinsic nature as dedicated degraders of collagen. *J. Proteomics* **2014**, 100, 102-114. DOI: 10.1016/j.jprot.2013.10.004
59. Osberger, T. J.; Rogness, D. C.; Kohrt, J. T.; Stepan, A. F.; White, M. C., Oxidative diversification of amino acids and peptides by small-molecule iron catalysis. *Nature* **2016**, 537 (7619), 214-219. DOI: 10.1038/nature18941
60. Bochi GV, Torbitz VD, de Campos LP, Sangoi MB, Fernandes NF, Gomes P, Moretto MB, Barbisan F, da Cruz IB, Moresco RN. In Vitro Oxidation of Collagen Promotes the Formation of Advanced Oxidation Protein Products and the Activation of Human Neutrophils. *Inflammation.* **2016** Apr;39(2):916-27. doi: 10.1007/s10753-016-0325-3. PMID: 26920846.

61. Davies, M. J., The oxidative environment and protein damage. *Biochimica Et Biophysica Acta-Proteins and Proteomics* **2005**, 1703 (2), 93-109. DOI: 10.1016/j.bbapap.2004.08.007
62. P Stadtman E R Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. *Free Radic Biol Med.* 1990;9(4):315-25. doi: 10.1016/0891-5849(90)90006-5.
63. Rabe, M.; Verdes, D.; Seeger, S., Understanding protein adsorption phenomena at solid surfaces. *Adv. Colloid Interface Sci.* **2011**, 162 (1-2), 87-106. DOI: 10.1016/j.cis.2010.12.007
64. Vroman, L.; Adams, A. L.; Fischer, G. C.; Munoz, P. C., Interaction of high molecular-weight kininogen, factor-xii, and fibrinogen in plasma at interfaces. *Blood* **1980**, 55 (1), 156-159.
65. Ponche, A.; Ploux, L.; Anselme, K., Protein/Material Interfaces: Investigation on Model Surfaces. In *Surface and Interfacial Aspects of Cell Adhesion*, 1st ed.; Carre, A.; Mittal, K. L., Eds. CRC Press: London, **2011**.
66. Orgel, J.; Irving, T. C.; Miller, A.; Wess, T. J., Microfibrillar structure of type I collagen in situ. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, 103 (24), 9001-9005. DOI: 10.1073/pnas.0502718103
67. Almalki, S. G.; Agrawal, D. K., Effects of matrix metalloproteinases on the fate of mesenchymal stem cells. *Stem Cell. Res. Ther.* **2016**, 7. DOI: 10.1186/s13287-016-0393-1
68. Dittmore A, Silver J, Sarkar SK, Marmer B, Goldberg GI, Neuman KC. Internal strain drives spontaneous periodic buckling in collagen and regulates remodeling. *Proc Natl Acad Sci U S A.* **2016**;113(30):8436-8441. doi:10.1073/pnas.1523228113
69. Manka, S. W.; Carafoli, F.; Visse, R.; Bihan, D.; Raynal, N.; Farndale, R. W.; Murphy, G.; Enghild, J. J.; Hohenester, E.; Nagase, H., Structural insights into triple-helical collagen cleavage by matrix metalloproteinase 1. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, 109 (31), 12461-12466. DOI: 10.1073/pnas.1204991109
70. Puma, P. L., Hubbard, A. L. Transcytosis: Crossing Cellular Barriers. *Physiol Rev* 83: 871-932, **2003**; 10.1152/physrev.00001.2003.
71. He, F.F., Gong, Y., Li, Z.Q., Wu L., Jiang H.J., Su H., Zhang C., Wang, Y.M. A New Pathogenesis of Albuminuria: Role of Transcytosis. *Cell Physiol Biochem* **2018**; 47: 1274-1286. doi: 10.1159/000490223
72. Cieplak, P.; Strongin, A. Y., Matrix metalloproteinases - From the cleavage data to the prediction tools and beyond. *Biochimica Et Biophysica Acta-Molecular Cell Research* **2017**, 1864 (11), 1952-1963. DOI: 10.1016/j.bbamcr.2017.03.010
- 73 Laronha, H.; Caldeira, J., Structure and Function of Human Matrix Metalloproteinases. *Cells* **2020**, 9 (5). DOI: 10.3390/cells9051076
74. Sipila, K. H.; Drushinin, K.; Rappu, P.; Jokinen, J.; Salminen, T. A.; Salo, A. M.; Kapyla, J.; Myllyharju, J.; Heino, J., Proline hydroxylation in collagen supports integrin binding by two distinct mechanisms. *J. Biol. Chem.* **2018**, 293 (20), 7645-7658. DOI: 10.1074/jbc.RA118.002200
75. Komsa-Penkova, R.; Koynova, R.; Kostov, G.; Tenchov, B. G., Thermal stability of calf skin collagen type I in salt solutions. *Biochimica et Biophysica Acta-Protein Structure and Molecular Enzymology* **1996**, 1297 (2), 171-181. DOI: 10.1016/S0167-4838(96)00092-1
76. S. Milev, DSC and Protein Stability: What Does The Enthalpy Change Mean? **2020**,08.25 <https://www.materials-talks.com/blog/2020/08/25/dsc-and-protein-stability-what-does-the-enthalpy-change-mean/>

