Sox9 Regulates Extracellular Matrix Stiffness during Vascular Smooth Muscle Cell ageing by increased secretion of LH3 via extracellular vesicles.

Maria Faleeva[1], Sadia Ahmad[1], Steven Lynham[2,] Meredith Whitehead[1], Susan Cox[3], Catherine M Shanahan[1*].

[1] School of Cardiovascular and Metabolic Medicine & Sciences, King's College London, London, UK
[2] Proteomics Facility, Centre of Excellence for Mass Spectrometry, King's College London, UK
[3] Randall Centre for Cell & Molecular Biophysics, Faculty of Life Sciences & Medicine, King's College London, London, UK

Corresponding Author:
Professor Catherine Shanahan
School of Cardiovascular and Metabolic Medicine & Sciences,
King's College London,
London,
SE5 9NU
UK

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Abstract:

Objective:
The differentiation of vascular smooth muscle cells (VSMCs) into an osteo-chondrogenic phenotype and consequent vascular calcification and increased extracellular matrix (ECM) stiffness are hallmarks of vascular ageing. Sox9 (SRY-Box Transcription Factor 9) is a major regulator of chondrogenesis that is also expressed in the vasculature during development and disease. Here, we investigated the relationship between VSMC ageing and Sox9-driven ECM regulation in VSMCs *in vitro* and *in vivo*.

Approach and Results:
We found that Sox9 expression in the aortic media was not associated with vascular calcification but strongly correlated with the senescence marker p16. Analysis of Sox9 expression *in vitro* showed that it was not a direct regulator of VSMC senescence but was highly mechanosensitive exclusively in senescent VSMCs, showing increased expression on stiff matrices. Manipulation of Sox9 via overexpression and depletion in VSMCs revealed that Sox9 regulates both ECM stiffness and organisation by orchestrating changes in collagen expression and reducing VSMC contractility. Importantly these ECM changes promoted VSMC phenotypic modulation such that senescent cells plated onto ECM synthesized from senescent cells depleted of Sox9 returned to a proliferative state and reduced p16 and p21 expression, as well as decreased the senescence associated secretory phenotype (SASP) marker IL6. We identified procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (LH3) as a novel Sox9 target, and key regulator of Sox9-driven ECM stiffness during VSMC senescence. LH3 is packaged into extracellular vesicles (EVs) and secreted into the ECM and we found that Sox9 also increases EV secretion, leading to increased LH3 deposition within the ECM.

Conclusions:
These findings identify Sox9 as a key driver of increased ECM stiffness during VSMC ageing through upregulation of LH3 deposition via increased EV release. They also highlight a crucial role for ECM structure and composition in regulating VSMC phenotype and identify a novel cycle where cellular senescence and increased ECM stiffening promote Sox9 expression which acts to further enhance ECM modifications thus accelerating vascular stiffening, ageing and senescence.

Introduction:

Vascular calcification is a life-threatening pathology caused by the abnormal deposition of calcium salts in the vessel wall(1). It occurs in both the vessel media and intima (1)(2) and contributes to the development of atherosclerosis (3), as well as arterial stiffening (1). Vascular calcification is a cell-mediated process and both forms are associated with modulation of vascular smooth muscle cells (VSMCs) to an osteo/chondrogenic phenotype. This is characterised by loss of contractile markers such as smooth muscle protein 22-alpha (SM22-α) and alpha- smooth muscle actin (α-SMA) (1)(2)(3) and increased expression of key transcription factors (TFs) associated with developmental osteo/chondrogenesis (4) including Runx2 and Sox9. This phenotype is also associated with increased extracellular vesicle (EV) release (5).

The transition of the VSMCs from their contractile to osteo/chondrogenic phenotype has been tightly linked with cellular senescence. Senescent VSMCs are increased in the calcified vessel wall and calcify more readily *in vitro* (6). Senescent cells are also pro-inflammatory, expressing a senescence-associated secretory phenotype (SASP), and secreting a large volume of cytokines, chemokines and interleukins (7), as well as EVs (8). EVs are involved in physiological and pathophysiological processes, mediating intracellular communication but also forming the nidus for mineralization. During senescence, there is a change in cargo loading of EVs, resulting in an increased abundance of calcification-promoting factors, including annexin A2, A6, and bone morphogenic protein 2 (BMP2) (9) (10). Upon secretion, these EVs also have the potential to modulate the phenotype of the neighbouring VSMCs and attenuate their contractile state (11).

Senescent cells also cause changes within the extracellular matrix (ECM), changing its composition, deposition, and structure. In the ageing aorta there is a decrease in elastin deposition and an increase in its fragmentation, increased collagen deposition and an increase in the crosslinking of collagen fibres with some of these changes also implicated in promoting calcification(12)(13). ECM proteins also play a role in extracellular cell signalling, as well as a role in maintaining the integrity of the vasculature. Interestingly, most of the changes in ECM have been attributed to endothelial dysfunction, with VSMC de-differentiation largely considered a reactive response to ECM changes, rather than an active contributor to these alterations (12).

Runx2 and Sox9 are master regulators of bone and cartilage differentiation and drive expression of matrisomal and other proteins required to form and promote, or not, the mineralisation of these specialist tissues. While the role of Runx2 in activating the expression of osteogenic genes during vascular calcification and ageing has been well characterized (14)(15), less is known about the expression and role of Sox9. Interestingly, Sox9 plays a key role during vascular development. In sclerotomal progenitor cells Sox9 is vital for cell fate specification between VSMCs and chondrocytes. Sox9 expression must be silenced via Notch signalling (16) to allow for the de-repression of myocardin, a key TF regulating expression of smooth muscle marker genes such as SM22-α and α-SMA (17) to enable VSMC differentiation. Maintenance of Sox9 expression facilitates the expression of numerous ECM genes such as Collagen2, 9, and 11 essential for chondrocyte differentiation (18) (19). Vascular injury and ageing can prompt a decrease of Notch signalling (20) and re-expression of Sox9 (16) (21). In the human vasculature Sox9 expression has been shown to increase in aged and calcified aortic tissue (22). A study in an arteriosclerosis mouse model, revealed a role for Sox9 in ECM-reorganization through its upregulation and co-localisation with Collagen 2 (21). *In vitro,* Sox9 expression has been demonstrated to induce VSMC de-differentiation by decreasing expression of contractile genes (21) (23). It has also been shown to increase VSMC

calcification (21), possibly via the regulation of proteoglycan 4 (PRG4) (24) in atherosclerotic mouse models. However, its role in the human vasculature has been largely overlooked.

In this study we explored the role of Sox9 in vascular ageing and calcification. We show that Sox9 expression in the vasculature correlates with VSMC senescence and demonstrate that Sox9 is mechanosensitive in aged VSMCs and a critical mediator of ECM stiffness through its activation of the collagen modifier LH3 and its role in driving its secretion in EVs.

Results:

Sox9 expression in the vessel media correlates with loss of contractile markers and ageing but not calcification.

To investigate the role of Sox9 *in vivo*, we performed immunohistochemistry (IHC) on human aortic tissue samples from young and old patients with and without vascular calcification. α-SMA and CD68, a marker of macrophage infiltration, were used to delineate the cell types present in the samples. Nuclear Sox9 expression was detectable in the intima, adventitia, and media of the vessel wall predominantly in VSMCs (Fig 1). Elevated expression of Sox9 was associated with decreased α-SMA expression with a strong negative correlation in the vessel media (Fig 1a,b), consistent with the role of Sox9 as a negative regulator of contractile gene expression. As expected, there was a strong correlation between patient age and calcification, and age and expression of p16 a marker of cell senescence (Fig1c-e). However, we found no association between Sox9 expression and calcification or patient age (Fig 1f,g). This was consistent with observations in the vessel media where some non-calcified areas displayed high levels of Sox9, whilst areas of high calcification were negative for Sox9 staining. However, we did find a correlation between Sox9 and the senescence marker p16, in the medial aortic layer (Fig 1h). p16 also showed a negative correlation with medial α-SMA consistent with VSMC phenotypic modulation during ageing (Fig 1i,j). IHC of Sox9 and p16 in serial sections revealed that all Sox9 positively stained nuclei also stained positively for p16, and typically occurred in an area of the media where vascular remodelling was occurring. This suggests that senescent VSMCs may be providing an environment that promotes elevated Sox9 expression.

Sox9 is mechanosensitive during VSMC senescence:
The association between p16 and Sox9 *in vivo* led us to investigate whether Sox9 is increased during cellular senescence. We cultured primary human VSMCs to replicative senescence using serial passaging and found that senescent VSMCs showed increased expression of Sox9 at the RNA level, but not at the protein level (Fig2a-c). Immunofluorescence (IF) however showed that there was more nuclear expression of Sox9 in senescent VSMCs compared to early passage counterparts(Fig 2d-e). To investigate whether Sox9 can promote VSMC senescence, Sox9 was overexpressed in young VSMCs using adenoviral transduction (Sox9 OE). This resulted in a decrease in contractile markers such as α-SMA at both the gene and protein level consistent with its role in regulating VSMC differentiation (S Fig1a-c). Overexpression of Sox9 increased the percentage of cells showing senescence associated β-galactosidase staining (S Fig 1d,e) however, minimal changes in p16 gene expression were observed with Sox9 overexpression. In the reverse experiment Sox9 depletion (Sox9 KO) in senescent cells had no effect on expression of senescence markers (S Fig 1f,g) suggesting that there is a complex relationship between sox9 and senescence and that other factors are likely to be involved in Sox9 regulation during VSMC ageing.

In chondrocytes Sox9 is mechanosensitive and shows increased expression in stiff environments (25). As vascular ageing and senescence are associated with increased cell and ECM stiffness, we next tested whether Sox9 might be mechanosensitive in VSMCs. Early passage (hereafter termed 'young' cells) and senescent VSMCs were plated onto hydrogels of different stiffness (Fig 2f-j). While young VSMCs showed no changes in Sox9 gene expression, senescent VSMCs showed markedly increased expression on stiff surfaces at both the gene and protein level (Fig2 f-i). Moreover, IF showed that when plated on soft matrices, Sox9 is diffusely localized in the cytoplasm, and this was most pronounced in young cells with senescent cells already showing nuclear

localisation. When plated on stiff matrices, Sox9 translocated into the nucleus in both young and senescent cells, indicating a potential increase in its transcriptional activity (Fig 2j). This was confirmed using RT-qPCR for Sox5 and Sox6, two transcriptional targets of Sox9, that also increased their expression on stiff surfaces (S Fig1 h-k). Western blotting revealed a second band of higher molecular weight for Sox9 present only on the stiff surfaces in both young and senescent VSMCs, which might be attributed to a posttranslational modification acting to target Sox9 to the nucleus (26).

Sox9 regulates VSMC extracellular matrix organisation and stiffness.

Sox9 has been shown to regulate an array of extracellular matrix (ECM) related genes in chondrocytes (19) and mouse VSMCs (21). Thus, we next wondered whether Sox9 may regulate ECM changes during vascular ageing. To examine the effects of ageing on matrix stiffness, ECM was synthesized from young and senescent VSMCs, the cells removed and the stiffness of the native ECM measured by atomic force microscopy (AFM). This revealed an increase in stiffness of ECM produced by senescent VSMCs compared to that synthesized by young cells (Fig3a). We next analysed the ECM by immunofluorescence (Fig 3b). Fibronectin staining revealed that ECM synthesized by young cells showed an organised fibrillar pattern. In contrast the senescent ECM showed a woven, matted appearance with disorganized fibers. Mapping the topography of the fluorescent signal of fibronectin also revealed that the senescent ECM adopts a flat appearance compared to the peaks and valleys seen in the young ECM.

To investigate the role of Sox9 in ECM stiffness and fibre organisation, we overexpressed or depleted Sox9 in young and senescent VSMCs respectively and analysed the resulting ECM (Fig 3). We found that Sox9 overexpression led to an increase in ECM stiffness and a decrease in the meshwork fibre alignment, creating an ECM resembling that produced by senescent VSMC. Its depletion in senescent cells resulted in decreased stiffness and more parallel fibres reminiscent of those observed in young ECM(Fig 3c,d). To understand the mechanism behind this, we tested whether Sox9 regulates fibre alignment through focal adhesions (FA), which anchor cells to the ECM. Overexpression of Sox9 resulted in a reduction of vinculin, a marker for FAs. In addition, FAs were only present around the perimeter of the cell membrane, like the FA localisation in senescent VSMCs (Fig 3e). Importantly, we could replicate the disorganised fibre alignment found in senescent VSMCs by treating young VSMCs with a ROCK inhibitor to reduce cellular contractility (Fig 3f). These findings suggest that Sox9 plays a crucial role in regulating ECM stiffness and fibre organisation in part via regulation of cellular contractility and FA localisation.

