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Unbalanced SOD2 Overexpression in Senescent Fibroblasts Triggers Altered Redox Signaling and Skin Ageing

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ABBREVIATIONS

2D-DIGE	two dimensional fluorescence difference gel electrophoresis
8-OHdG	8-hydroxydeoxyguanosine
AP-1	activator protein 1
APMA	4-aminophenylmercuric acetate
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related
ATZ	aminotriazole
BSA	bovine serum albumin
BSO	buthionine sulfoximine
CMV	cytomegalovirus
COX-2	cyclooxygenase-2
CPD	cumulative population doublings
CRE	cAMP responsive element
DAPI	4',6'-diamidino-2-phenylindole
DCFH-DA	dichlorofluorescein diacetate
DETAPAC	diethylenetriaminepentaacetic acid
DHE	dihydroethidium
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	ethylenediaminetetraacetic acid
ETC	electron transport chain
FCS	fetal calf serum
FKBP10	FK506-binding protein 10
GFP	green fluorescent protein
GPX	glutathione peroxidase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MKK	mitogen-activated protein kinase kinase

MMP-1	matrix metalloproteinase 1
MS	mass spectrometry
MT-MMP	membrane type matrix metalloproteinases
NAC	N-acetyl-cysteine
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa B
NP-40	nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PNPP	para-nitrophenylphosphate
PTRF	polymerase I and transcript release factor
ROS	reactive oxygen species
SASP	senescence associated secretory phenotype
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
TBST	mixture of Tris-Buffered Saline and Tween 20
TCP1	T-complex protein 1
TGF-β2	transforming growth factor beta 2
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRE	TPA responsive element
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet

1. INTRODUCTION

1.1. Fibroblast senescence is the key player for skin ageing

1.1.1. Skin ageing

Ageing is defined as the progressive decline in the structure and function of the organism which occurs during time thus enhancing organismic morbidity and mortality due to an incremental loss of cellular, tissue and organ homeostasis (Kirkwood and Austad 2000).

Skin ageing is a part of the overall organismal ageing with modifications in skin structure and function which consists of wrinkle formation, impaired wound healing and loss of tensile strength. The underlying mechanisms for ageing are not clear yet. Several theories tried to explain the mechanisms of ageing which could be classified in two groups: stochastic theories and deterministic theories. The stochastic theories predict that ageing is the effect of random damage and comprise the free radical theory of ageing (Harman 1956), the mutation theory (Szilard 1959) and the catastrophe theory (Orgel 1963). The free radical theory of ageing will be described in detail later [1.2.1] and proposes that ageing is caused by the damage induced by an increase in the concentrations of reactive oxygen species. The mutation theory of ageing postulates that age-related accumulations of mutations randomly inactivate genes that are important for the functioning of somatic cells of the adult and result in a decrease in organ function. The error catastrophe theory states that copying errors in DNA and the incorrect sequence of amino acids in protein synthesis could aggregate over the lifetime of an organism and eventually causing a catastrophic breakdown as shown as the process of ageing. The deterministic theories predict that ageing is intrinsically genetically controlled and comprise the metabolism theory (Speakman 2005) and cell division theory (Hayflick and Moorhead 1961), (Hayflick 1965). In the context of the metabolism theory of ageing, Rubner (1908) observed that the lifespan correlates inversely with body size in mammals, and he proposed that this is the consequence of basal metabolic rate. The cell division theory forwarded by Hayflick (Hayflick 1965) is decribed later [1.1.2] and proposes that human fibroblasts enter a growth arrest state after a limited number of cell divisions *in vitro*.

Skin ageing is in the sense distinct to ageing of other organs as skin represents the outermost layer of the organisms and, thus is exposed to a variety of environmental extrinsic attacks. Accordingly, overall skin ageing is the result of extrinsic ageing/photoageing, mainly due to ultraviolet irradiation, and the overlapping intrinsic ageing/chronologic ageing (Fisher *et al*. 2002, Ma *et al.* 2001, Reddy and Gilchrest 2011). The aged skin is atrophic and reveals transparent quality and loss of elasticity. The epidermis is thinner, while the dermoepidermal junction is flattened which leads to an increased fragility of the old skin. A decrease in dermal thickness is observed with a reduction in fibroblasts number and their proliferative capacity and a change in the organization of the collagen and elastic fibers (Gilchrest 1996). The major cellular component of the dermal compartment is represented by the fibroblasts that play an important role in maintaining the composition of the connective tissue in the skin which confers the elasticity and resilience of the skin. The dermal fibroblast is one of the key players for skin ageing as fibroblasts decrease in number during ageing (Freinkel 2001) and, in addition, they may undergo cellular or replicative senescence with a drastic change in their cellular and functional phenotype.

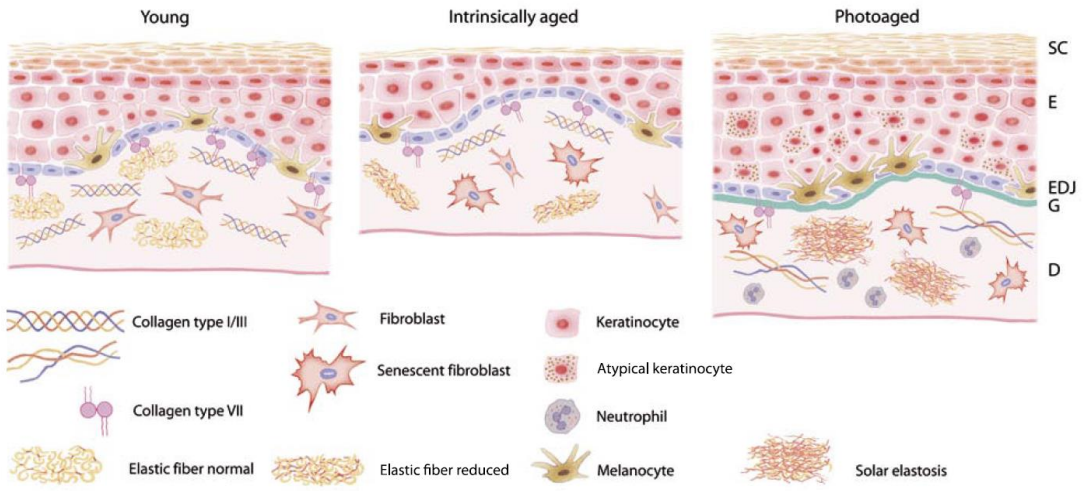

Fig. 1. Structure of young, intrinsically and photoaged skin.
The structure of the skin is modified during the intrinsic ageing process. The intrinsically aged skin has a thinner epidermis, an atrophic dermis with a modified organization and decreased subcutaneous fat (SC stratum corneum, E epidermis, EDJ epidermal dermal junction, D dermis). [adopted from Braun-Falco's Dermatology].

1.1.2. Cellular senescence of fibroblasts

Hayflick (Hayflick and Moorhead 1961, Hayflick 1965) showed for the first time that fibroblasts have a limited lifespan *in vitro*, and enter a growth arrest phase as replicative or cellular senescence after a finite number of cell divisions. Senescent fibroblast proliferation is arrested in G_1S phase, but the cells are metabolically active. Senescent fibroblasts reveal a modified morphology with enlarged size and flattened appearance.

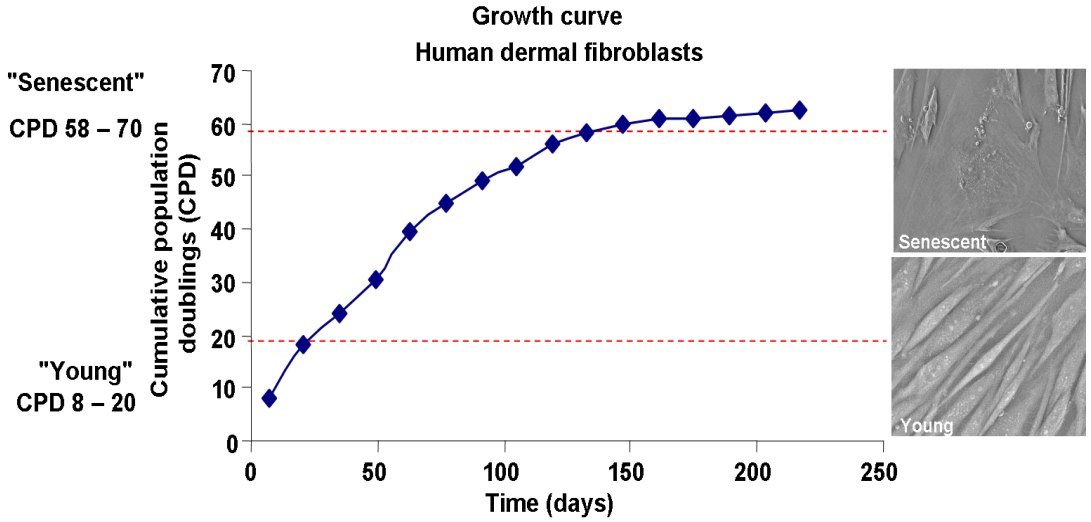

Fig. 2. Growth curve of a representative human fibroblast strain and morphology of young and senescent fibroblasts.
Fibroblasts at cumulative population doublings (CPD) between 8 to 20 which are intensively proliferating are considered to be "young" with a juvenile, spindle-shaped morphology and fibroblasts at CPD between 58 to 70 with drastically decreasing proliferation rates of <0.1 PD per week are considered to be "senescent" with enlarged morphology and flattened appearance. Microphotographs: original magnification 40x, scale bar: 50 µm.

A variety of cellular processes in senescent fibroblasts are altered compared to normal dividing fibroblasts. Their metabolism is active, but altered (Zwerschke *et al.* 2003, Hütter *et al.* 2004) and they are resistant to apoptosis (Wang 1995, Seluanov *et al.* 2001). They reveal a particular phenotype compared to normal fibroblasts with increased secretion of soluble factors involved in inflammation, ageing or tumour promotion constituting the senescence-associated secretory phenotype (SASP) (Coppé *et al.* 2008). These phenotypic changes of senescent fibroblasts markedly influence the microenvironment and affect the structure of the connective tissue of the skin. Although replicative senescence was studied *in vitro*, there is strong evidence for its significance for *in vivo* skin ageing. Senescent fibroblasts were found *in vivo* in

old skin (Dimri *et al.* 1995) and also in other aged human tissues like intervertebral discs (Gruber *et al.* 2007). Although in global transcriptome comparison between cellular senescence *in vitro* and ageing *in vivo* only limited similarities have been detected (Swindell *et al*. 2012, Wennmalm *et al*. 2005), central check point genes in senescence are highly expressed in ageing *in* vivo in humans, baboons and mice (Krishnamurthy *et al*. 2004, Ressler *et al*. 2006, Jeyapalan *et al*. 2007). One of these central check point genes is the $p16^{INK4a}$ gene which codes for a cyclin-dependent kinase inhibitor and is upregulated during replicative senescence of fibroblasts. It interacts with the cyclin-CDK complex and, subsequently, blocks kinase activity usually during G1, mediating the cell cycle arrest in G_1S phase (Sherr and Roberts 1999). Recently, a causal role of the cell cycle regulating gene $p16^{INK4a}$ in organismal ageing was shown by the rejuvenation effect of depletion of $p16^{INK4a}$ positive cells *in vivo* in a mouse model of premature ageing (Baker *et al.* 2011).

Replicative senescence occurs in fibroblasts *in vitro* due to the progressive telomere shortening during every cell division (Olovnikov 1973, Harley *et al*. 1990, Allsopp *et al.* 1992). The telomeres are repetitive nucleotide sequences located at the end of the chromosomes in most eukaryotic organisms (Blackburn 1991) [Fig. 3]. Due to the ‘end replication problem’ 50-200 bp at the end of the chromosome are lost during DNA replication and telomeres become shorter (Levy *et al.* 1992). Telomere attrition occurs with every cell division until they reach a critical size when the telomere ends are recognized as DNA damage and a subsequent DNA damage response is initiated in the cell. The DNA damage response mediated by ATM and ATR blocks the cellular division and the state of growth arrest with a change in gene expression pattern, and thus, senescence is established (di Fagagna *et al*. 2003).

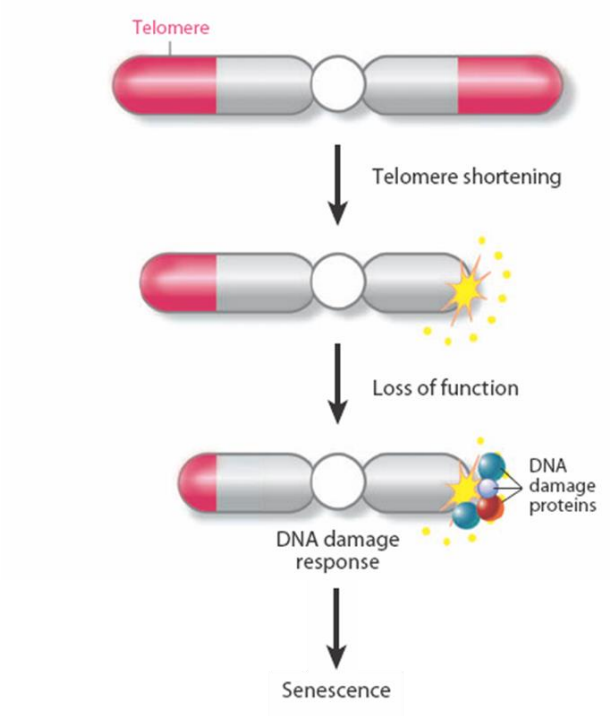

Fig. 3. Telomere shortening and DNA damage response during replicative senescence. *Telomeres are repetitive nucleotide sequences found at the end of the chromosome which become shorter with every cell division. When the telomere length reaches a critical size, a DNA damage response is initiated in the cell and the cell enters a state of cellular growth arrest. [Modified from (Armanios 2009)].*

Besides these changes, oxidative stress is observed in senescent fibroblasts, with an increase in reactive oxygen species (ROS) which causes so far have not been explained in detail (Lee *et al*. 2002, Stöckl *et al*. 2006). The increase in intracellular ROS levels constitutes the central component of the free radical theory of ageing.

1.2. Reactive oxygen species, oxidative stress and the free radical theory of ageing

1.2.1. Free radical theory of ageing

The free radical theory of ageing was forwarded by Denham Harman in 1956 and postulates that increased levels of reactive oxygen species (ROS) damage biomolecules and drive ageing (Harman 1956). Later, Harman modified the theory in order to emphasize the role of the mitochondria as the major source of ROS in the cell under physiological conditions (Harman 1972). However, this theory is

controversially discussed as the changes in ROS concentrations induced by different experimental approaches resulted in discrepant results in different organisms. Many studies using genetic approaches to overexpress antioxidative enzymes did not reveal an increase in life span in mice or invertebrates (for review see Muller *et al.* 2007, Perez *et al*. 2009). Notably, one study using mitochondrial overexpression of catalase in mice resulted in an increase in life span of 20% (Schriner *et al.* 2005). Collectively, oxidative stress may modulate ageing of cells and tissues. The molecular details are, however, not clarified so far.

1.2.2. Reactive oxygen species (ROS) and oxidative stress (OS)

1.2.2.1. Reactive oxygen species (ROS)

Reactive oxygen species define a group of small molecules that are produced during the normal metabolism of the cell. They contain oxygen with unpaired electrons conferring them the high chemical reactivity. The major origin of ROS production is the mitochondrial electron transport chain (ETC). At complex I and complex III, electrons leak from the ETC, react with molecular oxygen, generating the superoxide anion radicals which initiate the cascade of ROS production (Brand 2010). Superoxide anion radicals are produced inside the mitochondrial matrix and at the inner mitochondrial space. They possess a very high reactivity and, therefore, a short half-life at high concentrations (0.05 seconds at 0.1 mM) with a consequently short range of action of 40µm (Saran and Bors 1994), while the half-life is markedly longer at low superoxide anion concentrations (14 hours at 0.1 nM) (Fridovich 1983) with a longer range of action. Besides the mitochondria, there are other sites where superoxide anion radicals are generated within the cell like the membrane-associated enzyme NADPH oxidase. As superoxide has a low membrane permeability, its localization is mostly restricted to the compartment where it is generated (Winterbourn and Hampton 2008). However, a small amount of superoxide was found to be transported outside of the mitochondria via the voltage-dependent anion channel (VDAC) (Han *et al.* 2003).

Superoxide anion radicals are dismutated enzymatically by superoxide dismutases or spontaneously to molecular oxygen and hydrogen peroxide (H_2O_2). Hydrogen peroxide is a more stable reactive oxygen species, with a low reactivity,

longer half-life in biological systems and a high diffusibility. It is able to migrate up to 1.5 mm in aqueous solutions (Winterbourn and Hampton 2008) which is favored by the ability of being membrane permeable (Winterbourn and Hampton 2008). Thus, it is able to exert its effects at far distances from the site of generation. Due to its diffusion ability, H_2O_2 can occur in different compartments and, most likely during evolution, several cellular H_2O_2-detoxifying enzymes with different cellular localization have developed including: catalase, glutathione peroxidases and peroxiredoxins. These enzymes detoxify H_2O_2 to H_2O and molecular oxygen [Fig. 4].

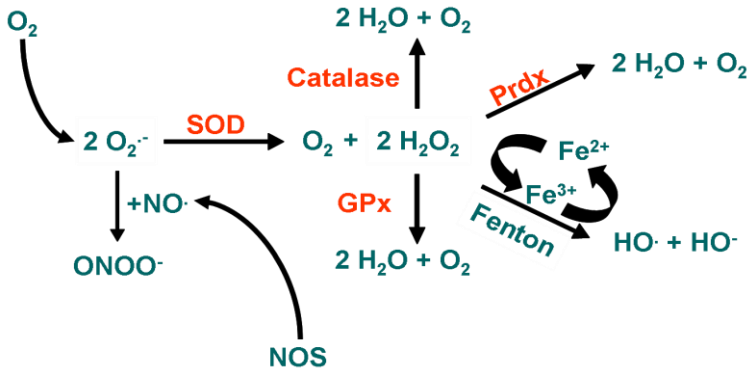

Fig. 4. ROS production and detoxification in human cellular systems.
During the mitochondrial electron transport chain, superoxide anion radicals ($O2^{\bullet-}$) are generated from molecular oxygen (O_2). Superoxide anion radicals ($O2^{\bullet-}$) reacts with nitric oxide ($NO^{\cdot}$) which results in peroxynitrite ($ONOO^{-}$) formation or is catalytically dismutated in the presence of the antioxidant enzyme superoxide dismutase (SOD) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). Hydrogen peroxide (H_2O_2) is further detoxified by catalase, glutathione peroxidases (GPx) or peroxiredoxins (Prdx) depending on its localization and concentration, resulting in water (H_2O) and molecular oxygen (O_2). In the presence of transition metal ions like iron ion (Fe^{2+}), the Fenton reaction takes place with generation of the highly toxic hydroxyl radical ($HO^{\cdot}$ and the hydroxyl anion (HO^{-}). Reactive oxygen species are characterized by a high reactivity, and, thus, they may readily react with biomolecules like proteins, lipids or nucleic acids, inducing structural modifications via oxidation.

1.2.2.2. Redox homeostasis, oxidative stress and redox signaling

Although initially ROS were considered to be harmful species, they are now, in addition, acknowledged as physiological mediators in the cellular processes. There is a certain physiological threshold for the cellular ROS concentrations which characterizes a balanced state known as redox homeostasis (Halliwell and Gutteridge 1985). The redox homeostasis or redox balance is achieved by fine tuning of ROS production via specific cellular systems and the ROS detoxifications via antioxidant defense system. The redox status of the cell may, however, have transient changes

during different physiological cellular processes such as apoptosis (Chandra *et al*. 2000) or cell adhesion (Chiarugi *et al*. 2003).

When the ROS production becomes increased or/and ROS detoxification is lowered, the redox status of the cell is disbalanced and oxidative stress occurs (Sies and Cadenas 1985). The redox status of the cell may, however, encounter changes which might be transient or permanent, depending on the cellular processes that happen in the cell (Dröge 2002). As mentioned before, due to their reactivity to biomolecules and to the structural modifications, ROS most likely modify the function of different biomolecules, mainly proteins involved in signal transduction. ROS are known to induce protein oxidation by different mechanisms (Berlett and Stadtman 1997). Two major mechanisms are oxidation of cysteine residues to disulfide or to sulfenic acids or oxidation to carbonyl derivatives. These changes in the protein structure lead to modifications in protein function, to activation or inactivation of the protein. Lipids can also be oxidized by ROS during lipid peroxidation, a chain reaction initiated by the hydroxyl radical. There are several end-products of lipid peroxidation that exert cytotoxic or genotoxic effects. Recently, a role in signal transduction as second messengers was attributed to these end-product of lipid peroxidation (Niki *et al.* 2005). ROS induce oxidative damage to DNA which triggers a response in order to repair the DNA lesion and limit mutagenesis, cytostasis or cytotoxicity (Cooke *et al*. 2003).