- 77 Johnson CM. Differential scanning calorimetry as a tool for protein folding and stability. *Arch Biochem Biophys*. 2013. Volume 531, Issues 1–2, March 2013, Pages 100-109, <https://doi.org/10.1016/j.abb.2012.09.008>
78. Chandrakasan, G.; Torchia, D. A.; Piez, K. A., Preparation of intact monomeric collagen from rat tail tendon and skin and structure of nonhelical ends in solution. *J. Biol. Chem.* **1976**, 251 (19), 6062-6067.
79. Rajan, N., et al., Preparation of ready-to-use, storable and reconstituted type I collagen from rat tail tendon for tissue engineering applications. *Nat Protocols*, **2007**. 1(6): p. 2753-2758.
80. Komsa-Penkova, R.; Spirova, R.; Bechev, B., Modification of Lowry's method for collagen concentration measurement. *J. Biochem. Biophys. Methods* **1996**, 32 (1), 33-43. DOI: 10.1016/0165-022x(95)00046-t
81. Kuznetsova, N.; Leikin, S., Does the triple helical domain of type I collagen encode molecular recognition and fiber assembly while telopeptides serve as catalytic domains? Effect of proteolytic cleavage on fibrillogenesis and on collagen-collagen interaction in fibers. *J. Biol. Chem.* **1999**, 274 (51), 36083-36088. DOI: 10.1074/jbc.274.51.36083

Figures

Figure 1. Overall design of the experiments on Col I remodeling by stem cells.

Figure 2. Morphology of ADSCS adhering on native (A-C) and oxidized (E-G) FITC-Col I viewed together with the underlining substratum (green). A and E present the overall morphology of cells adhering on native and oxidized collagen, respectively. B and D present the underlining substrates only of the same samples with artificially superimposed cell contours, while on C and G cell shapes are omitted. D and H show the plane substrates of both native and oxidized collagen, respectively, where no cell were added. The yellow arrows point the typical fibrillary collagen arrangements beneath the cells. The white arrows show the arrangements outside the cells along the cell's periphery. Bar 20 μm .

Figure 3 De-quenching of FITC-Col I in the presence of CH collagenase for 1h at 37° C.

Figure 4. Relative changes in the fluorescence intensity presented as ΔRPU upon addition of FITC-Col (blue) or oxidized FITC-Col I-OXI (green) to a solution of Collagenase CH as substrates for proteolytic digestion at time 0, 60 and 120 min at 37° C.

Figure 5. Relative changes in the fluorescence values of substratum associated (A) and spontaneously released (B) FITC-Col I after 24 h of incubation of samples with cells versus without cells. The data are presented as ΔRPU characterizing the proteolytic de-quenching caused by the cells. The effect of oxidation is evident comparing the ΔRPU for native FITC-Col I (left columns blue) and oxidized FITC-Col I (right columns, green).

Figure 6. DSC thermograms of native FITC-Col I (blue line) and oxidized FITC-Col I-OXI (red line).