Sox9 Regulated ECM regulates the VSMC Phenotype:

We next investigated whether the Sox9-modulated ECM could influence VSMC phenotype and senescence. To do this, we again synthesized ECM from young and senescent VSMCs with Sox9 overexpression or depletion, respectively. We then replated fresh young or senescent VSMCs on top of the decellularized ECM (Fig 4a). We found that young VSMCs plated on ECM synthesized from young VSMCs overexpressing Sox9 had increased expression of p16 and p21, as well as elevated IL6 an inflammatory SASP marker compared to cells plated on EGFP control young ECM. Senescent cells plated on matrices synthesised from senescent VSMCs depleted of Sox9 exhibited a downregulation of both p16 and p21, as well as IL6, consistent with a reduction in senescent indices in these cells (Fig 4b-d). Cell proliferation, measured via Edu incorporation, and DNA damage

analysis were consistent with the changes in senescent markers observed in the cells. Young VSMCs plated on young Sox9 overexpressing ECM had reduced cell proliferation and an increased number of DNA double-strand breaks shown by γH2Ax foci. Conversely, senescent cells plated on senescent Sox9 knockout ECM were able to re-enter the cell cycle as shown by increased Edu incorporation without an increase in DNA damage (Fig 4 e-g). Furthermore, we observed that the orientation of the VSMCs was highly dependent on the ECM. Both young and senescent VSMCs when plated on young control and senescent Sox9 knockout matrices exhibited parallel orientation in line with the fibronectin fibres. VSMCs plated on young Sox9 overexpression and senescent control ECM exhibited random orientation, frequently aligning at perpendicular angles with each other (Fig 4h). This change in cell orientation is likely due to a lack of fibronectin fibre definition and organization. To rule out that the changes in gene expression observed were not due to changes in matrix stiffness we performed RT-PCR on hydrogels of different stiffness for p16, p21 and IL6. None were mechanosensitive (S Fig 2a-f) while expression of the mechanosensitive proteins, α-SMA and SM22-a, did not show any changes in response to Sox9 mediated ECM modulation (S Fig 2i-l). This suggests that it is ECM composition that is driving these changes in gene expression.

Sox9 Regulates Protein Ratios Deposited in the ECM:

To gain insights into the specific components of the ECM that are directly regulated by Sox9, we next conducted Mass spectrometry analysis on young and senescent decellularized matrix with Sox9 overexpression and Sox9 knockout, respectively (Fig 5). Principle component analysis (PCA) (S. Fig 3a) and differentially expressed protein (DEP) clustering showed young Sox9 overexpression and senescent control ECM, and senescent Sox9 knockout and young control ECM, to a lesser extent, were closely related (Fig5a). We found that Sox9 overexpression resulted in a decrease in ECM-related proteins and an increase in proteins associated with extracellular exosomes and cytoplasmic proteins, while Sox9 knockout resulted in an increase in ECM related proteins and a decrease in exosome and cytoplasmic components (Fig5b). Volcano plots highlighted Col15a1 as having the highest negative log fold change in both the senescent control ECM and the in the young Sox overexpression as compared to the young control ECM(Fig 5d). The young Sox9 overexpression ECM contained a high number of upregulated Annexin (Anx) proteins, which are frequently associated with EV cargo. The senescent Sox9 knockout ECM exhibited a strong upregulation of Lysyl Oxidase Like 4 (LOXL4) a key modulator of collagen and elastin cross-links (Fig 5c).

Analysis of the differentially expressed matrisomal proteins in a protein- protein interaction (PPI) network revealed that the most downregulated proteins in the young Sox9 overexpression ECM were glycoproteins and collagens. Additionally, we observed a decrease in proteins such as Elastin microfibril interfacer 1 (EMILIN1) and Fibulin1/2 (FBLN1/2), which are associated with elastic fibre assembly and deposition, as well as basement membrane proteins such as Collagen 4a1-2 (Col4a1-2) and Nidogen 1/2(NID1/2), which were validated via RT-PCR and Immunofluorescence (S Fig 3b-e). Upregulated matrisomal proteins were involved with ECM remodelling such as Cathepsin C (CTSC) and Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3 (*Plod3*/LH3). Conversely, ECM synthesized from senescent VSMCs depleted of Sox9 showed an upregulation of collagens and basement membrane proteins and a downregulation of Annexins and other ECM remodelling proteins (Figure 5f). We did not see any changes in key chondrocyte Sox9 targets such as collagen 2 and 11.

Sox9 regulates the posttranslational modification of collagen fibrils via LH3:
Most interestingly, we observed that Sox9 upregulated collagen modifiers within the ECM that could potentially be responsible for the changes in ECM stiffness. *Plod3*/LH3, a protein with dual cross-linking and glycating properties, stood out as one of the few proteins that was upregulated in the young Sox9 overexpression ECM (Fig 6). To validate the changes in LH3 in response to Sox9, we overexpressed and depleted Sox9 in young and senescent VSMCs and examined LH3 expression using RT-PCR and Western blot. Results showed that *Plod3*/LH was upregulated in young Sox9 overexpressing VSMCs and downregulated in senescent Sox9 knockout VSMCs as compared to the young and senescent controls, respectively (Fig 6a-c). IF showed that LH3 was consistently localised to the Golgi apparatus with no change in localisation either with senescence or Sox9 expression (S Fig 4a). However, examination of the decellularized ECM using IF showed that LH3 deposition was markedly increased in the young Sox9 overexpression and senescent control conditions and decreased in senescent Sox9 knockout ECM (Fig 6 d-g). LH3 also showed a distinctive pattern change in the Sox9 overexpression and senescent control ECM, adopting an increased fibrillar deposition pattern (Fig 6g). This suggested that increased deposition of LH3 into the matrix, rather than increased intracellular protein synthesis, maybe involved in ECM modulation.

We next tested whether LH3 plays a role in Sox9-driven ECM stiffness, *Plod3* was depleted using RNAi in young Sox9 overexpressing and in senescent control VSMCs (S Fig 4b). AFM stiffness measurements revealed a marked reduction of ECM stiffness in matrices that were synthesised from young VSMCs treated with Sox9 overexpression adenovirus in combination with siPlod3 as compared to the young Sox9 overexpression ECM. There was also no difference in stiffness when compared to the young control ECM (Fig 7e). Knocking out Plod3 in senescent VSMCs resulted in a reduction of ECM stiffness as compared to the senescent control but displayed no change in stiffness as compared to the Sox9 knockout ECM (Fig 7f). This demonstrated that LH3 is a primary target driving Sox9-dependent ECM stiffness in ageing.

To validate our findings *in vivo*, we again conducted IHC on aortic patient tissue samples testing for LH3 and Sox9 (Fig 7). We found a correlation between elevated expression of LH3 and Sox9 in patient tissue samples expressing high levels of p16 (Fig 7a-c). Furthermore, LH3 deposition increases with patient's age, showing a higher deposition in patients over the age of 40 (Fig 7d).

LH3 deposition into the ECM is regulated via extracellular vesicles:
EVs carry cargo from the cytosol that deposit within the extracellular space (9). The proteomics dataset showed that the upregulated proteins deposited in the ECM with Sox9 overexpression and senescence were related to EVs (Fig 8a). Sub-classifying the proteins revealed that several of them were related to the regulation of intracellular transport, such as early endosome antigen 1 (EEA1) and Ras-related protein Rab21 (Rab21) (Fig 8b). As LH3 has been shown to be trafficked into the ECM in Collagen IV carriers (27), we hypothesized that EVs may drive the increased deposition of LH3 into the ECM in VSMC senescence. To validate the changes in EV secretion and deposition in response to Sox9 overexpression and VSMC senescence we a CD63 bead capture assay and IF for the EV marker CD63 to quantify exosome secretion (Fig 8c) and deposition into the ECM respectively (Fig 8d,e). These assays showed that Sox9 overexpression, as well as VSMC senescence, increased EV secretion and deposition, while Sox9 knockout resulted in the opposite results decreasing EV secretion and deposition. Using differential centrifugation to isolate different EV populations, we showed that LH3 was detectable in all EV subtypes including apoptotic bodies

(Abs), micro-vesicles (miEV) and small vesicles (sEVs) in both young and senescent VSMCs (Fig 8f), with a trend to increased LH3 loading into sEVs in senescent cells (S Fig5). To investigate the role of sEVs in the secretion of LH3, we inhibited EV secretion using 3-O-methylsphingomyelin (3-OMS) in both young and senescent VSMCs during ECM synthesis. We observed a reduction in LH3 deposition into the ECM, further validating its trafficking into the ECM via EVs. These findings support the notion that Sox9 drives increased release of EVs into the ECM during senescence, ultimately leading to the enhanced deposition of LH3 and the modulation of ECM mechanics.

Discussion:

This study explored the role and regulation of Sox9 in vascular ageing pathology. We show that Sox9 in VSMCs plays a crucial role in driving the senescent ECM phenotype. In aortic patient tissue samples, Sox9 expression correlated with the senescence marker p16 and reduced expression of α-SMA. Importantly, we showed that Sox9 is mechanosensitive in senescent VSMCs. Its expression increased with ECM stiffness and acted to drive further ECM stiffening and fibre misalignment by reducing cellular contractility. Sox9 also regulates ECM composition by downregulating collagen deposition and upregulating the collagen modifier LH3 and these ECM modifications act to accelerate VSMC senescence. For the first time we demonstrate that LH3, is upregulated in aged patient tissue samples, and plays a vital role in the regulation of ECM stiffness via its secretion in EVs.

Sox9 is a key regulator of VSMC phenotype during ageing

Our results have provided further confirmation of previous studies that highlight the role of Sox9 in regulating VSMC contractility (17) (21). We observed a negative correlation between Sox9 and α-SMA in *in vivo* human aortic tissue samples, as well as *in vitro* via Sox9 overexpression. However, our results produced contrasting results to previous studies looking at the interplay between Sox9 and vascular calcification (21). We found no correlation between samples showing high levels of medial calcification and Sox9 expression *in vivo*, and we verified this *in vitro* by showing no changes in calcification propensity between control and Sox9 overexpressing VSMCs (data not shown). We propose the differences in findings may be associated with the origins of cells used in the studies. Previous studies finding a positive correlation between Sox9 expression and calcification were conducted in mouse models, which are genetically susceptible to spontaneous cartilaginous metaplasia that is prevalent in mice and not present in humans (28). Indeed, we found in human VSMCs that Sox9 did not upregulate the chondrocyte markers Collagen 2 and 11 that have been co-localised with Sox9 in mouse aortic tissues (21). Our results are indicative of the probable interplay between Sox9 and Runx2 within the vasculature. Runx2 is a critical regulator of osteoblast differentiation (29) and a driver of vascular calcification(14)(30). It also has a tight interplay with Sox9, with both TF's exerting decreased transcriptional activation of one another (31). Thus, even though calcification and osteo/chondrogenic VSMC differentiation are tightly interconnected, the VSMCs within the microenvironments where calcification occurs potentially have a dominant Runx2 expressing phenotype, as opposed to Sox9. These findings highlight the complexity of the interplay between VSMC de-differentiation and calcification, and the need for further investigation into the regulatory mechanisms underlying these processes.

Instead, in our study, we observed a correlation between Sox9 expression and p16, a marker for cellular senescence, in *in vivo* human aortic tissue samples. However, our *in vitro* findings revealed an increase in Sox9 expression only on the gene level, not on the protein level, in senescent VSMCs. Interestingly, we found that Sox9 expression was mechanosensitive only in senescent VSMCs, with increased expression and transcription levels observed on stiff surfaces. This observation is in line with previous studies that have shown Sox9 to be mechanosensitive in chondrocytes (25). As senescent VSMCs synthesize ECM that is stiffer and with changes in key components of the basement membrane than their young counterparts, our findings suggest that the environment created by senescent VSMCs in the aorta is conducive to increased expression of Sox9. The lack of protein-level changes in Sox9 expression in isolated senescent VSMCs *in vitro* further highlights the

importance of mechanosignalling and extracellular cues that are required for its effective transcription.