By their potential to induce structural modifications to different molecules, ROS are considered to act as second messengers relaying signals in a variety of cellular processes (Dröge 2002). Based on this ability of ROS to regulate cellular processes, the term of redox regulation has been used. There are several reports of redox regulation of specific signal transduction pathways such as NF-κB, AP-1 or p53 (Sun and Oberley 1996).

The cellular redox status is conferred by the balance between oxidants and the antioxidant system. The antioxidant system consists of small molecular antioxidants such as glutathione or thioredoxin and enzymes like catalase, glutathione peroxidases or superoxide dismutases.

1.3. Superoxide dismutases

Superoxide dismutases are major ubiquitous antioxidant enzymes. In humans, there are 3 distinct isoforms for SOD according to their localization. Superoxide dismutase1/SOD1 or CuZnSOD found in the cytoplasm and in the mitochondrial inner space is a dimer with Cu^{2+} and Zn^{2+} as cofactors in the active site. SOD2 or MnSOD found in the mitochondria is a tetramer with Mn^{2+} in the active site, while SOD3 or CuZnSOD found in the extracellular space is also a tetramer, but with the Cu^{2+} and Zn^{2+} as cofactors in the active site.

SOD2 represents the first line of defense against mitochondrial oxidative stress. This enzyme was shown to be essential for survival as deletion of SOD2 in mice results in neonatal lethality (Li *et al*. 1995),(Lebovitz *et al*. 1996). Interestingly, SOD2 heterozygous deficient mice did not show any change in lifespan (Tsan *et al*. 1998, Treiber *et al*. 2011). However, they reveal a high degree of oxidative stress in mitochondria like reduced glutathione levels, reduced aconitase and NADH oxidoreductase activity, increased carbonyl groups, increased levels of the oxidative DNA-lesion 8-hydroxydesoxyguanosine (8-OHdG), all markers indicative for oxidative stress (Williams *et al*. 1998). In addition, SOD2 heterozygous deficient mice have an enhanced incidence of lymphoma and adenocarcinoma (Van Remmen *et al*. 2003). Interestingly, SOD2 deletion exclusively targeted to the connective tissue in mice results in a premature ageing phenotype (Treiber *et al*. 2011). Notably, genetic manipulation of SOD2 in different organisms showed conflicting results, indicating that SOD2 and the redox balance might play distinct roles in ageing of different organisms and organs. SOD2 deletion in yeast results in decreased chronological and replicative lifespan (Unlu and Koc 2007, Longo *et al*. 1996, 1999), in flies results in marked reduction in lifespan (Kirby *et al*. 2002, Duttaroy *et al.* 2003). Surprisingly, in C. elegans, SOD2 deletion extends lifespan (Van Raamsdonk and Hekimi 2009, 2012). The impact of SOD2 function at the organismal level is apparent as it influences the redox state of the cell in most studied organisms. For skin, one study shows that SOD2 is a part of the adaptive response to UVA exposure, indicating that SOD2 is a stress reponse gene (Poswig *et al*. 1999). However, the interindividual differences in the expression of SOD2 may be responsible for the accumulation of either superoxide or hydrogen peroxide leading to a different interindividual

susceptibility for ageing and tumor formation (Poswig *et al*. 1999). The vital role of SOD2 for the skin is emphasized by the effects of SOD2 deletion in the connective tissue in mice. Mice with this deficiency reveal a phenotype with skin ageing (Treiber *et al.* 2011, Velarde *et al.* 2012). Together, these data underline the importance of the redox balance and, in particular, of SOD2 for the structure and function of the skin during ageing, however, the mechanisms by which the redox balance is altered are not precisely understood. As described before, the redox balance and SOD2, in particular, may influence different signaling pathways which are redox-sensitive. Two important signaling pathways that are regulated by ROS are activator protein 1 (AP-1) and nuclear factor κB (NF-κB) (Schenk *et al.* 1994, Toledano and Leonard 1991, Sen and Packer 1996). The AP-1 transcription factor has a vital role in skin physiology and pathology as the expression of many AP-1 target genes is modulated during different physiological and pathological processes that take place within the skin (Angel *et al*. 2001). The oxidative stress-regulated transcription factor NF-κB is also involved in skin ageing (Bigot *et al*. 2012), and the inhibition of its pathway at least delays premature ageing due to DNA damage in mice (Adler *et al.* 2008, Tilstra *et al.* 2012) and protects against skin photoageing (Tanaka *et al*. 2010). NF-κB was shown to mediate the collagen downregulation that occurs during ageing in dermal fibroblasts in the skin (Bigot *et al.* 2012). In this thesis, the focus was on the AP-1 signaling pathway since the expression of the connective tissue degrading matrix metalloprotease-1 (MMP-1) is mainly controlled by this pathway (Fisher *et al.* 2002, Wenk *et al.* 1999, Brenneisen *et al.* 1998).

1.4. AP-1 signaling pathway

1.4.1. AP-1 transcription factor

Activator protein 1 (AP-1) is a transcription factor involved in several cellular processes like cell proliferation, death, survival and differentiation (Shaulian and Karin 2002). AP-1 is represented by a protein dimer that upon activation translocates to the nucleus where it is further transactivated by post-translational modifications and subsequently, binds to specific DNA sequences in the promoter region of target genes, inducing their transcription. There is a large group of AP-1 subunits that might form different combination of homo- or heterodimers. AP-1 subunits are divided in the

3 groups JUN, FOS, ATF and MAF family. The JUN family comprises c-JUN, JUN-B, JUN-D, FOS family comprises c-FOS, FOS-B, FRA-1 and FRA-2, ATF family comprises ATFa, ATF-2, ATF-3, ATF-4, B-ATF (Eferl and Wagner 2003). Upon AP-1 activation, AP-1 subunits dimerize with each other and depending on the combination, lead to activation of a particular subset of AP-1 target genes. AP-1 activation has been suggested to be further enhanced by specific mechanisms like transactivation which occurs via post-translational modifications of the AP-1 subunits. A key event during AP-1 activation is phosphorylation of c-JUN at serines 63 and 73 as transactivation step that facilitates transcriptional activity of cJUN that is known to be mediated by c-JUN N-terminal kinase (JNK). Other AP-1 subunits have also phosphorylation sites in their transactivational domain which favor for AP-1 activation (Karin *et al.* 1997).

The activated AP-1 dimer that might also be transactivated binds to specific DNA sequences known as AP-1 binding sites included in the promoter of target genes. Two well-known AP-1 binding sites are the TPA-responsive element TGACTCA (TRE) and cAMP response element TGACGTCA (CRE) (Eferl and Wagner 2003).

The pattern of AP-1 activation is complex depending on the stimuli that result in a specific composition and transactivation of the AP-1 dimer will subsequently lead to the activation of a certain subset of AP-1 target genes.

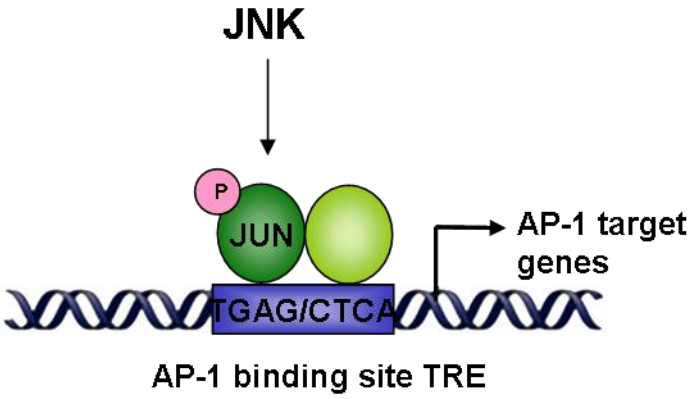

Fig. 5. JNK- mediated AP-1 transactivation.
JNK kinase will induce phosphorylation of cJUN subunit of the AP-1 dimer which results in transactivation of AP-1 transcription factor.

1.4.2. JNK kinases

c-JUN NH_2-terminal kinases (JNKs) are protein kinases belonging to the family of mitogen-activated protein kinases (MAPK) kinases that bind to and phosphorylate cJUN or ATF-2 or JUN-D resulting in increased transcriptional activity. There are 3 isoforms with JNK1, JNK2 and JNK3. JNK1 and JNK2 are ubiquitously expressed, while JNK3 is expressed predominantly in the brain (Mohit *et al.* 1995). JNKs are known to be stress-activated kinases which respond to cellular stresses like environmental genotoxic or oxidative stress, radiation, growth factors. JNKs were found to play a role in cell proliferation as fibroblasts that lack JNK1 or both JNK1 and JNK2 reveal a defect in proliferation (Sabapathy *et al.* 1999,Johnson and Lapadat 2002).

JNKs regulation occurs via 13 upstream protein kinases known as MKKs (MAPK kinases) which are induced by multiple stimuli (Johnson and Lapadat 2002).

1.4.3. AP-1 target genes

There are several genes in fibroblasts that contain AP-1 binding sites in their gene promoter and become activated when AP-1 is switched on. One major AP-1 target gene in fibroblasts that is involved in skin ageing is matrix metalloproteinase 1 (MMP-1) (Angel *et al.* 1987a, Brenneisen *et al.* 1998, Wenk *et al.* 1999). It contains AP-1 binding sites in its promoter. Other AP-1 target genes dependent on cJUN which are known to be upregulated during senescence or ageing are MCP-1 (Martin *et al.* 1997),(Lim and Garzino-Demo 2000), vimentin (Rittling *et al.* 1989), COX-2 (Allport *et al*. 2001,Yamaguchi *et al*. 2005), fibronectin-1 (DeNardo *et al.* 2005), and TGFβ2 (Malipiero *et al.* 1990).

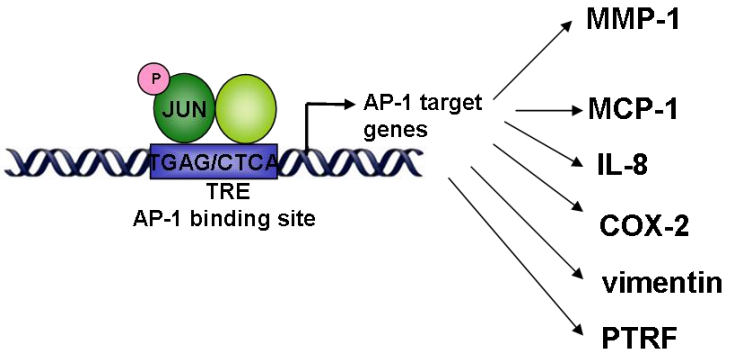

Fig. 6. AP-1 target genes.
AP-1 activation results in the induction of AP-1 target genes. A few AP-1 target genes are listed below, genes that are known to be upregulated during senescence.

Monocyte chemoattractant protein 1 (MCP-1) is a chemoattractant cytokine or chemokine and recruits monocytes, T cells and dendritic cells to the inflammation site. MCP-1 is implicated in the pathogenesis of inflammatory diseases like psoriasis, rheumatoid arthritis or atherosclerosis, but also in tumor progression (Yadav *et al.* 2010).

Vimentin represents a structural protein, a part of the cellular cytoskeleton, playing a role in supporting and anchoring the position of the organelle inside of the cell and in conferring flexibility to the cell. Recent reports revealed that vimentin is involved in adhesion, migration and cell signaling (Ivaska *et al.* 2007).

1.5. Matrix metalloproteinase 1/ interstitial collagenase (MMP-1)

Matrix metalloprotease 1 (MMP-1) or interstitial collagenase is an AP-1 target gene involved in skin ageing (Chung *et al.* 2000, Scharffetter-Kochanek *et al.* 2000) and tumor invasion (Westermarck and Kähäri 1999). Degradation of the connective tissue and the extracellular matrix is a hallmark in skin ageing. MMP-1 is responsible for this degradation, histologically termed basophilic degeneration. MMP-1 is a member of the matrix metalloprotease family of proteolytic enzymes consisting of at least 29 members with 23 expressed in humans (Somerville *et al.* 2003, Iyer *et al.* 2012). MMPs are synthesized inside the cell as inactive proMMP zymogens. Most of them consist of the protease domain and the ancillary or helper domain which are connected by a flexible proline-rich hinge peptide. The protease domain consists of a signal peptide, the prodomain and the catalytic module. The active site of the enzyme contains a catalytic zinc ion. In the inactive zymogen form the active site with the zinc

ion is blocked by the interaction with an unpaired conserved cysteine residue included in the prodomain. The activation of the zymogen occurs by disruption of the cysteine-zinc pairing and may be induced by a conformational change or proteolysis which may be induced by the protease plasmin, oxidative stress or by other MMPs (Ra and Parks 2007).

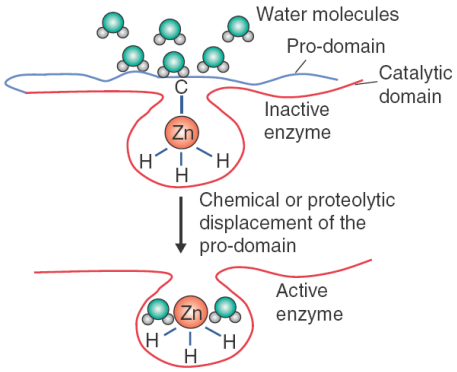

Fig. 7. Mechanism of activation of pro-MMPs
In the inactive form proMMP the active site of the enzyme is blocked by a peptid chain known as pro-domain. During activation, the pro-domain is removed proteolytically or chemically and, thus, the enzyme becomes activated.
(Figure taken from Somerville et al. 2003).

Inhibitors of MMPs constitute an alternative mechanism of MMP regulation. These inhibitors are found in the extracellular spaces and body fluids. The most important inhibitors are the tissue inhibitors of metalloproteinases (TIMPs) which are disulfide-bonded proteins of 20-30 kDa that directly interact with the MMP active site through a small number of their amino acids (Hu *et al*. 2007).

Most of the MMPs are secreted in the extracellular space as soluble MMPs. However, some of them may be membrane-anchored referred to as membrane type MMPs or MT-MMPs. In most cases, the activation occurs after secretion of the zymogen to the extracellular space (Page-McCaw *et al*. 2007, Somerville *et al*. 2003).

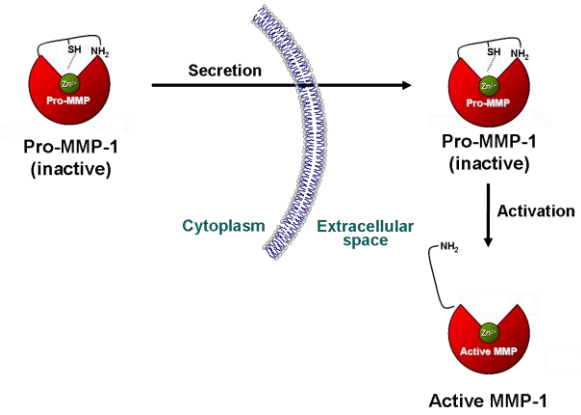

Fig. 8. Secretion and activation of MMP-1.
The inactive proMMP-1 form is synthesized inside of the cell and, subsequently, secreted outside of the cell. In the extracellular space, proMMP-1 is proteolytically processed and, thus, activated to the active form of the enzyme, MMP-1.

The active form is able to cleave proteins of the extracellular matrix and, thus plays a role in tissue remodeling. MMP-1 or collagenase cleaves interstitial collagens type I and type III which results in an altered composition of the skin with loss of resilience and tensile strength, impaired wound healing and wrinkle formation in intrinsic and extrinsic skin ageing.

1.6. Aim of the study

Skin ageing has not been characterized and understood fully in molecular detail. The overall aim of this study is to analyze the molecular mechanisms that mediate the altered redox signaling in senescent human dermal fibroblasts involved in the process of skin ageing. In detail, the following questions were addressed:

1. What is the role of SOD2 in MMP-1 induction in cellular senescence and skin ageing?
2. What are the cellular and molecular mechanisms of SOD2-mediated oxidative stress?
3. What are the signaling pathways regulated by SOD2-mediated oxidative stress involved in the induction of MMP-1 and degradation of the connective tissue as a hallmark of skin ageing?

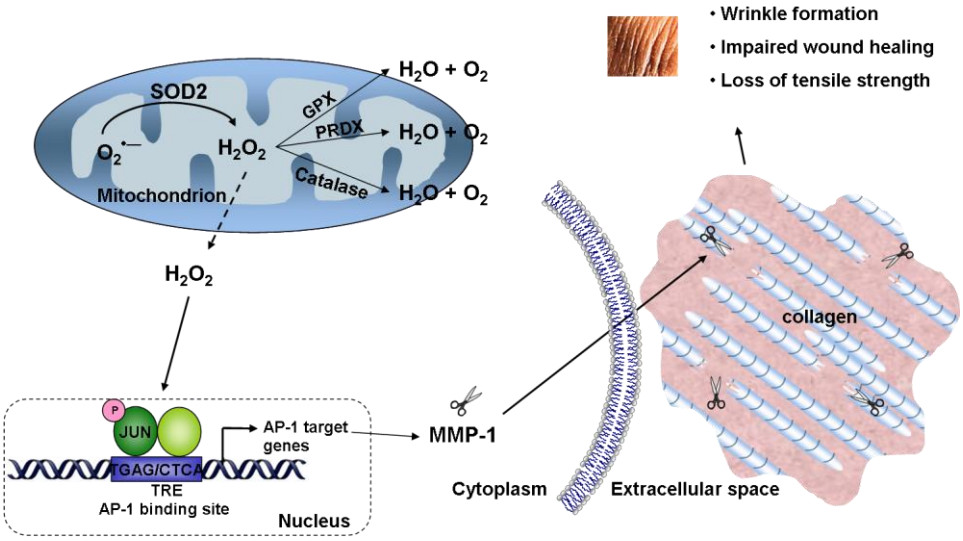

Fig. 9. Hypothesis: increased intracellular levels of H_2O_2 induce MMP-1 via AP-1 activation.
The hypothesis of this study predicts that SOD2 overexpression in senescent fibroblasts yielding an imbalanced increase in H_2O_2 may induce AP-1 transactivation by cJUN phosphorylation and subsequent induction of AP-1 target genes. Among them, MMP-1 is particularly interesting for skin ageing as it may induce the cleavage of interstitial collagens type I and III. These processes are involved in the wrinkle formation, impaired wound healing and loss of tensile strength which are features of skin ageing.

2. MATERIALS AND METHODS

2.1. Cell culture and drug treatment

Materials

DMEM – 12-604F, Lonza

FBS superior – S0615, Biochrom AG

Penicillin 10 000 U/mL, Streptomycin 10 000 µg/mL - Biochrom, cat no. A2213

L-Glutamine 200 mM – Biochrom, cat no. KO283

Sodium chloride 0.9% - Braun

PBS Dulbecco without calcium and magnesium – Biochrom, cat no. L1825

Accutase – PAA, cat no. L11-007

Human foreskin fibroblasts collected from 3 donors of 1 year old age were cultivated until replicative senescence. Cells were grown in Dulbecco's modified Eagle's medium DMEM supplemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine and maintained in a humidified 5% CO_2 - 95% air atmosphere at 37°C. They were passaged when they reached 90% confluence. The replicative age of fibroblasts was quantified by cumulative population doublings (CPDs) which is calculated after counting cells in a Fuchs-Rosenthal haemocytometer:

$$CPD_n = \sum_{i=1}^{i=n} (\ln(a_i / a_0) / \ln 2)$$

a_i = Cell number per dish at the end of each passage.

a_0 = cell number seeded per dish at the beginning of each passage.

Early passage fibroblasts were defined as ‘young’ at cumulative population doublings (CPD) 8 and 20. Senescent fibroblasts at CPDs greater than 58 with a proliferation rate lower than 0.1 PD per week showed more than 95% positive cells for senescence associated biomarker beta-galactosidase and upregulation of senescence marker $p16^{INK4a}$.

Drug treatment

Young or senescent fibroblasts were serum starved overnight in DMEM with 0.1% FCS and subsequently treated with different reagents: buthionine sulfoximine/ BSO (Sigma, cat no. A7250), aminotriazole/ ATZ (Sigma, cat no. A8056), N-acetyl-cysteine/ NAC (Sigma, cat no. A7250), hydrogen peroxide/ H_2O_2 (JT Baker, cat no. 7047), SP600125 (Sigma, cat no. S5567) at the concentrations and the time points as indicated in the result section.

