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Heparan sulfate affects elastin deposition in fibroblasts cultured from donors of different ages.

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ABSTRACT

Heparan sulfate (HS), due to its presence on the cell surface and in the extracellular milieu and its ability to modulate cell signaling, has a fundamental role in both physiological and pathological conditions. Since decades we have demonstrated the occurrence of interactions between glycosaminoglycans and elastic fibers. In particular, we have recently shown that HS is present inside elastic fibers and plays a role in the assembly and stability of elastin coacervates. Since elastin represents, within the extracellular matrix, the component most severely affected during ageing, and changes in the synthesis and post-translational modifications of HS have been described, possibly influencing cellular behavior and protein interactions, the present study has investigated, in two different *in vitro* experimental models, the role of HS on elastin deposition and assembly. Results demonstrate that:1) biological effects of HS are partly dependent on the physico-chemical characteristics of the GAGs; 2) HS does not affect attachment, viability and growth of human dermal fibroblasts; 3) HS does not modify elastin gene expression nor elastin synthesis, but favors alpha-elastin aggregation and, independently from the age of donors, elastin assembly; 4) HS significantly increases the expression of fibulin 5 and these effects are especially evident in fibroblasts isolated from ageing donors. These data provide a better understanding of the biological role of HS and put forward new perspectives on the possibility to restore and/or preserve the elastic component with ageing.

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INTRODUCTION

Heparan sulfate (HS) is a glycosaminoglycan (GAG) composed of alternating units of hexuronic acid and glucosamine, which is variously sulfate-substituted at different positions. Proteoglycans carrying HS chains are ubiquitously expressed at the cell surface and in the extracellular matrix and, due to their highly negative charges, interact with numerous proteins, including growth factors and extracellular-matrix proteins [1]. The pleiotropic role of HS sustains the increased interest in this GAG and the hope to generate HS-based glycotherapeutics [2]. HS has been shown to be involved in a variety of patho-physiological processes, including angiogenesis, ageing, inflammation and cancer, by regulating cell survival, division, adhesion, migration and differentiation [3] and by interacting with various protein domains as in the case of amyloid deposition [4] and tropelastin assembly [5-7]. We have previously demonstrated that isolated HS chains interact with recombinant tropoelastin and with peptides encoded by specific exons (EDPs) of the human tropoelastin gene, lowering the coacervation temperature and favoring the formation of ordered structures of tropoelastin or of EDPs [5]. Moreover, it has been demonstrated that HS controls fibrillin-1 interactions, possibly modulating tropoelastin binding on the cell surface [8]. Consistently, we have demonstrated that, within connective tissues as in the human dermis, HS containing proteoglycans are associated with the amorphous component of elastic fibers [5], supporting the concept that HS–elastin interactions may play a role in elastin fibrogenesis and/or in elastin stability.

Loss of elasticity is a well known paradigm of ageing connective tissue [9], therefore maintenance of the morpho-functional properties of the extracellular matrix, and of elastic fibers in particular, would represent the gold standard of any approach aiming to preserve the elastic compliance of organs and tissues.

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Aim of the present investigation was to focus on the role of HS on elastin deposition and assembly. For this purpose, a natural HS (n-HS) and a semi-synthetic HS (ss-HS) of different molecular weight were used in two different *in vitro* models (i.e. isolated molecules and cultured fibroblasts). In addition, results have been compared with those obtained using a very-low-molecular-mass heparin (VLMM-H), since all these molecules belong to the same family of GAGs, although their structural diversity, molecular mass and sulfation degree may differently affect GAG-protein interactions and cell behavior [2,10].

EXPERIMENTAL PROCEDURES

Chemicals

All reagents were of analytical grade and suitable for ultrastructural analysis, molecular biology and cell culture.

Alpha elastin (EPC, USA) was obtained by fractionation from soluble elastin and the molecular weight ranges between 10 and 60 kDa.

GAGs were prepared for research use by Opocrin (Modena-Italy) and consisted in a natural HS (n-HS), a semi-synthetic HS (ss-HS) and a Very-Low-Molecular-Mass heparin (VLMM-H) (Table 1). For a detailed characterization of GAGs, molecular weight was assessed by HPLC SEC methods according to E.P.7 (Heparin Low Molecular Mass) for VLMW-H, or to OPOCRIN internal method for n-HS and ss-HS (see Table 1); SO3-/COO- ratio was measured on the basis of potentiometric evaluations [11], whereas Activated Partial Thromboplastin Time (APTT) was calculated using the method described by Basu and coworkers [12].

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Cell cultures

Human skin fibroblasts from 6 adult (36 ± 7 years) and 3 aged (83 ± 2 years) healthy donors, undergoing surgical procedures for traumatic events, were used between the 3rd and 8th passage *in vitro*. Cells were obtained after informed consent, according to the procedures approved by the Ethical Committee of the Medical Faculty of the University of Modena and Reggio Emilia.

Fibroblasts were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (Australian FBS, Gibco, Invitrogen Corporation, NY, USA), as already described [13]. Cells from each donor were kept separate during all experimental procedures.

Cells were treated with the three different GAGs provided by Opocrin (Modena-Italy), as mentioned in the previous paragraph. In a series of preliminary experiments, GAGs were added to cell cultures at the final concentration of 10, 50 and 100 μM in order to find the concentration that was low but, at same time, sufficient to be present in excess in the extracellular milieu, independently from the amounts of GAGs synthesized by cells.

Cell growth and viability

Cells were plated in duplicate into 35 mm culture dishes at a density of 1.5 x 10^5 cells/dish. Every day, cells were observed at the inverted microscope (Leica DM-IL), counted with a Neubauer chamber and, in the remaining dishes, the medium, with and without GAGs, was replaced.

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RNA preparation and quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from fibroblasts using the RNeasy Protect cells Mini kit (Qiagen, Valencia, CA). Quality and quantity of RNA were checked by spectrophotometry and by agarose gel electrophoresis. Total RNA (3 μg) was reverse transcribed using Superscript III (Invitrogen) and Oligo dT_{18} primers (Invitrogen) according to the manufacturer's instructions; conversion was confirmed by PCR with *CLK2* primers. A negative control was carried out to ensure no DNA contamination. Ten fold diluted cDNA samples were further amplified on an iCycler (BioRad) using SYBR® GreenER™qPCR SuperMix (Invitrogen), according to the manufacturer's instructions. For real-time qRT-PCR the following primer sets were used: for elastin (*ELN*) sense 5'-CAGCTAAATACGGTGCTGCTG-3', antisense 5'-AATCCGAAGCCAGGTCTTG-3' with an efficiency of 98.6%; for fibulin 5 (*FBLN5*) sense 5'-CGGCACATACTTCTGCTCCTG-3', antisense 5'-GCTCACATTCGTTGATGTCTTGG-3' with an efficiency of 105%; for CDC-like kinase 2 (*CLK2*) sense 5'-ACCTACAACCTAGAGAAGAAGC-3', antisense 5'-GGCGAGTGGAGACAATGG-3' with an efficiency of 105% (Table 1 Supp). Thermal cycling parameters were set to 50°C for 2 min, 95°C for 3 min, 45 cycles at 95°C for 30 sec, an annealing temperature of 59°C for 30 sec and 72°C for 30 sec, followed by melting curve analysis with a temperature ranging from 95 to 55°C.

