Dissertation

Functional and phenotypic changes of dermal stem cells in human skin aging

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Functional and phenotypic changes of dermal stem cells in human skin aging

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BS	Bloom syndrome
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
CMT2	Charcot-Marie-Tooth type 2
CS	Cockayne syndrome
D	day
DAPI	4′,6-diamidino-2-phenylindole
DCM	dilated cardiomyopathy
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
DPBS	Dulbecco's phosphate-buffered saline
EDMD	Emery–Dreifuss muscular dystrophy
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
Fb	fibroblast
FBS	fetal bovine serum
FGF2	fibroblast growth factor 2
FITC	fluorescein isothiocyanate
FPLD	familial partial lipodystrophy
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HBSS	Hank's balanced salt solution
HGPS	Hutchinson-Gilford progeria syndrome
HRP	horseradish peroxidase
IF	immunofluorescence
kDa	kilo dalton
LMNA	lamin A (gene)
M	molarity

MAD	mandibuloacral dysplasia
MSC	mesenchymal stem cell
MHC	myosin heavy chain
NGFR	nerve growth factor receptor
NTC	non-template control
Oct4	octamer binding transcription factor 4
P	passage
P75NTR	p75 neurotrophin receptor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF-BB	platelet derived growth factor-BB
PMSF	phenylmethanesulfonylfluoride
PPDs	population doublings
PS	penicillin-streptomycin
RD	restrictive dermopathy
rpm	revolutions per minutes
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SKP	skin-derived precursor
SMA	smooth muscle actin
SMC	smooth muscle cell
Sox2	sex determining region Y-box 2
T_A	annealing temperature
TGF	transforming growth factor
VSMC	vascular smooth muscle cell
WB	western blot
WS	Werner syndrome
XP	xeroderma pigmentosum

1 Summary

Adult stem cells are of increasing interest in medical research and regenerative medicine. For a long time adult stem cells were regarded as stem cells with a limited and tissue-specific regeneration potential. However, recent research has revealed the multilineage differentiation capacity of several adult stem cell populations, which has brought adult stem cells into focus as a research target and a potential for novel treatments.

Skin-derived precursor (SKP) cells are a dermis-derived population of adult stem cells which were shown to possess multilineage differentiation potential to develop into, e.g., fibroblasts, adipocytes, smooth muscle cells and neuronal cells. This feature turns SKP cells into a highly interesting population of adult stem cells for research and medicine. Existing isolation protocols for SKP cells require skin biopsies. However, such biopsies are an invasive and costly procedure. Within this project a new isolation method for SKP cells, which limits the need for skin biopsies to isolate adult stem cells, was developed. Pre-existing primary human fibroblast cultures, which are easily available and expendable, were shown to be a source for SKP cells. Importantly, this further allows for the isolation and analysis of SKP cells from disease states when biopsies are hard to obtain but primary fibroblast cultures are available. In addition, new possibilities for the use of SKP cells in medical approaches are created.

Since the homeostasis of our body needs to be maintained for a lifetime, adult stem cells are required for regenerative processes. As we age, the number and the potential of adult stem cells decreases. The aging process and related changes in SKP cells were investigated within this project. The analysis of SKP cells derived from young and old probands showed that skin aging results from increased senescence in the stem cell pool and, consequently, from a decreased differentiation potential in aged skin stem cells. Prolonged in vitro culture times of SKP cells mimicked this process and its consequences, nevertheless the stem cell potential was maintained in some cells during the aging process.

With the newly developed isolation method, it became possible to analyze SKP cells from Hutchinson-Gilford progeria syndrome patients (HGPS). HGPS is a severe premature aging disease, which is caused by a mutation in the *LMNA* gene in the majority of cases. This mutation leads to the expression of a truncated prelamin A protein, called progerin. The accumulation of progerin is presumably responsible for severe problems at the cellular level, leading to the fatal consequences of the disease.

Since lamins are known to be involved in aging processes, the role of lamins and abnormal lamin forms in aging was investigated. An accumulation of lamins, including abnormal lamin variants, could be observed in SKP cells, both with increasing age of the donor and during the aging process in vitro. Prelamin A, a lamin A precursor, was shown to accumulate during aging and correlated strongly with senescence and a decreasing differentiation potential. These consequences of prelamin A accumulation might be caused by analogous mechanisms as in HGPS cells, since progerin and prelamin A share a strong similarity.

Therefore, prelamin A can serve as a biomarker for the assessment of adult stem cells, in order to ascertain their stem cell and differentiation potential. This is particularly important in regard to a future use of SKP cells in regenerative medicine.

2 Introduction

Adult stem cells are responsible for tissue maintenance, repair and regeneration. These functions are granted by the self-renewing capacities of the stem cells. Aging and aging-related diseases are caused by the decline of stem cell number and function. Resulting defects affect the organism and lead to fatal incidences. Knowledge about aging processes in stem cells is not only required to treat or ameliorate these effects but is also interesting for regenerative medical approaches.

2.1 Aging

Aging is not only a process which affects each of us on a personal level but increasingly societies as a whole. This is due to the fact that for the first time in history elderly people will outnumber children and that our society is getting older and older based on an increasing life expectancy (Figure 1). The segment of the population which is 60 years old or older is twice as high as it was in 1980 and by 2050 we expect to have more than 2 billion people living worldwide who are aged 60 plus (WHO, 2014a).