2.2. Human skin samples

Five mm skin biopsies from healthy individuals were collected after informed consent. Skin samples were frozen in liquid nitrogen and stored at -80ºC. Two independent age groups were studied: young donors between 13 and 22 years and old donors ranging between 61 and 82 years. All protocols were approved by the Ethics Committee at the University of Ulm according to the Declaration of Helsinki Principles.

2.3. 2D-DIGE/MS

Two dimensional fluorescence difference gel electrophoresis (2D-DIGE) was performed in cooperation with Laboratory of Cellular Biochemistry and Biology, University of Namur, Belgium according to (Chrétien *et al*. 2008). Briefly, protein extracts were prepared by cell lysis with DLA buffer (7M urea, 2M thiourea, 30 mM Tris, 4% CHAPS, pH 8.5) and subsequently labeled with the fluorescent dyes cyanin. The internal control consisted of a mixture of equal parts of protein extracts from young and senescent fibroblasts. The labeled samples were run on a 2D gel and protein spots were scanned and analyzed by mass spectrometry. The analysis of the protein spots by mass spectrometry (MS) was performed in collaboration with Department of Biochemistry and Molecular Biology, University of Odense, Denmark.

2.4. Western blot

Materials

0.9% NaCl	Braun
Tris	USB, 758251
Glycine	USB, 16407
SDS	Serva, 20765
Methanol	Sigma, 32213
Sodium chloride	Sigma, 31434
Glycerol	AppliChem, A3739
Triton X-100	Serva, 37240
EDTA	AppliChem, A3553
Sodium fluoride	Sigma, S7920
Sodium orthovanadate	Sigma, S6508
Protease inhibitors	Roche, 11836153001
Tween 20	Serva, 37470
Blotting Grade Blocker Non-Fat Dry Milk	Biorad, cat no. 170-6404
20x Lumiglo Reagent and 20x Peroxide	Cell Signaling, cat no. 7003

Buffers

Lysis buffer

20 mM Tris HCl pH8.0

137 mM NaCl

10% glycerol

1% Triton X-100

2mM EDTA

100 mM NaF (protease inhibitor)

2 mM Na_3VO_4 (protease inhibitor)

Running buffer 1x

25 mM Tris

250 mM Glycine

1% SDS

Transfer buffer 1x

240 mM Tris

190 mM glycine

0.185% SDS

20% methanol

TBS (Tris-buffered saline) 1x

150 mM NaCl

10 mM Tris

pH 7.4

TBST

150 mM NaCl

10 mM Tris pH 7.4

0.05% Tween 20

Blocking solution

5% non-fat dry milk in TBST

Bio-Rad Protein Assay Bradford – cat no. 500-0006, Bio-Rad

Antibodies

p16 – mouse monoclonal antibody – 554070, Becton Dickinson

FKBP10 – rabbit polyclonal antibody – 98767, Santa Cruz Biotechnology

TCP-1 subunit eta – goat polyclonal antibody – sc13889, Santa Cruz Biotechnology

SOD2 – rabbit polyclonal antibody – sc-30080, Santa Cruz Biotechnology

Catalase – rabbit monoclonal antibody – 2058-1, Epitomics

Glutathione peroxidase 1 – rabbit polyclonal antibody – ab22604, Abcam

Phosphorylated cJUN – mouse monoclonal antibody – sc-822, Santa Cruz Biotechnology

cJUN – rabbit polyclonal antibody – sc-1694, Santa Cruz Biotechnology

Vimentin – mouse monoclonal antibody – MAB3400, Millipore

PTRF – mouse monoclonal antibody – 611258, BD

Actin – goat polyclonal antibody – sc-1615-HRP, Santa Cruz Biotechnology

Anti- rabbit secondary antibody conjugated to horseradish peroxidase – Dianova

Anti-mouse secondary antibody conjugated to horseradish peroxidase – Dianova

Cells were washed with 0.9% NaCl and then lysed with lysis buffer, and detached with a cell scrapper. The lysates were incubated 10 minutes on ice in order to favor the lysis of cells and centrifuged for 15 minutes at 4ºC to remove the dead cells and the cell debris. The supernatant was collected, snap frozen in liquid nitrogen and stored at -80°C until processed. The protein concentration in protein extracts was measured by Bradford assay (BioRad) according to the recommended manufacturer's protocol. Samples were loaded and run on SDS-PAGE (polyacrylamide) gels, and proteins were electrotransferred from the gel to nitrocellulose membranes using a semi-dry transfer method (1 hour, 20V). The nitrocellulose membrane was blocked in blocking buffer for 1hour at room temperature. Membranes were probed with antibodies diluted in blocking buffer at different concentrations (1:10 000 for SOD2, 1:5000 for catalase, 1:1000 for GPX-1, phosphorylated cJUN, cJUN, PTRF, 1:500 for vimentin) and incubated overnight at 4ºC. After 30 minutes washing with TBST (0.05% Tween20), membranes were incubated with appropriate secondary antibodies at a concentration of 1:10000 and washed again for 30 minutes in TBST (0.005% Tween20). Thereafter membranes were developed using a chemiluminescence detection device and the Lumiglo chemiluminescent substrate.

2.5. Immunofluorescence staining

Materials

PBS	Biochrom, L1825
Methanol	Sigma, 32213
Triton X-100	Serva, 39795.01
Bovine serum albumin (BSA)	PAA, K41-001
DAPI	Sigma, D8417
Antibody diluent	Dako, S3022

Antibodies

SOD2 – rabbit polyclonal antibody – sc-30080, Santa Cruz Biotechnology

Catalase – rabbit monoclonal antibody – 2058-1, Epitomics

Anti-mouse Alexa 555 – Invitrogen

Anti-rabbit 488 – Invitrogen

2.5.1. Immunofluorescence staining of fibroblasts

Fibroblasts were seeded on glass slides at a cell density of 10^5 cells/slide for young fibroblasts and $5x10^4$ cells/slide for senescent fibroblasts and incubated for 24 hours. Fibroblasts were washed with PBS, fixed with 100% methanol, permeabilized with 0.5% Triton X-100, blocked with 5% BSA in PBS, incubated with antibodies against SOD2 (Santa Cruz, cat no. sc-30080) or catalase (Sigma Aldrich, cat no C0979), washed in PBS and subsequently, incubated with secondary antibodies conjugated with fluorescent dyes Alexa 555 and Alexa 488 and washed again. DAPI (1.4µM) was used for nuclei staining. Photomicrographs were taken with a Zeiss Axiophot microscope and corresponding software (Zeiss).

2.5.2. Immunofluorescence staining of cryosections from human skin samples

5µM cryosections from human skin collected from young and old donors were fixed with 4% paraformaldehyde, blocked with 5% BSA, incubated with antibodies against SOD2 (cat no sc-30080, Santa Cruz) or phosphorylated-cJUN (Santa Cruz, sc-822), washed with PBS and incubated with secondary antibodies conjugated with fluorescent dyes Alexa 555 and Alexa 488. DAPI (1.4µM) was used for nuclei staining. Photomicrographs were taken with an Axio Imager.M1 microscope (Zeiss) with a AxioCam MRc digital camera (Zeiss) and analysed by AxioVision LE; Rel.4.3. software (Zeiss).

2.6. SOD activity assay

Materials

Triton X-100, reduced	Sigma, X100RS
Potassium dihydrogen phosphate	AppliChem, A3620
Potassium monohydrogen phosphate	AppliChem, A3589
Diethylenetriaminepentaacetic acid (DETAPAC)	Sigma, D1133
Catalase from bovine liver	Sigma, C1345
Xanthine	Sigma, X7375
Bovine superoxide dismutase	Sigma, S7571

Sodium cyanide (NaCN)	Sigma, S3296
Cytochrome c	Sigma, C2037
Xanthine oxidase	Sigma, X4875

Buffers

0.1 M monopotassium phosphate (KH_2PO_4) pH 7.0

0.166M dipotassium phosphate (K_2HPO_4) pH 7.0

DETAPAC 1mM

10 U/mL catalase

11.8mM xanthine dissolved in 0.1N sodium hydroxide (NaOH)

50 mM sodium cyanide (NaCN)

500 µM cytochrome C

0.1U/mL xanthine oxidase

Reaction mixture

0.722 mM DETAPAC

2 U/mL catalase

0.33 mM xanthine

SOD activity was quantified by measuring SOD ability to scavenge superoxide anion radicals generated by xanthine/xanthine oxidase. Production of superoxide anion radicals in the system was quantified based on their capability to reduce cytochrome c. The reduced form of cytochrome c has a maximum of absorbance at 550nm which may be monitored spectrophotometrically according to (McCord and Fridovich 1969). Young and senescent fibroblasts were lysed with freshly prepared lysis buffer (1% reduced Triton X-100 in 0.1M KH_2PO_4 pH 7). Protein extracts collected from identical number of young and senescent fibroblasts (10^5 cells) were added to the reaction mixture, incubated for 30 minutes in the dark. Cytochrome c (50µM) was added into the sytem and optical density (OD) was monitored for 2 minutes to study possible interferences with cytochrome c reduction. Xanthine oxidase (0.01 U/mL) was added to induce production of superoxide and OD of the mixture was measured during 8 minutes. Slope of the curve corresponding to the change in OD was calculated for

each well. Inihibition of cytochrome c reduction induced by SOD was estimated according to the formula:

% inhibition of sample = (slope[blank] – slope[sample])/slope[sample] x 100

One unit SOD is defined as the amount of SOD that induces 50% inhibition of the rate of cytochrome c reduction. The results were expressed as units per 10^6 cells. SOD2 activity was measured separately after inhibition of SOD1 with 5mM NaCN. The optical density was monitored using a Tecan Saphire II microplate reader.

The procedure was identical for detection of SOD2 activity in human skin samples. The human skin samples after epidermis removal were lysed and 200µg protein extracts were taken for analysis.

2.7. Measurement of mitochondrial superoxide anion concentrations by the specific dye MitoSOX

Materials

MitoSOX Invitrogen, M36008

Buffers

FACS buffer

MitoSOX is a dye that accumulates selectively in the mitochondria and is rapidly oxidized by superoxide to a highly fluorescent compound (excitation wavelength 396 nm, emission wavelength 510 nm). Fibroblasts were seeded in 6-well plates at a cell density of 10^5 cells/well and 24 hours later were stained with MitoSOX 5µM for 30 minutes, in the dark, at 37ºC. At the end of the incubation time, the fibroblasts were washed with PBS, detached and transferred to FACS tubes in FACS buffer. The fluorescence was monitored using flow cytometry (FACS Canto II, BD Biosciences) for 10^4 cells at excitation wavelength of 396 nm and emission of 510 nm.

2.8. Catalase activity assay

Catalase activity was measured spectrophotometrically based on the decomposition of H_2O_2 due to its absorbance at 240 nm. Protein lysates were prepared from young and senescent fibroblasts, exogenous H_2O_2 was added into the system and the decrease in absorbance at 240 nm was monitored every 10 seconds

for 2 minutes in quarz cuvettes using the Ultrospec 3000 UV/visible spectrophotometer (Pharmacia Biotech). One unit catalase activity was defined as the amount of enzyme which is able to decompose 1 µmol H_2O_2 per minute at 25ºC and pH 7.0.

2.9. Glutathione peroxidase assay

GPx buffer

0.1 M Tris buffer (pH 8.0)

0.5 mM EDTA (pH 8.0)

200 µM NADPH

2 mM glutathione

1 U/mL glutathione reductase

Glutathione peroxidases are selenoproteins that catalyze the reduction of various peroxides by oxidation of reduced glutathione (GSH) to oxidized (GSSG) glutathione. The oxidized glutathione is subsequently reduced by glutathione reductase (GR) and NADPH forming NADP and, thus recycling GSH.

$$R\text{-}OOH + 2\ GSH \rightarrow R\text{-}OH + H_2O + GSSG$$

$$GSSG + NADPH/H^+ \rightarrow 2\ GSH + NADP^+$$

The method for measuring GPx activity (Paglia and Valentine 1967) monitors the rate of NADPH oxidation to $NADP^+$ which results in a decrease in absorbance as measured at 340 nm. The reaction is initiated with t-butyl-hydroperoxide as a substrate and external addition of glutathione and glutathione reductase. Young and senescent fibroblasts were lysed using the same conditions as mentioned before [see 2.4. Western blot]. Twenty µg protein lysates were diluted in 100 µl reaction buffer and incubated for 3 minutes at 37ºC. Absorbance of the mixture was determined in the Ultrospec 3000 UV/ visible spectrophotometer (Pharmacia Biotech) at 340 nm in quarz cuvettes for 150 seconds. The reaction was started with t-butyl-hydroperoxide (14µM) which was added to the system. The activity of the enzyme was calculated by the slope of time versus absorption and expressed as units GPx activity per mg protein. One unit of enzyme was defined as the amount of enzyme needed to reduce 1µmol NADPH per minute at 25ºC.

2.10. Measurement of H_2O_2 concentrations by genetically encoded biosensor HyPer

Materials

pHyPer-cyto Evrogen, FP941

pHyPer-dMito Evrogen, FP942

AdCyto.HyPer

AdMito-HyPer

Buffers

FACS buffer

The expression cassette of pHyPer-cyto (cat no. FP941, Evrogen; (Belousov *et al.* 2006) and pHyPer-dMito (cat no. FP942, Evrogen) vectors were cloned into the adenovirus vector plasmid backbone pGS66 as described before (Schiedner *et al*. 2006). Briefly, the HyPer adenoviral vector encodes the HyPer protein that consists of the H_2O_2-sensitive regulatory domain of OxyR coupled to the yellow fluorescent protein (YFP). H_2O_2 will oxidize the 2 cysteine residues within the regulatory domain of OxyR, thus inducing a switch in YFP fluorescence from 488 nm to 405 nm. The higher the shift in fluorescence, the higher the H_2O_2 concentrations are. Young and senescent fibroblasts at a cell density of 10^5 cells/well were cultivated in 6-well plates for 24 hours and transduced with the AdCyto-HyPer and respectively AdMito-HyPer vectors at multiplicity of infection (MOI) of 5000 for 48 hours. At the end of incubation time, the cells were washed with PBS, detached and transferred to FACS tubes in FACS buffer. The fluorescence was monitored by flow cytometry (FACS Canto II, BD Biosciences) at excitation wavelength of 405 nm (reduced form of HyPer) and 488 nm (oxidized form of HyPer) and emission of 540 nm. The ratio of relative fluorescence units (RFU) at 488 nm and 405 nm was calculated for 10^4 cells.

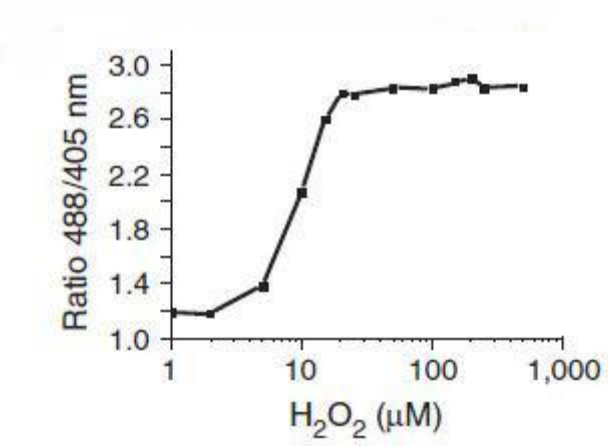

Fig. 10. HyPer response to hydrogen peroxide
Standard curve for the ratio response of the HyPer construct reveals the non-linear response for hydrogen peroxide (Belousov et al. 2006).

2.11. Measurement of ROS concentrations by DHE staining in skin cryosections

Materials

Dihydroethidium (DHE)	Invitrogen, D11347
Catalase from bovine liver	Sigma, C1345
N-acetyl-cysteine	Sigma, A7250
DAPI	Sigma, D8417

Ten µM-thick fresh cryosections collected from skin from young and old individuals were incubated with DHE (2µM) diluted in PBS for 20 minutes, 37ºC, in the dark. Nuclei were stained with DAPI. To identify specific ROS, the cryosections were coincubated with DHE and the ROS scavengers catalase (750 U) or N-acetyl-cysteine (5 mM). Sections were fixed with 4% paraformaldehyde for 10 minutes at 4°C and fluorescence was monitored with a fluorescence microscope (Axio Imager.M1, Zeiss). Identical exposure time was used for all cryosections when taking the pictures. In order to avoid autofluorescence background, the exposure time where no fluorescence was observed in the young skin treated with PBS and set as threshold.

2.12. MMP-1 activity assay

Materials

SensoLyte® Plus 520 MMP-1 Assay Kit *Fluorimetric and Enhanced Selectivity* AnaSpec, 72012

MMP-1 and pro-MMP-1 in the biological samples are captured by a mouse monoclonal antibody immobilized on an ELISA plate. APMA (4-aminophenylmercuric acetate) is used to activate the inactive form proMMP-1 into MMP-1. The fluorogenic peptide 5-FAM/QXL™520FRET peptide which functions as a MMP-1 substrate is added into the system. The proteolytic activity of MMP-1 is measured by the increase in fluorescence. Young, senescent fibroblasts and treated senescent fibroblasts were serum starved for 12 hours in DMEM with 0.1% FCS and the medium was collected and stored at -80ºC until the assay was performed. MMP-1 activity was monitored in supernatants collected from fibroblasts according to the manufacture recommendations. Briefly, 100 µl supernatant was added per well in the 96-well plate coated with the antibody raised against MMP-1 and pro-MMP1. For every assay, a standard curve with recombinant pro-MMP-1 was run together with the samples. The samples were incubated for 2 hours at room temperature with gentle shaking (100rpm). Samples were discarded from the wells and the wells were washed 4 times with washing buffer 1x. To activate proMMP-1 to MMP-1, 100µl APMA (4-aminophenylmercuric acetate) 1 mM was added in each well and incubated for 3 hours at 37ºC. The APMA was discarded and the fluorogenic substrate was added to the wells – 100 µl of MMP-1 substrate (5-FAM/QXL™520FRET peptide) and incubated for 2 hours at room temperature in the dark. The fluorescence was monitored with the Mithras LB940 microplate reader (Berthold Technologies) and an excitation wavelength of 490 nm and emission wavelength of 520 nm.

2.13. Activity of AP-1 by lentiviral reporter vectors

Materials

Cignal Lenti AP-1 Reporter (luc)	SA Biosciences, CLS-011L
Cignal Lenti Negative Control (luc)	SA Biosciences, CLS-NCL
Cignal Lenti Renilla Control (luc)	SA Biosciences, CLS-RCL
SureENTRY™ Transduction Reagent	SA Biosciences, 336921
Dual Glo Luciferase Assay System	Promega, E2940

Activation of AP-1 binding site TPA responsive element (TRE) was measured by luciferase reporter gene assays. Young and senescent fibroblasts seeded in 6-well plates at a cell density of 10^5 cells per well were transiently cotransduced with a lentiviral vector encoding the firefly luciferase under the control of AP-1 binding site TRE (Cignal Lenti AP-1 Reporter, MOI 10) and a lentiviral vector encoding Renilla luciferase driven by CMV promoter (Cignal Lenti Renilla Control, MOI 5). As a negative control, a vector encoding firefly luciferase, but not the TRE element (Cignal Lenti Negative Control, MOI 10) was used. The cells were incubated with the lentiviral vectors for 48 hours. 24 hours before the end of the incubation time, medium was replaced with serum free medium to avoid possible interference of components of serum with AP-1 pathway. Activity of firefly and Renilla luciferase was monitored using the Dual Glo Luciferase Assay System, Promega according to the manufacturer's protocol. Briefly, cells were lysed with 300 µl Luciferase reagent and incubated for 10 minutes at room temperature and the luminescence was measured for firefly luciferase. Subsequently, 300 µl Stop&Glo reagent was added and incubated for 10 minutes at room temperature and the luminescence was measured for Renilla luciferase. Results were expressed as ratio of relative luminescence units (RLU) generated by firefly luciferase per RLU generated by Renilla luciferase. Luminescence was measured at 0.5 seconds per well, in the LumiNunc™ plates (white 96-well plates, Nunc, 236105), using the Mithras LB940 microplate reader (Berthold Technologies).