In each sample, gene expression was normalized to the housekeeping gene *CLK2* and compared with control samples, using the Pfaffl method [14]. Analyses were performed in triplicate, keeping cell dishes separate.

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Western blot

Confluent fibroblasts were cultured for 48 h in DMEM + 10% FBS in the presence or absence of 10 µM n-HS, ss-HS and VLMM-H.

For Western blots, cells were mechanically detached from plastic dishes. Total cell lysates were analyzed for protein concentration using the Bradford technique [15]. Equal amounts of proteins were then resolved by 10% polyacrylamide gel under reducing conditions. Elastin (ELN) and fibulin 5 (FBLN5) were detected by Western blot using anti-ELN rabbit polyclonal antibody (Abcam, Cambridge, UK) (1/200) and anti-FBLN5 goat polyclonal antibody (Santa Cruz Biotechnology, USA) (1/1000) in blocking buffer (2.5% non-fat milk in TBST). After three washes with TBST, membranes were incubated with suitable secondary antibodies HRP-conjugated (Abcam, Cambridge, UK). Subsequently, Western blots were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to manufacturer's protocols. Densitometric analysis of protein bands was performed using the ImageQuant TL v2005 software (GE Healthcare).

Immunofluorescence

Human dermal fibroblasts, cultured on glass coverslips (Falcon, Becton Dickson, NJ) for 7 days, were fixed in ice cold methanol for 30 min at -20°C. After blocking with 1% bovine serum albumin (BSA) in PBS, cells were incubated for 2 h at room temperature with the following antibodies diluted 1:100 in PBS: rabbit polyclonal anti-elastin (Abcam, Cambridge, UK), goat polyclonal anti-fibulin-5 (Santa Cruz Biotechnology, USA); mouse monoclonal anti-HS (Seikagaku Corp, Japan), rat monoclonal anti-heparan sulfate proteoglycan (Chemicon, USA). After washes with PBS, fibroblasts were incubated with secondary antibodies conjugated with either

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goat anti-rabbit IgG-TRITC (Sigma) diluted 1:200, rabbit anti-goat IgG-FITC (Sigma) diluted 1:100, goat anti-mouse IgG-FITC (Sigma) diluted 1:100, rabbit anti-rat IgG-TRITC (Sigma) diluted 1:100 for 40 min at room temperature. After a final wash, cells were visualized with a Leica TCS SP2 confocal microscope.

Negative staining

Human alpha elastin with a molecular weight range between 10-60 kDa (EPC, USA) was used at a final concentration of 1mg/ml in Tyrode's physiological solution and was examined after incubation at 37°C in the presence or not of n-HS, ss-HS and VLMM-H (Opocrin) added in a GAGs:elastin ratio 1:10. This concentration and the ratio between elastin and GAGs were selected on the basis of previously reported data in order to maximize the effects of glycosaminoglycans on elastin aggregation [5]. However, in a set of experiments, we have tested also at higher and lower HS concentrations (i.e. 1 mg/ml or 50 and 10 μg/ml). Incubations in sterile conditions were performed for 24h up to 7 days at 37°C. At different time points, samples were mounted on copper grids covered with formvar and carbon. Grids were observed by transmission electron microscopy (Jeol JEM 1200EX) after staining with 1% uranyl acetate in water.

Turbidimetry measurements

Coacervation experiments were performed on a Cary UV50 spectrometer equipped with a Peltier temperature controller. In order to use concentrations comparable to those of negative staining experiments, α-elastin was dissolved in 50 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.5 to a final concentration of 1 mg/ml. Temperature was increased from 10 °C, at a rate of 1 °C/min, up to 90 °C and absorbance was

monitored at 440 nm. The coacervation temperature (T_c) was determined as the temperature at 50% maximal absorbance. The effect of HS on coacervation was assessed upon the addition of 10, 50 and 100 µg/ml of n-HS, ss-HS or VLMM-H.

RESULTS

Molecular *in vitro* model: HS and alpha-elastin coacervation and aggregation

In order to preliminary evaluate the direct effect of HS on elastin assembly, experiments were performed by simply adding HSs to a solution of alpha-elastin followed by incubation for few hours up to several days.

As expected, alpha-elastin in physiological solution at 37°C formed structures that, when examined by negative staining electron microscopy, consisted of 4-5 nm thick filaments with tendency to form aggregates as well as bundles (figure 1 A_{1-3}). In the presence of n-HS, alpha-elastin always formed huge and rather compact bundles of filaments mainly organized in a parallel order (figure 1 B_{1-3}). Bundles were also visible after addition of ss-HS, although their shape appeared less linear with an irregular and twisted behavior (figure 1 C_{1-3}), suggesting that single filaments could be not so tightly packed. Addition of VLMM-H gave rise to loose waving fibrils as well as to irregular aggregates (figure 1 D_{1-3}). Once formed, these structures appeared quite stable in solution. The efficiency of HS supplementation on alpha-elastin coacervation was quantitatively evaluated by turbidimetry assay. In these experimental conditions, the coacervation temperature of α-elastin, at the concentration of 1 mg/ml (i.e. comparable to that used in the negative staining experiments), is around 80°C and decreases to approximately 70°C in the presence of 10, 50 and 100µg/ml n-HS (Figure 1 E-G), without marked variations depending on the dose of this GAG (Figure 1E). By contrast, ss-HS lowered the coacervation

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temperature of α-elastin to 79°C, 75°C and 70°C after addition of 10, 50 and 100µg/ml of HS, respectively (Figure 1 F). To be noted that, there were no changes in the coacervation temperature after addition of VLMM-H, at all doses used (Figure 1G).