Sox9 induced ECM changes influence VSMC ageing and inflammation

We also demonstrated the significant role of Sox9 in regulating ECM fiber alignment, ECM stiffness, and the phenotype of VSMCs. Our findings show that decreased cellular contractility, as a result of Sox9 expression, contributed to ECM fiber alignment, and we provide novel evidence that the Sox9-driven ECM compositional changes can feedback and regulate the phenotype of VSMCs. While previous studies have examined the role of ECM in cell signalling, stem cell differentiation (32), adhesion, and migration (33), the role of ECM in regenerating cells from a pathological state has remained understudied. Replating senescent cells on an ECM synthesized from Sox9-depleted VSMCs enables, to some extent, the rejuvenation of cells with reduction in senescence markers and re-entry back into the cell cycle. We also showed the vital role of ECM alignment in VSMC orientation. Cells plated on fibrous ECMs, such as those synthesized from senescent and young Sox9-overexpressing VSMCs, were unable to form proper alignments with neighbouring cells and fibres. Correct VSMC alignment is crucial in the vasculature, as it affects the mechanical properties of vascular tissues, influencing their ability to withstand and respond appropriately to mechanical stresses and strains (34). Our findings underscore the impact of Sox9-modulated ECM on the phenotype of VSMCs and suggest at its potential role in vascular remodelling.

Our investigation also explored the compositional changes driven by Sox9 that increase ECM stiffness. Previous studies (18)(19) identified Sox9 as a potent activator of numerous ECM proteins in chondrocytes, leading us to anticipate an increase in collagen deposition. However, we found that increased Sox9 expression in VSMCs results in the downregulation of a wide range of collagens and glycoproteins. This observation highlights previous research showing that Sox9 can serve both as an activator and a repressor (35), as well as a regulator of both ubiquitous and chondrocyte-specific genes (36). Our findings suggest that Sox9's transcriptional targets vary significantly depending on the cell type in which it is expressed. In VSMCs, Sox9 acts as a repressor for the transcription of various basement membrane proteins, whose decreased expression can negatively impact both the phenotype of VSMCs (37) and the tensile strength of the vessel wall (38). While previous studies have shown the strong co-localization of Sox9 with chondrocyte-related proteins such as Collagen 2 within the aorta of mice models (21), it is important to note that Sox9 has been linked to fibrotic diseases (39) (40), characterized primarily by an increase in ECM protein deposition. As Sox9 is known to increase overall ECM deposition, there should undoubtedly be an increase in collagen deposition. However, mass-spectrometry analysis and normalization techniques limit us to examining the normalized protein ratios within a sample, precluding any evaluation of the overall amount of starting material. Further work is now required to understand how the complex compositional and structural changes induced by Sox9 contribute to the VSMC rejuvenation in response to ECM changes.

LH3 and EVs as key modulators of ECM stiffening

Our proteomics revealed LH3 as a novel gene regulated by Sox9 in VSMCs. LH3 plays an important role in collagen cross-linking and glycation both intra- (41) and extracellularly (42). However, its function in VSMCs remains largely unexplored. Our results demonstrate that with Sox9

overexpression and cellular senescence, LH3 deposition is increased within the ECM. Furthermore, our *in vivo* IHC showed an increased deposition of LH3 in aged tissues, which correlated strongly with Sox9 and p16 expression levels. Our *in vitro* work demonstrated that Sox9-regulated ECM stiffness is primarily driven by LH3, as its depletion resulted in a reduction of ECM stiffness, similar to the depletion of Sox9. Previous research has shown LH3 to be trafficked into the extracellular space via Collagen IV carriers (27). Our analysis of EV secretion from young and senescent VSMCs also revealed that LH3 is loaded onto all EV types. These findings suggest that the increased deposition of LH3 in the ECM is due to both Sox9-mediated intracellular expression and increased secretion of EVs. This highlights LH3 as a potential therapeutic target for further investigation in cardiovascular pathologies. Further research is also warranted to understand the precise mechanism by which LH3 regulates the stiffness of the senescent VSMC ECM.

In conclusion, our study reveals the crucial role of Sox9 in driving the senescent ECM phenotype by regulating ECM stiffness, fibre alignment, and composition. Our findings suggest that the elevated expression of Sox9 in senescent VSMCs strongly depends on mechanosignalling cues from the extracellular environment. We demonstrate how Sox9 regulates the ECM composition, driving it towards a senescent phenotype by downregulating essential basement membrane proteins and upregulating LH3. The study provides novel evidence that the Sox9-driven ECM changes can feedback and regulate the phenotype of VSMCs as well as highlights the underappreciated role of LH3 as a driver of ECM stiffness. These findings provide new insights into the complex interplay between ageing, cellular senescence, ECM composition, and VSMCs in vascular disease.

Experimental Procedures:

Immunohistochemistry:

All human materials were handled in compliance with the Human Tissue Act (2004, UK) and with ethical approval from the research ethics committee (REC reference: 13/LO/1950). Human aortic tissue samples used are shown on Supplementary Table 1. Immunohistochemistry was performed on 7um thick paraffin-embedded human aortic tissue sections. These sections were deparaffinized, rehydrated prior to heat-mediated antigen retrieval in sodium citrate buffer. Endogenous peroxidase activity was quenched by 3% H2O2, in methanol and non-specific binding blocked with 10% serum from the source species for the secondary antibody followed by a primary antibody incubation overnight at 4°C (Sox9, abcam, ab185966,; p16, (abcam, ab241543), α-SMA (Sigma-Aldrich' A5691), LH3 (Proteintech, 1:100). Biotinylated secondary antibody was added, followed by avidin-biotin complex (ABC) reaction (Vector Labs kits PK-6102 & PK-6010). Staining was developed with 3, 3'-diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories; SK-4100) and these sections were counterstained with Hematoxylin. The slides were mounted with DPX followed by dehydration and images were taken using a Leica ICC50 W Microscope, a minimum of eight images were taken of the tissue sample per aortic region (intima, media, adventitia). Percentage of positively stained nuclei was quantified using ImageJ software, with brown nuclei regarded as positive and blue/purple as negative. Percentage of positive extracellular staining was quantified via threshold detection method on ImageJ.

Cell culture:

VSMCs were isolated from explants of human aortic tissues. Three different donors were used for this study; 35-year old female (04.35F.11A), 38-year old female (03.38F.11A), and 33-year old female (05.33F.5A). Cells were cultured at 37°C and 5% CO_2 in M199 media (Gibco), supplemented with 20% fetal bovine serum and 1% Penicillin-Streptomycin-Glutamine (PSG) (100x). Replicative senescence was induced by passaging the cells until growth arrest, typically occurring between passage 20-25. All experiments were performed using these 3 biological replicates unless stated otherwise. Early passage VSMCs (termed "young VSMCs) were collected at passage 6-13. VSMCs would reach replicative senescence in passages 25-30.

Extracellular matrix synthesis:

Decellularized ECM was synthesized as previously described (43). Plates or cover slides were coated with 0.2% filtered gelatin solution at 37°C for 1 hour, cross-linked with 1% glutaraldehyde for 30 minutes at room temperature, and quenched with 1M ethanolamine for another 30 minutes at room temperature. Cells were seeded at high confluency and cultured in 20% FBS, 1% PSG M199 media supplemented with 50ug/mL L-ascorbate for 9-12 days. The ECM was decellularized by incubating the cells for 5 minutes with the Extraction Buffer (0.1% Triton, 20mM NH4OH, diluted in PBS), then washed three times with PBS to remove cell debris.

Adenoviral Constructs and Transfections:

Sox9 was overexpressed in young VSMC via a recombinant Enhanced Green Fluorescent Protein (EGFP)-CMV-Sox9 (VectorBuilder) and knocked out in senescent VSMC via a recombinant EGFP-SOX9 shRNA (short hairpin RNA against Sox9) adenovirus (Vectorbuilder). Control young and senescent VSMCs were treated with either EGFP (VectorBuilder) or Scramble shRNA control adenovirus (VectorBuilder). Cells were transduced in serum free M199 media containing the adenovirus for 40 minutes, and then diluted in M199 media with 20% FBS and 0.1% PSG for three days. Cells synthesising matrices were re-transduced every 5 days with L-ascorbate

supplementation for a total of 9 days. 100% transduction efficiency was assessed via EGFP fluorescence imaging.

RNA Extraction, Reverse Transcription, and qPCR:
RNA from cultured VSMCs was isolated using RNA STAT-60 phenol-chloroform. cDNA was synthesized using Mu-MLV reverse transcriptase with dNTPS, random and oligo primers, and RNAse inhibitor. qPCR (quantitative PCR) was performed using qPCRBIO SyGreen Mix and run in a StepOnePlus Real Time PCR system. PCRs were performed in 20uL reaction volumes, with the thermocycler program running 95°C for 10 minutes followed by 40 cycles at 95°C for 5 seconds and 60°C for 30 seconds. The following primers used; 18S (QuantiTec Primer Hs-RRN18s), *Sox9* (Sox9, 5'- AGCTCTGGAGACTTCTGAACGAGA -3', and 5' – ACTTGTAATCCGGGTGGTCCTTCT -3'), *PLOD3* (PLOD3, 5'- TCTACACACGGCTCTACC-3' and 5'- ACCACTTCATCTAAAGCCC-3'), *COL4A1* (COL4A1, 5'-GGCAGCCAGGACCTAAA-3', and 5'-CCCATTCCACCAACAGAT-3'), *COL4A2* (COL4A2, 5'-ACAAGTCCTACTGGCTCTCTA-3', and 5'-GGCCTCACACACAGAAC-3', and *NID2 (*NID2, 5'-TTCCTTCTCCTGCCGTTGTC-3', and 5'- TGCTGTTGTTCACAGGGTGT-3'). Expression levels of target genes were normalised to 18S expression values in young VSMC using the 2-ΔΔCt method. Ct values were normalized to those of young or senescent cells plated on plastic in hydrogel and ECM cell re-plating experiments.

Protein Extraction and Western blotting:
ECM and cell lysate was homogenized in RIPA buffer with protease inhibitor cocktail, sonicated on ice, and centrifuged for 10 minutes at 7,500 at 4°C. 50% glycerol, 0.1% SDS, 30% 1M Tris Buffer pH=6.9, Bromophenol blue 6 mM, and 10% 10mM dithiothreitol (DTT) was added to the samples prior to boiling them for 10 minutes at 95°C. Proteins were separated via SDS-PAGE, transferred to Polyvinylidine Fluoride (PVDF) membrane, and blotted with 5% BSA in PBS-Tween (0.1%) (PBS-T) for one hour at room temperature. Membranes were incubated overnight at 4°C with the primary antibodies: Sox9 (Abcam, ab185966, 1:1000), A-SMA (Abcam, ab7818, 1:5000), LH3 (Proteintech, 11027-1AP, 1:1000). IRDye 800CW conjugated secondary antibodies (LI-COR Biosciences) were incubated for one hour at room temperature and visualized using an images (Odyssey; LI-COR Biosciences). Protein bands were quantified through ImageJ, normalized to either GAPDH or beta-actin.

Hydrogel Setup:
Hydrogel recipes measuring 10kPa and 250kPa were made up from 10.75% and 15% acrylamide and 9% and 1.2% bisacrylamide, respectively and stiffness was confirmed using AFM. Glass coverslips were coated with 3-aminopropyl trimethoxysilane (97%) for 2 minutes, washed thoroughly, and fixed for 30 minutes at room temperature with 1% glutaraldehyde with ddH_2O. Coverslips were dried face up overnight. Per each volume of acrylamide solution, $1/100^{th}$ of the volume of APS (10%) and $1/1000^{th}$ of the volume of TEMED was added. The solution was mixed thoroughly and aliquoted out onto glass slides. The fixed coverslips were placed onto the hydrogel solution facedown. The hydrogel solution was allowed to solidify and adhere onto the glass coverslips. The glass coverslips were then inverted, hydrogel slide up, and subjected to ultraviolet light (UV) for 5 minutes with a 2% of sulfa SANPAH solution (Thermo scientific, 22589). The hydrogels were washed several times under sterile conditions and incubated with 3.3% of Rat Tail Collagen 1 (Gibco A10483) solution at 4°C for 2 hours. The collagen solution was then removed and cells were plated onto the hydrogels as normal.

Immunofluorescence:
VSMCs and ECM were fixed in 4% paraformaldehyde in PBS, and permeabilized with 0.5% PF-40. Permeabilization step was omitted in case of ECM staining. Cells were then blocked at room temperature with 3% BSA in PBS, and incubated with the primary antibodies; Fibronectin (ab2413, 1;500), Sox9 (ab185966, 1:100), CD63, (Santa Cruz, s-15,363, 1:200), LH3 (Proteintech, 11027-1-AP, 1:250), Vinculin (Sigma Aldrich,V9264, 1:500), gamma-H2AX (Cell Signaling, 2577,1:200) diluted in the blocking solution overnight at 4°C. Cells were then washed and incubated in the dark with the fluorophore conjugated secondary antibody (Donkey anti-mouse/ rabbit, A10036/ A10040 ,1:400). Cells were incubated with Phalloidin (Sigma Aldrich, 1:400), diluted in PBS, post-secondary antibody staining. The nuclear staining was obtained with DAPI (Sigma Aldrich, D9542) at room temperature. Coverslips were mounted onto slides using Mowiol media and visualized using Z-stacks of ECM and VSMCs was acquired using the Nikon Eclipse Ti Inverted Spinning Disk Confocal System. The images and fluorescence intensity were analysed by calculating the corrected total cell fluorescence (CTCF) on FIJI.