2.14. Detection of AP-1 activation by Transcription Factor ELISA

Materials

TransAM® AP-1 Family	Active Motif, 44296
Sodium fluoride	Sigma, S7920
β-Glycerophosphate	Fluka, 50020
Para-nitrophenyl phosphate (PNPP)	Sigma, 71768
Sodium orthovanadate	Sigma, S-6508
HEPES 1M	Gibco, 15630-056
Sodium orthomolybdate	AppliChem, A2193
EDTA	AppliChem, A3553
NP-40	Fluka, 74385

Buffers

Phosphate Inhibitor Buffer – PIB

125 mM NaF

250 mM β-glycerophosphate

250 mM PNPP

25 mM sodium orthovanadate

Hypotonic buffer

20 mM HEPES pH 7.5

5 mM NaF

10 µM sodium orthomolybdate

0.1 mM EDTA

10% NP-40

2.14.1. Preparation of nuclear extracts

Nuclear extracts were prepared as recommended in the manual of the kit TransAM® AP-1 Family. Nuclear extracts were prepared according to the manufacturer's protocol. Briefly, cells were washed with ice cold PBS/PIB and scraped from the dish. Cell suspension was centrifuged at 300 gx, at 4ºC for 5

minutes. The cell pellet was resuspended in 1 ml hypotonic buffer, incubated on ice for 15 minutes and Nonidet P-40 was added at a concentration of 10%. After centrifugation at 14 000 rpm, 4°C for 30 seconds, supernatants containing the cytoplasmic fraction were collected. Nuclear pellet was resuspended in 50 µl Complete Lysis Buffer and incubated for 30 minutes on ice on an orbital shaker. Supernatants containing the nuclear extracts were collected after centrifugation at 14 000 rpm, 4ºC for 10 minutes. Protein concentrations of nuclear extracts were measured by the Bradford assay (BioRad). Two µg nuclear extracts prepared from young and senescent fibroblasts were loaded per well. The Transcription Factor ELISA was performed according to the instructions in the manual and analyzed for phosphorylated cJUN. The incubation time with the substrate was 7 minutes for the phosphorylated cJUN antibody.

2.15. Silencing of distinct genes by small interfering RNAs (siRNAs)

Materials

ON-TARGET plus SMART pool cJUN	Dharmacon, L-003268-00-0005
ON-TARGET plus SMART pool SOD2	Dharmacon, L-009784-00-0005
ON-TARGET plus Non-targeting pool	Dharmacon, D-001810-10-05
DharmaFECT 4 siRNA Transfection Reagent	Dharmacon, T-2004-01

Senescent fibroblasts were transfected with 25 nM ON-TARGET siRNA against cJUN or SOD2 in 6-well plates. After 24 hours, protein lysates were prepared and expression of silenced proteins was assessed by Western blot analysis. For measurement of MMP-1 activity, 24 hours after first addition of siRNA, the medium was removed and fresh serum free medium with siRNA at the same concentration was added. At the end of the incubation time, medium was collected and MMP-1 total activity was analyzed.

2.16. Detection of MCP-1 in supernatants

Materials

Human CCL2/MCP-1 Quantikine ELISA kit - R&D, DCP00

The kit consists of a quantitative sandwich ELISA assay. The samples were supernatants collected from fibroblasts that were incubated with serum free medium. The assay was performed according to the manufacturer's recommendations provided in the data sheet.

2.17. Lentivirally – mediated overexpression of SOD2 and mitochondria – targeted catalase

Materials

Lentiviruses containing vector with human SOD2 cDNA

Lentiviruses containing vector with human catalase cDNA with mitochondrial leader sequence

Control lentiviruses containing vector with GFP

Polybrene – Sigma, 107689

Generation of lentiviral vectors

In order to generate lentiviral vectors, a third generation packaging system was used (Addgene) in cooperation with Department of Gene Therapy of the University of Ulm (Head: Prof. Stefan Kochanek) in accordance with S2 regulations. This system consisted of the 3 vectors pCS (the lentiviral vector where the target cDNA was inserted), pMDLg/pRRE (packaging plasmid), pRSV-Rev (packaging plasmid) and pMD2.G (envelope plasmid). Hek293T cells were cotransfected with these 4 plasmids using the PEI (polyethylenimine) method and the supernatants containing the lentiviruses were collected after 72 hours of transfection, subsequently centrifuged and further frozen at -80°C.

The human SOD2 cDNA was analysed by restriction analysis, cut by restriction enzymes from a plasmid (Wenk *et al.* 1999), and inserted into the pCS vector. The human MCAT cDNA containing the human catalase cDNA with a mitochondrial targeting sequence was taken from a plasmid that was a gift from Department of

Biological Chemistry, University of California, USA (Schriner *et al.* 2005). The MCAT cDNA was analysed, cut and subsequently inserted into the pCS vector.

Fibroblasts were seeded in 6-well plates at a cell density of 10^5 cells/well for young fibroblasts and $5x10^4$ cells/well for senescent fibroblasts. After 24 hours incubation at 37ºC, the fibroblasts were transduced with the corresponding lentiviruses of 2 ml lentiviruses per well and polybrene at a concentration of 8µg/ml in order to increase the transduction efficiency (Kutner *et al.* 2009). As a control, a lentiviral preparation containing GFP protein was used. The fibroblasts were incubated for 72 hours at 37ºC with the lentiviruses. At this time point, protein lysates were prepared in order to perform Western blot analysis to test for the protein expression. To monitor MMP-1 activity, the medium containing lentiviral particles was discarded at 72 hours time point and fresh medium was added and the cells were incubated for another 24 hours. At the end of the incubation time, the supernatant was collected and MMP-1 activity tested. All lentiviral transductions were performed according to S2 regulation of the genetic engineering act.

2.18. Statistical analysis

Quantitative data are presented as mean ± standard deviation (SD). Statistical significance was determined by the unpaired two-tailed Student's test. P values less than 0.05 were considered statistically significant. Statistical significance was graphically indicated as (*) for $p<0.05$, (**) for $p<0.01$ and (***) for $p<0.001$.

3. RESULTS

3.1 Several proteins are differentially expressed in replicative senescent compared to young fibroblasts as revealed by 2D-DIGE and mass spectrometry

In order to study the role of SOD2 in MMP-1 induction in cellular senescence and skin aging, I first set out to identify differentially expressed proteins in replicative senescent versus young fibroblasts. A proteomic approach was used with 2D fluorescence difference gel electrophoresis (2D-DIGE) and mass spectrometry (MS) (Table I). Antioxidant enzymes were found to be upregulated in replicative senescent human dermal fibroblasts. Interestingly, manganese superoxide dismutase (SOD2) was found to be upregulated 13-fold on protein level in senescent (CPD70) compared to young (CPD20) fibroblasts. The increase in SOD2 expression in senescent fibroblasts was validated in three independent fibroblast strains using immunoblotting (Fig. 11).

Protein	Fold expression	Function	Gene group
SOD2	13.3	Antioxidative enzyme	Damage protection
Peroxiredoxin2	1.6	Antioxidative enzyme	Damage protection
Peroxiredoxin5	1.8	Antioxidative enzyme	Damage protection
Peroxiredoxin3	1.7	Antioxidative enzyme	Damage protection
Aldose reductase	4.5	Aldehyde reductase activity	Damage protection
Annexin A5	9.2	Anticoagulant protein	Apoptosis
Annexin A1	6.4	Calcium/phospholipid-binding protein	Cell communication
Annexin A2	4.5	Calcium-regulated membrane-binding protein	Cell communication
Annexin A6	3.0	Calcium/phospholipid-binding protein	Cell communication
Polymerase I and transcript release factor	3.8	Initiation of transcription	RNA synthesis
T-complex protein1 subunit eta	3.9	Protein folding	Protein biogenesis
FK506-binding protein 10	3.7	Protein folding	Protein biogenesis
Vimentin	10.9	Intermediate filament protein	Cell structure
Alpha-actinin-4	3.7	F-actin crosslinking protein	Cell structure

Table I. Differentially expressed proteins in senescent compared to young fibroblasts.
Proteins with different cellular functions were upregulated in senescent (CPD70) compared to young (CPD20) fibroblasts collected from one young donor of 1 year using 2-dimensional fluorescence difference gel electrophoresis (2D-DIGE) coupled with identification of protein spots by mass spectrometry (MS) [in collaboration with Laboratory of Cellular Biochemistry and Biology, University of Namur and Department of Biochemistry and Molecular Biology, University of Odense, Denmark]. Shown are proteins with an increase in protein levels of at least 1.5-fold.

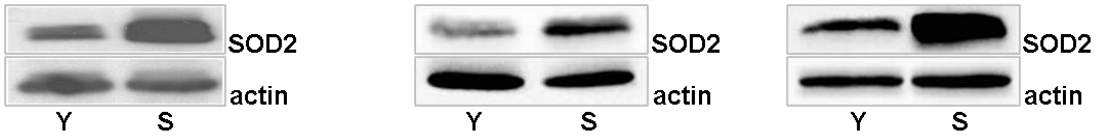

Fig. 11. Protein expression of SOD2 in young and senescent fibroblasts in 3 independent fibroblasts strains
SOD2 protein expression is highly increased in replicative senescent fibroblasts in 3 independent fibroblasts strains isolated from 3 independent donors as shown by Western blot.(Y: young, S: senescent).

Several proteins were found to be up-regulated in senesent fibroblasts as compared to young fibroblasts. The following proteins were increased: Annexins A5, A1, A2 involved in cell communication, vimentin and alpha-actinin-4 essential for maintaining cell structure, polymerase I and transcript release factor (PTRF) which play an important role in RNA synthesis, T-complex protein 1 subunit eta (TCP1) and FK506-binding protein 10 (FKBP10) involved in protein biogenesis. To coroborate the results of the 2D-DIGE experiments, immunoblotting was performed for selected proteins. The expression of SOD2, PTRF, FKBP10, vimentin and TCP-1 was examined in fibroblasts of three independent fibroblasts strains. Upregulation of SOD2 (Fig. 11), polymerase I and transcript release factor (PTRF) (Fig. 12) and vimentin (Fig. 13) was confirmed in senescent fibroblasts by Western blot analysis.

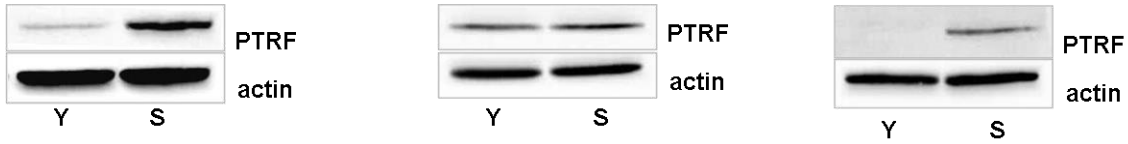

Fig. 12. PTRF protein expression in young and senescent fibroblasts
PTRF protein expression was increased in lysates prepared from senescent fibroblasts compared to young fibroblasts as revealed by Western blot analysis in three independent fibroblasts strains collected from donors of 1 year. (Y: young, S: senescent)

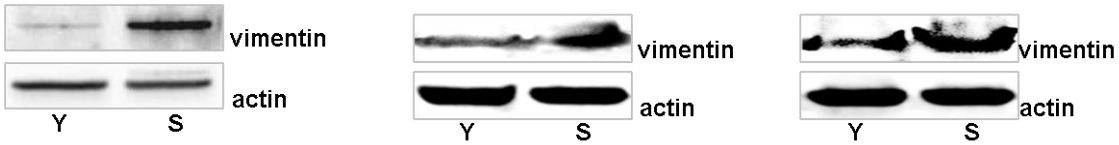

Fig. 13. Protein expression of vimentin in young and senescent fibroblasts
The protein expression of vimentin reveals a high increase in lysates prepared from senescent compared to young fibroblasts as shown by Western blot analysis of three independent strains. (Y: young, S: senescent).

The induction of FK506-binding protein 10 was confirmed in one fibroblast strain with no change in the second and decrease in the third fibroblast strain indicating that the expression of this protein might be cell strain-dependent (Fig. 14).

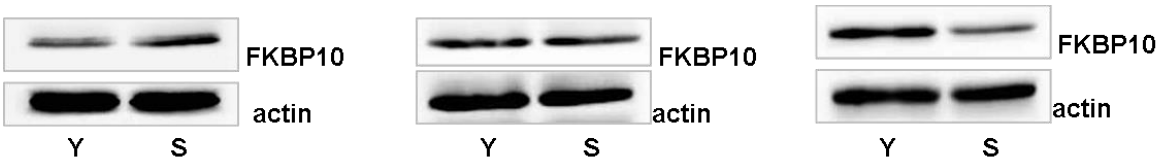

Fig. 14. FKBP10 protein expression in young and senescent fibroblasts
FKBP10 protein expression was increased in lysates prepared from senescent fibroblasts compared to young fibroblasts as revealed by Western blot analysis in 3 independent fibroblasts strains (Y: young, S: senescent).

By contrast to results derived from the 2D-DIGE/MS, TCP-1 was not found to be upregulated in senescent fibroblasts by Western blot analysis (Fig. 15).

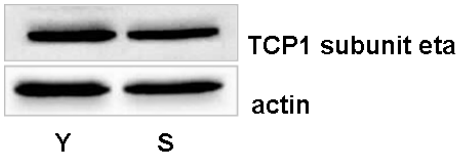

Fig. 15. Protein expression of TCP-1 subunit eta in young and senescent fibroblasts.
TCP-1 protein expression is not changed in senescent compared to young fibroblasts as shown by Western blot analysis.The result is representative for 3 independent experiments.

3.2. SOD2 protein expression and activity are increased in senescent human dermal fibroblasts in vitro and in vivo

SOD2 upregulation was confirmed by Western blot analysis with a 2-fold increase in senescent fibroblasts from 3 distinct fibroblasts strains (Fig. 11), and by immunostaining with mitochondrial staining in senescent compared to young fibroblasts (Fig. 16).

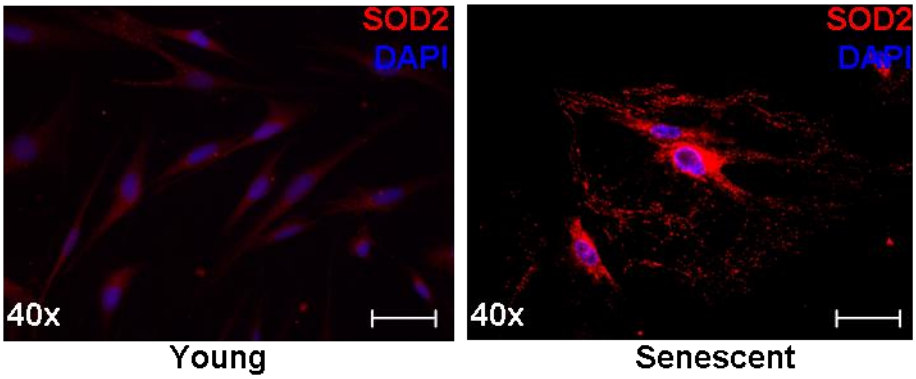

Fig. 16. SOD2 protein expression in young and senescent fibroblasts.
SOD2 protein expression is highly increased in senescent compared to young fibroblasts as shown by fluorescent staining with a particular stippled mitochondrial staining as revealed by immunofluorescence microscopy (DAPI in blue, SOD2 in red, scale bar 50µm).

Upregulation of SOD2 in senescent fibroblasts was further confirmed by a 1.5-fold increase in SOD2 activity in senescent versus young fibroblasts (Fig. 17).

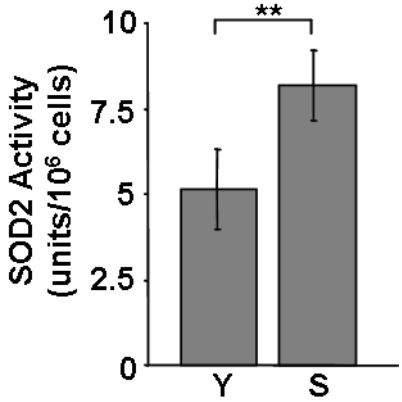

Fig. 17. SOD2 activity in young and senescent fibroblasts.
*SOD2 activity is 1.5-fold increased in whole cell lysates prepared from replicative senescent compared to young fibroblasts using the cytochrome c assay. The results represent 1 of 4 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001 (Y: young, S: senescent).*

To investigate whether these *in vitro* findings translate into skin ageing *in vivo*, SOD2 protein expression in human skin samples from young and old individuals was analysed using immunostaining. Skin cryosections from 5 young individuals (3-22 years) versus 5 old individuals (61-82 years) were assessed with immunostaining with antibodies against SOD2. Skin samples from old individuals revealed statistically significant increased number of cells positive for SOD2 (Fig. 18), quantified by counting (Fig. 19).

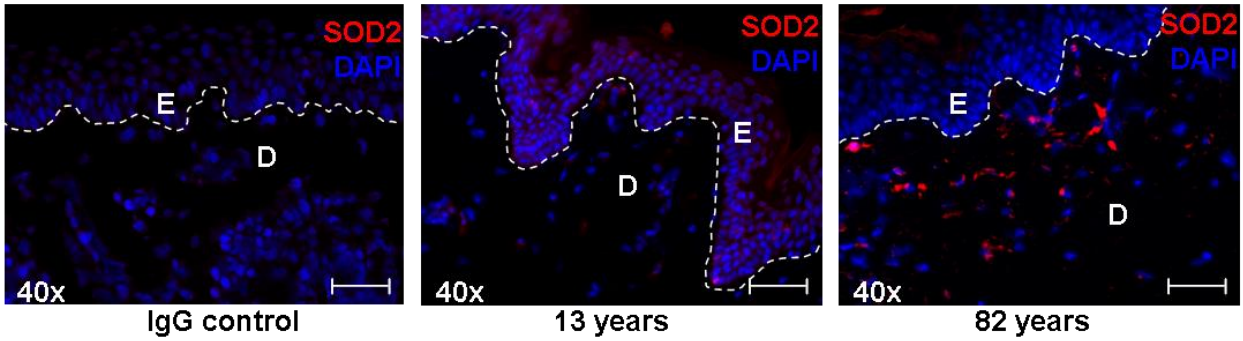

Fig. 18. SOD2 protein expressing cells in young and old skin.
The number of SOD2 expressing dermal cells is significantly increased in cryosections prepared from human skin samples collected from old compared to cryosections from human skin samples from young donors. The increase in SOD2 protein expression displays as a strong red fluorescence signal in fibroblasts localized in the dermis. The nucleus is stained with DAPI (blue) (D dermis, E epidermis, scale bar 50µm).

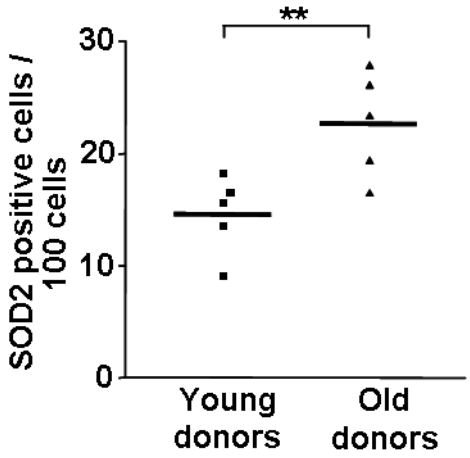

Fig. 19. Quantification of SOD2 protein expression in young and old skin.
*The number of dermal SOD2 positive cells expressed as percentage of total cell number is increased in the cryosections of 5 old compared to 5 young donors. SOD2 positive cells were counted and normalized per total dermal cell number as indicated by DAPI staining (blue). Positive cells were counted in 50 high power fields per donor. Results are expressed as percentage of positive cells per total cell number. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001.*

To complement the immunofluorescence data, immunoblotting of total skin lysate with the SOD2 antibody was performed. Similar to the data with immunostaining in figure 18, SOD2 expression is increased on the protein level in aged human skin.

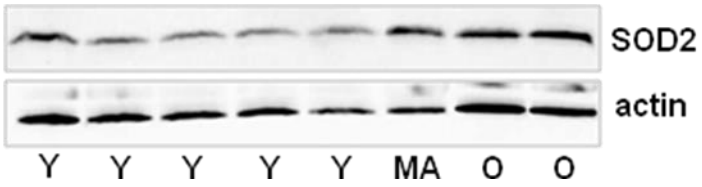

Fig. 20. SOD2 protein expression in young and old skin with immunoblotting.
SOD2 expression in 5 young, one middle-aged and two old individuals was determined semiquantatively using immunoblotting. The SOD2 expression increased clearly in the old skin (Y: 18 – 26 years, O: 78 – 79 years, MA: 43 years).

To further analyze the relevance of SOD2-expression in old versus young skin, the enzymatic activity of SOD2 was investigated in 8 tissue samples from old compared to young skin identical to the immunoblotting. Whereas total SOD-activity including activity of SOD1 and SOD2 revealed a relatively stable activity from young to middle-aged skin with 6-8 units/mg protein and decreased activity of 4 units/mg protein in old donors, the activity of SOD2 in young skin with 3-6 units/mg protein showed higher variability with a clear decrease of down to 30% with 2 units /mg protein in old skin (Fig. 21).