Since ss-HS had a dose-dependent effect on the coacervation temperature of α-elastin, we investigated by negative staining the morphology of elastin aggregates in the presence of different concentrations of the GAG (Figure 1 H - K). Although, no significant differences were observed depending on the experimental conditions, at higher HS concentrations, bundles appeared slightly more densely packed. As a whole, data suggest that different amounts of ss-HS may influence the coacervation temperature, i.e. the efficiency of the reaction, with minimal consequences on the sovramolecular organization of the aggregates.

In vitro **cell culture model: fibroblast's response to HS treatment**

Cell growth and cell viability

Since elastin deposition depends on cell density as well as on the presence of matrix molecules in the extracellular milieu [16], the possible influence of HS on cell adhesion was tested by adding n-HS, ss-HS as well as VLMM-H at different concentrations (i.e. 10, 50 and 100 µM) to the culture medium at the time of fibroblast plating and cell number was evaluated after 24 hours. No significant differences were observed in comparison with cells plated in the absence of GAGs, between treatments, or depending on donor's age (data not shown).

In a separate set of experiments, the effects of HS on cell proliferation have been assessed upon supplementation of GAGs (at the same concentrations as in the previous experiment) to the culture medium, after 24 hours from plating. Cells were

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counted every day until confluence. At passages used in these experiments, all fibroblast strains, independently from donor's age, exhibited good growth capabilities and no significant changes were observed upon addition of GAGs nor between different treatments (data not shown).

Furthermore, in order to verify if prolonged HS supplementation was associated to cytotoxic effects, GAGs were added to confluent fibroblasts and cell number evaluated during the next 10 days. Significant differences in the number of cells or in their morphology were never observed (data not shown).

HS immunostaining in fibroblasts from adult donors

To confirm that HS supplementation, even at the lower concentration of 10 μM, lead to higher amounts of this GAG on the cellular monolayer, we have immunodetected HS positive epitopes comparing fibroblasts grown in the presence and in the absence of added GAGs. By confocal microscopy, anti-HS antibodies revealed an increased labeling in cell cultures supplemented with 10 μM n-HS (figure 2B), compared to control fibroblasts (figure 2A). Similar results were obtained upon addition of ss-HS (data not shown), suggesting that, even at this very low concentration, HS is abundantly present in the medium and may interact with cells at their surfaces.

Moreover, since all cells have been carefully washed before fixation, the more intense fluorescence observed upon HS supplementation indicate that either added and newly synthesized HS-containing GAGs are tightly associated to cells and to the extracellular matrix formed *in vitro* by fibroblasts.

Effect of HS on elastin and fibulin 5 expression in fibroblasts from adult donors

In cell cultures, deposition of morphologically recognizable elastic aggregates can be

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observed after confluence as a network of interwoven filaments deposited on and among cells (figure 3C). The organization of these aggregates was affected by the type of GAG added to the culture medium (figure 3D-F). In particular, both n-HS (figure 3D) and ss-HS (figure 3E) appeared to favor the formation of the elastin network with thicker and more densely packed filaments. By contrast, VLMM-H induced the formation of more globular structures and a less organized meshwork (figure 3F).

Therefore, similarly to previously described observations (figure 1), HS appear to favor the coalescence of tropoelastin molecules into interwoven strands. In particular, supplementation of n-HS and ss-HS gave rise to similar results, whereas addition of VLMM-H exerted little effects.

In order to discriminate if the elastin network deposited upon addition of n-HS and ss-HS is due for instance to an effect on elastin assembly or on elastin synthesis, Western blot and RT-PCR analyses have been performed on fibroblast cell cultures from adult donors (figure 3A-B).

The addition of n-HS, ss-HS and VLMM-H did not exert any significant change in elastin mRNA expression in comparison to untreated cells (Figure 3A). These results were in agreement with those at protein level, the elastin present in the cellular monolayer being quantified by Western blot (figure 3B).

Data indicate that HSs have negligible effects on both elastin mRNA and protein levels. Therefore, from these *in vitro* experiments, it can be suggested that HSs do not influence tropoelastin synthesis, rather they would seem to favor tropoelastin assembly.

Since fibulin 5 is an important matrix molecule functioning as a scaffold for elastic fibers [17,18] the effect of HS on this protein has been investigated. At mRNA level, a

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significant increase of fibulin 5 gene expression was always evident after treatment with n-HS, whereas ss-HS and VLMM-H only moderately affected the different cell strains (figure 4A). At protein level, either by Western blot (figure 4B) and by immunofluorescence (figure 4C-F) a significant increase of fibulin 5 accumulation was measured after addition of n-HS and ss-HS as well as of VLMM-H, suggesting that accumulation of this protein could be the result of increased mRNA expression and of molecular interactions.

Effect of HS on elastin and fibulin 5 expression in fibroblasts from aged donors

Since elastin and fibulin 5 are known to be negatively affected by ageing, the biological role of HS was investigated also in fibroblasts from donors more than 80 years old. First of all we have investigated if the expression of HS is modified, depending on the age of donors, Figure 5 shows by immunostaining the accumulation of HS in fibroblasts from adult (figure 5A) and old donors (figure 5B). Cultures from aged subjects exhibited a reduced number of positive epitopes scattered on the cell surface. Consistently, also the expression of HS-containing proteoglycans, as perlecan, (figure 5C-D) was significantly reduced in cells from aged donors.

On these premises we have investigated the effects of HS supplementation on the expression of elastin and fibulin 5. As shown in figure 6, fibulin 5 expression at mRNA (figure 6A) and protein (figure 6B) levels were significantly down-regulated in fibroblasts from aged compared to those from adult donors. This trend was completely reversed upon addition of ss-HS and the effects appeared well evident at mRNA and protein levels (figure 6B). Moreover, elastic fiber deposition, as observed by confocal microscopy (figure 6 panels E and F), appeared increased in cell cultures

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treated with ss-HS (figure 6F), in the absence of any significant change in elastin mRNA (figure 6C) and protein (figure 6D) expression, indicating that indirect mechanisms could be responsible for elastin accumulation.

CONCLUSIONS

Since decades it has been demonstrated that glycosaminoglycans (GAGs) are normal constituents of elastic fibers [19], suggesting that they may contribute to deliver tropoelastin molecules from cells to growing elastic fibers [20] modulating the stability of these polymers [21]. In ageing and in pathologic conditions, GAGs and GAGs-containing proteoglycans, similarly to other matrix constituents, go through changes in their expression and post-translational modifications [22,23]. Moreover, they suffer from unbalanced degradative processes, being responsible for altered interactions between matrix proteins and between cells and matrix [24,25], thus contributing to morpho-functional alterations of connective tissues, as observed for instance in photoageing [26] and in aged blood vessels [27].