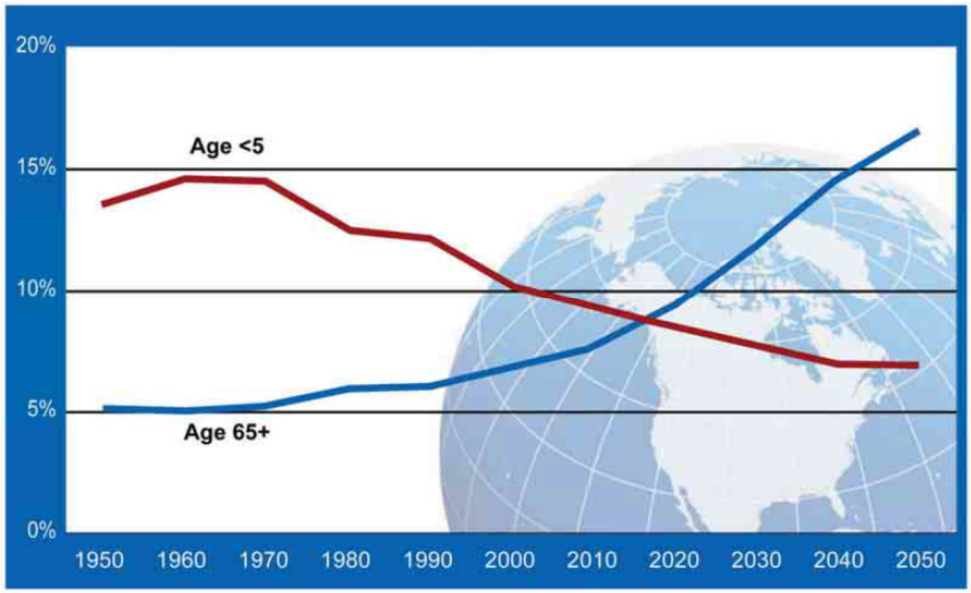

Figure 1: For the first time in history elderly people outnumber children under 5 years old due to increased life expectancy and decreased births rates. Young children and older people as a percentage of the global population 1950-2050. Taken from: Global Health and Aging, WHO, Source: United Nations. World Population Prospects: The 2010 Revision, WHO (2011).

The aging populations have crucial impact on global health. While our standard of living and health care are getting better, people start to suffer and die more from non-communicable diseases as for example cardiovascular diseases or cancer than from communicable diseases (WHO, 2011). Currently, ischaemic heart disease and stroke are the two leading causes of death in the world (Figure 2) (WHO, 2014b).

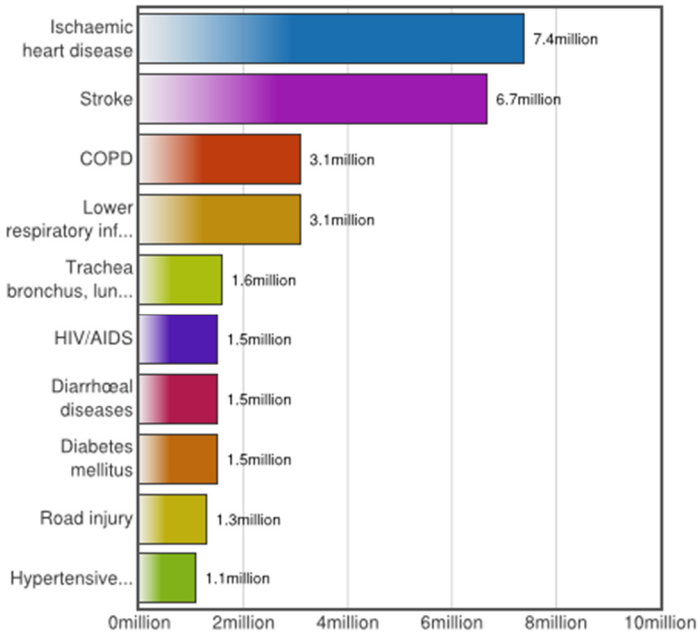

Figure 2: Ischaemic heart disease and stroke are the leading causes of death. The 10 leading causes of death worldwide are shown. Ischaemic heart disease and stroke affect a total number of 14.1 million cases. Modified from: "The top 10 causes of death", Fact sheet N°310, WHO (2014b).

While politics need to find strategies to cope with the challenges of an increasingly over-aging society, science can contribute to understand the process behind aging and to find solutions for age-related health problems.

2.1.1 General aging

With the fusion of egg and sperm, the organism starts to develop and forms a complete functional body at the time of birth. During the first 20 years of our life we reach the peak of our performance in most body related issues (Stewart, 2005). After that, in our mid-twenties, first symptoms of aging are noticeable and the decline of some of our body functions begins.

During the course of our life this is more and more characterized by symptoms as skin wrinkling, hair loss, loss of sportive performance, weakness, loss of mental functions and health impairments. Although every one of us is undergoing this process, the aging of our body depends on several different factors. Some of these factors can be influenced by our behavior such as physical activity and diet (Daniel and Tollefsbol, 2015), smoking (Koh et al., 2002), exposure to UV light (Montagna and Carlisle, 1979; Pandel et al., 2013) and toxic substances or our general stress levels, while others are determined by our genetic makeup (Brooks-Wilson, 2013). The parts of the aging process that occur inevitable are called intrinsic aging.