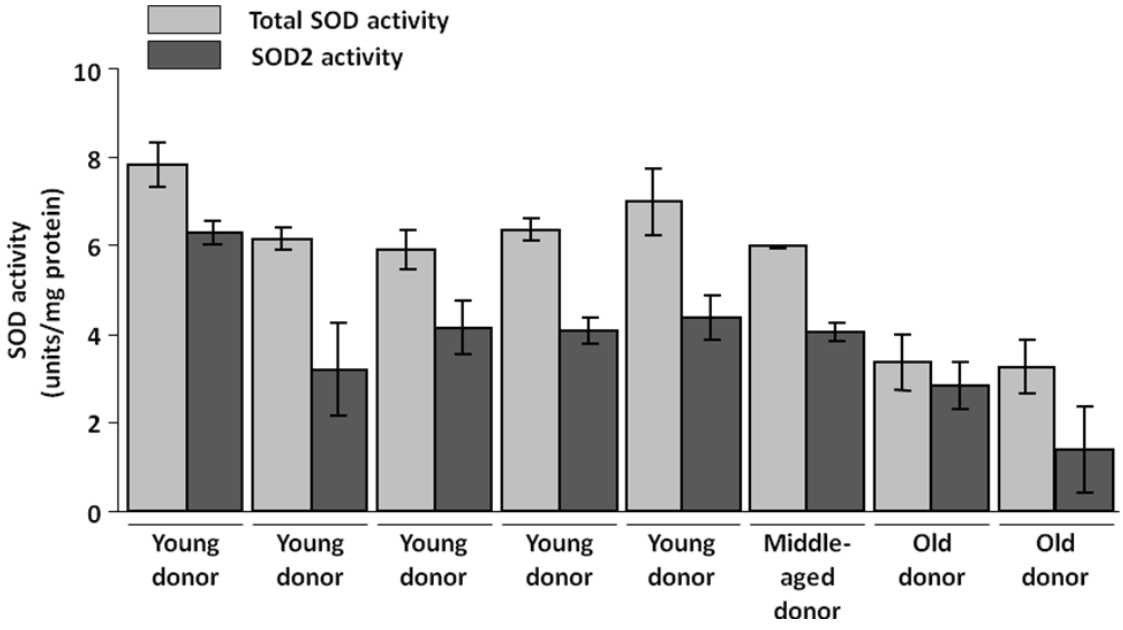

Fig. 21. SOD1 and SOD2 activity in young and old skin.
Total superoxide dismutase activity (SOD1 and SOD2) in human skin samples from young and old donors reveals a stable activity until middle aged donors with a sharp decrease in old donors. SOD2 activity shows high variability with a decrease in old donors. Age groups: young: 18-26 years [n=5], middle aged: 43 years [n=1], old: 78-79 years [n=2].

Together, these data reveal that SOD2 expression and activity are increased during cellular senescence *in vitro* and that the number of SOD2 expressing cells is increased in old skin *in vivo* modulating the cellular redox balance.

3.3. Mitochondrial superoxide concentrations are increased and H_2O_2-detoxifying enzymes reveal minor changes in expression and activity in senescent fibroblasts

In order to get insight into the putative modulation of the cellular redox balance, the level of superoxide anion radicals was analyzed using the fluorescent probe MitoSOX in replicative senescent fibroblasts. Mitochondrial superoxide anion radical concentrations were 5-fold increased in senescent compared to young fibroblasts (Fig. 22).

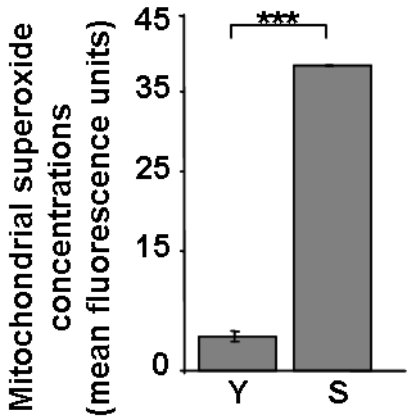

Fig. 22. Mitochondrial superoxide levels in young and senescent fibroblasts.
*Mitochondrial superoxide anion concentrations expressed as mean fluorescence units are highly increased in senescent compared to young fibroblasts as shown by MitoSOX staining w quantification by flow cytometry. The results represent 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001 (Y: young, S: senescent).*

SOD2 overexpression in senescent fibroblasts coupled with the increased concentrations of superoxide anion radicals may suggest an increased generation of hydrogen peroxide. Therefore, enzymes responsible for H_2O_2 detoxification were further analyzed. Catalase protein expression and activity are slightly changed in senescent fibroblasts (Fig. 23), while expression of glutathione peroxidase 1 (GPX-1) and total activity of glutathione peroxidases show a 2-fold increase in senescent versus young fibroblasts (Fig. 24).

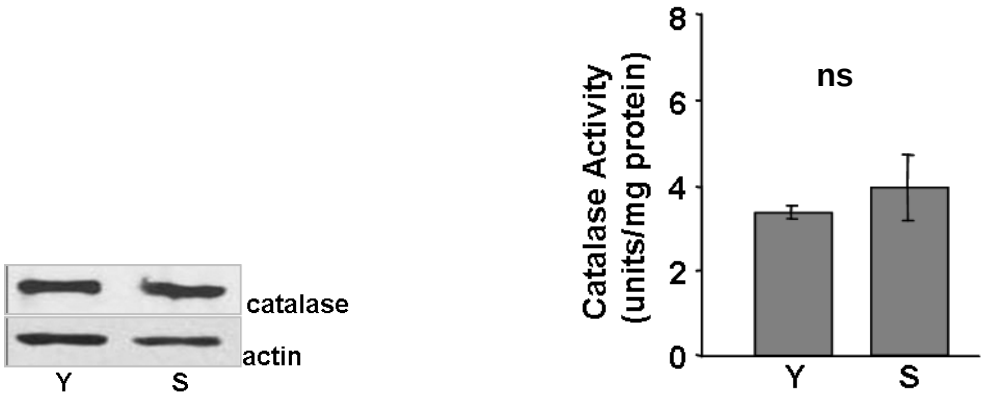

Fig. 23. Catalase expression and activity in young and senescent fibroblasts.
*Catalase protein expression is not changed in lysates from senescent compared to young fibroblasts as shown by Western blot analysis. Catalase activity reveals no significant difference between young and senescent fibroblasts. The results represent 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001, ns: non significant (Y: young, S: senescent).*

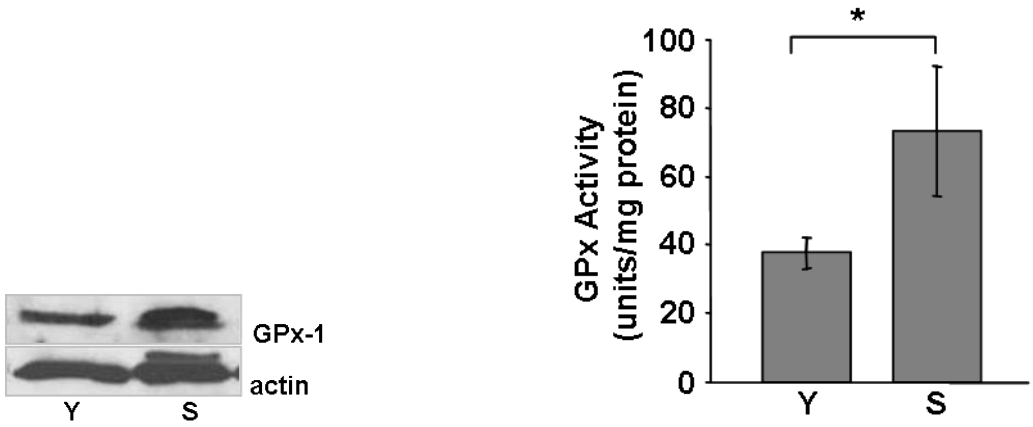

Fig. 24. GPX-1 expression and activity in young and senescent fibroblasts.
*Glutathione peroxidase 1 (GPX-1) protein expression is 2-fold increased in lysates from senescent compared to young fibroblast. GPX activity is enhanced in lysates prepared from senescent compared to young fibroblasts. The results represents 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001 (Y: young, S: senescent).*

In addition, the H_2O_2-detoxifying enzymes peroxiredoxin 2, 3 and 5 were found to be 1.6-, 1.7- and 1.8-fold upregulated in senescent compared to young fibroblasts on protein level (Table I). The weak upregulation of the H_2O_2-detoxifying enzymes together with marked SOD2 overexpression and increased mitochondrial superoxide anion radical concentrations might point to the selective accumulation of H_2O_2 in senescent fibroblasts.

3.4. Hydrogen peroxide concentrations are increased in senescent fibroblasts in vitro and in vivo

In order to verify the hypothesis that the SOD2 upregulation in senescent fibroblasts results in an altered redox balance with an increase in H_2O_2, intracellular H_2O_2 levels were compared between young and senescent fibroblasts with the biosensor HyPer as detailed in Materials and Methods. Mitochondrial and cytoplasmic H_2O_2 levels were increased in senescent compared to young fibroblasts (Fig. 25). The HyPer system reveals only a small numerical increase in H_2O_2 (Fig. 25), since the HyPer response to H_2O_2 is not linear. The observed increase by 5% in the HyPer fluorescence ratio 488nm/405nm rather reflects a high increase in H_2O_2 levels. To evaluate whether the *in vitro* findings are concordant to the situation of the skin *in vivo*, total ROS load was analyzed in human skin samples with the redox-sensitive dye dihydroethidium (DHE). When staining cryosections from 5 young and 5 old individuals with DHE, an increase in fluorescence in dermal skin fibroblasts in samples from old compared to young individuals was detected (Fig. 26). To identify the involved reactive oxygen species *in vivo*, skin cryosections derived from old donors were coincubated with DHE and the H_2O_2 scavenger catalase. A strong decrease in fluorescence was observed after catalase treatment of the sections, pointing to hydrogen peroxide as the main ROS detected in senescent fibroblasts *in situ* in skin (Fig. 27). The antioxidant N-acetyl-cysteine (NAC) was used to support ROS specificity of the DHE staining. NAC treatment completely quenched DHE fluorescence (Fig. 27). Together, these data show an increase in ROS levels and, in particular, of H_2O_2 in fibroblasts *in vitro* and most likely in skin *in vivo*.

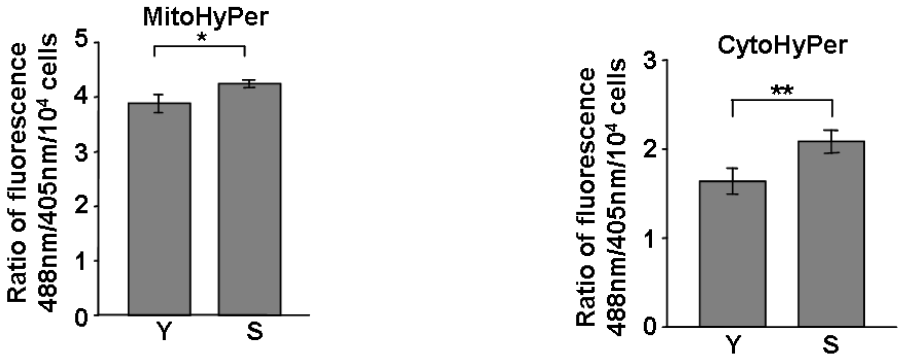

Fig. 25. Mitochondrial and cytoplasmic H_2O_2 concentrations in young and senescent fibroblasts.
*Mitochondrial and cytoplasmic H_2O_2 levels are significantly enhanced in senescent compared to young fibroblasts as measured with the biosensor HyPer and expressed as ratio of mean fluorescence units 488 nm/405 nm in 10^4 cells. The results represent 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001 (Y: young, S: senescent).*

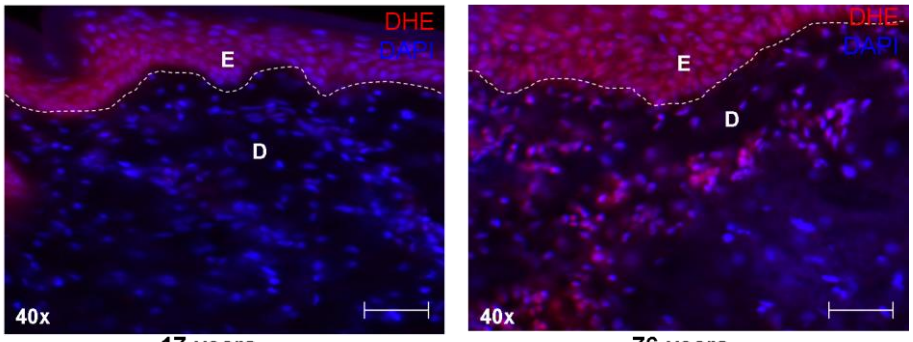

Fig. 26. ROS concentrations in human skin samples collected from young and old donors.
ROS levels are enhanced in dermal cells in cryosections from human skin samples collected from an old donor (76 years) compared to a young donor (17 years) as shown by the fluorescence signal after staining the skin cryosections with the redox-sensitive dihydroethidium (DHE). Nuclei were stained with DAPI (scale bar 50µm).

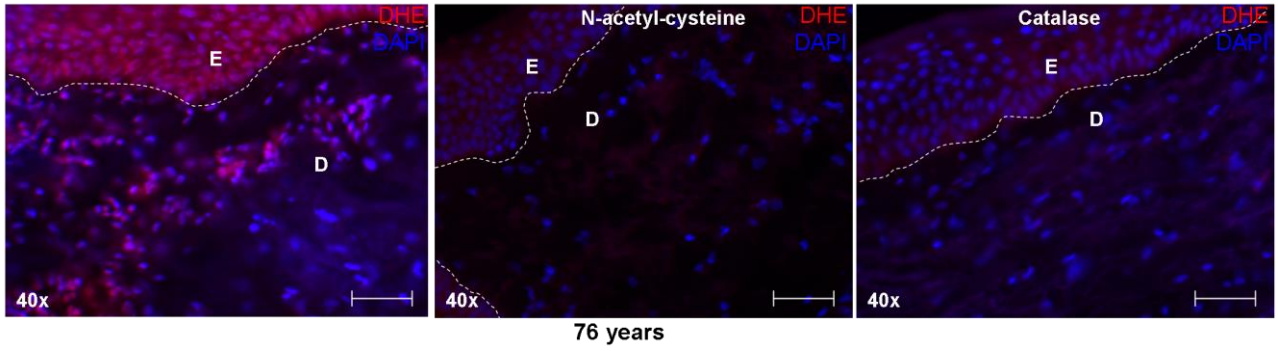

Fig. 27. Enhanced H_2O_2 concentrations and specificity of DHE staining in human skin samples collected from old donors.
The enhanced H_2O_2 levels in cryosections prepared from human skin samples collected from an old donor were shown indirectly by external addition of the H_2O_2-detoxifying enzyme catalase (750U) to the cryosection incubated with DHE with a subsequent decrease of the fluorescence signal. The fluorescence signal was completely quenched when N-acetyl-cysteine (5 mM) was added to the cryosection incubated with DHE showing the specificity of the DHE staining for ROS (scale bar 50µm).

3.5. MMP-1 is H_2O_2-regulated and cJUN-dependent in senescent fibroblasts

MMP-1 expression is upregulated during skin ageing (Varani *et al*. 2000) and may be induced by changes in the redox status of the cell (Nelson *et al*. 2006). To assess whether MMP-1 is upregulated during replicative senescence of fibroblasts, MMP-1 levels were measured in supernatants obtained after serum starvation. Senescent fibroblasts (CPD65) were found to generate active MMP-1, while young fibroblasts (CPD11) failed to produce any active MMP-1 (Fig. 28). Total MMP-1 levels measured after activation of the pro-MMP1 form in the same supernatants were found to be 13-fold increased in senescent compared to young fibroblasts (Fig. 28).

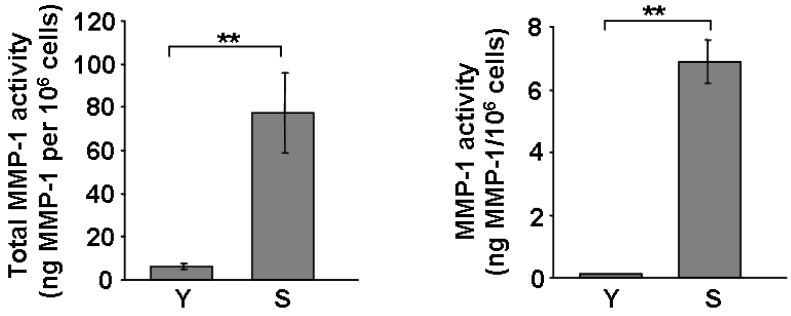

Fig. 28. Total and native MMP-1 activity in young and senescent fibroblasts.
*Total and native MMP-1 activity is increased in supernatants from senescent compared to young fibroblasts. The results represent 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001 (Y: young, S: senescent).*

As MMP-1 expression and activity may be induced by ROS and, in particular, H_2O_2 (Nelson *et al.* 2006), the impact of intracellular total ROS and H_2O_2 levels on MMP-1 activation in senescent fibroblasts was analyzed. To further investigate this question, total intracellular ROS levels were decreased by treatment with the antioxidant N-acetyl-cysteine (NAC). N-acetyl-cysteine (NAC) provides cysteine for glutathione biosynthesis and increases intracellular glutathione capable to detoxify ROS. NAC induced a dose-dependent decrease in MMP-1 activity in senescent fibroblasts after incubation with 1, 5 or 10 mM NAC for 24 hours (Fig. 29). According to the hypothesis that ROS and, in particular, H_2O_2, are generated continuously in senescent fibroblasts, resulting in persistent signaling with MMP-1 induction. To scavenge the continuous flux of ROS/H_2O_2, treatment with NAC was repeated every 3 hours during 12 hours. A substantial decrease by 2-fold in MMP-1 activity in supernatants from senescent fibroblasts was observed (Fig. 29). These data reveal

that MMP-1 induction in senescent fibroblasts is dependent on the redox homeostasis of the cell.

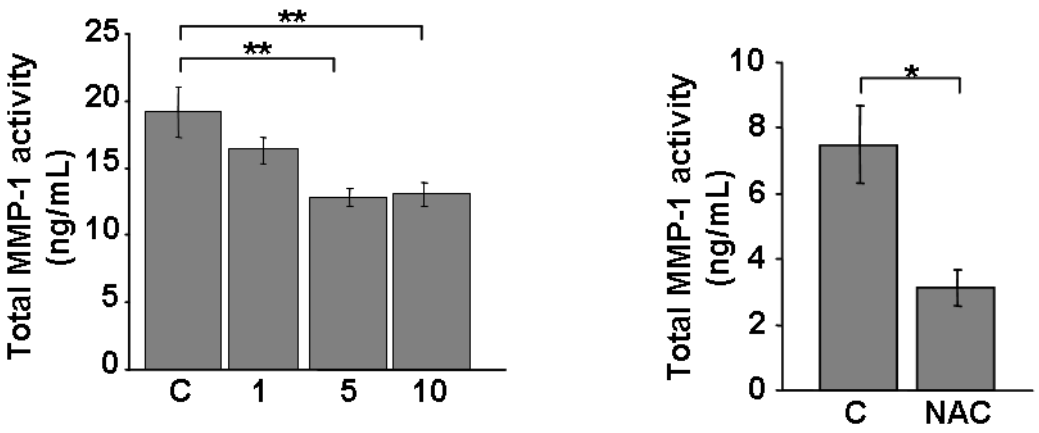

Fig. 29. MMP-1 activity decreases after quenching of ROS.
*Senescent fibroblasts were treated with 1, 5 or 10 mM N-acetyl-cysteine for 24 hours. A decrease in total MMP-1 activity was observed in the supernatants and significantly for 5 and 10 mM NAC. The decrease in MMP-1 activity is stronger when 1 mM NAC is added 3 times during the 24 hours incubation time. The results represent 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001 (Y: young, S: senescent).*

To support the hypothesis that H_2O_2 is responsible for MMP-1 activation, intracellular H_2O_2 levels were experimentally elevated by inhibition of H_2O_2 detoxification. For this purpose, the H_2O_2-detoxifying enzymes glutathione peroxidase and catalase were blocked with the inhibitors buthionine sulfoximine (BSO) for glutathione peroxidase and aminotriazole (ATZ) for catalase (Fig. 30). The concentrations of the reagents used in this study were selected according to cytotoxicity assays with a viability of > 80% as shown before (Brenneisen *et al.* 1997).