Due to their negligible turnover, and the consequent susceptibility to endogenous and exogenous noxae, elastic fibers represent, within connective tissues, the component most severely affected by ageing [9,28].

Although heparan sulfates (HS) has been shown to interact with a number of proteins, changing their structural conformation and causing their aggregation, as recently demonstrated in the process of amyloidogenesis [4], we have recently demonstrated that this GAG is able to promote the coacervation of tropoelastin molecules and of specific tropoelastin peptides [5]. However, it has to be elucidated if HS effects on elastin assembly are due only to direct interactions of the GAG with specific elastin domains and/or if they may also elicit changes in cellular behavior.

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Therefore, in the present study, the role of HS on tropoelastin deposition and assembly has been investigated on different *in vitro* experimental models. Moreover, since HS-containing proteoglycans can be degraded *in vivo* to products of various size with distinct physical-chemical and biological properties on cells and on matrix components [1-3], HS of different size and sulfation ratio were assessed.

Results from the molecular i*n vitro* model indicate that the coacervation temperature of alpha-elastin is modified by the presence of both n-HS and ss-HS, although n-HS, even at the lowest concentration, was already very efficient, whereas ss-HS lowered the coacervation temperature in a dose-dependent way, suggesting that structural HS characteristics may have a specific impact on macromolecule interactions. Moreover, morphologic evaluation of elastin aggregates, after incubation at 37°C of the same amount of alpha-elastin as in the coacervation experiments, showed that the presence of HS favored the formation of filamentous structures that appeared more elongated and densely organized after n-HS supplementation. By contrast, the effect of VLMM-H was more heterogeneous in terms of structural organization of elastin aggregates, as it was negligible also in terms of coacervation process activation. These data are in agreement with the hypothesis that: i) HS specifically interacts with tropoelastin [5-7], ii) interactions may occur also in the absence of living cells [29]; iii) interactions seem to depend, at least in part, on the structural characteristics of the HS chain.

Therefore, HSs with longer chains and/or lower sulfation rate seem to be very efficient in favoring tropoelastin assembly. Interestingly, it has been demonstrated that sulfation of HS increases with age [21], suggesting that this modification might have detrimental consequences on connective tissues [27]. Although, further investigations are required in order to discriminate on the precise contribution of

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molecular weight and sulfation characteristics on the rheological properties sustaining HS-elastin interactions, present data highlight the specificity of these interactions, depending not only on tropoelastin sequences, as previously demonstrated [5], but also on HS characteristics.

However, the role of HS on elastin fibrillogenesis may also represent the result of changes in cell behavior and in the cellular response to exogenously added HS.

To test this hypothesis, we have set up a series of experiments using human dermal fibroblasts cultured in the presence of the same GAGs used in the molecular *in vitro* model.

First of all, results demonstrate that HS supplementation, differently to what has been described for smooth muscle cells [30], does not appear to interfere with cell adhesion, nor with cell proliferation or cell viability of dermal fibroblasts and therefore it can be excluded that effects on matrix molecules could be the consequence of changes in cell density or cell number. Moreover, data indicate that cells (i.e. smooth muscle cells and fibroblasts) may behave differently, depending on the environment or on their "functional imprinting".

Morphological evaluations of the elastic meshwork formed *in vitro* by dermal fibroblasts, support the positive effects of HS on elastin assembly. Surprisingly, elastin synthesis and expression were not modified by any HS added to the system, suggesting that HSs act in the extracellular compartment favoring elastin accumulation and assembly, without up-regulating mRNA nor protein expression, consistently with data from molecular *in vitro* data. However, in a cell culture system, other matrix molecules, beside elastin and HS, may influence the formation/organization of the elastin network.

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As recently demonstrated for fibrillin-1 [8], GAG supplementation may influence synthesis and interactions of various molecules within elastic fibers. One possible candidate could be fibulin 5, a protein known to bind to tropoelastin promoting its coacervation [31] and inducing elastic fiber assembly [17,18]. We have recently provided *in vitro* evidence that elastin secretion/deposition in the extracellular space undergoes an age-dependent decline irrespective of the level of mRNA expression or of the amount of protein detectable by Western blot in the cellular monolayer [32] and that reduced FBLN5 expression may be responsible for the low deposition of elastin aggregates in cultures of *in vitro* aged fibroblasts, as well as in fibroblasts isolated from old donors [32]. Moreover, in the present study we have provided evidence that in fibroblasts cultured from ageing donors there is a marked reduction of HS as well as of HS containing proteoglycans, consistently with the modified properties of the aged cellular and extracellular environment [23-24].

The stimulatory effects of HS supplementation on fibulin 5 expression, clearly evident also in fibroblasts isolated from ageing donors, may contribute to the increased elastin staining observed on the cell surface upon HS treatment, even in the absence of changes in elastin mRNA and protein expression in the cellular monolayer.

To be noted that although n-HS was the most effective at mRNA levels, also ss-HS and VLMW-H induced fibulin 5 protein accumulation at similar values. These data indicate that, at least in part, HS-mediated effects could be due to interactions with "heparin-binding domains", and that HS glycosaminoglycans might exert similar modulatory properties on fibulin 5 expression and accumulation [10].

Since fibulin 5 is a well known ageing marker [32,33], being significantly down-regulated with ageing, it is of great interest the possibility that HS is capable to reinduce the synthesis of this protein, thus contributing to the elastin accumulation we have

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observed in the extracellular matrix of fibroblasts from ageing donors. Although *in vivo* experiments are necessary to fully evaluate the potential role of HS as a rejuvenation compound by investigating the effects of the GAG on the formation and functional properties of mature crosslinked elastic fibers, never the less, present data highlight the pleiotropic role of HS, also in the ageing context.

Moreover, in accordance with previous data [32,34], in order to have a structurally organized elastic component, it is necessary the coordinated expression, deposition and assembly of elastin as well as of other molecules as fibulin 5 and GAGs with appropriate size and physical-chemical characteristics. This concept is of paramount importance *in vivo*, where changes in the characteristics of matrix components, occurring for instance with age, have profound consequences on functional parameters related to stiffness, tissue elasticity and compliance.

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No competing financial interests exist.

REFERENCES

1. Dreyfuss JL, Regatieri CV, Jarrouge TR, Cavalheiro RP, Sampaio LO, Nader HB. Heparan sulfate proteoglycans: structure, protein interactions and cell signaling. An Acad Bras Cienc 2009; 81: 409-429.