Edu Cell Proliferation:
To measure cell proliferation, VSMCs were depleted from FBS for 24h to restart the cell cycle. Following depletion, cells were incubated with 10uM Click-iT Edu (ThermoFisher, C10337) for 1 hour in 20% FBS, 1% PSG supplemented M199 media. Edu visualization was achieved according to manufacturer protocol prior to any further immunofluorescence staining.

CD63 Beads Assay:
Quantification of CD63 positive sEV secretion was performed by the CD63 bead assay and flow cytometry analysis as previously described (44). $1x10^8$ 4µm aldehyde-sulphate beads (Invitrogen, A37304) were added to filter sterilised 150µl MES buffer (Sigma, 76039). Beads were spun for 10 minutes at 3000g and washed twice more with MES buffer. Beads were incubated with 35µg of CD63 antibodies (BD Biosciences, 556019) overnight at room temperature with shaking. The beads were washed thrice with 4% filter sterilised BSA-PBS and resuspended in storage buffer (0.1% Glycine, 0.1% NaN_3 in PBS). Young and senescent VSMC were plated at a concentration of 15,000 cells per well. The following day they were washed thoroughly to remove residual exosomes and transduced with the control EGFP, Sox9 overexpression and, knockout adenovirus in 2.5% exosome-free FBS M199 media for three days. The media was then collected, spun for 5 minutes at 2500g and transferred to a new 24-well plate. The bead stock solution was washed once with 2% BSA-PBS, with 1µL of bead suspension per each media sample. Beads were incubated in the media overnight at 4°C with gentle rocking. Cells were trypsinized and incubated with Solution13 (ChemoMetec) for a cell viability assay using the NC3000 cell nucleocounter (ChemoMetec). The beads were washed twice with 2% BSA-PBS and incubated with phycoerythrin (PE)-conjugated light sensitive anti-CD81 antibody for 1 hour at room temperature. The beads were quantified using FACs analysis with a BD Accuri C6 flow cytometer. Data analysis was conducted using FlowJo software, with the number of sEVs normalised to the cell number that was calculated during the cell viability assay.

Proteomics and bioinformatic analysis:
Decellularized ECM protein samples were de-glycosylated as previously described (45) Samples were precipitated overnight in acetone at -20°C and the protein pellet airdried in a speed vacuum. They were then incubated with chondroitinase, heparinase, keratanase, and 3 de-branching enzymes +1 O-deglycosidase diluted in the de-glycosylation buffer (Tris 200mM, NaAc 200mM,

EDTA 100mM) at 25°C for two hours and then at 37°C overnight with shaking. Samples were air dried once again and incubated with PNGaseF diluted in H_2O^{18} for 48 hours at 37°C with shaking. Sample purification and trypsin digestion was then undertaken for peptide extraction. Samples were injected to be analyzed by LC-MS/MS in triplicate. Raw mass spectrometry data was processed into peak list files using Proteome Discoverer (ThermoScientific, v2.5) and processed using Sequest (Eng et al; PMID 24226387) search algorithms against the Uniprot Human Taxonomy database with a 1% FDR stringency. The overall intensity value for each peptide was compared across the sample set to further calculate changes in protein abundance.

Differentially expressed proteins were identified using DEP and Limma packages in R (Fold change set to 1, minimum P-value 0.05). David Gene Ontology (GO) was used to identify the categories that the differentially expressed proteins belonged to. Proteins belonging to the classification of "extracellular space", "extracellular region", "basement membrane", and "extracellular matrix" were extracted, pooled together, and further sub-classified in Cytoscape app 3.8.0 with GeneMANIA app. The same process was repeated for all proteins initially classified under "extracellular exosome". Protein-protein interaction maps were built in Cytoscape, with proteins exhibiting minimal interactions with the network being discarded.

Atomic Force Microscopy:

Atomic Force Microscopy (AFM) data acquisition was done on JPK Nanowizard 3. For AFM imaging, a tetrahedral shaped cantilever with a silicone tip with a spring constant of 0.081 M/m, tip height of 4-5µm (HYDRA-6v-200WG, Applied NanoStructures, CA) was used. The matrix stiffness was recorded via Contact Mode, measuring the force spectroscopy, in liquid. The set point was 5nN with a Z-length of 14 microns and extend speed of 10µm/second. The AFM data was processed via the JPK Nanowizard 3 data processing software.

Vesicle Isolation:

EVs were isolated and purified by ultracentrifugation (UC) as previously described (44). VSMCs were washed twice with Earl's balanced salt solution (EBSS) and the media changed to Dulbecco's modified eagle medium (DMEM) supplemented with 0.1% BSA and 1% PSG. After 24h the media was collected and centrifuged at 1000g to pellet apoptotic bodies, at 10,000g to pellet micro-vesicles, and 100,000g by UC to pellet small EVs. Pellets were washed and resuspended in PBS.

Statistical Analysis:

All results are from three different VSMC donor isolates. All results are presented as +/-Standard error of mean (SEM). Statistical analysis was performed in GraphPad software. Normality tests were conducted for all datasets via the Shapiro-Wilk test prior to comparison analysis. Multiple groups were compared via the 1-way ANOVA with Tukey test, whilst two independent groups were compared via the Student *t* test. Simple linear correlation was conducted to determine the p value and R squared fit between two different proteins in histological samples. *p-value< 0.05, **p-value<0.01, ***p-value<0.001, ****p-value<0.0001.

Figure Legends:

Figure 1: Sox9 expression in VSMCs correlates with increased cellular senescence and decreased expression of a-Smooth muscle actin (a-SMA), but not with patient age or calcification. (a) Sox9, a-SMA, and CD68 staining in the medial layer of aorta from human subjects. (b) Sox9 positive nuclei have been correlated with a-SMA. (c) Von Kossa and p16 staining in media. (d&e) Graphs correlating Von Kossa and p16 positive nuclei with patient age. Correlation of Sox9 positive nuclei with (f) calcification, (g) patient age, and (h) p16 positive nuclei. Correlation of a-SMA with (i) p16 positive nuclei and (j) patient age. Red arrows highlight positively stained nuclei for Sox9 and p16, n=16. Correlation was performed using a pearson test. (k) IHC of serial sectioning staining for Sox9 and p16 within the identical area of the media.

Figure 2: Sox9 is mechanosensitive in senescent VSMCs. (a,b,c) Sox9 gene and protein expression in young and senescent VSMCs, quantified by RT-qPCR and Western blotting, n=6. (d,e) Quantification of Corrected Total Cell Fluorescence (CTCF) and immunofluorescence of Sox9 in young and senescent VSMCs, n=18. Unpaired Student's t test, **p-value<0.01, ***p<0.005. Sox9 (f,g) Gene and (h,i) protein expression, quantified by RT-qPCR and western blotting, of young and senescent VSMCs plated on hydrogels of 10kPa, 250kPa stiffness, and plastic coated with collagen (P+C), n=6, n=3, respectively. One-way ANOVA with Tukey post hoc test, *p-value<0.05, ***p<0.005, ****p<0.0001. (j) Immunofluorescence of Sox9 when plated on hydrogels of 10kPa stiffness and P+C in young and senescent VSMCs. Sox9 in grey and green, nuclear DAPI staining in blue.

Figure 3: VSMC senescence and Sox9 expression regulate the ECM. (a) Atomic force microscopy (AFM) measurements of ECM stiffness synthesized from young and senescent VSMCs. Normality rejected via Shapiro-Wilk test, Unpaired Mann-Whitney test, ****p-value<0.0001. Stiffness measurements taken from ECM synthesized from three different donors; 35F, 38F, 33F. (b) Representative fibronectin immunofluorescence staining of young and senescent ECM. Topology of fibronectin staining presented to the right-hand side of the immunofluorescence image. (c) AFM measurements of ECM synthesized from young VSMC transfected with EGFP (Young Ctrl), Sox9 overexpression adenovirus (Sox9 OE), and Sox9 adenoviral knockout (Sox9 KO). Normality rejected via Shapiro-Wilk test, Unpaired Mann-Whitney test, ***p-value<0.005, ****p-value<0.0001. (d) Representative immunofluorescence staining of fibronectin in ECM synthesized from Young Ctrl, Young Sox9 OE, Senescent VSMCs transduced with EGFP (Senescent Ctrl), and Senescent cells with Sox9 knockout (Sox9 KO). (e) Representative immunofluorescent images of vinculin (VCL) in Young Ctrl, Sox9 OE, and Senescent Ctrl. VCL in grey/red, EGFP in green, nuclear staining (DAPI) in blue. (f) Representative immunofluorescence staining of fibronectin in ECM synthesized from young VSMCs with no treatment (Young N/T) and young VSMCs treated with Rock Inhibitor (Young RI).

Figure 4: Composition of Sox9-regulated ECM regulates VSMC phenotype. (a) Schematic of experimental protocol. Young VSMCs were plated on decellularized ECM originally synthesized from young VSMCs transduced either with EGFP (Young Ctrl) or Sox9 overexpression adenovirus (Sox9 OE). Senescent VSMCs were plated on decellularized ECM originally synthesized from senescent VSMCs transduced either with shEGFP (Senescent Ctrl) or Sox9 knockout adenovirus (Sox9 KO). Gene expression, quantified by RT-qPCR, of p16, p21, and IL6 from (b) young and (c) senescent VSMCs plated on transduced ECMs. N=4, Unpaired Student's t-test, *p-value<0.05. (d) Representative IF and quantification (%) of (e) Edu and (f) γH2AX when VSMCs plated on transduced ECMs. Edu, γH2AX, and nuclear DAPI staining in green, magenta, and blue, respectively,

n=20. One-way ANOVA with Tukey post hoc test, *p-value<0.05, ***p-value<0.005, ****p-value<0.0001. (g) Representative IF staining of young and senescent VSMCs when plated on transduced ECMs, with fibronectin (Fn) in red, Phalloidin in magenta, Vinculin (VCL) in green, and nuclear staining (DAPI) in blue. White line indicates relative fibronectin fiber alignment.

Figure 5: Sox9 drives the ECM composition towards a senescent-like phenotype (n= 6 injections for each condition from 35-year-old female (35F) and 38-year-old female (38F)). (a) Heatmap of clustered differentially expressed proteins (DEP), showing the similarly of protein expression between ECM synthesized from young VSMCs treated with control EGFP adenovirus (Young Ctrl) and senescent VSMCs with Sox9 knockout adenovirus (Sox9 KO), and between young VSMCs transduced with Sox9 overexpression adenovirus (Sox9 OE) and senescent VSMCs transduced with control shEGFP adenovirus (senescent Ctrl). (b) Gene ontology (GO) analysis of differentially expressed proteins (DEP) in Sox9 OE ECM as compared to Young Ctrl, and Sox9 KO as compared to Senescent Ctrl. (c) Pie chart and Venn-diagram of downregulated DEP both in the Sox9 OE and Senescent Ctrl ECM. (d) Volcano plots of DEP when comparing Senescent Ctrl against Young Ctrl, Sox9 OE against Young Ctrl, and Sox9 KO against Senescent Ctrl. (f) Protein-protein interaction network showing interactions between significantly upregulated and downregulated proteins in Sox9 OE ECM as compared to the Young Ctrl ECM, and in Sox9 KO ECM as compared to the Senescent Ctrl ECM.

Figure 6: *Plod3*/LH3 in VSMCs expression and deposition into the ECM is regulated by Sox9 expression. (a) Gene expression, quantified via RT-qPCR, of *Plod3* in young VSMCs transduced with EGFP control adenovirus (Young Ctrl), Sox9 overexpression adenovirus (Sox9 OE), and senescent VSMCs transduced with shEGFP control adenovirus (Senescent Ctrl) and Sox9 knockout adenovirus (Sox9 KO), n=5. Quantification and representative Western blot of LH3 protein expression in Young Ctrl, Sox9 OE, Senescent Ctrl, and Sox9 KO VSMCs (b,c) cell lysate, n=5, and (d,e) ECM, n=3. (f,g) Quantification and representative immunofluorescence of fluorescent LH3 signal when deposited into the ECM synthesized from Young Ctrl, Sox9 OE, Senescent Ctrl, and Sox9 KO VSMCs, n= 25. One-way ANOVA with Tukey post hoc test, *p-value<0.05, ***p-value<0.005, ****p-value<0.0001. (h) Atomic force microscopy (AFM) stiffness measurements (kPa) in decellularized ECM synthesized from (e) Young Ctrl, Sox9 Sox9 OE, and Sox9 OE with Plod3 knockout (siPlod3) and (i) Senescent Ctrl, Sox9 KO, and Plod3 knockout (Senescent siPlod3). Measurements were taken from ECM synthesized from three isolates, 35F, 38F, and 33F. Shapiro-Wilk test for normality distribution was rejected. One-way ANOVA Kruskal-Wallis test, ****p-value <0.0001.