2.1.2 Skin aging

The phenotype of aging skin is characterized by dryness, wrinkling, loss of elasticity, hair greying and loss, atrophy and reduction in wound healing (Nishimura et al., 2005; McCullough and Kelly, 2006). During life, homeostasis of the skin is maintained by stem cells that promote self-renewal and wound healing (Fuchs, 2008). With the decline of stem cells in aging the skin undergoes visible changes. The junctions between the skin layers (see 2.3.1) flatten by more than a third, the connective tissue of the skin losses plasticity and reduces its thickness, promoting a wrinkled and aged phenotype that is increasingly vulnerable to insult (Kurban and Bhawan, 1990; Waller and Maibach, 2005; Farage et al., 2013). The collagen turnover in the skin is reduced due to a loss of collagen producing fibroblasts, the remaining collagen fibers disorganize and straighten to diminished elasticity (Waller and Maibach, 2006). Taken together aged skin is thinner, more rigid and more prone to injury. While a large part of these aging processes is caused by extrinsic aging and external factors, intrinsic factors do play an important role as well and contribute to the aging process.

2.1.3 Cellular aging

Aging is defined on a cellular level as the accumulation of damages and a resulting loss of function (Kirkwood, 2005; Vijg and Campisi, 2008). Processes that are known to be correlated with aging have recently been collected and defined as nine different hallmarks of aging (López-Otín et al., 2013). These are 1) genomic instability which is reflected by accumulation of genetic damage such as mutations in genomic and mitochondrial DNA and nuclear architecture changes, 2) telomere attrition, 3) epigenetic alterations, 4) loss of proteostasis, 5) deregulated nutrient-sensing, 6) mitochondrial dysfunction, 7) cellular senescence,

8) stem cell exhaustion, and 9) altered intercellular communication. As shown in Figure 3, some of these hallmarks cause others and lead to consequences that are responsible for the resulting phenotype.

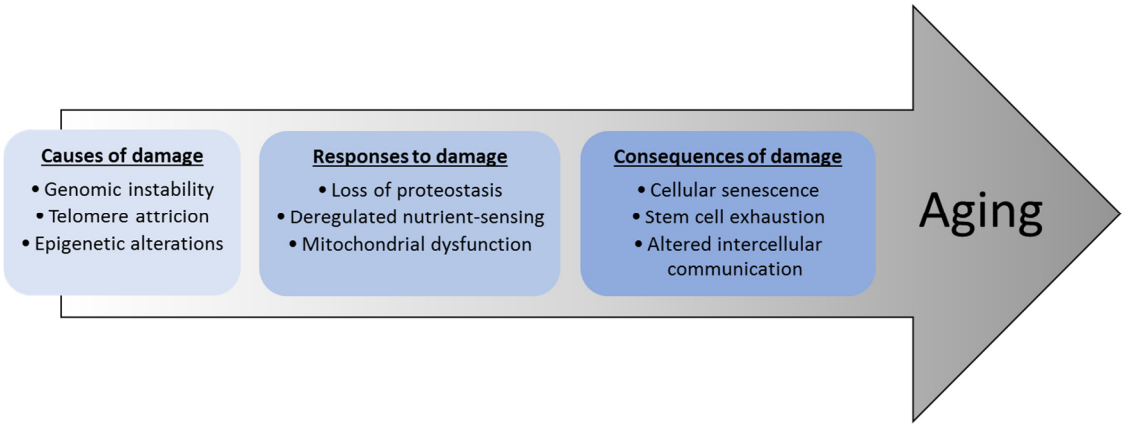

Figure 3: Hallmarks of aging: causes, responses and consequences of damage. While some hallmarks happen due to intrinsic factors, others are caused by these mechanisms e.g. stem cell exhaustion. Content according to López-Otín et al. (2013).

Since all of the factors influence each other, it is challenging to dissect the connections between the factors that cause cellular aging. To gain further insights into the mechanisms leading to these hallmarks, especially stem cell exhaustion, would be an important step in the treatment of aging related diseases and problems.

2.1.4 Senescence

Senescence is defined as the irreversible arrest of the cell cycle. It was described first, about 50 years ago, as a process which limits the growth and proliferation of cells in cultures and it was speculated then that this observation was either reflecting the aging process or a mechanism to prevent the development of cancer (Hayflick, 1965; Sager, 1991). Findings of the last decades support both theories and prove that senescence can prevent the proliferation of damaged cells and can protect the organism from cancer in earlier years. In contrast, the accumulation of damage leads to increasing amounts of senescent cells in aged tissue which contributes to the aging process (Campisi, 2013).

The most obvious signs of senescence are irreversible growth arrest and an enlarged morphology, further signs among others are the expression of senescence-associated β-galactosidase and the expression of p16INK4a, a cyclin-dependent kinase inhibitor (Dimri et al., 1995; Ohtani et al., 2004; Campisi, 2011).