2. Coombe DR, Kett WC. Heparan sulfate-protein interactions: therapeutic potential through structure-function insights. Cell Mol Life Sci 2005; 62: 410-424.

3. Nadanaka S, Kitagawa H. Heparan sulphate biosynthesis and disease. J Biochem 2008;144: 7-14.

4. Motamedi-Shad N, Monsellier E, Chiti F. Amyloid formation by the model protein muscle acylphosphatase is accelerated by heparin and heparan sulphate through a scaffolding-based mechanism. J Biochem 2009; 146: 805-814.

5. Gheduzzi D, Guerra D, Bochicchio B, Pepe A, Tamburro AM, Quaglino D, Mithieux S, Weiss AS, Pasquali-Ronchetti I. Heparan sulphate interacts with tropoelastin, with some tropoelastin peptides and is present in human dermis elastic fibers. Matrix Biol 2005; 24: 15-25.

6. Tu Y, Weiss AS. Transient tropoelastin nanoparticles are early-stage intermediates in the coacervation of human tropoelastin whose aggregation is facilitated by heparan sulfate and heparin decasaccharides. Matrix Biol 2010; 29: 152-159.

7. Broekelmann TJ, Kozel BA, Ishibashi H, Werneck CC, Keeley FW, Zhang L, Mecham RP. Tropoelastin interacts with cell-surface glycosaminoglycans via its COOH-terminal domain. J Biol Chem 2005; 280: 40939-40947.

8. Cain SA, Baldwin AK, Mahalingam Y, Raynal B, Jowitt TA, Shuttleworth CA, Couchman JR, Kielty CM. Heparan sulfate regulates fibrillin-1 N- and C-terminal interactions. J Biol Chem 2008; 283: 27017-27027.

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9. Robert L, Robert AM, Fülöp T. Rapid increase in human life expectancy: will it soon be limited by the ageing of elastin? Biogerontology 2008; 9: 119-133.

10. Casu B, Naggi A, Torri G. Heparin-derived heparan sulfate mimics to modulate heparan sulfate-protein interaction in inflammation and cancer. Matrix Biol 2010; 29: 442-52.

11 Mascellani G, Rasconi A, Brugnoli E, Bianchini P. Potentiometry applied to the characterization and analysis of polysaccharides for pharmaceutical use. Farmaco Prat. 1988; 43:165-75.

12 Basu D, Gallus A, Hirsh J, Cade J. A prospective study of the value of monitoring heparin treatment with the activated partial thromboplastin time. N Engl J Med. 1972 ; 287:324-7.

13. Quaglino D, Boraldi F, Barbieri D, Croce A, Tiozzo R, Pasquali-Ronchetti I. Abnormal phenotype of in vitro dermal fibroblasts from patients with Pseudoxanthoma elasticum (PXE). Biochim Biophys Acta 2000; 1501: 51-62.

14. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29: 2002-2007.

15 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976, 72: 248-54.

16. Mecham RP, Lange G, Madaras J, Starcher B. Elastin synthesis by ligamentum nuchae fibroblasts: effects of culture c onditions and extracellular matrix on elastin production. J Cell Biol 1981; 90: 332-8.

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17. Yanagisawa H, Davis EC, Starcher BC, Ouchi T, Yanagisawa M, Richardson JA, Olson EN. Fibulin-5 is an elastin-binding protein essential for elastic fibre development in vivo. Nature 2002; 415: 168-171.

18. Nakamura T, Lozano PR, Ikeda Y, Iwanaga Y, Hinek A, Minamisawa S, Cheng CF, Kobuke K, Dalton N, Takada Y, Tashiro K, Ross JrJ, Honjo T, Chien KR. Fibulin-5/DANCE is essential for elastogenesis in vivo. Nature 2002; 415: 171-175.

19. Baccarani-Contri M, Vincenzi D, Cicchetti F, Mori G, Pasquali-Ronchetti I. Immunocytochemical localization of proteoglycans within normal elastin fibers. Eur J Cell Biol 1990; 53: 305-312.

20. Pasquali-Ronchetti I, Bressan GM, Fornieri C, Baccarani-Contri M, Castellani I, Volpin D. Elastin fiber-associated glycosaminoglycans in beta-aminopropionitrile-induced lathyrism. Exp Mol Pathol 1984; 40: 235-245.

21. Bartold PM, Boyd RR, Page RC. Proteoglycans synthesized by gingival fibroblasts derived from human donors of different ages. J Cell Physiol 1986; 126: 37-46.

22. Wu WJ, Vrhovski B, Weiss AS. Glycosaminoglycans mediate the coacervation of human tropoelastin through dominant charge interactions involving lysine side chains. J Biol Chem 1999; 274: 21719-217.

23. Carrino DA, Sorrell JM, Caplan AI. Age-related changes in the proteoglycans of human skin. Arch Biochem Biophys 2000; 373: 91-101.

24. Carrino DA, Onnerfjord P, Sandy JD, Cs-Szabo G, Scott PG, Sorrell JM, Heinegård D, Caplan AI. Age-related changes in the proteoglycans of human skin. Specific cleavage of decorin to yield a major catabolic fragment in adult skin. J Biol Chem 2003; 278: 17566-72.

For Peer Review

25. Hornebeck W, Maquart F. Proteolyzed matrix as a template for the regulation of tumor progression. Biomed. Pharmacother 2003; 57: 223-230.

26. Iriyama S, Matsunaga Y, Takahashi K, Matsuzaki K, Kumagai N, Amano S. Activation of heparanase by ultraviolet B irradiation leads to functional loss of basement membrane at the dermal-epidermal junction in human skin. Arch Dermatol Res 2011; 303:253-61

27. Feyzi E, Saldeen T, Larsson E, Lindahl U, Salmivirta M. Age-dependent modulation of heparan sulfate structure and function. J Biol Chem 1998; 273: 13395-8.

28. Pasquali-Ronchetti I, Baccarani-Contri M. Elastic fiber during development and ageing. Microsc Res Tech 1997; 38: 428-435.

29. Kozel BA, Ciliberto CH, Mecham RP. Deposition of tropoelastin into the extracellular matrix requires a competent elastic fiber scaffold but not live cells. Matrix Biol 2004; 23: 23-34.

30. Kruse R, Merten M, Yoshida K, Schmidt A, Völker W, Buddecke E. Cholesterol-dependent changes of glycosaminoglycan pattern in human aorta. Basic Res Cardiol 1996; 91: 344-52.