Figure 7: LH3 deposition increases in the medial aortic layer with age and Sox9 expression and regulates extracellular matrix (ECM) stiffness. (a) Immunohistochemistry of Sox9, p16, and LH3 staining in the aortic medial layer. The red arrows highlight positive staining of Sox9 and p16. Positive staining of p16, Sox9, and LH3 in brown. Correlation of LH3 positive staining (%) with (b) p16 and (c) Sox9 Positive nuclei. Correlation determined by Spearman's correlation, n=16. (d) Bar chart of positive percentage of LH3 staining in patient samples less than 40 years of age and over 40 years of age, Unpaired Student's t test, *p-value <0.05.

Figure 8: Sox9 regulates LH3 deposition into the ECM via increased extracellular vesicle (EV) secretion. (a) Pie chart and Venn-diagram depicting categories and percentages of upregulated differentially expressed proteins (DEP) found both in the ECM synthesized from senescent VSMCs transduced with EGFP (Senescent Ctrl) and young VSMCs transduced with Sox9 overexpression

adenovirus (Sox9 OE). (b) Protein-protein-interaction of upregulated DEP found in Sox9 OE ECM related to extracellular vesicles through gene ontology. (c) Quantification of exosome secretion in young VSMCs transduced with EGFP control adenovirus (Young Ctrl), Sox9 OE, Senescent Ctrl, and Sox9 knockout adenovirus (Sox9 KO), n=4. (d,e) Quantification and representative IF images of CD63 deposition in the Young Ctrl, Sox9 OE, Senescent Ctrl, and Sox9 KO ECM, n=20. One-way ANOVA with Tukey post hoc test, *p-value<0.05, ***p-value<0.005, ****p-value<0.0001. (f) Western blot of LH3 protein expression in three different types of extracellular vesicles; Apoptotic bodies (AB), micro-vesicles (miV), and small EV (sEV). (g,h) Representative IF images and quantification of LH3 deposition into young and senescent ECM either with or without sEV secretion inhibitor 3-O-methylsphingomyelin (3-OMS), n=10. Two-way ANOVA, *p-value<0.05, **p-value<0.001.

S Table 1: Table describing the age, gender, American Heart Association (AHA) classification of atherosclerotic plaques, and level of calcification within the Medial aortic area. Lack of calcification is denoted by - , whist severity of calcification is graded from + to + + + +.

S Fig1: Sox9 regulates Vascular Smooth Muscle Cell Phenotype. (a) Gene expression, quantified via RT-qPCR of TAGLN, in young VSMCs transfected with control EGFP adenovirus (Young Ctrl) and Sox9 overexpressing adenovirus (Sox9 OE), n=4. (b,c) Quantification of protein expression and Western blot of α-SMA from young Ctrl and Sox9 OE cell lysate samples, n=4 (d,e) Beta galactosidase quantification and representative images of young Ctrl and Sox9 OE VSMCs, n=15. The red arrows highlight positively stained blue cells. Unpaired Student's t test, **p-value<0.005, ****p-value<0.0001. (f) Western blot of Sox9 expression in young Ctrl, Sox9 OE, senescent VSMCs transduced with shEGFP control adenovirus (Senescent Ctrl), and Sox9 knockout adenovirus (Sox9 KO). (g) Gene expression, quantified by RT-qPCR, of p16 in Young Ctrl, Sox9 OE, Senescent Ctrl, and Sox9 KO, n=4. Gene expression, quantified by RT-qPCR, of Sox5 in (h) young and (i) senescent VSMCs plated on matrices of different stiffnesses and plastic coated with collagen (P+C). Gene expression, quantified by RT-qPCR, of Sox8 of (j) young and (k) senescent VSMCs plated on hydrogels of 10kpa and 250kpa, and P+C, n=4. Genes normalized to young and senescent VSMCs plated on plastic (indicated by the dotted line).One-way ANOVA with Tukey post hoc test, *p-value<0.05.

S. Figure 2: Gene expression of p16 in (a) young and (b) senescent VSMCs when plated on hydrogels of stiffness 10kPa and 250kpa, and plastic coated with collagen (P+C). Gene expression, of p21 in (c) young and (d) senescent VSMCs when plated on hydrogels of stiffness 10kPa and 250kpa, and P+C. Gene expression of IL6 in (e) young and (f) senescent VSMCs when plated on hydrogels of stiffness 10kPa and 250kpa, and P+C. Gene expression of α-SMA in (g) young and (h) senescent VSMCs when plated on hydrogels of stiffness 10kPa and 250kpa, and P+C. Gene expression of SM22-*a* in (i) young and (j) senescent VSMCs when plated on hydrogels of stiffness 10kPa and 250kpa, and P+C. N=7, One-way ANOVA with Tukey post hoc test, *p-value<0.05. Gene expression of (k) *a*-SMA and (l) SM22-*a* from young and senescent VSMCs plated on ECM synthesized from young VSMCS treated with EGFP control adenovirus (Young Ctrl), Sox9 overexpressing adenovirus (Sox9 OE), senescent VSMCs treated with shEGFP control adenovirus (Senescent Ctrl), and Sox9 knockout adenovirus (Sox9 KO). N=4, Unpaired Student's t-test. All gene expressions were quantified via RT-qPCR.

S Figure 3: (a) Principal component Analysis (PCA) plot of proteomics samples. Gene expression, quantified via RT-qPCR of (b) Collagen (Col)4a1, (c), Col4a2, and (d) nidogen2 (Nid2) from young

VSMCs transduced with EGFP control adenovirus (Young Ctrl), Sox9 overexpression adenovirus (Sox9 OE), and senescent cells transduced with shEGFP control adenovirus (Senescent Ctrl). N=4, One-way ANOVA with Tukey post hoc test, **p-value<0.01, ***p-value<0.005, p****<0.00001. (e) Immunofluorescence of Collagen 4 (Col IV) in ECM synthesized from Young Ctrl, Sox9 OE, Senescent Ctrl, and senescent VSMCs transduced with Sox9 knockout adenovirus (Sox9 KO).

S Figure 4: (a) Representative immunofluorescence images of LH3 in young VSMCs transduced with EGFP control adenovirus (Young Ctrl), Sox9 overexpression (Sox9 OE), and senescent VSMCs transduced with shEGFP control adenovirus (Senescent Ctrl), and Sox9 knockout (Sox9 KO). LH3 is in grey and red, EGFP in green, and nuclear staining (DAPI) in blue. (b) Western blot depicting LH3 knockout (siPlod3) with Sox9 overexpression (Sox9 OE) and Sox9 knockout (KO) from protein lysate in young and senescent Vascular Smooth Muscle Cells.

S. Figure 5: Protein quantification of LH3 from three types of extracellular vesicles in young and senescent Vascular Smooth muscle cells.

Author Contributions:
MF, SC and CMS contributed to conception; MF, MW, SC and CMS to experimental design; MF and SA to acquisition of data; MF and SA to analysis and interpretation of data. MF and CMS wrote and revised the manuscript and all authors provided final approval of the submitted version.

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Conflict of Interest:
The authors declare that they have no conflicts of interest.

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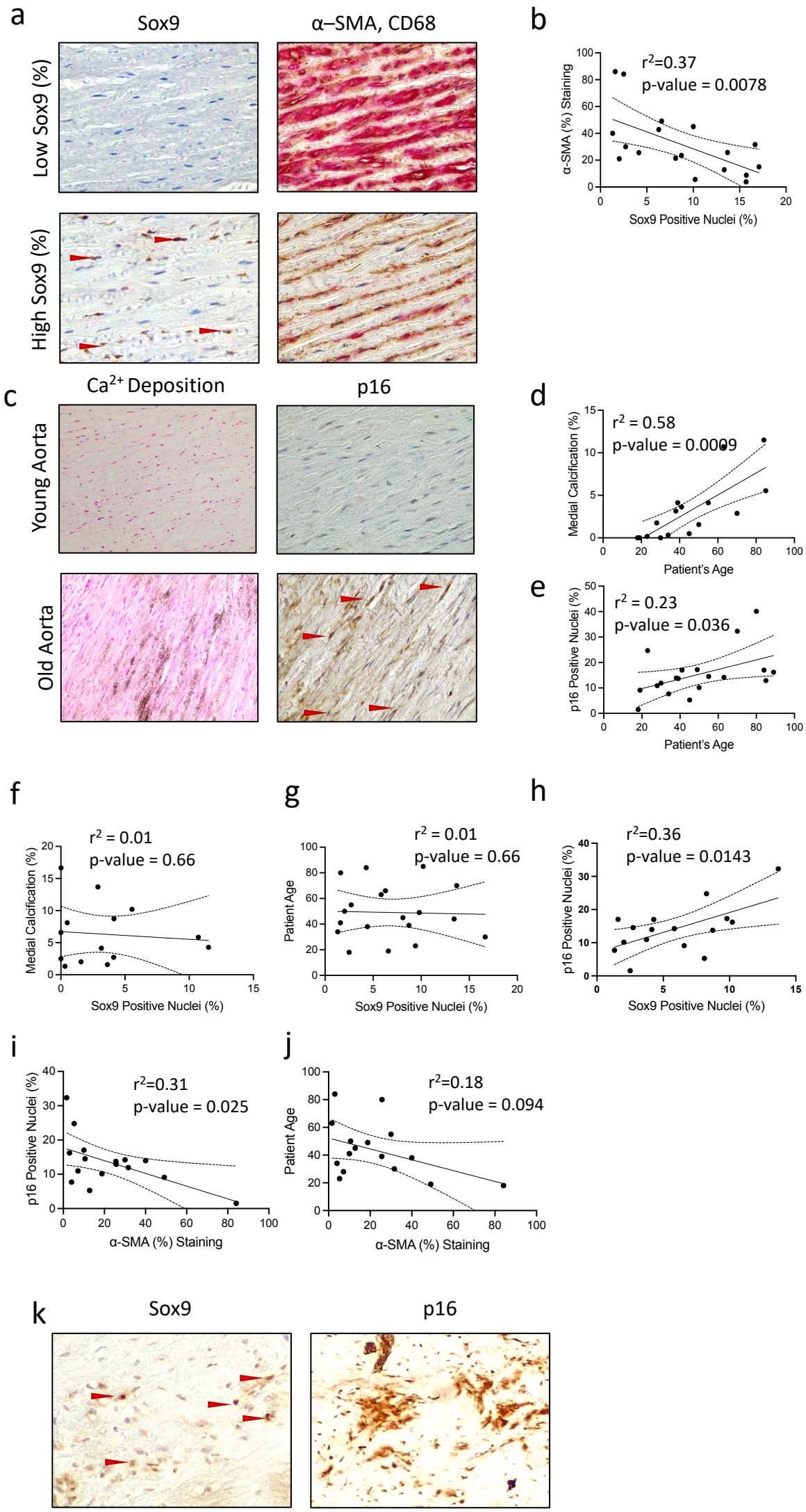


Figure 1: Sox9 expression in VSMCs correlates with increased cellular senescence and decreased expression of a-Smooth muscle actin (a-SMA), but not with patient age or calcification. (a) Sox9, a-SMA, and CD68 staining in the medial layer of aorta from human subjects. (b) Sox9 positive nuclei have been correlated with a-SMA. (c) Von Kossa and p16 staining in media. (d&e) Graphs correlating Von Kossa and p16 positive nuclei with patient age. Correlation of Sox9 positive nuclei with (f) calcification, (g) patient age, and (h) p16 positive nuclei. Correlation of a-SMA with (i) p16 positive nuclei and (j) patient age. Red arrows highlight positively stained nuclei for Sox9 and p16, n=16. Correlation was performed using a pearson test. (k) IHC of serial sectioning staining for Sox9 and p16 within the identical area of the media.