The phenotype of growth limitation and cell cycle arrest is established and maintained by the p16-pRB and p53 tumor suppressor pathways (Figure 4). Both pathways are able to stop the cell cycle upon signals such as ionizing radiation or telomere dysfunction, which lead to a DNA-damage response, or upon oncogenic signals such as the presence of oncogenic Ras (Campisi and d'Adda di Fagagna, 2007; Rayess et al., 2012).

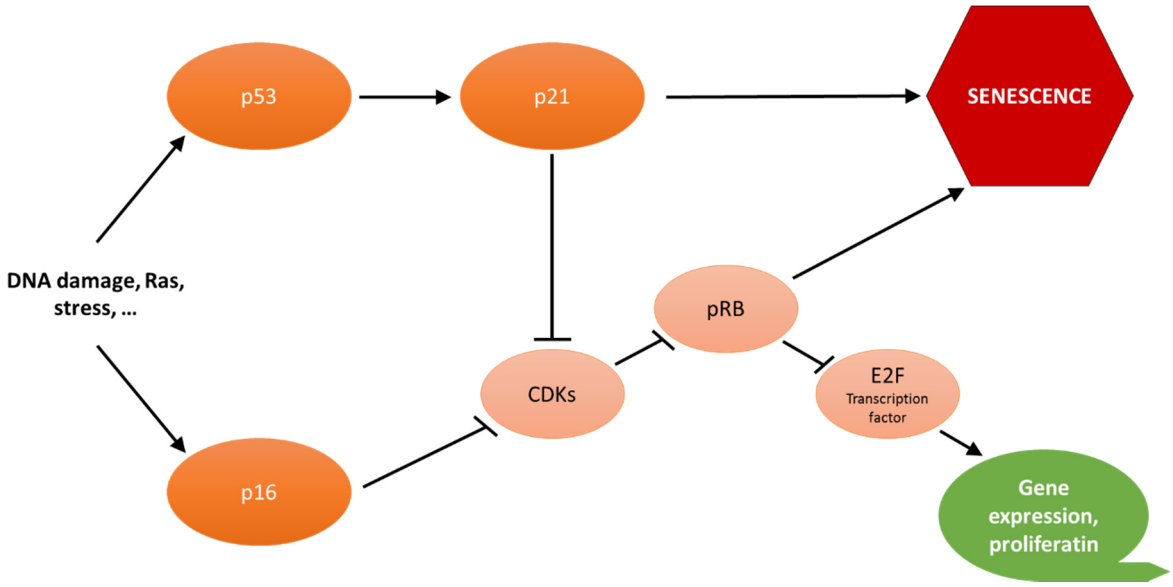

Figure 4: p53 and p16-pRB signaling pathways induce senescence. The interacting pathways are activated by signals such as DNA damage response, the presence of oncogenic Ras or other stress factors. p53 expression triggers p21 expression and induces senescence directly. p16 and p21 act as cyclin-dependent kinase (CDK) inhibitors and prevent the phosphorylation of pRB and therefore its inactivation. pRB suppresses the E2F transcription factor which is required for cell cycle progression and leads to senescence. Consequently, the expression of p16 and p21 indicates senescence in cells. Adapted from Campisi and d'Adda di Fagagna (2007).

Even though senescent cells arrest their cell cycle, they maintain their metabolism and in the case of many cell types even resist apoptotic signals. Therefore, these cells remain alive in cultures (Serrano et al., 1997; Hampel et al., 2004).

When linking senescence to aging it could not only be shown that the number of senescent cells increases with age, but also that they can be found at sites of age-related pathologies such as atherosclerosis (Erusalimsky and Kurz, 2005; Campisi and d'Adda di Fagagna, 2007). Moreover, it has been found that p16 expression increases in some adult stem cells with age, for instance in hematopoietic stem cells in mice, and influences the stem cell function (Janzen et al., 2006). The fact that most adult stem cells are capable to undergo senescence might be responsible for the decline of the stem cell numbers with age as well as for reduced stem cell functions (Molofsky et al., 2006; Sharpless and DePinho, 2007; Oh et al., 2014). To gain further insight into senescence of adult stem cells would be therefore important for understanding these mechanisms.

2.1.5 Accelerated aging diseases

Accelerated aging diseases, also called progeroid syndromes, are rare genetic disorders that resemble physiological aging and reflect premature aging processes. Symptoms of aging are observed on clinical and molecular levels in the patients and lead in many cases to a diminished life expectancy. The resemblance to normal physiological aging has potential to elucidate the underlying mechanisms of aging. However, most progeroid syndromes are segmental and do not affect the whole organism. Consequently, they do not reflect all facets of biological aging (Puzianowska-Kuznicka and Kuznicki, 2005).

The known progeroid syndromes are monogenic and mainly affect either DNA-repair related genes or lamin genes as well as genes coding for proteins that are involved in lamin processing or binding (Eriksson et al., 2003; Capell and Collins, 2006; Navarro et al., 2006).

Syndromes that are caused from mutations in DNA-repair related genes are amongst others the Werner syndrome (WS), Bloom syndrome (BS), Cockayne syndrome (CS) and xeroderma pigmentosum (XP) (Navarro et al., 2006).

Lamin related syndromes are for example restrictive dermopathy (RD) or mandibuloacral dysplasia (MAD) (Broers et al., 2006). The best-studied syndrome of the lamin related progeroid diseases is the Hutchinson–Gilford progeria syndrome (HGPS) which shows a mutation in the lamin A/C gene (see 2.2.2 HGPS).