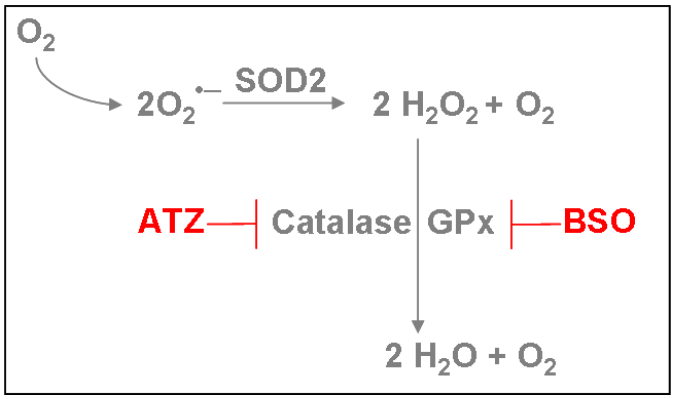

Fig. 30. Inhibition of H_2O_2 detoxification.
Buthionine sulfoximine (BSO) indirectly inhibits the synthesis of glutathione peroxidase 1 (GPX-1) by inhibition of the enzyme gamma-glutamylcysteine synthetase involved in glutathione synthesis.

Aminotriazole (ATZ) inhibits catalase. BSO and ATZ impaired H_2O_2 detoxification resulting in H_2O_2 accumulation.

Treatment with 50 µM BSO or 50 µM ATZ for 12, 24 and 48 hours induced a time-dependent increase in total MMP-1 activity in supernatants from senescent fibroblasts (Fig. 31), revealing that MMP-1 upregulation is H_2O_2-mediated in senescent fibroblasts.

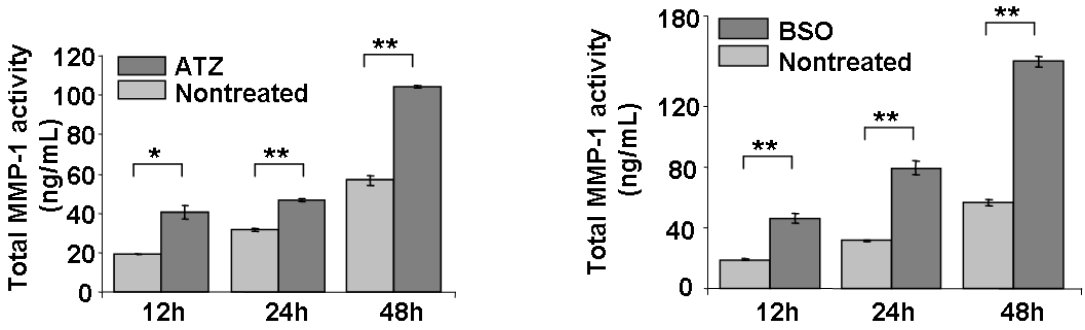

Fig. 31. MMP-1 activity is increased by accumulation of intracellular H_2O_2 in senescent fibroblasts.
*Senescent fibroblasts were treated with 50 µM BSO or with 50µM ATZ for 24, 48 and 72 hours. Total MMP-1 activity was increased in supernatants from senescent fibroblasts treated at all incubation periods. Results represent 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001.*

MMP-1 expression is known to be regulated mainly via the AP-1 pathway as the MMP-1 promoter contains the AP-1 binding site TPA-responsive element (TRE). The major AP-1 subunit of the AP-1 dimer is cJUN. To study whether MMP-1 induction in senescent fibroblasts is mediated by a cJUN-containing AP-1 dimer, cJUN protein was silenced in senescent fibroblasts by small interfering RNA (siRNA) as shown by Western blot analysis (Fig. 32). Downregulation of cJUN in senescent fibroblasts resulted in a marked decrease in MMP-1 activity in supernatants from cJUN silenced senescent fibroblasts (Fig. 32). This finding indicates that MMP-1 activation occurs via the AP-1 pathway, in particular, by a cJUN-containing AP-1 dimer.

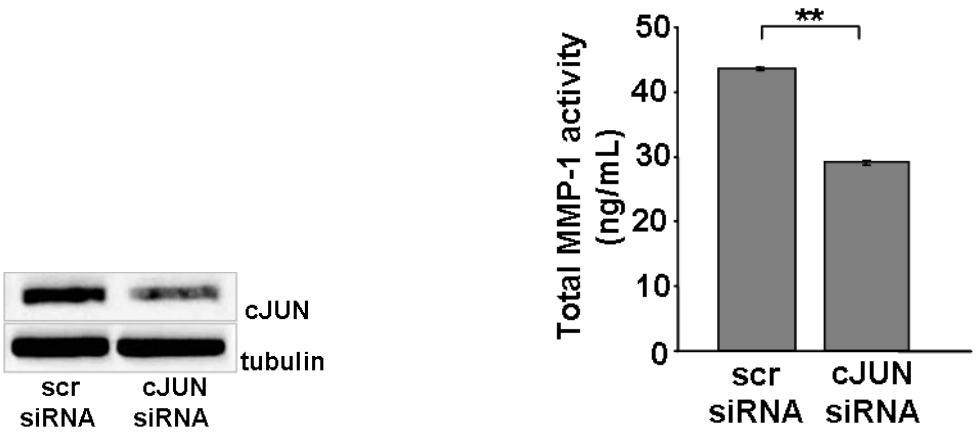

Fig. 32. MMP-1 activation is cJUN-dependent in senescent fibroblasts.
*cJUN protein expression was downregulated using small interfering RNAs in senescent fibroblasts as quantified from the Western Blots. cJUN downregulation in senescent fibroblasts results in a 1.5-fold decrease in MMP-1 activity in supernatants from senescent fibroblasts treated with non-targeting siRNA. The results represent 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001.*

3.6. The AP-1 pathway is activated via cJUN phosphorylation in senescent fibroblasts and in old skin and redox-sensitive in senescent fibroblasts

In order to analyze AP-1 signaling in senescent fibroblasts in more detail, activation of the AP-1 binding site TPA-responsive element (TRE) was monitored in young and senescent fibroblasts. Using a lentiviral approach, transient transduction with a vector encoding the firefly luciferase reporter gene under the control of TRE revealed a 2-fold increase in TRE activation in senescent compared to young fibroblasts (Fig. 33). In order to normalize the transduction efficiency, a lentiviral vector encoding Renilla luciferase driven by CMV promoter was used [Materials and Methods section, 2.13]. The activity of the CMV promoter is similar in young and senescent fibroblasts as shown earlier as previously showed by our group.

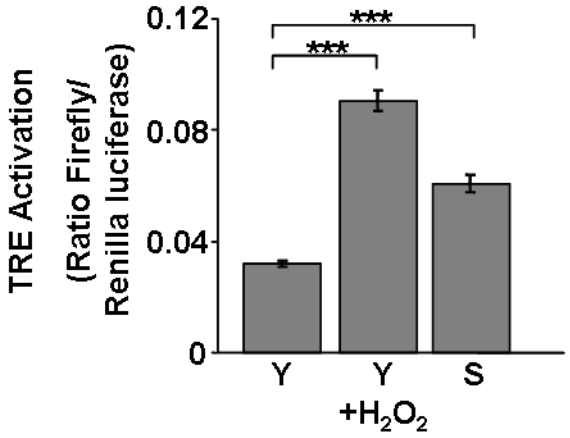

Fig. 33. AP-1 binding site TRE is stronger activated in senescent compared to young fibroblasts.
*TRE activity is enhanced by 2-fold in senescent (CPD68) compared to young (CPD9) fibroblasts as revealed by the ratio firefly to Renilla luciferase activity. As a positive control, young fibroblasts treated with H_2O_2 show a 3-fold activation of TRE. Control experiments were performed simultaneously with an empty vector lacking the TRE element in order to prove the specificity of TRE detection by this method. The results represent 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001 (Y: young, Y+H_2O_2 : young treated with H_2O_2, S: senescent).*

Transcription is induced via the TRE element after binding of the activated AP-1 dimer. Since phosphorylation of the c-JUN subunit with enhanced transactivation of AP-1 controls AP-1 activation, phosphorylation of cJUN in young and senescent fibroblasts was analysed. Western blot analysis revealed 2.5-fold higher levels of phosphorylated c-JUN in lysates from senescent compared to young fibroblasts (Fig. 34).

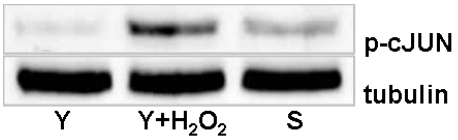

Fig. 34. Protein expression of phosphorylated cJUN in young, senescent and H_2O_2-treated young fibroblasts.
Phosphorylated cJUN increased by 2.5-fold in senescent (CPD67) compared to young (CPD16) fibroblasts as shown by Western blot analysis. Young fibroblasts treated with H_2O_2 served as a positive control and reveal 6-fold increased levels of phosphorylated cJUN as shown by a corresponding strong band (Y: young, Y+H_2O_2: young treated with H_2O_2, S: senescent). A representative Western blot out of 3 independent experiemnts is depicted.

To further study the functionality of phosphorylated cJUN, a transcription factor ELISA was used and the levels of nuclear phosphorylated cJUN capable of binding to the TRE element were measured in nuclear extracts from young and senescent

fibroblasts. A 2-fold increase was found in nuclear extracts from senescent compared to young fibroblasts (Fig. 35).

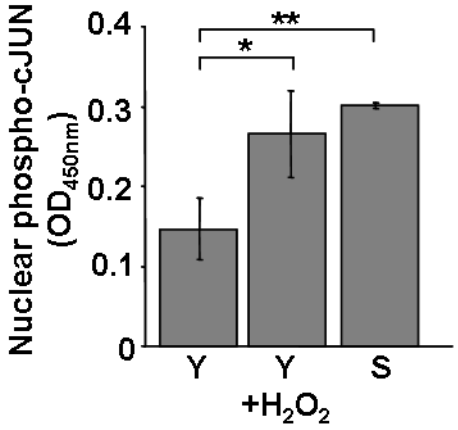

Fig. 35. Nuclear levels of phosphorylated cJUN in young, senescent and H_2O_2-treated young fibroblasts.
*Nuclear levels of phosphorylated cJUN capable of binding to the AP-1 binding site TRE are increased by 2-fold in senescent compared to young fibroblasts as shown by the cJUN specific Transcription Factor ELISA. As positive control, H_2O_2-treated young fibroblasts show a 1.8-fold increase in phosphorylated cJUN. The results represent 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001 (Y: young, Y+H_2O_2: young treated with H_2O_2, S: senescent).*

To translate the *in vitro* findings to the situation *in vivo*, phosphorylation of c-JUN was studied in human skin samples from young and old donors (3 donors for each age group) by immunostaining. Cryosections collected from old individuals revealed a distinctly stronger fluorescence in more dermal fibroblasts compared to the cryosections from young donors (Fig. 36), extending the *in vitro* results of enhanced phosphorylation of cJUN in senescent fibroblasts to skin *in vivo*.

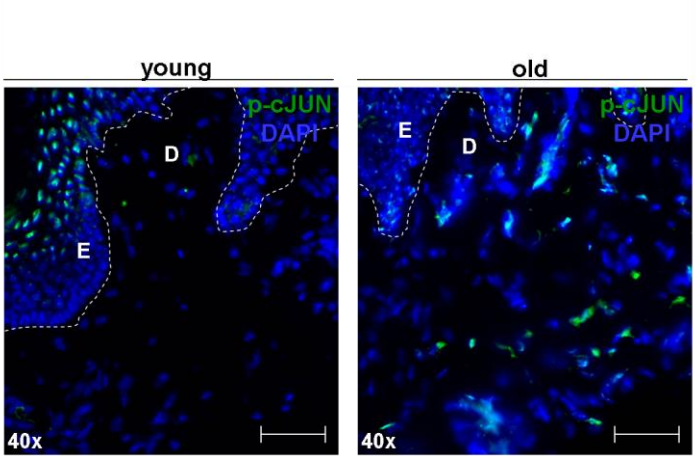

Fig. 36. Expression of phosphorylated cJUN in young and old human skin.
Numbers of phophorylated cJUN positive nuclei of dermal fibroblasts are increased in cryosections prepared from human skin samples collected from old donors compared to young donors as indicated by an increase in fluorescence intensity and positively labelled cell numbers in immunostained skin sections. Nuclei are stained with DAPI. Representative microphotographs of young and old individuals are depicted. Similar results were obtained in 3 independently studied individuals of both age groups. Scale bar 50µm.

As the AP-1 pathway is known to be redox-sensitive (Meyer *et al.* 1994, Toone *et al.* 2001), the causal relationship between redox-regulation of cJUN phosporylation and subsequent AP-1 activation was assessed in senescent fibroblasts. For this purpose, the antioxidant N-acetyl-cysteine was applied to effectively scavenge ROS levels in senescent fibroblasts. NAC at concentrations of 1, 5 and 10 mM strongly decreased phosphorylation of cJUN in lysates from senescent fibroblasts (Fig. 37). These results indicate that cJUN phosphorylation is redox-sensitive and is triggered by enhanced ROS concentrations in senescent fibroblasts.

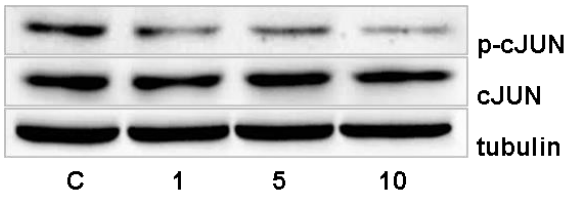

Fig. 37. Protein expression of phosphorylated form and nonphosphorylated form of cJUN after treatment of senescent fibroblasts with N-acetyl-cysteine.
The cytoplasmic levels of phosphorylated cJUN markedly decreased after treatment of senescent fibroblasts with N-acetyl-cysteine at different concentrations as shown by the decrease in the intensity of the phosphorylated cJUN specific staining in the Western blot analysis (C: nontreated, 1: treatment with 1 mM NAC, 5: treatment with 5 mM NAC, 10: treatment with 10 mM NAC).The results represent 1 of 3 independent experiments.

To verify that H_2O_2 mediates AP-1 transactivation, intracellular H_2O_2 levels were increased by exogenous addition of H_2O_2. H_2O_2 treatment at a concentration of 250µM for 1 hour produced a strong increase in cellular levels of phosphorylated cJUN in young fibroblasts (Fig. 34). This finding was confirmed by the cJUN specific Transcription Factor ELISA in nuclear extracts from young fibroblasts subjected to identical conditions (Fig. 35). In line with these data, the TRE element was 3-fold activated after 7 hours following treatment of young fibroblasts with 250µM H_2O_2 (Fig. 33). Together, these findings indicate that H_2O_2 is able to induce AP-1 transactivation by cJUN phosphorylation with subsequent activation of TRE.

3.7. AP-1 pathway is induced by the stress-responsive JNK in senescent fibroblasts

To further analyze the mechanism underlying AP-1 transactivation and MMP-1 induction in senescent fibroblasts, I investigated whether c-JUN N-terminal kinase (JNK) is responsible for this activation. Senescent fibroblasts were treated with the JNK inhibitor SP600125 at a concentration of 5µM for 48 hours. Inhibition of JNK in senescent fibroblasts almost completely abrogated cJUN phosphorylation as shown by Western blot analysis (Fig. 38) and a subsequent strong decrease in MMP-1 activity in supernatants (Fig. 39).

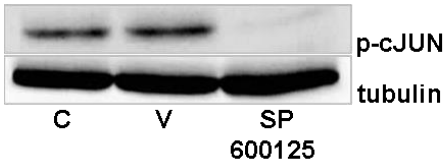

Fig. 38. Protein expression of phosphorylated cJUN after treatment of senescent fibroblasts with the JNK inhibitor SP600125.
Phosphorylated cJUN protein levels are markedly decreased in senescent fibroblasts treated with the JNK inhibitor SP600125 at 5µM for 48 hours compared to the non-treated and the vehicle-treated (DMSO) senescent fibroblasts serving as negative controls. Results are depicted by Western blot analysis. A representative Western blot is depicted out of 3 independent experiments with similar results (C: nontreated, V: treatment with vehicle, SP600125: treatment with SP600125).

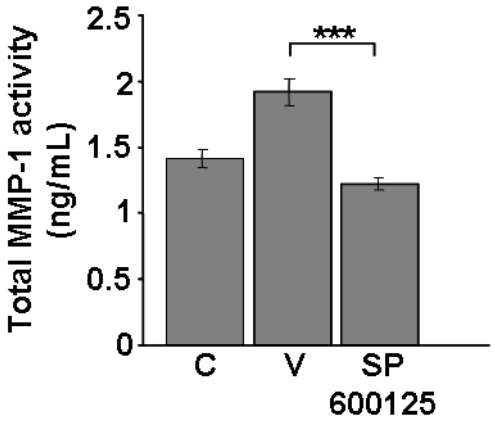

Fig. 39. Total MMP-1 activity in senescent fibroblasts after treatment with JNK inhibitor SP600125.
*Total MMP-1 activity in senescent fibroblasts treated with the JNK inhibitor SP600125 at 5µM for 48 hours is decreased compared to the vehicle (DMSO)-treated senescent fibroblasts. The results represent 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001 (C: nontreated, V: treatment with vehicle, SP600125: treatment with SP600125).*

To support AP-1 high transactivation in senescent fibroblasts which most likely is responsible for enhanced MMP-1 induction, the status of the three other AP-1 target genes monocyte chemoattractant protein 1 (MCP-1), vimentin and PTRF was analyzed in senescent fibroblasts. An upregulation of MCP-1 in supernatants from senescent compared to young fibroblasts was found (Fig. 40).

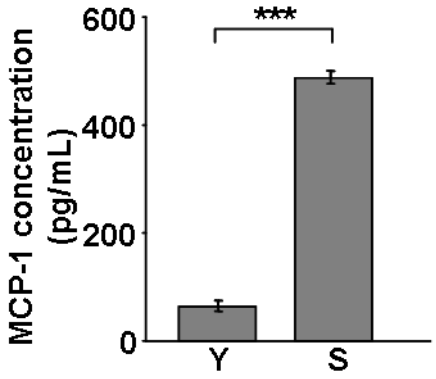

Fig. 40. MCP-1 concentration in young and senescent fibroblasts.
*MCP-1 concentration is highly enhanced in supernatants from senescent compared to young fibroblasts as measured by a MCP-1 specific ELISA. The results represent 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001 (Y: young, S: senescent).*

Vimentin and PTRF revealed higher expression levels in lysates prepared from senescent versus young fibroblasts as shown by Western blot analysis (Fig. 12, 13).

Most interestingly, the expression of all three AP-1 target genes MCP1, a secreted protein with macrophage recruiting activity, vimentin and PTRF could be suppressed by JNK inhibition using the JNK inhibitor SP600125 under the same conditions as used for MMP-1 (Fig. 41, 42, 43).

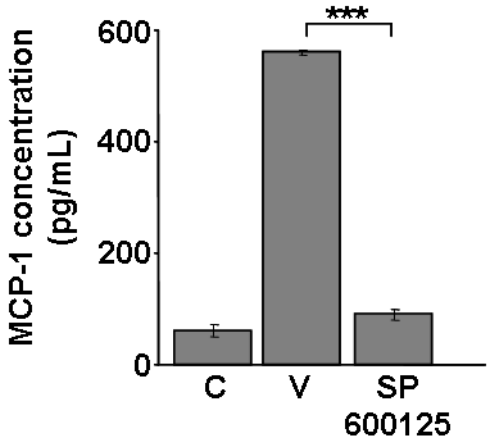

Fig. 41. MCP-1 concentration in senescent fibroblasts treated with the JNK inhibitor SP600125.
*The MCP-1 concentration is highly decreased in supernatants from senescent fibroblasts treated with the JNK inhibitor SP600125 5µM for 48 hours compared to DMSO-treated senescent fibroblasts as measured by ELISA. The results represent 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001 (C: non-treated, V: treatment with vehicle, SP600125: treatment with SP600125).*

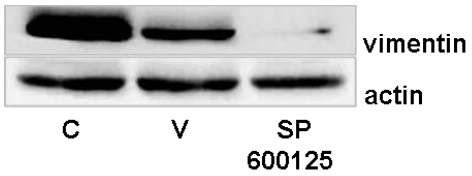

Fig. 42. Protein expression of vimentin in senescent fibroblasts treated with the JNK inhibitor SP600125.
Protein expression of vimentin is strongly decreased in senescent fibroblasts treated with SP600125 compared to DMSO-treated and non-treated senescent fibroblasts as revealed by Western blot analysis (C: non-treated, V: treatment with vehicle, SP600125: treatment with SP600125). The results represent 1 of 3 independent experiments.

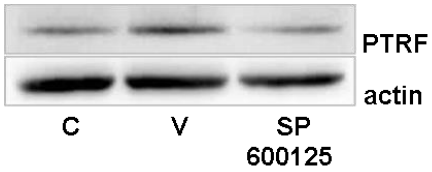

Fig. 43. Protein expression of PTRF in senescent fibroblasts treated with SP600125
PTRF protein expression is decreased in senescent fibroblasts treated with the JNK inhibitor SP600125 compared to DMSO-treated but not to non-treated senescent fibroblasts as revealed by Western blot analysis (C: nontreated, V: treatment with vehicle, SP600125: treatment with SP600125). The results represent 1 of 3 independent experiments.