31. Hirai M, Ohbayashi T, Horiguchi M, Okawa K, Hagiwara A, Chien KR, Kita T, Nakamura T. Fibulin-5/DANCE has an elastogenic organizer activity that is abrogated by proteolytic cleavage in vivo. J Cell Biol 2007; 176: 1061-1071.

32. Boraldi F, Annovi G, Tiozzo R, Sommer P, Quaglino D. Comparison of ex vivo and in vitro human fibroblast ageing models. Mech Ageing Dev 2010; 131: 625-35.

33. Kadoya K, Sasaki T, Kostka G, Timpl R, Matsuzaki K, Kumagai N, Sakai LY, Nishiyama T, Amano S. Fibulin-5 deposition in human skin: decrease with ageing

and ultraviolet B exposure and increase in solar elastosis. Br J Dermatol 2005; 153: 607-612.

34. Jacob MP, Sauvage M, Osborne-Pellegrin M. Regulation of elastin synthesis. J Soc Biol 2001; 195: 131-141.

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Table 1: Glycosaminoglycans' characteristics

	n-HS	ss-HS	VLMW-H
Preparation	Natural heparan sulphate extracted from horse spleen according to OPOCRIN patent WO8904328	Semi-synthetic heparan sulphate obtained from heparin, by N-desulfatation followed by N-acetylation according to OPOCRIN patent EP 0557887 B1, examples 1-2	Very Low Molecular Mass Heparin obtained by peroxyradicalic depolymerization of heparin according to OPOCRIN patent US4,933,326
Mw (kD)	29,5	10,3	3,1
SO_3^- / COO^-	0,8	1,4	2,3
Spec. rotation [a^{20}_D]	+ 74°	+ 44°	+ 43°
Anticoagulant activity U/mg (APTT test)	< 1	5	6

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LEGEND TO FIGURES

Figure 1. Transmission electron microscopy of negatively stained alfa-elastin aggregates after 24h incubation at 37°C in the absence of glycosaminoglycans (A_{1-3}) and in the presence of n-HS (B_{1-3}), ss-HS (C_{1-3}) and VLMM-H (D_{1-3}) at a GAG:elastin ratio of 1:10. Since GAGs-elastin interactions may lead to different types of aggregates, three representative images are provided for each experimental condition. Panels E-G reported coacervation experiments using alpha elastin (αE) at the concentration of 1mg/ml and n-HS, ss-HS and VLMM-H at 10, 50 100 μg/ml. Only n-HS and ss-HS appeared to reduce the coacervation temperature. Panels H-K illustrate the aggregates formed upon addition of 10 (H), 50 (I), 100 (J) and 1000 (K) μg/m of ss-HS to alpha-elastin. Bar 1μm

Figure 2. Confocal microscopy showing the immunostaining for heparan sulfate in human dermal fibroblasts grown in the absence (A) or in the presence of 10 μM n-HS (B). Bar: 10 μm

Figure 3. Elastin expression in fibroblasts cultured for 7 days in presence or in absence of 10μM n-HS, ss-HS and VLMM-H. Elastin mRNA (A) and protein (B) expression have been evaluated by RT-PCR and Western blot, respectively. Data are expressed as mean values of two experiments performed in triplicate with all different fibroblasts cell lines and indicate the fold increase of treated compared to control cells set at one. In panel B, a representative Western blot is also shown. Confocal microscopy (C-F) showing the immunostaining for elastin in untreated fibroblast cell culture (C) and upon addition of n-HS (D), ss-HS (E) and VLMM-H (F).

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In the presence of n-HS (D) and ss-HS (E) a meshwork made of thick and densely packed aggregates can be observed, while addition of VLMM-H (F) causes elastin deposition in thinner and sometimes globular aggregates. Bar: 10 μm

Figure 4. Fibulin 5 expression in fibroblasts cultured for 7 days in presence or in absence of 10μM n-HS, ss-HS and VLMM-H. Fibulin 5 mRNA (A) and protein (B) expression have been evaluated by RT-PCR and Western blot, respectively. Data are expressed as mean values of two experiments performed in triplicate with all different fibroblasts cell lines and indicate the fold increase of treated compared to control cells set at one. In panel B, a representative Western blot is also shown. *$p<0.05$ vs control. Confocal microscopy (C-F) showing the accumulation of fibulin-5 in untreated fibroblast cell culture (C) and upon addition of n-HS (D), ss-HS (E) and VLMM-H (F). The positive effect of HS supplementation is clearly visible. Bar: 10 μm

Figure 5. Confocal microscopy showing the immunostaining for heparan sulfate (A-B) and for HS containing proteoglycans (C-D) in human dermal fibroblasts grown from adult (A,C) and old (B,D) donors. Bar: 10 μm

Figure 6. Fibulin 5 (A-B) and elastin (C-D) expression in fibroblasts from adult (C_{adl}) and old (C_{old}-ss-HS) donors. ss-HS supplementation on fibroblasts from aging donors (C_{old} +ss-HS) is ineffective on elastin expression, but significantly increases fibulin 5 expression at protein (B) and mRNA (A) levels. Data are expressed as mean values of two experiments performed with all different fibroblast strains and indicate the fold increase of treated and untreated C_{old} cells compared to fibroblasts from adult donors (C_{adl}). Confocal microscopy (E-F) showing the elastin network on cells from old

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donors untreated (E) and treated with ss-HS (F). *p<0.05 C_{old} vs C_{adl}; # p<0.05 C_{old}-ss-HS vs C_{old}+ss-HS. Bar: 10 μm.