Since the relation of lamins and premature aging is known the further analysis of their function in aging and especially in stem cell aging seems promising.

2.2 Lamins

Lamins are type V intermediate filament proteins that form the nuclear lamina, a protein network that connects the inner nuclear membrane with the chromatin (Aebi et al., 1986). A-type lamins, with the two main forms lamin A and lamin C, derive from alternative splicing of the *LMNA* gene transcript while the B-type lamins B1 and B2 derive from two different genes (Worman, 2012). B-type lamins are expressed in nearly all somatic cells while A-type lamins are known to be expressed in more differentiated cells (Dechat et al., 2008). Lamins seem to play important roles in integrity and function of the nucleus and DNA replication (Shumaker et al., 2003).

A CAAX-box motive is located at the C-terminal end of the precursor forms of lamin A, lamin B1 and lamin B2. In contrast to lamin A, lamin C is shorter and does not possess a CAAX motive at the C-terminus (Fisher et al., 1986). Therefore, only lamin A and lamin B undergo several post-translational modifications at this site. At first, the cysteine of the CAAX-box motive is farnesylated, afterwards the AAX residues are cleaved and the farnesylated cysteine is methylated (Sinensky et al., 1994). B-type lamins do not undergo further steps and remain farnesylated and carboxymethylated, while the lamin A precursor, which is called prelamin A, is modified by cleavage of the last 15 amino acids of the C-terminus to obtain the mature lamin A protein (Young et al., 2006). This final cleavage step is catalyzed by the zinc metalloproteinase ZMSPTE24 and removes as well the farnesylated and carboxymethylated cysteine residue (Corrigan et al., 2005). The order of these steps is important since, for example, lacking of the farnesylation leads to a stop of the downstream modification steps and prevents the formation of the mature lamin A (Rusinol and Sinensky, 2006). This occurs for instance, when cells are treated with farnesyltransferase inhibitors (FTIs), a medication for Hutchinson-Gilford progeria syndrome. Another stop in the processing occurs in the case of HGPS. The truncated mutant protein cannot be cleaved and remains permanently farnesylated (see Figure 6, 2.2.2 HGPS). Nevertheless, all forms of these proteins are permanently incorporated in the nuclear lamina. They therefore influence the nuclear stability and, as hypothesized, the chromatin organization and gene expression (Worman, 2012). However, the exact role of lamins remains unclear at the moment.

2.2.1 Laminopathies

The *LMNA* gene and the lamin proteins play important roles in the cell. Several hundreds of mutations (300-400) have been found only in the *LMNA* gene (Butin-Israeli et al., 2012; Reddy and Comai, 2012). Mutations in *LMNA* and related genes are associated with approximately 12-15 different diseases at the moment (Worman, 2012; Schreiber and Kennedy, 2013). These so called laminopathies can affect a multitude of different tissues such as muscle, nerves, bones, adipose tissue and skin. Manifestations can vary from myopathy, cardiomyopathy and lipodystrophy to neuropathy and premature aging (Capell and Collins, 2006; Worman and Bonne, 2007). While some of the diseases affect only one tissue, others affect a combination of tissues and organs. Among others they include Emery–Dreifuss muscular dystrophy (EDMD), dilated cardiomyopathy (DCM), autosomal recessive Charcot-Marie-Tooth type 2 (CMT2), Dunnigan type familial partial lipodystrophy (FPLD), mandibuloacral dysplasia (MAD), restrictive dermopathy (RD) and Hutchison–Gilford progeria syndrome (HGPS) (Broers et al., 2006). In addition, some mutations of B-type lamins lead to neurological disorder or lipodystrophies (Hegele et al., 2006; Padiath et al., 2006). The severity of some of these diseases reflects the importance of the lamin proteins for the cell.

2.2.2 HGPS

HGPS is a rare disease which occurs sporadically at an incidence of 1 per 4–8 million births (Capell and Collins, 2006). Because of the severity of the disease and the similarities to physiological aging HGPS it is the best studied laminopathy to date. The symptoms start to appear at the age of one year and include a general phenotype of accelerated aging. First symptoms are failure to thrive, hair loss, skin problems and lipodystrophy, further symptoms in life are for instance a short stature, alopecia, the lack of subcutaneous fat, skeletal abnormalities and arteriosclerosis (Figure 5) (Hennekam, 2006; McClintock et al., 2007).

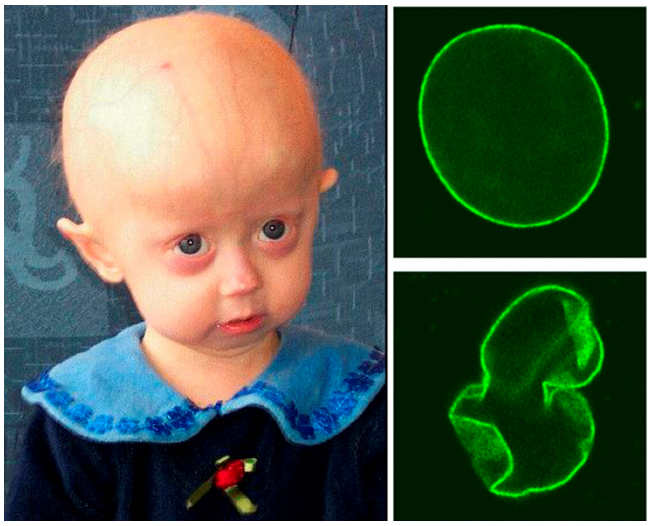

Figure 5: Phenotype of the Hutchinson-Gilford progeria syndrome. Phenotype of a young patient (left panel). Images of a non-affected nucleus (upper panel) and an HGPS-affected nucleus (lower panel) with typical blebbing of the nuclear membrane. Taken from: Scaffidi et al. (2005).