Figure 1

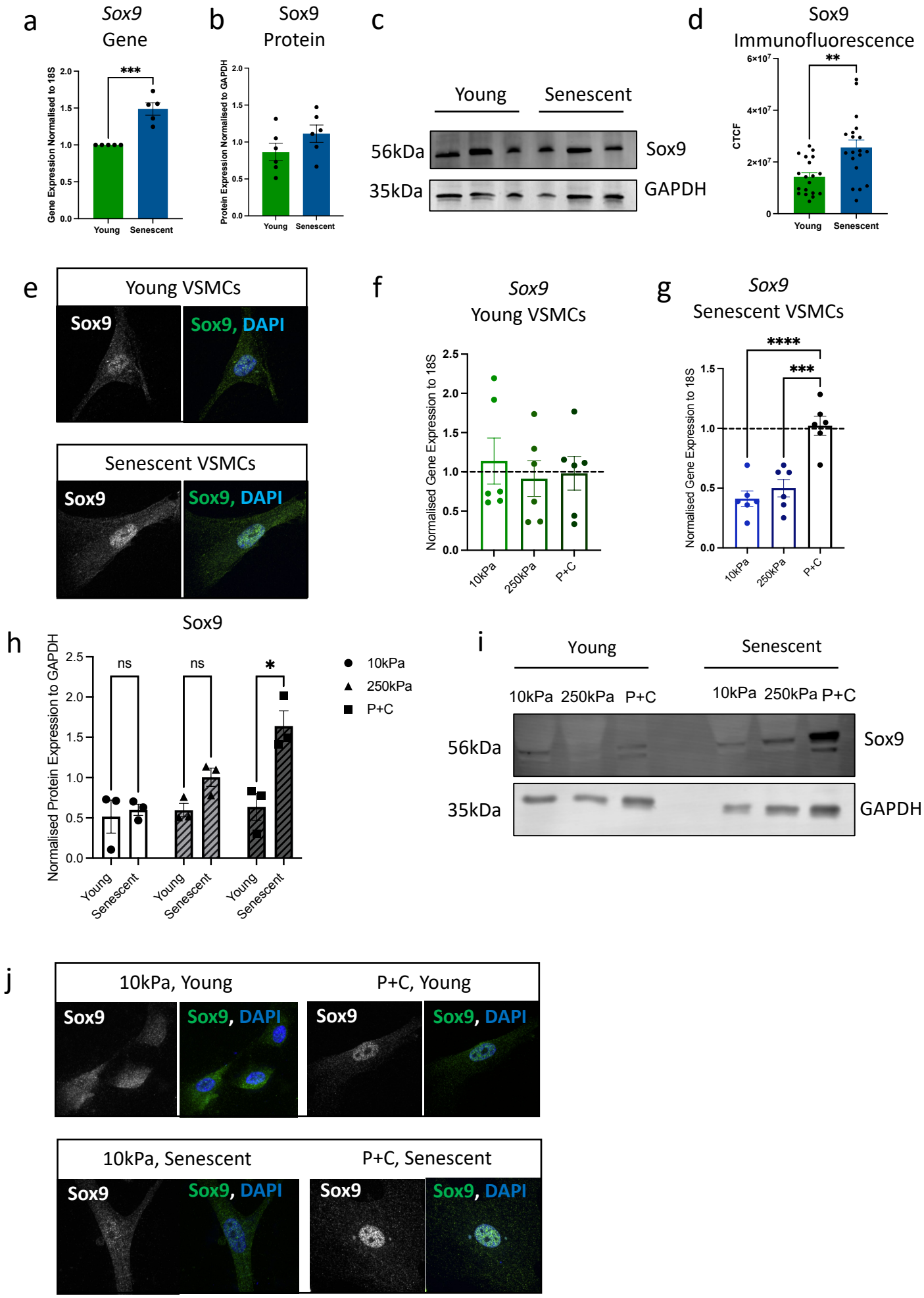


Figure 2: Sox9 is mechanosensitive in senescent VSMCs. (a,b,c) Sox9 gene and protein expression in young and senescent VSMCs, quantified by RT-qPCR and Western blotting, n=6. (d,e) Quantification of Corrected Total Cell Fluorescence (CTCF) and immunofluorescence of Sox9 in young and senescent VSMCs, n=18. Unpaired Student's t test, **p-value<0.01, ***p<0.005. Sox9 (f,g) Gene and (h,i) protein expression, quantified by RT-qPCR and western blotting, of young and senescent VSMCs plated on hydrogels of 10kPa, 250kPa stiffness, and plastic coated with collagen (P+C), n=6, n=3, respectively. One-way ANOVA with Tukey post hoc test, *p-value<0.05, ***p<0.005, ****p<0.0001. (j) Immunofluorescence of Sox9 when plated on hydrogels of 10kPa stiffness and P+C in young and senescent VSMCs. Sox9 in grey and green, nuclear DAPI staining in blue.

Figure 2

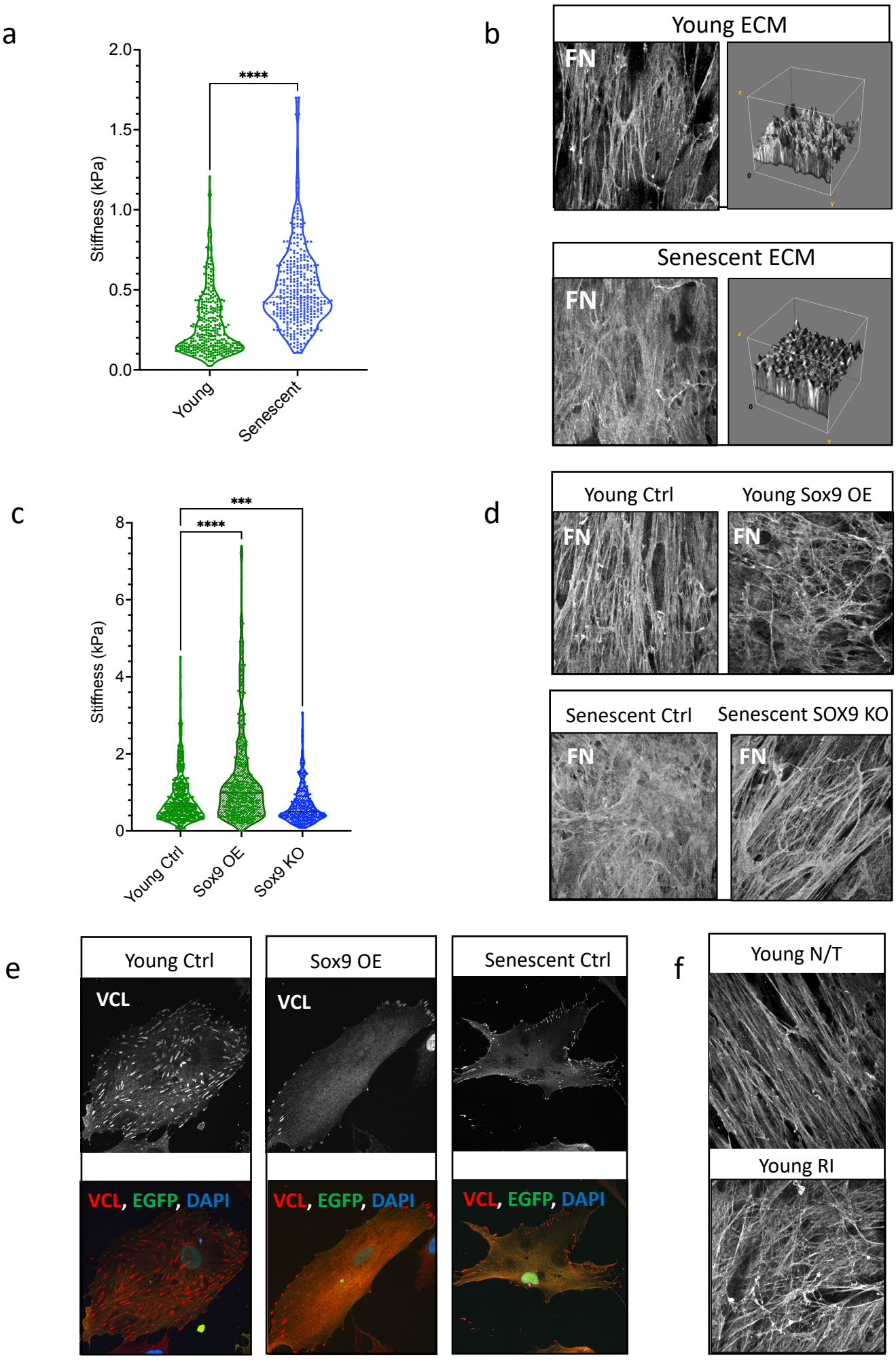


Figure 3: VSMC senescence and Sox9 expression regulate the ECM. (a) Atomic force microscopy (AFM) measurements of ECM stiffness synthesized from young and senescent VSMCs. Normality rejected via Shapiro-Wilk test, Unpaired Mann-Whitney test, ****p-value<0.0001. Stiffness measurements taken from ECM synthesized from three different donors; 35F, 38F, 33F. (b) Representative fibronectin immunofluorescence staining of young and senescent ECM. Topology of fibronectin staining presented to the right-hand side of the immunofluorescence image. (c) AFM measurements of ECM synthesized from young VSMC transfected with EGFP (Young Ctrl), Sox9 overexpression adenovirus (Sox9 OE), and Sox9 adenoviral knockout (Sox9 KO). Normality rejected via Shapiro-Wilk test, Unpaired Mann-Whitney test, ***p-value<0.005, ****p-value<0.0001. (d) Representative immunofluorescence staining of fibronectin in ECM synthesized from Young Ctrl, Young Sox9 OE, Senescent VSMCs transduced with EGFP (Senescent Ctrl), and Senescent cells with Sox9 knockout (Sox9 KO). (e) Representative immunofluorescent images of vinculin (VCL) in Young Ctrl, Sox9 OE, and Senescent Ctrl. VCL in grey/red, EGFP in green, nuclear staining (DAPI) in blue. (f) Representative immunofluorescence staining of fibronectin in ECM synthesized from young VSMCs with no treatment (Young N/T) and young VSMCs treated with Rock Inhibitor (Young RI).