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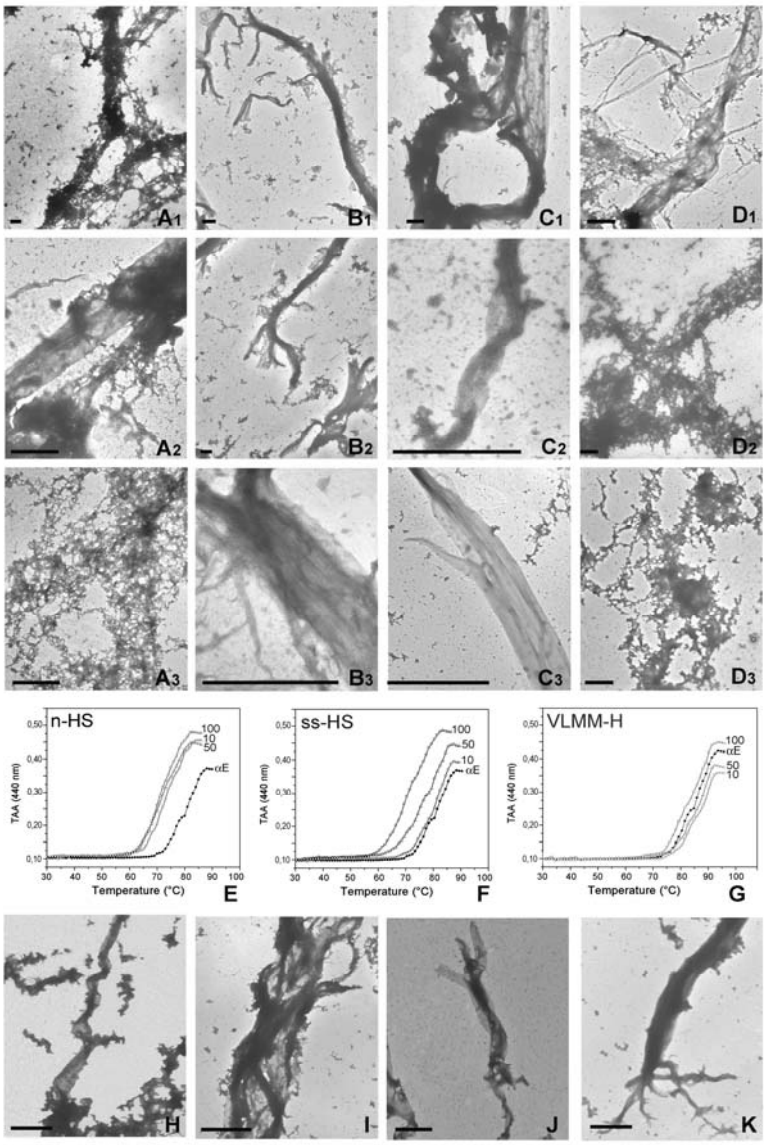

Figure 1
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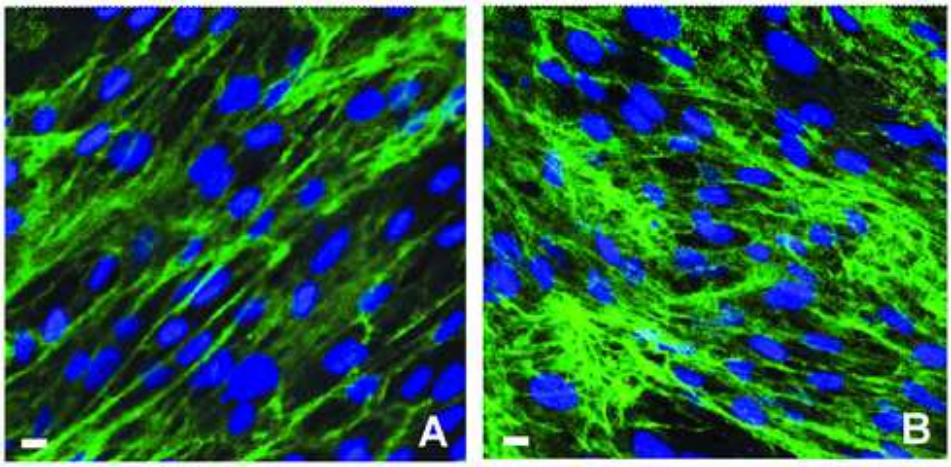

Figure 2
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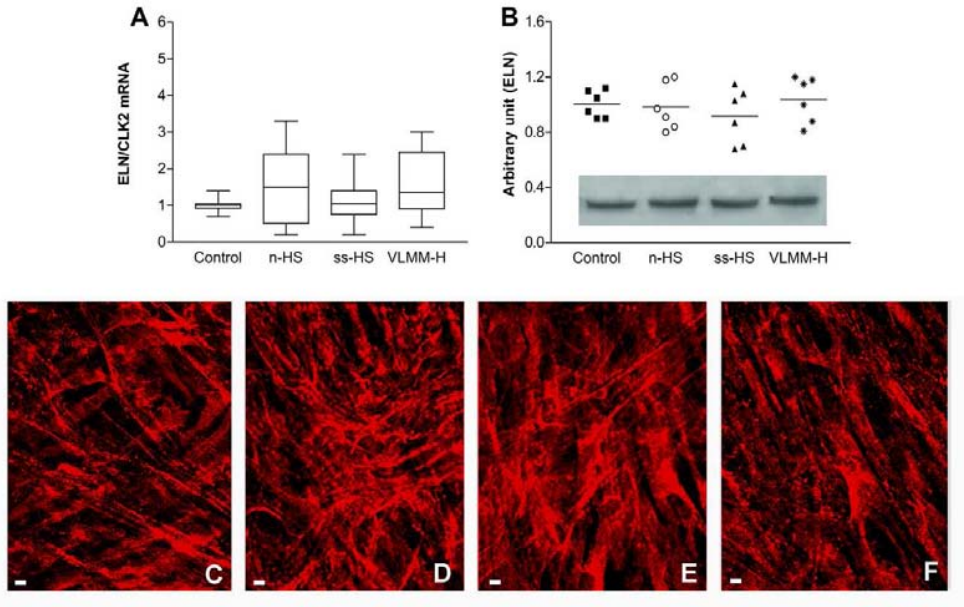

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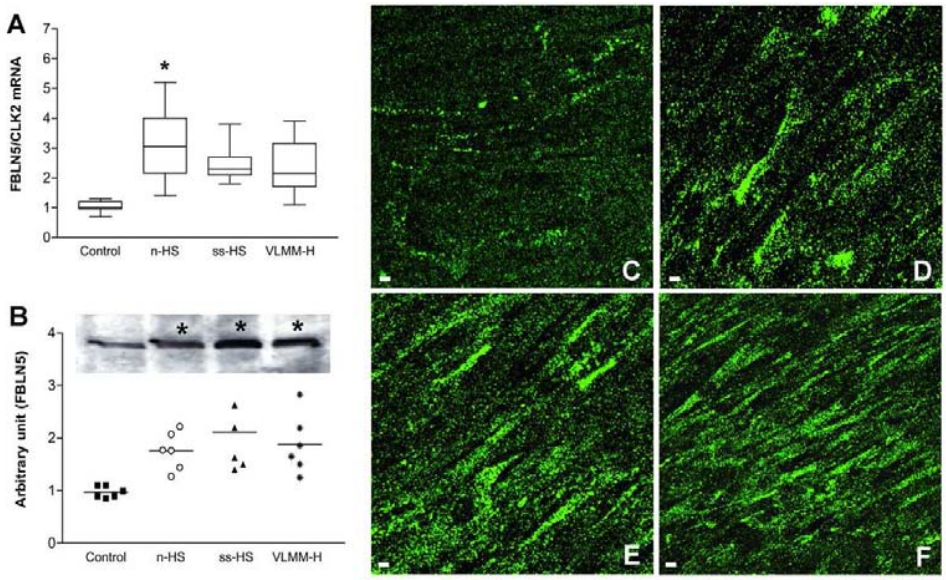