HGPS is an autosomal-dominant disease and is caused by a single de novo point mutation in the *LMNA* gene. Most of the patients carry a C→ T substitution at position 1824 in the gene (G608 mutation within exon 11). This leads to the formation of a cryptic splice site and a splicing variant that is lacking 50 amino acids in the region of the C-terminus (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). The resulting protein is called progerin (LAΔ50). While the normal lamin A can be cleaved by ZMSPTE24, progerin is lacking this cleavage site due to the deletion, which leads to a permanently farnesylated and carboxymethylated protein (Figure 6). Progerin is predominantly localized at the periphery of the nucleus while the non-mutated lamin A protein is also present in the nuclear interior. (Glynn and Glover, 2005). This is most likely the cause for abnormalities such as changes in the nuclear shape e.g. blebbing of the nuclei, thickening of the nuclear lamina, loss of peripheral heterochromatin, and clustering of nuclear pores (Goldman et al., 2004).

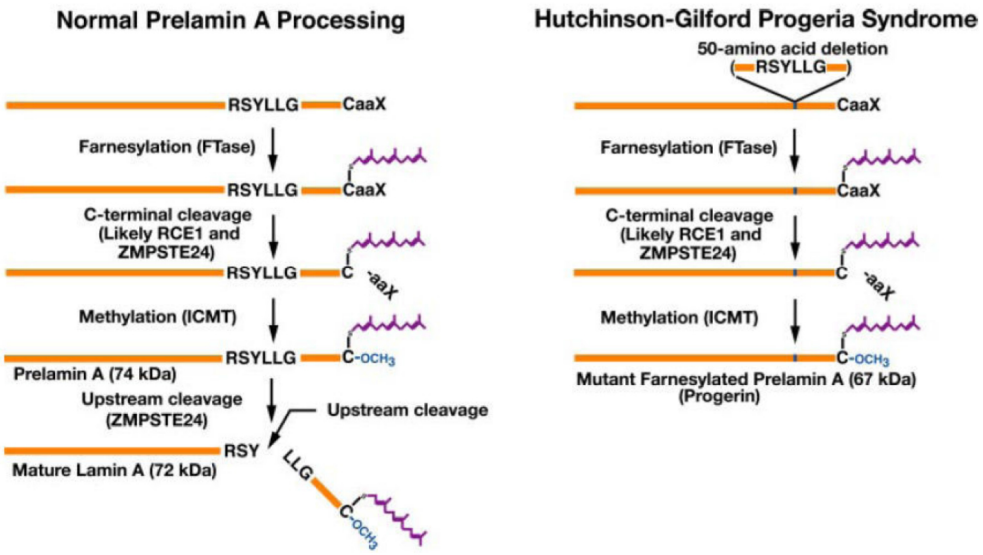

Figure 6: Lamin A processing in normal cells and in HGPS cells. The lamin A precursor undergoes a farnesylation step at the CAAX motive followed by cleavage of AAX and carboxymethylation. The so-called prelamin A is then cleaved by ZMPSTE24 and 15 amino acids as well as the farnesylated and methylated C-terminus are removed. In the case of HGPS the lamin A precursor undergoes the first three processing steps. Due to the 50 amino acid deletion the cleavage site for ZMPSTE24 is lacking which leads to a stop of the processing at the last step. The resulting protein, called progerin, remains farnesylated at the C-terminus. Taken from:Young et al. (2006).

Due to the defects which are caused by only one mutation, most patients die in the second decade of their life, at around 13 years of age. The main causes of death are coronary artery disease or strokes due to widespread arteriosclerosis (Brown, 1992). This is presumably caused by the toxic accumulation of progerin in the cells. Therapies with farnesyltransferase inhibitors (FTIs) are currently tested. FTIs cause a stop in the processing before the peptide is farnesylated, which is assumed to reduce the toxicity of the mutant protein (Young et al., 2006).

Pathologies that are similar to HGPS (such as atypical Werner syndrome, restrictive dermopathy and mandibular dysplasia) seem to have similar causes. All of them base on the accumulation of prelamin A; either of the non-mutated variant of the lamin A precursor or of mutated prelamin A forms, such as progerin (Agarwal et al., 2003; Navarro et al., 2005; Young et al., 2006). It seems, that the permanently farnesylated protein causes problems by accumulating and influencing the nuclear structure and a multitude of nuclear and DNA

related processes (Goldman et al., 2004; Shumaker et al., 2006; Cao et al., 2007; Lattanzi et al., 2007).