Figure 3

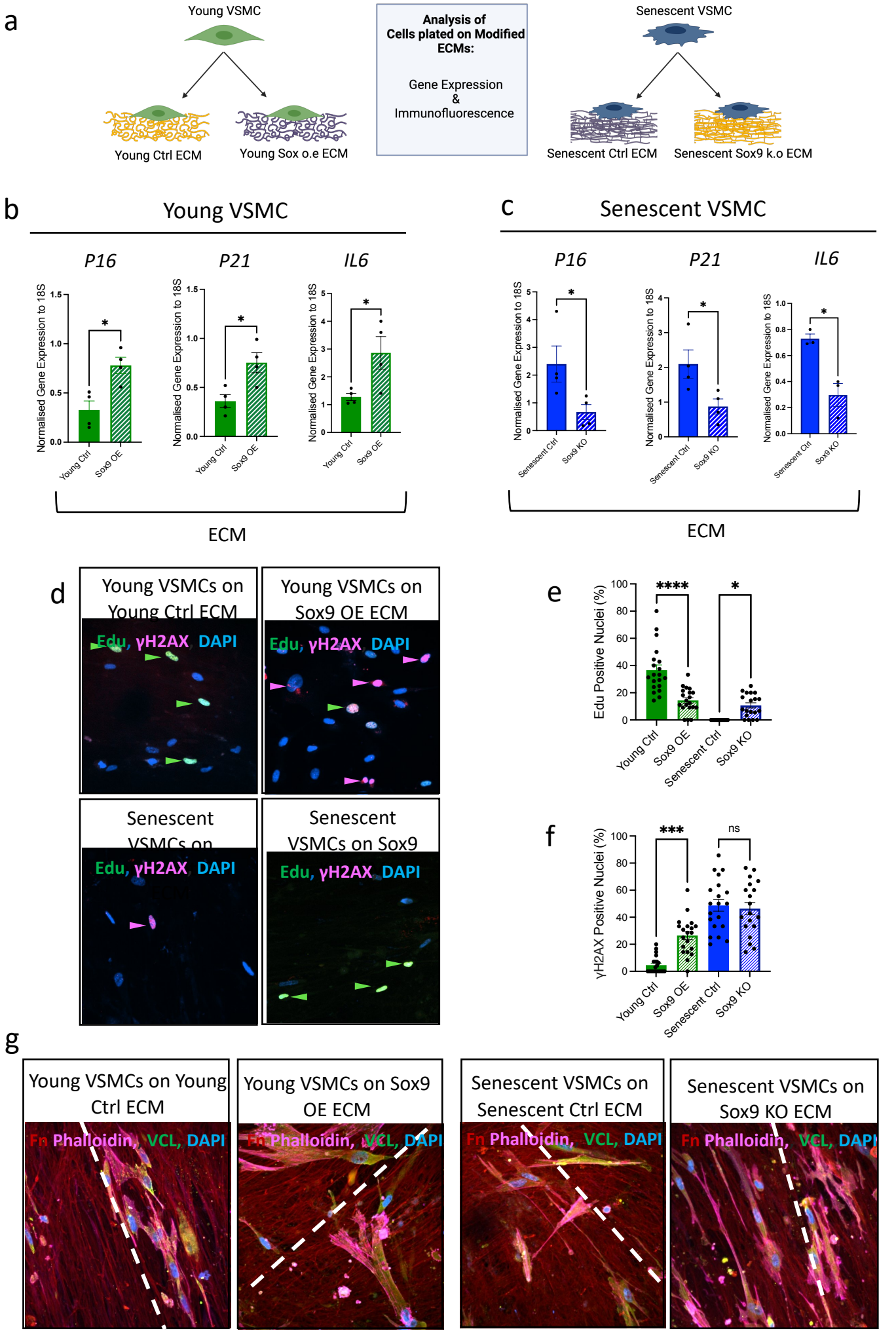


Figure 4: Composition of Sox9-regulated ECM regulates VSMC phenotype. (a) Schematic of experimental protocol. Young VSMCs were plated on decellularized ECM originally synthesized from young VSMCs transduced either with EGFP (Young Ctrl) or Sox9 overexpression adenovirus (Sox9 OE). Senescent VSMCs were plated on decellularized ECM originally synthesized from senescent VSMCs transduced either with shEGFP (Senescent Ctrl) or Sox9 knockout adenovirus (Sox9 KO). Gene expression, quantified by RT-qPCR, of p16, p21, and IL6 from (b) young and (c) senescent VSMCs plated on transduced ECMs. N=4, Unpaired Student's t-test, *p-value<0.05. (d) Representative IF and quantification (%) of (e) Edu and (f) γH2AX when VSMCs plated on transduced ECMs. Edu, γH2AX, and nuclear DAPI staining in green, magenta, and blue, respectively, n=20. One-way ANOVA with Tukey post hoc test, *p-value<0.05, ***p-value<0.005, ****p-value<0.0001. (g) Representative IF staining of young and senescent VSMCs when plated on transduced ECMs, with fibronectin (Fn) in red, Phalloidin in magenta, Vinculin (VCL) in green, and nuclear staining (DAPI) in blue. White line indicates relative fibronectin fiber alignment.

Figure 4

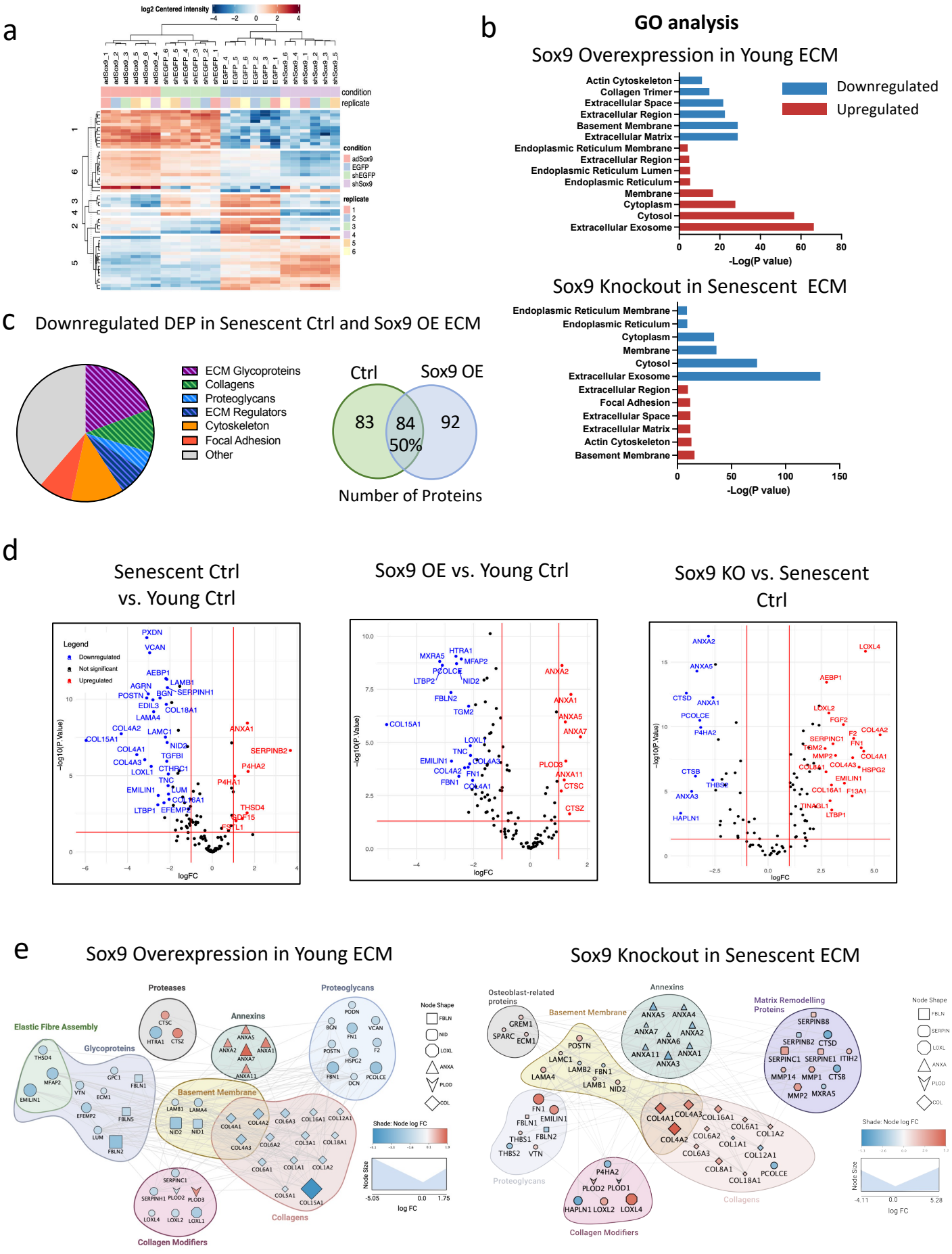


Figure 5: Sox9 drives the ECM composition towards a senescent-like phenotype (n= 6 injections for each condition from 35-year-old female (35F) and 38-year-old female (38F)). (a) Heatmap of clustered differentially expressed proteins (DEP), showing the similarly of protein expression between ECM synthesized from young VSMCs treated with control EGFP adenovirus (Young Ctrl) and senescent VSMCs with Sox9 knockout adenovirus (Sox9 KO), and between young VSMCs transduced with Sox9 overexpression adenovirus (Sox9 OE) and senescent VSMCs transduced with control shEGFP adenovirus (senescent Ctrl). (b) Gene ontology (GO) analysis of differentially expressed proteins (DEP) in Sox9 OE ECM as compared to Young Ctrl, and Sox9 KO as compared to Senescent Ctrl. (c) Pie chart and Venn-diagram of downregulated DEP both in the Sox9 OE and Senescent Ctrl ECM. (d) Volcano plots of DEP when comparing Senescent Ctrl against Young Ctrl, Sox9 OE against Young Ctrl, and Sox9 KO against Senescent Ctrl. (f) Protein-protein interaction network showing interactions between significantly upregulated and downregulated proteins in Sox9 OE ECM as compared to the Young Ctrl ECM, and in Sox9 KO ECM as compared to the Senescent Ctrl ECM.

Figure 5

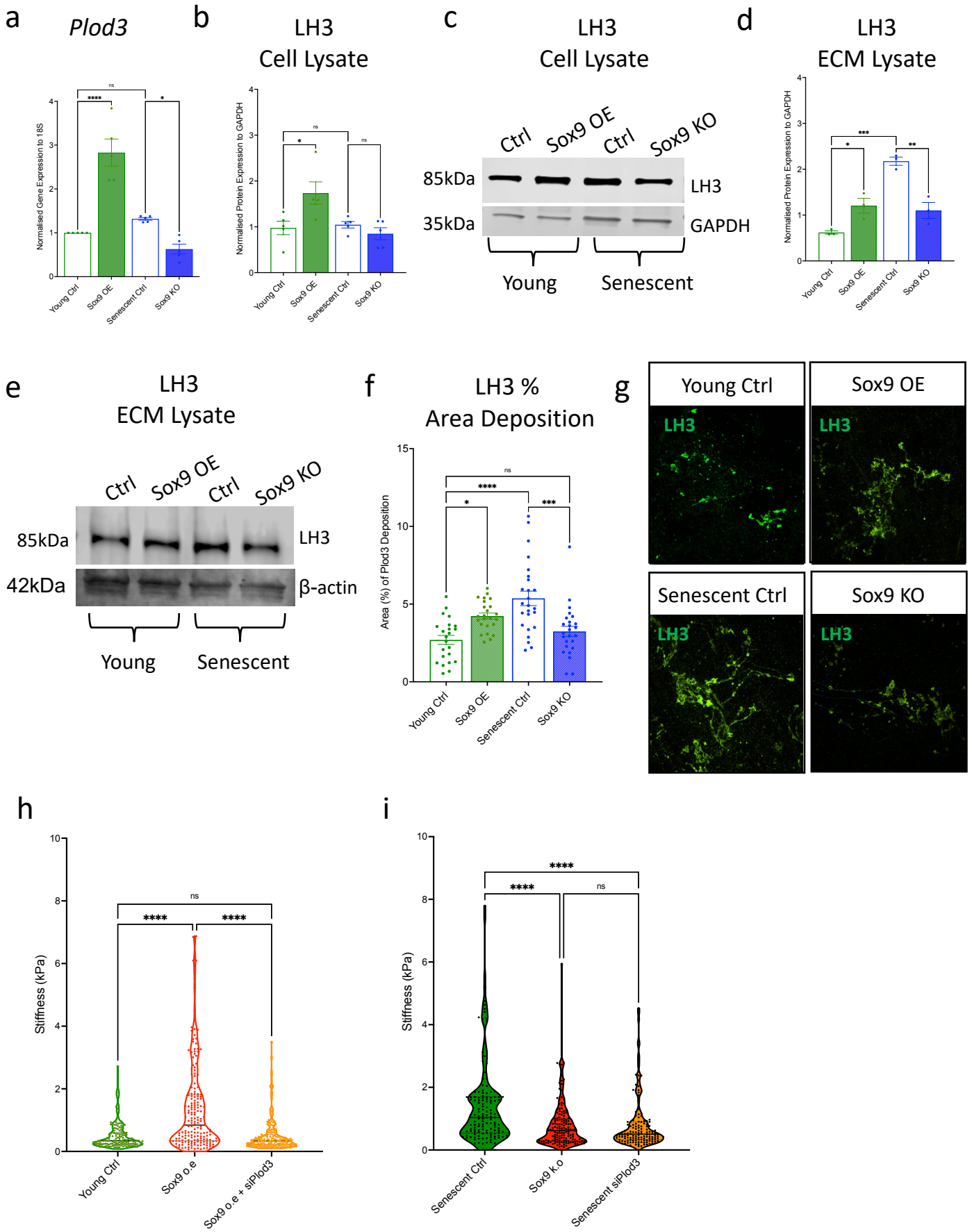


Figure 6: *Plod3*/LH3 in VSMCs expression and deposition into the ECM is regulated by Sox9 expression. (a) Gene expression, quantified via RT-qPCR, of *Plod3* in young VSMCs transduced with EGFP control adenovirus (Young Ctrl), Sox9 overexpression adenovirus (Sox9 OE), and senescent VSMCs transduced with shEGFP control adenovirus (Senescent Ctrl) and Sox9 knockout adenovirus (Sox9 KO), n=5. Quantification and representative Western blot of LH3 protein expression in Young Ctrl, Sox9 OE, Senescent Ctrl, and Sox9 KO VSMCs (b,c) cell lysate, n=5, and (d,e) ECM, n=3. (f,g) Quantification and representative immunofluorescence of fluorescent LH3 signal when deposited into the ECM synthesized from Young Ctrl, Sox9 OE, Senescent Ctrl, and Sox9 KO VSMCs, n= 25. One-way ANOVA with Tukey post hoc test, *p-value<0.05, ***p-value<0.005, ****p-value<0.0001. (h) Atomic force microscopy (AFM) stiffness measurements (kPa) in decellularized ECM synthesized from (e) Young Ctrl, Sox9 Sox9 OE, and Sox9 OE with Plod3 knockout (siPlod3) and (i) Senescent Ctrl, Sox9 KO, and Plod3 knockout (Senescent siPlod3). Measurements were taken from ECM synthesized from three isolates, 35F, 38F, and 33F. Shapiro-Wilk test for normality distribution was rejected. One-way ANOVA Kruskal-Wallis test, ****p-value <0.0001.