Together, these results support the hypothesis that AP-1 enhanced transactivation in senescent fibroblast occurs via the JNK pathway.

3.8. SOD2 is mainly responsible for MMP-1 activation in senescent fibroblasts via enhanced H_2O_2 generation

To test the hypothesis that SOD2 overexpression triggers MMP-1 activation in senescent fibroblasts, SOD2 expression was silenced by small interfering RNAs (siRNAs) as shown by Western blot analysis (Fig. 44). The SOD2 silenced senescent fibroblasts showed a 1.5-fold decrease in MMP-1 activity in the supernatants compared to senescent fibroblasts treated with scrambled non-targeting siRNA (Fig. 45).

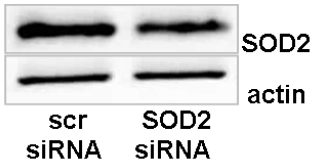

Fig. 44. SOD2 protein expression in senescent fibroblasts treated with SOD2 siRNA.
SOD2 protein expression is downregulated in senescent fibroblasts treated with SOD2 siRNA as revealed by Western blot analysis compared to senescent fibroblasts treated with non-targeting scrambled siRNAs. The results represent 1 of 3 independent experiments.

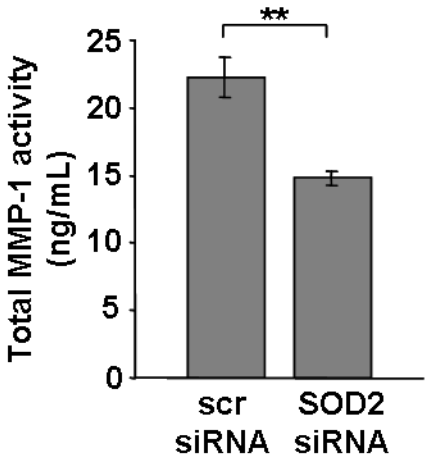

Fig. 45. Total MMP-1 activity in SOD2 silenced senescent fibroblasts.
*Total MMP-1 activity is decreased in supernatants from SOD2 silenced senescent fibroblasts compared to senescent fibroblasts treated with non-targeting scrambled siRNAs. The results represent 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001.*

To further coroborate the data, a complementary approach was used by overexpressing SOD2 in young fibroblasts using lentiviral vector containing the human SOD2 cDNA. Young fibroblasts with SOD2 overexpression (Fig. 46) revealed higher MMP-1 activity in supernatants compared to young fibroblasts treated with the control vector (Fig. 47).

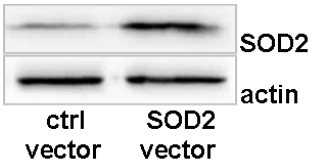

Fig. 46. SOD2 protein expression in lentivirally-transduced young fibroblasts.
SOD2 protein expression is enhanced in young fibroblasts transduced with the SOD2 encoding lentivirus compared to the young fibroblasts transduced with the control vector. The results represent 1 of 3 independent experiments.

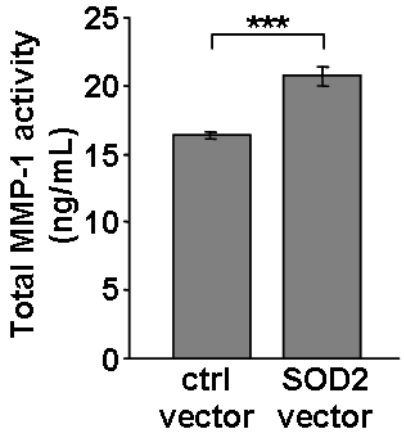

Fig. 47. Total MMP-1 activity in SOD2 lentivirally-transduced young fibroblasts.
*Total MMP-1 activity is increased in supernatants from young fibroblasts transduced with the SOD2-encoding lentivirus compared to young fibroblasts transduced with the control lentivirus. The results represent 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001.*

To assess whether mitochondrial H_2O_2 is responsible for MMP-1 activity catalase expression targeted to the mitochondria was lentivirally-induced in senescent fibroblasts. Senescent fibroblasts transduced with the mitochondrial catalase (MCAT) lentiviral vector revealed enhanced catalase expression in the mitochondria as shown by Western blot analysis (Fig. 48a). The activity of catalase was increased by 75% in senescent fibroblasts after transduction with the mitochondrially targeted lentiviral vector MCAT (Fig. 48b). Notably, a marked decrease in MMP-1 activity down to 25% was found in supernatants from senescent fibroblasts transduced with the MCAT vector when compared to senescent fibroblasts transduced with the control lentiviral vector (Fig. 48c). Together, these data support the hypothesis that SOD2 overexpression and H_2O_2 induce and mediate MMP-1 expression in senescent human dermal fibroblasts.

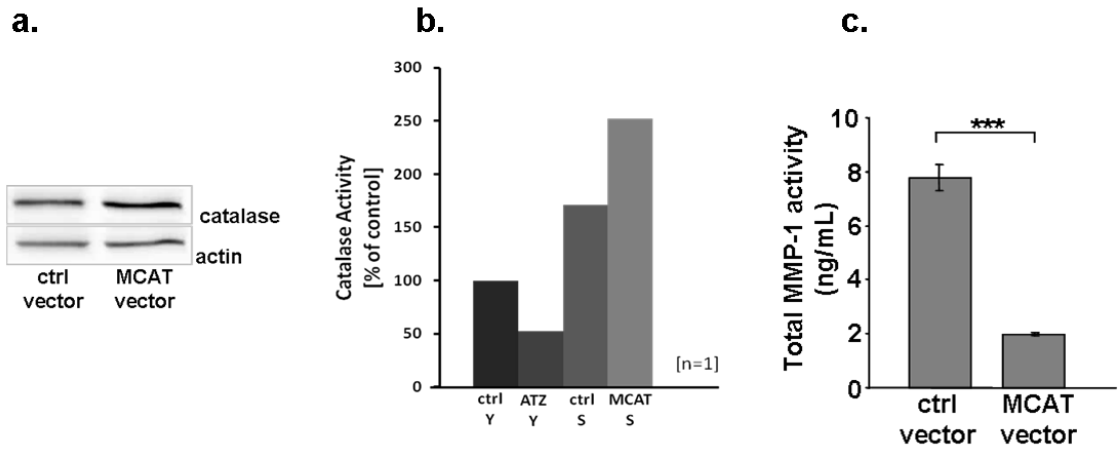

Fig. 48. Mitochondrially-targeted catalase expression results in decreased total MMP-1 activity in senescent fibroblasts

*Catalase protein expression (a) and activity (b) are increased in lysates prepared from senescent fibroblasts transduced with MCAT-encoding lentivirus compared to senescent fibroblasts treated with control lentivirus. Young control fibroblasts (ctrl Y) reveal a lower catalase activity compared to senescent (ctrl S) fibroblasts of the same strain, while ATZ reduces catalase activity by about 50% as revealed in (b). Senescent fibroblasts as shown in (a) were used to detect total MMP-1 activity which was found to be decreased (c). The results shown in (a) and (c) represent 1 of 3 independent experiments. Statistical analysis was performed by using Student's t test, *p<0.05, **p<0.01 and ***p<0.001 (Y: young, S: senescent)*

In summary these results strongly suggest a causal relationship between SOD2-generated enhanced hydrogen peroxide induced oxidative stress and the AP-1 mediated induction of the matrix-degrading interstitial collagenase/MMP-1 in human dermal senescent fibroblasts *in vitro* and also an unbalanced redox signaling in skin ageing *in vivo*.

4. DISCUSSION

According to the Free Radical Theory of Ageing, first framed by Denham Harman in 1956 (Harman 1956) the continuous generation of reactive oxygen species with accumulating damage to cellular macromolecules is postulated to be at least in part responsible for ageing of cells, tissue and the organism. In this thesis the cellular and tissue redox state was analyzed by measuring antioxidative enzyme activity and reactive oxygen species in replicative senescent fibroblasts as well as in human skin from young and old donors.

The detection of ROS due to their short lifetime and high reactivity is difficult. A main approach is the detection of intermediates like carbonylated proteins or oxidative DNA damage. In recent years, the use of oxidation-dependent fluorochromes like dihydroethidine (DHE) has been established. DHE intercalates into the double-stranded DNA and after oxidation gives rise to increased fluorescence. DHE, similar to other fluorescent probes due to the target-unspecific oxidation by reactive oxygen species is not able to identify and detect any specific ROS (Zielonka and Kalyanaraman 2010). The fluorescence signal for DHE is additionally depending on the total DNA available for intercalation. I have used the combination of specific ROS quenching enzymes like superoxide dismutase and catalase allowing more reliably the identification of distinct reactive oxygen species. I found that SOD2 expression and activity was increased in senescent fibroblasts *in vitro* leading to enhanced H_2O_2 concentration and subsequently to the AP-1 mediated induction of distinct target genes including MMP-1 among others. In order to assess whether enhanced H_2O_2 levels occur also in fibroblasts in the skin of aged individuals, catalase as hydrogen peroxide detoxifying enzyme and N-acetylcysteine as a broad ROS scavenger were used on skin sections. As depicted in Figure 27, the number of DHE-positive cells is high in old skin and, in addition, the intensity per cell is higher compared to young skin. Due to the necessity to use 10µm cryosections without drying and fixation, the focus of the microphotographs of transduced cells is poor compared to pictures from 5µm sections. A critical point may also be that the generation of ROS could simply be due to the manipulation of the skin during cryosection preparation. Even though this possibility cannot be fully excluded, cryosections of young and old individuals have been treated identically, while the

difference in the numbers of DHE-positive cell is markedly increased in senescent skin. With the technical restriction of the DHE staining *in vivo* in mind, this result may suggest that ROS levels are increased in old skin similar to *in vitro* senescent fibroblasts.

4.1. SOD2 is upregulated during replicative senescence in dermal fibroblasts in vitro

Cellular senescence of fibroblasts is thought to be a key process driving ageing of the connective tissue, and it also constitutes a major component during skin ageing (Campisi 1998). The aim of this study was to analyse of molecular and cellular processes underlying replicative senescence of fibroblasts and skin ageing. The Free Radical Theory of Ageing postulates that oxidative stress - due to the inbalance between oxidative and antioxidative cellular mechanisms - results in continuous cell damage eventually leading to tissue and organ ageing. Therefore, the role of manganese superoxide dismutase (SOD2) on matrix-metalloproteinase-1/collagenase (MMP-1) induction in cellular senescence and skin ageing was addressed.

SOD2 expression was observed to be 13-fold upregulated during replicative senescence in human dermal fibroblasts applying 3 complementary methods. Using 2-dimensional fluorescence difference gel electrophoresis (2D-DIGE) coupled with mass spectrometry (MS) a 13-fold induction was detected in replicative senescent dermal fibroblasts at CPD70 compared to young quiescent fibroblasts at CPD20 (Table I). This result was verified using immunoblotting (Fig. 11) of three independent fibroblast strains and fluorescence based immunostaining in human skin *in situ* (Fig. 16). These less sensitive and semiquantitative methods revealed an induction of about 4-fold depending on the fibroblast strain. This is in line with the observation that the SOD2 expression in fibroblasts reveals a high interindividual variability (Poswig *et al.* 1999). Previous studies on SOD2 expression in replicative senescent fibroblasts are discrepant to our results as increased SOD2 mRNA levels were reported in replicative senescent fibroblasts (Saretzki *et al*. 1998, Shelton *et al*. 1999), but also unchanged SOD2 mRNA levels (Allen *et al.* 1999). These differences might be due to technical differences in the sensitivity of the method used as well as to interindividual

variability in SOD2 expression. Interestingly, in a global proteomic analysis of fibroblasts from young and old donors, Boraldi (Boraldi *et al.* 2003) detected increased SOD2 levels in fibroblasts from old donors, while the *in vitro* growth capacity of fibroblasts seems not to be age-dependent (Maier *et al*. 2007).

The 13-fold SOD2 induction in replicative senescent fibroblasts was not reflected by its activity with only a 1.5-fold increase (Fig. 17). This discrepancy in expression and activity may be explained by posttranscriptional and posttranslational mechanisms and the correct assembly of the SOD2 subunits within the mitochondria potentially regulating overall SOD2 activity. Availability of the cofactor manganese, transport of the monomers into the mitochondria or misincorporation of iron instead of manganese into the mature protein may be possible regulatory mechanisms occurring during synthesis and assembly (Culotta *et al*. 2006). Nitration and oxidation inactivating the SOD2 protein (MacMillan-Crow *et al.* 1996) might also at least in part contribute to the low SOD2 activity as enhanced nitration was shown to be associated with ageing (Kanski *et al.* 2003).

SOD2 is an indispensable antioxidative enzyme since lack of SOD2 is not compatible with life. General deficiency of SOD2 in all organs is prenatally lethal in mice with different genetic background (Li *et al.* 1995, Lebovitz *et al*. 1996), however, overexpression alone or in combination with other ROS-detoxifying enzymes does not modulate life-span in different model systems (Jang *et al*. 2009, Ladiges *et al.* 2009). Since SOD2 overexpression in fibroblasts results in enhanced hydrogen peroxide levels (Wenk *et al.* 1999), ROS homeostasis can only be achieved if H_2O_2-detoxifying enzymes like catalase, glutathione peroxidases or peroxiredoxines would adaptively increase, which was not the case in the experiments of my thesis. Transgenic SOD2 overexpressing mice generate less superoxide anions (Silva *et al.* 2005). These mice do not reveal features of premature ageing. However, in the SOD2 overexpression mouse model, H_2O_2 levels have not been studied, and it is unclear whether H_2O_2 – detoxifying enzymes are upregulated to compensate increased H_2O_2 concentrations in the SOD2 overexpressing mouse model. Therefore, it remains unclear whether the result that no features of premature ageing occurred in the SOD2- overexpressing murine model contradicts our hypothesis that increased hydrogen peroxide levels due

to enhanced SOD2 expression and activity result in premature ageing in general. Also skin ageing was not addressed in this publication.

Interestingly, in contrast to other tissue-specific deficiencies of SOD2 in transgenic mice, connective tissue specific SOD2-deficient mice reveal signatures of premature ageing (Treiber *et al*. 2011). Unexpectedly, heterozygous SOD2 deficient mice reveal marked and persisting molecular and cellular damage without any reduction in physiological function and lifespan under normal conditions. For the heterozygous SOD2 deficient mouse reduction of SOD2 gene dose to 50% is accompanied by dramatic structural damage with impairment of molecular, cellular and organ functions. SOD2+/- mice on the C57BL/6x129/Ola background exhibit, at least in part, similar cardiomyopathies with oxidative damage in proteins (Strassburger *et al.* 2005), DNA (Wenzel *et al*. 2008) and mitochondria (Treiber *et al*. 2011). On the molecular level, DNA-lesions like 8-hydroxydeoxyguanine (8oxodG) in nuclear DNA, end products of oxidative stress like 8-isoprostane and others are increased in the SOD2+/- mice (Van Remmen *et al.* 2003, Strassburger *et al.* 2005, Wenzel *et al.* 2008), while the life span as well as biomarkers of ageing like cataract formation, impaired immune response, and others are not changed in SOD2 +/- mice compared to wild type mice (Van Remen et al. 2003, Treiber *et al.* 2011). Notably, the incidence of lymphomas is increased by 100% in SOD2+/- mice (Van Remmen *et al.* 2003), suggesting that possibly the mutational rate is increased in SOD2 +/- mice.

To investigate whether the enhanced SOD2 expression and activity in senescent fibroblasts (Fig. 16, 17) reflects the situation *in vivo* in human skin, first the expression of SOD2 was analysed using immunostaining to detect SOD2-positive cells in human dermis from young compared to old humans. In old human skin from individuals ranging from 61-82 years about 60% more cells in the dermis are SOD2 positive compared to young skin from individuals between 13 and 22 years of age (Fig. 18, 19). Since the biological activity of SOD2 is also regulated on the posttranslational level, the enzymatic activity of SOD2 was measured in skin biopsies from humans. Unexpectedly, and in contrast to the *in vitro* data for senescent human dermal fibroblasts with an increase in SOD2 activity by 1.5-fold (Fig. 17), in skin from old donors the SOD2 activity decreased compared to young skin by 30%. In the literature, limited data on SOD2 activity are available for the cell-poor dermal tissue.

In human dermis, no change was observed by Lopez-Torres (Lopez-Torres *et al.* 1994). In the report published by Cho (1994) in the Korean Journal of Dermatology, the SOD2-activity was measured in scar and control human skin tissue of different age groups. Similar to the results in this thesis, the activity of SOD2 decreased to about 50% with age. Unfortunately, in this paper in the age group from 41-60 years, only one sample was assessed. The decrease in SOD2-activity in human dermal fibroblasts is in line with the age-dependent loss of activity in leukocytes reported by Fujimoto (Fujimoto *et al.* 2010) for 69 healthy participants aged 20-52 years. There are different possible explanations for the discrepancy between the *in vivo* and *in vitro* SOD2 activity. First, dermal fibroblasts are propagated until replicative senescence *in vitro* and, for cultured human dermal fibroblasts a high interindividual variability in age related activity has previously been reported (Poswig *et al.* 1999). By contrast, dermal fibroblasts rest in a quiescent state in the skin *in vivo* that, in turn, is regulated by SOD2 activity (Sarsour *et al*. 2008). At least for murine fibroblasts it was shown that the SOD2 activity regulates proliferative and quiescent states by modulating cellular ROS levels. It is tempting to speculate, that *in vivo* SOD2 is necessary for maintaining the quiescent non-proliferative state of dermal fibroblasts. Since in old skin increased ROS levels are driving the fibroblasts into $p16^{INK4a}$ dependent senescence (Ressler *et al*. 2006), increased SOD2 activity would only be necessary within quiescent fibroblasts. Such a predicted mosaicism in SOD2 activity of single cells would not be detectable by currently available methods for the detection of SOD2 activity as applied in this thesis. It cannot be excluded that the disrupted balance between senescent and quiescent dermal fibroblasts as occuring in ageing skin is responsible for its impaired physicomechanical properties with wrinkle formation as well as the reduction of resilience and tensile strength, key hallmarks of skin ageing. Contact-inhibited quiescent and non-proliferating senescent fibroblasts *in vitro* clearly reveal significant differences in ROS generation and MMP expression compared to young fibroblasts (West *et al*. 1989, Hütter *et al*. 2002).

In contrast to blood and skin, the SOD2 activity peaks in the age range from 76-85 years and declines thereafter in skeletal muscle (Pansarasa *et al*. 1999). During development from the fetal to the adult state an increase in SOD2 activity has been detected in liver and skeletal muscles (Asikainen *et al*. 1998). This is in line with

the work from Christofalo´s group who observed an increase in SOD2 activity between fetal and postnatal fibroblasts (Allen *et al*. 1995). It seems very clear that age-associated changes in antioxidant enzyme activity appear to depend on species/animal strain, sex and tissue studied as reviewed by Rikans and Hornbrook for mice and rats (Rikans and Hornbrook 1997).

A second reason for the dysbalance between expression and activity levels of SOD2 per mg protein is the fact that SODs are inactivated by peroxynitrite through oxidation and nitration (Szabo *et al*. 2007). Peroxynitrite is generated by the reaction between nitric oxide ($NO^{\cdot}$) and superoxide anion radicals ($O2^{-\cdot}$). Furthermore, histologically the number of dermal fibroblasts decreases with age and the dermis develops to a hypocellular state in ageing (Kurban and Bhawan 1990) accompanied by a marked destruction of extracellular matrix proteins. Therefore, it is possible that the ratio of SOD2 activity to total protein content is not exactly reflecting the differences between young and old skin. Here, the direct quantification of SOD2 positive cells after immunofluorescence staining with SOD2 antibodies (Fig. 18, 19) represents an appropriate experimental approach at least to indicate the imbalance in SOD2 protein levels. Interestingly, the number of mitochondria decreases with age for example in liver cells in humans (reviewed in Bratic and Larsson 2013). Although there are no data available on the changes in the number of mitochondria in skin with age *in vivo*, a measured increase in SOD2 protein content or activity in skin would implicate an even higher increase per mitochondrium and per cell as measured. Currently not available techniques which would reliably measure SOD2 activity on a cellular level in skin *in situ* would help to detangle the apparent discrepancy in the overall SOD2 activity in fibroblasts *in vitro* and in skin *in situ*.