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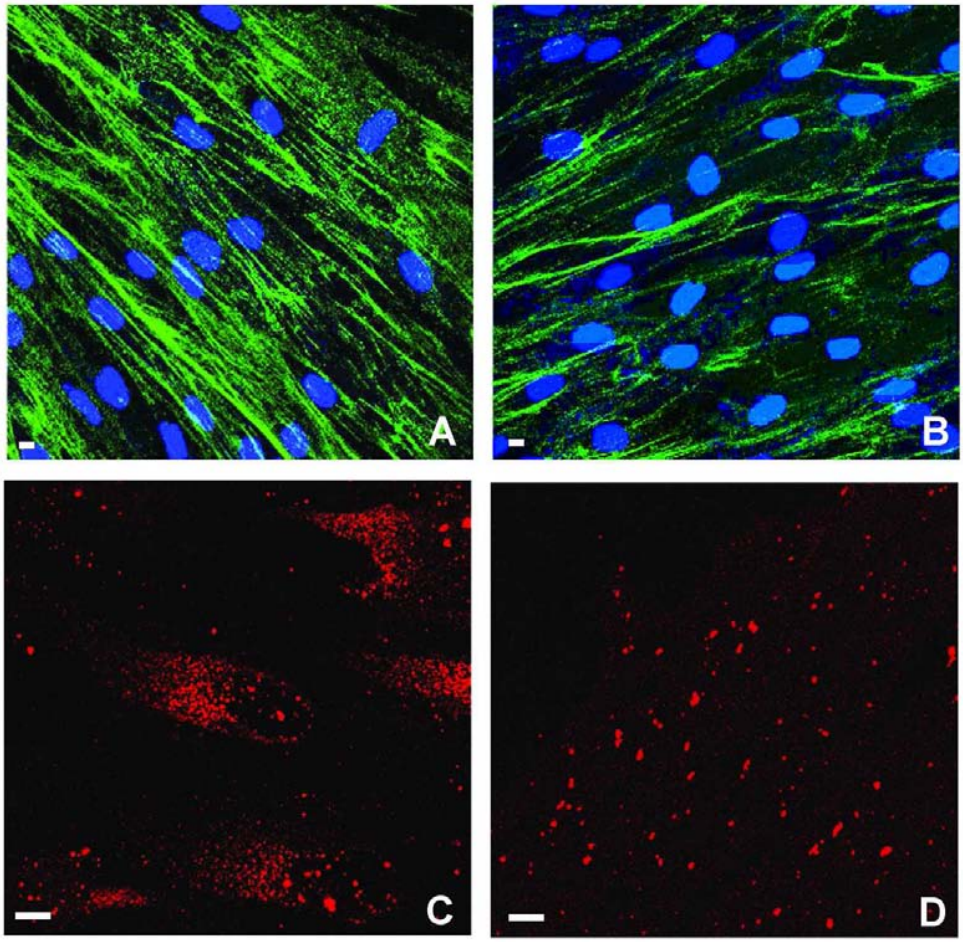

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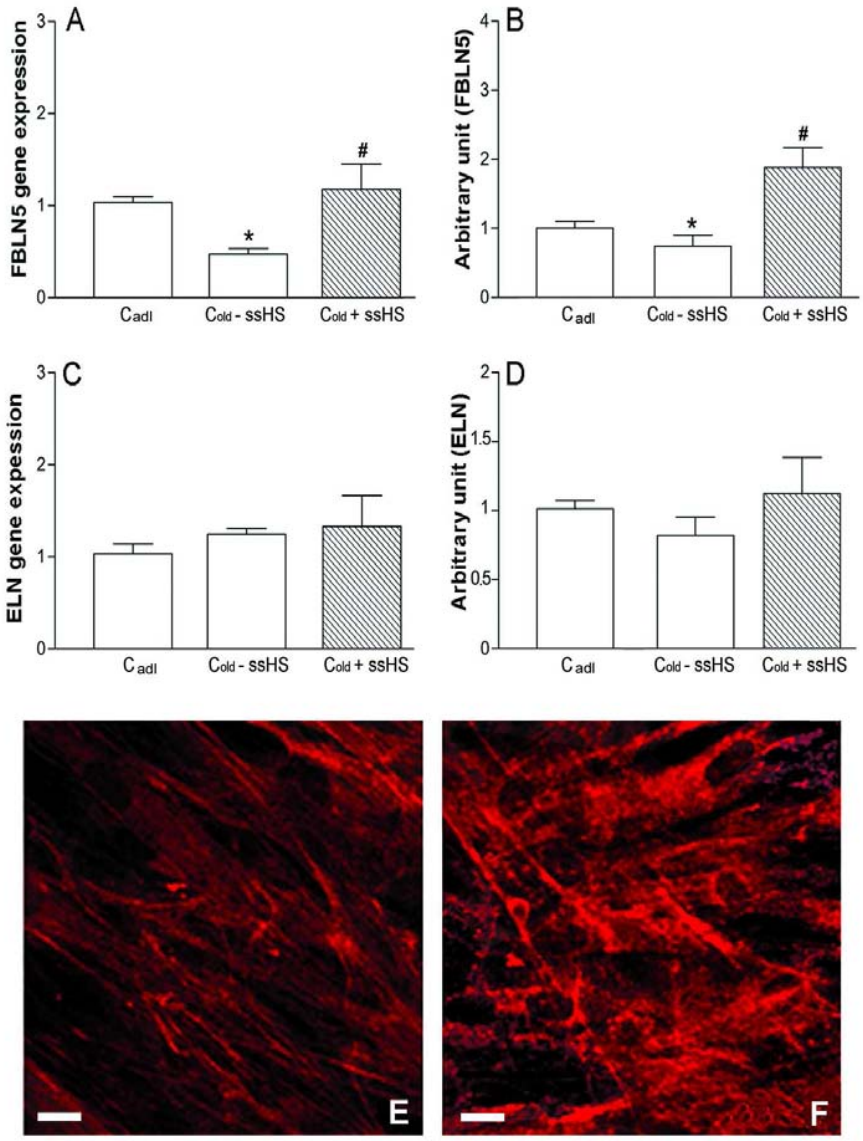

Figure 6
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