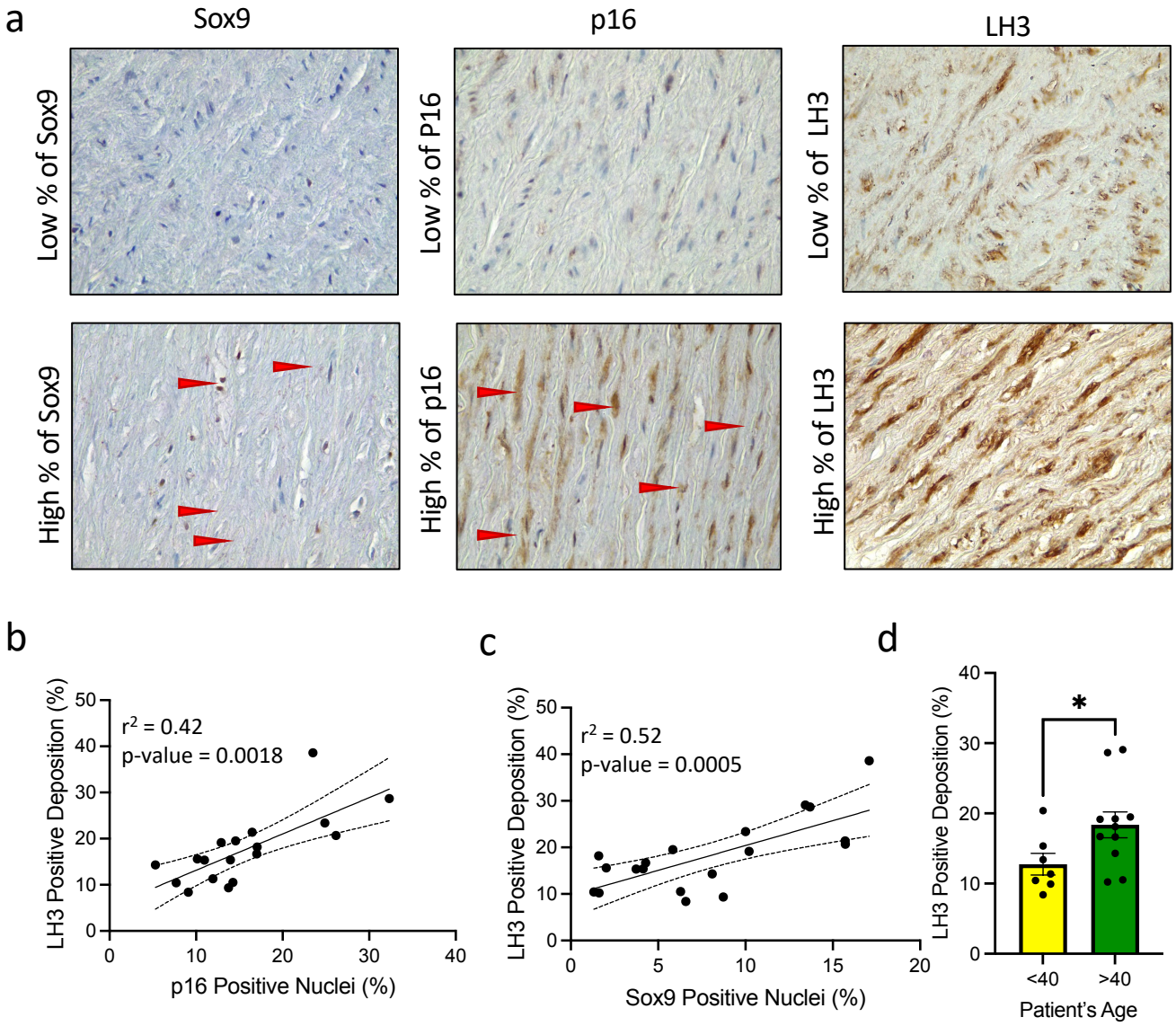


Figure 7: LH3 deposition increases in the medial aortic layer with age and Sox9 expression and regulates extracellular matrix (ECM) stiffness. (a) Immunohistochemistry of Sox9, p16, and LH3 staining in the aortic medial layer. The red arrows highlight positive staining of Sox9 and p16. Positive staining of p16, Sox9, and LH3 in brown. Correlation of LH3 positive staining (%) with (b) p16 and (c) Sox9 Positive nuclei. Correlation determined by Spearman's correlation, n=16. (d) Bar chart of positive percentage of LH3 staining in patient samples less than 40 years of age and over 40 years of age, Unpaired Student's t test, *p-value <0.05.

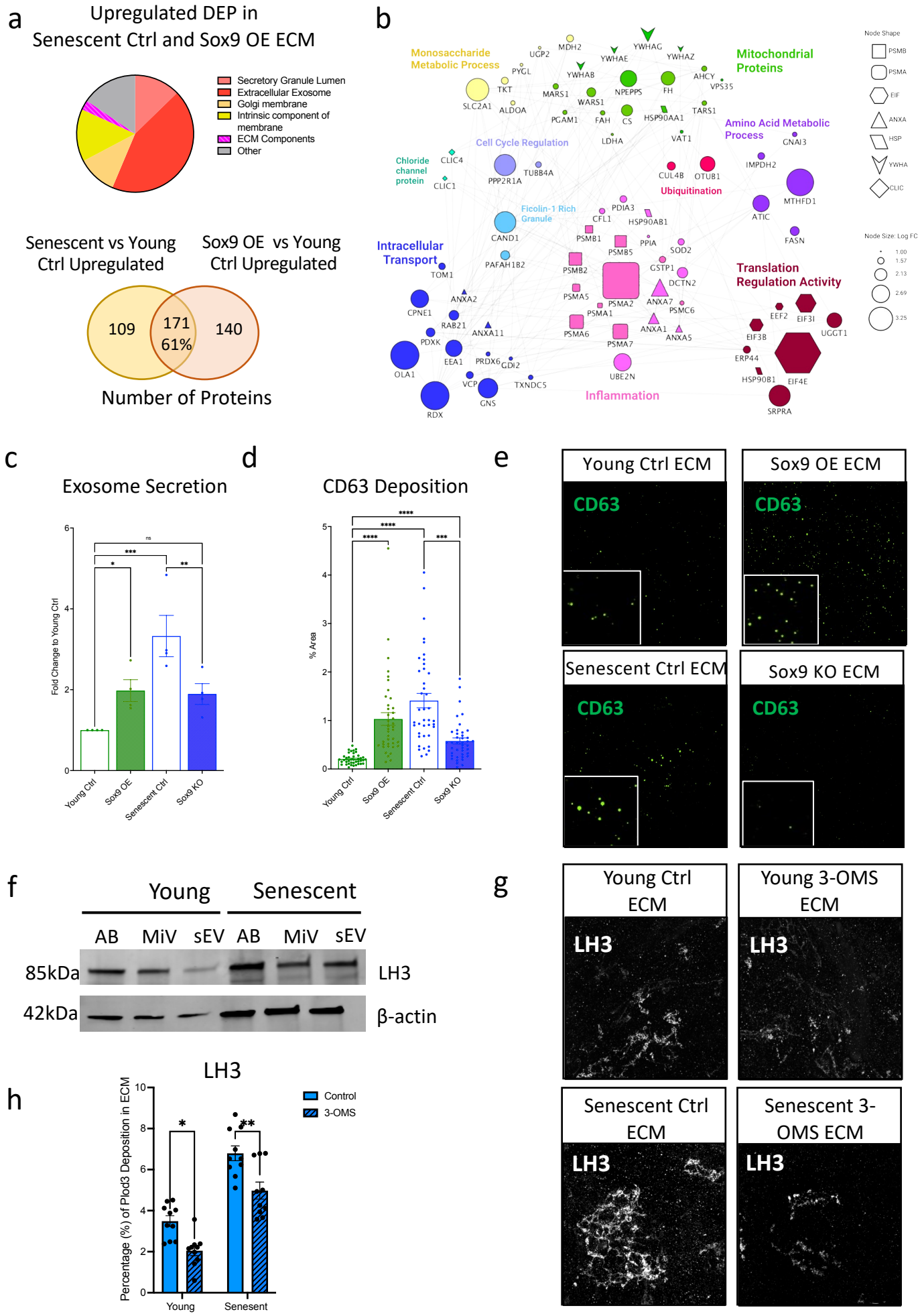
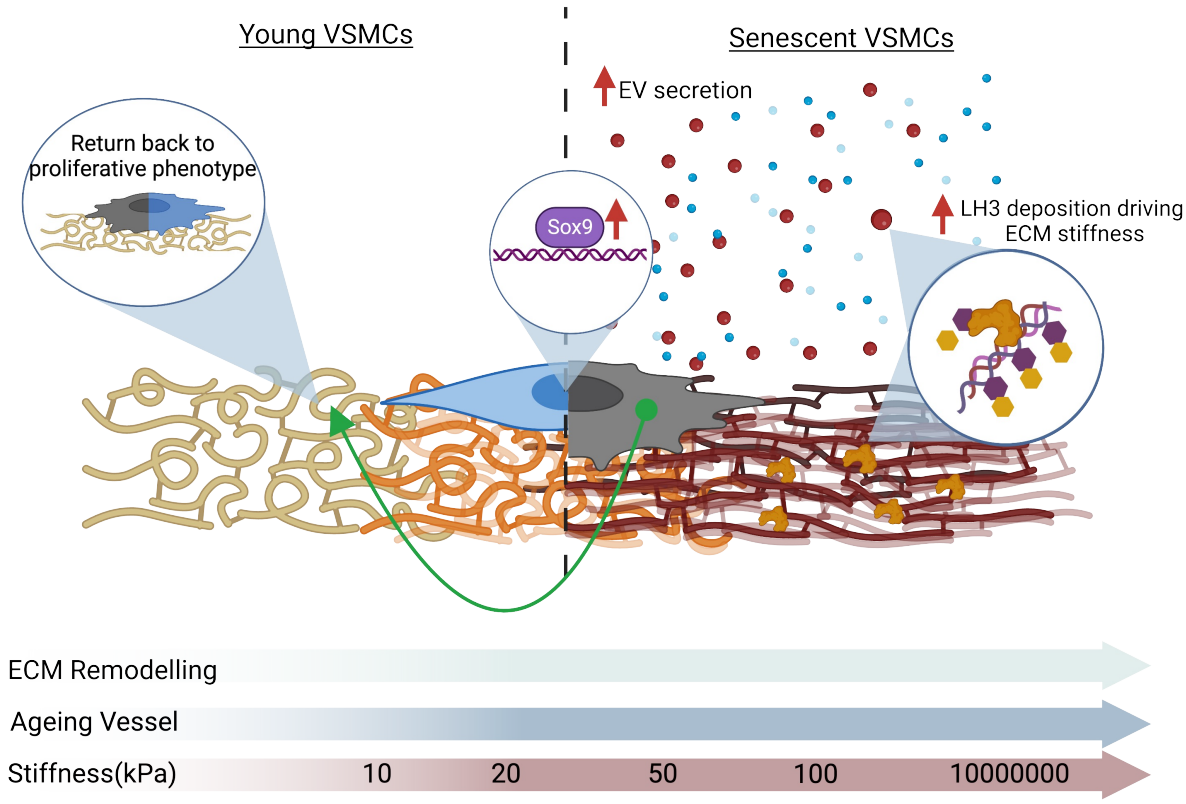


Figure 8: Sox9 regulates LH3 deposition into the ECM via increased extracellular vesicle (EV) secretion. (a) Pie chart and Venn-diagram depicting categories and percentages of upregulated differentially expressed proteins (DEP) found both in the ECM synthesized from senescent VSMCs transduced with EGFP (Senescent Ctrl) and young VSMCs transduced with Sox9 overexpression adenovirus (Sox9 OE). (b) Protein-protein-interaction of upregulated DEP found in Sox9 OE ECM related to extracellular vesicles through gene ontology. (c) Quantification of exosome secretion in young VSMCs transduced with EGFP control adenovirus (Young Ctrl), Sox9 OE, Senescent Ctrl, and Sox9 knockout adenovirus (Sox9 KO), n=4. (d,e) Quantification and representative IF images of CD63 deposition in the Young Ctrl, Sox9 OE, Senescent Ctrl, and Sox9 KO ECM, n=20. One-way ANOVA with Tukey post hoc test, *p-value<0.05, ***p-value<0.005, ****p-value<0.0001. (f) Western blot of LH3 protein expression in three different types of extracellular vesicles; Apoptotic bodies (AB), micro-vesicles (miV), and small EV (sEV). (g,h) Representative IF images and quantification of LH3 deposition into young and senescent ECM either with or without sEV secretion inhibitor 3-O-methylsphingomyelin (3-OMS), n=10. Two-way ANOVA, *p-value<0.05, **p-value<0.001.

Figure 8


Young VSMCs
Senescent VSMCs
EV secretion
Return back to proliferative phenotype
Sox9
LH3 deposition driving ECM stiffness
ECM Remodelling
Ageing Vessel
Stiffness(kPa)
10
20
50
100
10000000