The role of senescence *in vivo* was long under debate. For the *in vitro* situation it was long known that the expression of $p16^{INK4a}$ plays an important role in the initiation and maintenance of cellular senescence (Stein *et al*. 1999). After detecting senescent cells *in vivo* in ageing baboons (Jeyapalan *et al*. 2007), Ressler *et al* showed that $p16^{INK4a}$ positive senescent cells also gradually accumulate during skin ageing (Ressler *et al*. 2006). This was confirmed and extended by Waaijer *et al*. (Waaijer *et al*. 2012) who detected a direct correlation between biological age and number of $p16^{INK4a}$ positive cells in human skin. Extending this correlation to the

functional relevance of $p16^{INK4a}$ in senescence and ageing, Baker (Baker *et al*. 2011) showed that the depletion of $p16^{INK4a}$ positive cells *in vivo* results in functional rejuvenation of the mouse phenotype in premature ageing BubR transgenic mice. Since $p16^{INK4a}$ is induced by reactive oxygen species (Chen 2000), a logical connection between ROS increase and senescence *in vivo* is now corroborated. According to the results of increased hydrogen peroxide levels in skin, this H_2O_2 increase could drive fibroblasts into cellular senescence and it can be hypothesized that ROS-mediated $p16^{INK4a}$ driven senescence might also play an important role in ageing *in vivo*.

4.2. SOD2 upregulation in senescent fibroblasts might reflect an adaptive response due to enhanced superoxide anion radical levels

SOD2 upregulation may represent an adaptive response to a high concentration of superoxide anion radical which was found to be 10-fold increased within the mitochondria in senescent fibroblasts [Fig. 22]. Superoxide anion radicals induce SOD2 upregulation as shown by rotenone treatment which causes an artificial increase in mitochondrial superoxide production by inhibition of the mitochondrial complex I of the electron transport chain. The exact mechanisms underlying the observed increase in mitochondrial superoxide concentrations in senescent fibroblasts have not been elucidated so far. As the sites for mitochondrial superoxide production are complex I and complex III, previous reports revealed that inhibition of complex I by rotenone (Noppe *et al*. 2009) or inhibition of complex III by antimycin A (Stöckl *et al*. 2006) results in induction of stress-induced senescence in fibroblasts as confirmed by the increase in senescence-associated beta galactosidase activity, a biomarker for senescent cells (Dimri *et al*. 2005). Additionally, the mitochondrial redox state has been hypothesized to modulate SOD2 transcription in mouse NIH/3T3 cells (Kim *et* al. 2005).

4.3. The redox balance is altered in senescent fibroblasts with increased concentrations of superoxide anion radicals and hydrogen peroxide

Enhanced SOD2 expression and activity due to increased mitochondrial superoxide anion radicals concentrations eventually leads to an increased generation

of H_2O_2 in senescent fibroblasts. Physiologically, H_2O_2 generation is counterbalanced by several H_2O_2-detoxifying enzymes localized in different cellular compartments. Here, I show that H_2O_2-detoxifying enzymes like the glutathione peroxidase 1 (GPx-1) with a 2-fold upregulation (Fig. 24) and peroxiredoxins 2, 3 and 5 with a minimal induction (Table I) cannot fully compensate for the H_2O_2 increase in senescent fibroblasts thus leading to a significant accumulation of H_2O_2 in senescent fibroblasts. In fact, I was able to detect enhanced H_2O_2 concentrations in the mitochondria and the cytoplasm of senescent fibroblasts using the previously reported HyPer detection technique (Belousov *et al*. 2006). H_2O_2 is known to act as a signaling molecule due to its diffusibility and reactivity. H_2O_2 may diffuse and migrate to long intra- or inter-cellular distances compared to other ROS (Winterbourn and Hampton 2008, Giorgio *et al*. 2007). The key ability that confers its signaling function is its reactivity leading to proteins oxidation, eventually resulting in their activation or inactivation, which modulate distinct cellular processes (Dickinson and Chang 2011). In senescent fibroblasts, H_2O_2 is generated in the mitochondria as a byproduct of SOD2 activity coupled with enhanced superoxide anion radical levels that results in a continuous flux of H_2O_2 which diffuses outside of the mitochondria and reacts stochastically with proteins involved in signaling pathways. Thus, the signaling is switched to a redox-sensitive activation of stress-related pathways in senescent fibroblasts. This activation will lead to a major change in the cellular and secretory phenotype which has been described as “senescence associated secretory phenotype” (SASP) (Coppé *et al*. 2010).

Using the bacterial oxyR based hydrogen peroxide sensor HyPer (Belousov *et al*. 2006) for the first time evidence is provided that the hydrogen peroxide levels are enhanced in mitochondria *in vitro* (Fig. 25). Although in the graph of the fluorescence ratio only a difference of 10% between young and senescent fibroblasts is visible, when related to the non-linear standard curve of the 488/405nm ratio, this reflects a much higher concentration difference for hydrogen peroxide. Additionally, superoxide anion radical levels were measured using mitochondrially targeted hydroethidine (MitoSOX) (Fig. 22). The MitoSOX technique confirms earlier work by Hütter (Hütter *et al*. 2002). Interestingly, using dihydroethidine on skin sections increased ROS levels were observed in human aged skin *in vivo* (Fig. 26). Using catalase as

hydrogen peroxide quencher on human skin sections, the DHE fluorescence was fully suppressed suggesting that most likely H_2O_2 is increased in fibroblasts in the skin *in situ* derived from old individuals. However, the detection of specific ROS *in situ* awaits further confirmation by so far not available techniques.

4.4. The AP-1 transactivation pathway is activated in senescent fibroblasts as redox-sensitive signaling pathway

Among different redox-sensitive pathways, the focus of this thesis was the activating protein 1 (AP-1) pathway as this pathway is involved in MMP-1 induction and the stress response. The major event that occurs when AP-1 becomes transactivated in response to stress is the phosphorylation of the cJUN subunit which is mediated by the cJUN-N terminal kinase (JNK). Senescent fibroblasts reveal enhanced AP-1 binding to the TPA-responsive element (TRE) (Fíg. 33) as shown by transduction with a lentiviral reporter construct. cJUN is phosphorylated at higher levels in senescent compared to young fibroblasts (Fig. 34) a finding which is in accordance with the literature (Dasgupta *et al*. 2010). Measuring the levels of phosphorylated nuclear cJUN a 2-fold increase in nuclear phosphorylated active cJUN was observed in senescent fibroblasts (Fig. 35). These results are corroborated in human skin *in vivo* with enhanced phosphorylated cJUN in fibroblasts in old skin (Fig. 36). These results corroborate and extend data by Chung (Chung *et al*. 2000) who showed that cJUN kinase activity is increased in old skin by 4-fold and cJUN is the main AP-1 component elevated in old human skin *in vivo*. From the data presented it can be concluded that MMP-1 induction in senescent human fibroblasts is the endpoint of a redox controlled signaling cascade. This signaling cascade is initiated by enhanced SOD2 activity with increased intracellular H_2O_2 concentrations which via cJUN phosphorylation and activation with subsequent AP-1 activation, nuclear translocation and binding to the TPA-responsive element in the MMP-1 gene and other genes and their enhanced transcription eventually promote cellular senescence and ageing.

4.5. AP-1 target genes are activated in senescent fibroblasts

As AP-1 activity is induced by cJUN phosphorylation in senescent fibroblasts, known AP-1 target genes were analysed. MMP-1 (Fig. 39) (Angel *et al*. 1987b, Ozanne *et al*. 2006), MCP-1 (Fig. 41) (Lim and Garzino-Demo 2000), vimentin (Fig. 42) (Rittling *et al*. 1989) and PTRF (Fig. 43) were found to be JNK-dependently upregulated in senescent fibroblasts. These AP-1 dependent genes complement already known AP-1 target genes such as COX-2 (Yamaguchi *et al*. 2005, Zdanov *et al*. 2007) and cyclin D1 (Yamaguchi *et al*. 2005, Lucibello *et al*. 1993) that were found to be upregulated in senescent fibroblasts.

Vimentin was detected to be 11-fold increased in senescent fibroblasts in the 2-DIGE experiment (Table I). Vimentin constitutes an intermediate filament protein of the cytoskeleton and is involved in attachment, migration and signaling (Ivaska *et al*. 2007). Vimentin is increased in senescent fibroblasts (Nishio *et al*. 2001) and is a major target of age-related glycation (Kueper *et al*. 2007). Based on the observed increase in vimentin in the 2-DIGE, the question was addressed whether vimentin expression is regulated by c-Jun phosphorylation. Notably, the JNK inhibitor SP600125 almost completely suppressed the vimentin expression in senescent fibroblasts (Fig. 42) coroborating the hypothesis that vimentin indeed is regulated by the JNK pathway in senescent fibroblasts. These findings further substantiate the role of the ROS-dependent JNK pathway in cellular senescence in fibroblasts.

Polymerase I and transcript release factor (PTRF) was increased in senescent fibroblasts. PTRF is a structural protein of the caveolae and plays a role in membrane-related signaling. PTRF has been found to regulate oxidative-stress induced senescence (Bai *et al*. 2011, Volonte and Galbiati 2011). PTRF is increased in senescent fibroblasts as revealed by immunoblotting (Fig. 12). Inhibiting the JNK pathway using SP600125 slightly decreased the PTRF expression.

In addition, the regulation of monocyte chemotactic protein-1 (MCP-1), a secreted chemokine involved in immune cell function and differentiation was analysed in regard to the JNK-dependent MCP-1 induction. The MCP-1 gene is induced in replicative senescent fibroblasts (Shelton *et al*. 1999) and it is known to be increased in human serum with increased protein concentrations with age (Seidler *et al*. 2010).

In fibroblasts, hydrogen peroxide as well as UVA-irradiation up-regulate MCP-1 (Hazane-Puch *et al*. 2010). In addition, MCP-1 regulation by the JNK pathway has been reported in diabetic mesangial cells (Wu *et al*. 2009).

NF-κB was identified as a crucial transcriptional factor regulating SOD2 induction and it has been reported that the most important regulatory level is the control of transcription (St Clair *et al*. 2002). NF-κB is a redox-sensitive transcription factor (Bowie and O'Neill 2000, Gloire *et al*. 2006). These findings may provide indirect evidence and help to explain my results in regard to the ROS-related adaptive SOD2 response as discussed here. In addition, Kim and coworkers (Kim *et al*. 2005) showed that the mitochondrial antioxidant MitoQ decreased SOD2 transcripts in a model of murine sod2 gene overexpression in human cells.

4.6. MMP-1 activation in senescent fibroblasts is induced by SOD2 overexpression via enhanced H_2O_2 concentrations

One of the many AP-1 target genes known to be crucial for skin ageing is interstitial collagenase/ matrix metalloproteinase 1 (MMP-1) (Angel *et al*. 1987b, Ozanne *et al*. 2006). MMP-1 is known to be one component of the secretory phenotype of senescent fibroblasts (Coppé *et al*. 2010). MMP-1 is redox-dependently modulated in senescent fibroblasts and is specifically responsive to H_2O_2 (Dasgupta *et al*. 2010). These data are in line with previous findings reporting redox regulation of the MMP-1 gene by H_2O_2 (Brenneisen *et al*. 1997) most likely via the JNK pathway (Nelson *et al*. 2006). In this thesis, SOD2-induced MMP-1 upregulation was shown to be JNK-mediated as it could almost completely be suppressed with the JNK inhibitor SP600125. Induction of MMP-1 and other stress-related genes in conjunction with the altered phenotype of senescent fibroblasts is, at least in part, the result of enhanced SOD2 activity, with subsequent increase in H_2O_2 as a second messenger. In this thesis, it was shown that enhanced activity of SOD2 is causal for MMP-1 induction in senescent fibroblasts. This SOD2-dependent MMP-1 regulation was confirmed by SOD2 silencing siRNAs or lentivirally-mediated SOD2 overexpression. This coroborates previous results from our laboratory (Wenk *et al*. 1999) and of SOD2-dependent activation of MMP-1 in other experimental systems (Nelson *et al*. 2003). The study by Wenk and coworkers (Wenk *et al*. 1999) revealed that SOD2 induced

overexpression in a transformed fibroblasts cell line coupled with forced generation of superoxide anion by UVA exposure, procedures which lead to enhanced intracellular H_2O_2 concentrations, finally result in a strong increase in MMP-1 mRNA levels. In analog experiments, Nelson and coworkers (Nelson *et al*. 2003) showed that SOD2 induced overexpression in a human fibrosarcoma cell line increased MMP-1 promoter activity and MMP-1 expression could be reversed by the H_2O_2-detoxifying enzyme catalase. By contrast, SOD2 overexpression is not induced by external factors in replicative senescent fibroblasts, indicating that mechanisms are responsible which occur during *in vitro*. However, the induction of MMP-1 is comparable in these three models, indicating a possible common mechanism - most likely mediated by H_2O_2. The role of H_2O_2 in SOD2-induced MMP-1 activation in senescent fibroblasts was substantiated by overexpression of catalase within the mitochondria of senescent fibroblasts resulting in an increase in catalase activity by 50% [Fig. 48b], while the concomitantly measured MMP-1 activity decreased down to 25% [Fig. 48c]. These data identify hydrogen peroxide as the main mediator of MMP-1 induction in senescent fibroblasts. siRNA knock-down was used to decrease the expression of SOD2 that according to the hypothesis is responsible for the induction of MMP-1 in senescent fibroblasts. Since off-target effects of siRNA technology have been described (Jackson and Linsley 2010), control fibroblasts were transduced with scrambled siRNA sequences and the effect on MMP-1 total activity was analysed. Interestingly, silencing the Sod2 gene resulted in reduced MMP-1 induction when compared to fibroblasts transduced with scrambled siRNA sequences. These data confirm our hypothesis that enhanced SOD expression via the increase of H_2O2 is responsible for enhanced MMP-1 activity.

For the redox sensitivity of AP-1, it was reported that the binding activity of AP-1 could be enhanced by overexpression of SOD2 in HT1080 fibrosarcoma cells and reverted by coexpression with catalase independent of mitochondrial or cytosolic overexpression (Nelson *et al*. 2003). Similarly, mitochondrial or cytosolic expression of catalase in HepG2 cells protected against oxidative stress induced injury (Bai *et al*. 1999). Overexpression of catalase targeted to the mitochondria in mice resulted in a 20% increase in lifespan, suggesting the key role of H_2O_2 as a second messenger molecule in ageing at organismal level (Schriner *et al*. 2005). The mice with

mitochondrial overexpression of catalase revealed an increased lifespan, decreased severity in ageing-related pathologies like cardiac or eye-related diseases and decreased expression and activity of aconitase and 8-hydroxydeoxy-guanosine as markers for oxidative stress. Unfortunately, this study by Schriner (Schriner *et al*. 2005) remains inconclusive due to the mosaicism of catalase expression only in the heart and biochemical concerns related to the hydrogen peroxide detoxifying abilities of catalase under low ROS load (Alexeyev 2009). The lifespan extension decreased after further backcrossing indicating the influence of strain specific modifier genes. In other studies, the non-targeted expression of catalase or the combined expression of SOD2 plus catalase in mice had no effect on lifespan (Perez *et al*. 2009). Interestingly, acatalasia described as Takahara´s disease occurs without any disease symptoms in humans (Aebi and Suter 1971). This observation points to compensatory antioxidative and redox control mechanisms adaptively being induced upon the loss of catalase.

Overall, SOD2 enhanced activity triggers the activation of a subset of stress-associated AP-1 target genes which confers altered signaling and the senescence-specific phenotype. The messenger molecule that transports the signal from the mitochondria to the nucleus is H_2O_2.

4.7. Altered redox signaling is confirmed in old skin in situ

Most interestingly, the oxidative stress-inducing inbalance in SOD2 activity *in vitro* could be confirmed, at least in part, in old skin *in vivo*. During ageing, the number of senescent fibroblasts in the dermis gradually increases (Dimri *et al*. 1995), and their altered signaling and the senescence-associated secretory phenotype (SASP) contributes to skin pathophysiology. The *in vitro* findings of increased expression with low activity of SOD2 in combination with enhanced H_2O_2 concentrations were evaluated in skin samples from old compared to young donors *ex vivo*. The data extend previous reports of increased protein oxidation seen as protein carbonylation in old skin (Sander *et al*. 2002) and activation of the stress-associated signaling pathway JNK (Chung et *al*. 2000, Shin *et al*. 2005). Based on these results, putative anti-ageing strategies might include antioxidants that target only senescent fibroblasts in the skin and re-establish the redox balance and, thus, redirect the signaling to

revert the senescent phenotype. A possible intervention may be liposomes that encapsulate antioxidants such as N-acetyl-cysteine and harbour specific ligands on the surface that are able to interact with receptors specific for senescent fibroblasts. Alternatively, senolytic strategies to selectively deplete H_2O_2 overproducing senescent fibroblasts would be a promising intervention to preserve tissue architecture and function.

The results of my thesis contribute to extend the knowledge on the role of SOD2 imbalances and the concomitantly altered signaling pathways with the induction of matrixmetalloprotease-1/interstitial collagenase as the effector of dermal connective tissue degradation: the hallmark of skin ageing. In addition, the essential role of mitochondrial hydrogen peroxide was shown in senescent fibroblasts using the mitochondrially targeted catalase overexpression. For the first time, differences in the ROS load between young and senescent fibroblasts were substantiated using the HyPer biological sensor for hydrogen peroxide in fibroblasts *in vitro* and dihydroethidine staining of human skin from young and old individuals *in situ*.

4.8. Perspectives

This study identifies the key role of SOD2 and ROS in ageing *in vitro* and *in vivo*, supporting the free radical theory for skin ageing. SOD2 plays a major role during cellular senescence *in vitro* and, possibly, in skin ageing *in vivo* resulting, in an altered redox state as a major determinant of ageing. As fibroblasts not only occur in skin but also in other organs, they are relevant for ageing of connective tissue rich organs like the bone, vessels and the muscles. Therefore, antioxidant anti-ageing strategies might be relevant not only for skin ageing, but also for diseases that reveal degeneration of the connective tissue caused by cellular senescence of fibroblasts in different organs. It will be interesting to elucidate the molecular base why superoxide anions increase in senescent fibroblasts and whether this is due to mitochondrial DNA mutations, transcriptional or translational deficiences of components of the electron transfer chain. It will also be of utmost interest to assess the hypothesis whether enhanced $O_2^{\bullet -}$ and/or H_2O_2 levels are responsible for the permanent growth arrest and the installment of the p16-dependent senescence programme in senescent fibroblasts. There may even be a link to the senescence associated secretory

phenotype which, in addition, to MMP-1 and MCP-1 not only drives tissue degradation but also results in enhanced inflammation. The results of this thesis therefore open new questions and strategies to develop senolytic approaches to prevent connective tissue ageing and its pathologies.

5. SUMMARY

In this thesis, the molecular mechanisms underlying cellular senescence of human dermal fibroblasts were addressed *in vitro* and in aged skin *in vivo* with particular focus on redox-regulation of matrix metalloproteinase-1. Changes in the redox balance in replicative senescent fibroblasts affect signal transduction pathways leading to the expression of proteases finally execute tissue degradation during skin ageing. Expression and to a lesser extent activity of the mitochondrial antioxidant enzyme manganese superoxide dismutase (SOD2) was upregulated in replicative senescent fibroblasts *in vitro*. In aged human skin SOD2 expression was enhanced with a reduction in SOD2 activity probably due to inactivation of SOD2 by reactive oxygen species. Using the HyPer biosensor and reactive oxygen species sensitive fluorescence probes elevated intracellular concentrations of hydrogen peroxide were detected in senescent fibroblasts. Hydrogen peroxide was found to induce matrix metalloproteinase-1 (MMP-1) expression, secretion, and activity in supernatants of senescent fibroblasts. To study whether enhanced SOD2 activity with accumulation of H_2O_2 induce MMP-1 *in vitro* targeted overexpression and knock-down strategies of SOD2 and downstream signaling molecules were used showing that MMP-1 expression of senescent fibroblasts is modulated by intracellular oxidative stress. Accordingly, MMP-1 expression was induced by enhanced intracellular hydrogen peroxide concentrations as a consequence of SOD2 overexpression in replicative senescent fibroblasts. Vice versa, targeted mitochondrial overexpression of catalase reduced MMP-1 activation in senescent fibroblasts. MMP-1 is upregulated in senescent fibroblasts via the redox-sensitive AP-1 signaling pathway as shown by H_2O_2-dependently activation of the AP-1 binding site TRE, and by H_2O_2-dependent activation of JNK with subsequently enhanced phosphorylated cJUN levels. Increased cJUN phosphorylation, a signature for AP-1 activation, was also detected in aged human skin. In conclusion, imbalanced SOD2 expression and activity, at least in part, lead to the accumulation of intracellular hydrogen peroxide, which subsequently activates signal transduction pathways and regulates genes that promote tissue degradation and ageing *in vitro* and *in vivo*.

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