The theory, that progerin might directly contribute to arteriosclerosis as the main death cause of the patients, is supported by the striking finding that progerin accumulates mainly in vascular cells as seen in skin sections from HGPS patients (McClintock et al., 2006)

2.2.3 Lamins as aging markers

With the knowledge about lamin-related mutations leading to HGPS and other premature aging diseases, a link between the presence of lamins or of abnormal lamin forms and the normal physiological aging process seems possible. In fact, it was shown that progerin is expressed in healthy skin, namely in fibroblasts and in keratinocytes, and accumulates with age (McClintock et al., 2007). While progerin mRNA levels remain low, the mutant protein itself is accumulating. Nevertheless, the mRNA levels of progerin are significantly lower (160-fold) in healthy control cells compared to HGPS cells but increase in late passages of cells in vitro (Rodriguez et al., 2009).

It has been hypothesized that the transcription of the non-mutated *LMNA* gene in healthy individuals occasionally leads to the same error as caused by the mutation in HGPS. This might result in the increasing levels of progerin expression in aging cells of healthy elderly people (McClintock et al., 2007). Although no mutation is existent in normal individuals, this might happen due to the sporadic use of a cryptic splice site in the *LMNA* gene which, in contrast, is permanently activated and used in HGPS. However, it seems that this event not necessarily occurs more often with age but that aged cells might be more susceptible to the presence of progerin and less able to counterbalance the negative effects (Scaffidi and Misteli, 2006).

In addition, it was shown that adult human mesenchymal stem cells are affected by the expression of progerin, leading to a change in the differentiation potential (Scaffidi and Misteli, 2008).

Interestingly, it has also been shown that very low levels of progerin are expressed in coronary arteries of healthy young individuals and that expression levels increase at an average of 3.34 % per year age (Olive et al., 2010). This finding suggests a link between cardiovascular disease and the progerin accumulation in progeria. Since progerin leads to fatal arteriosclerosis in the case of HGPS patients, the fact that progerin and abnormal lamin species

are existent in the normal aging population might be an important finding for the understanding and treatment of vascular disease and arteriosclerosis.

Due to the age-dependent accumulation of progerin in skin and vascular tissue it was suggested that progerin could be used as a biomarker for aging (McClintock et al., 2007). This is in particular interesting since progerin and prelamin A cause similar effects when they accumulate in disease states such as HGPS, RD and MAD (see above 2.2.1 Laminopathies, 2.2.2 HGPS) (Lattanzi, 2011). These findings lead to the hypothesis that non-mutated prelamin A might also play a role in physiological aging.

In fact, prelamin A can be detected in aging vascular smooth muscle cells (VSMCs) but not in the ones of young probands. The nuclear accumulation of prelamin A causes increased levels of DNA damage in aging VSMCs and its presence is linked to senescence (Ragnauth et al., 2010).

These findings raise the questions how lamin precursors might influence the aging processes and age-related diseases, which cells are primarily affected, and, if future treatments for progeroid syndromes could also be useful to treat age-related health problems of the general population.

2.3 Stem cells

Stem cells are commonly defined as cells that are able to divide unlimited into both, new stem cells or cells that can differentiate into various cellular lineages (Fuchs and Segre, 2000). The first divisions of a fertilized oocyte results in cells of which every single one can grow into a new complete organism. This cellular totipotency is lost during the morula stage with eight cells. Afterwards, the blastocysts inner cell mass contains undifferentiated pluripotent embryonal stem cells which can still differentiate into ectodermal, mesodermal and endodermal tissue cells (Wobus and Boheler, 2005). Even after our organism is mature we do possess tissues that are in a constant state of replacement, based on adult stem cells maintaining the homeostasis (Figure 7).

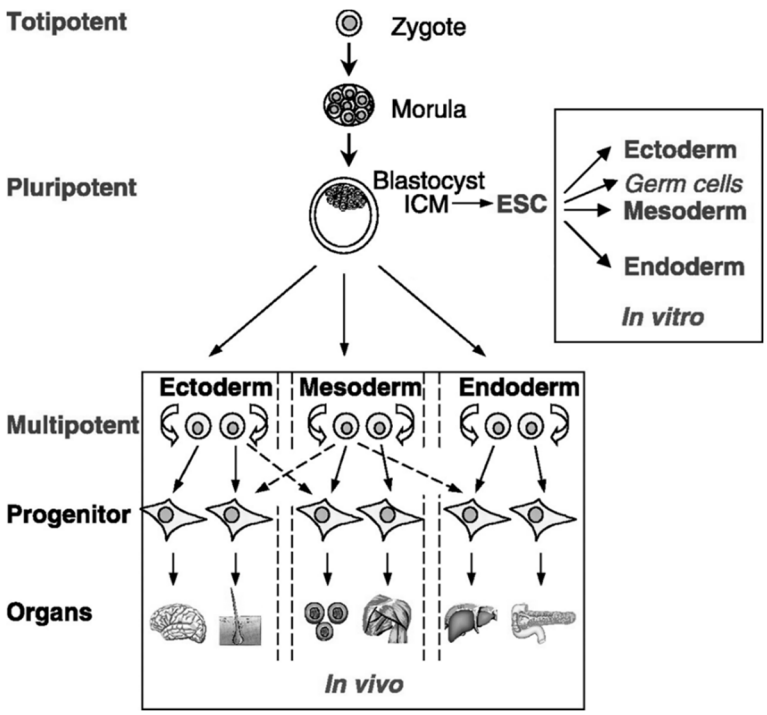

Figure 7: Stem cell hierarchy and development. Only stem cells from the early organism are totipotent while embryonic stem cells (ESC) of the inner cell mass (ICM) of the blastocyst are pluripotent and still able to differentiate into the three different lineages. At the adult stem cell stage some multipotent stem cells might still be able to differentiate into more than one lineage as indicated. Modified from: Wobus and Boheler (2005).

Embryonic stem cell research started in the 1970s with the discovery of mouse pluripotent embryonic stem cells and in the late 1990s the properties of human pluripotent embryonic stem cells were described (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). These cells can contribute to all lineages as well as become germ cells. Embryonic stem cells express the transcription factor Oct4 which is necessary for the pluripotency maintenance (Nichols et al., 1998). Other important interacting transcription factors that were identified are Sox2 and Nanog, which act together with Oct4 in contributing to pluripotency (Tomioka et al., 2002; Boyer et al., 2005; Chen and Daley, 2008).

The discovery of these important transcription factors contributed to the development of induced pluripotent stem (iPS) cells. The factors Oct4, Sox2, c-Myc and Klf4 together were shown to be able to reprogram mouse embryonic and adult fibroblast as well as human dermal fibroblasts into pluripotent cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Park et al., 2008).

Despite having a huge potential and showing promising results in regenerative medicine approaches some reasons exist why the medical use of embryonic and iPS cells is questionable. In the case of embryonic stem cells ethical reasons are a major obstacle for usage. Besides, safety concerns due to possible immunological reactions in allogenic transplantations and the risk of cancer development restrain the use of human embryonic cells in therapeutic approaches (Erdo et al., 2003; Ben-David and Benvenisty, 2011; Fu and Xu, 2012). While iPS cells represent a solution to avoid allogenic origin and infection risks, it was shown that these cell are genomic instable and that tumorigenicity could not be excluded (Ben-David and Benvenisty, 2011; Fu and Xu, 2012).

In contrast to embryonic stem cells the potential of the adult stem cell population was underestimated for a long time. Many tissues in the body, for example skin, blood, small intestine or hair, undergo permanent self-renewal. These processes are maintained by adult stem cells residing in tissues and organs. For long time it was widely believed that adult stem cells only possess a limited differentiation potential into cells of the original tissue in contrast to embryonic stem cells. However, some adult stem cells were shown to possess even more plasticity than expected since they can differentiate into cell types which differ from their tissue origin. Multipotent adult stem cells have been isolated not only from blood and bone marrow but also from many different body sites including brain, muscle, skin, heart, liver and testis (Leeb et al., 2010; Alvarez et al., 2012). Some adult stem cells are believed to reside in so called stem cell niches in the tissue. If necessary the cells can stay in a quiescent state for long periods of time until they are needed for maintenances or regeneration of the tissue e.g. after muscle injury (Fuchs and Segre, 2000).

In regard to regenerative medicine, adult stem cells have several advantages compared to embryonic or iPS cells: They are ethically unproblematic, they can be directly isolated from the patient and they represent an autologous source of cells, which minimizes immunologic problems. In addition, they are not likely to proliferate uncontrollable into tumors.

2.3.1 The skin and its stem cells

The skin is one of the largest organs of the human body and exhibits very important physiological functions. It is built by different layers and various cell types (Figure 8) (Junqueira, 2005). Essentially, skin consists of three layers, the epidermis, the dermis and the hypodermis.

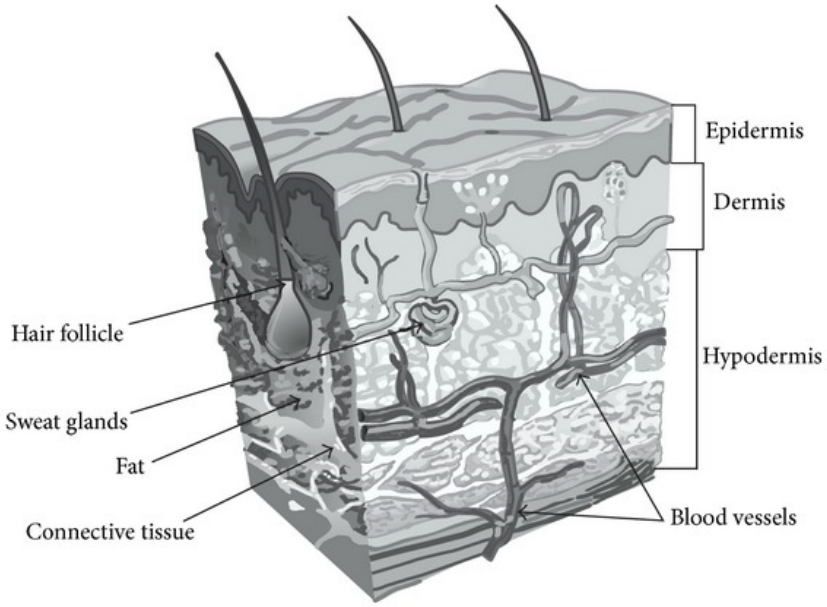

Figure 8: Layers of the skin. The skin is built from three different layers, the epidermis consisting of keratinocytes and melanocytes, the dermis consisting mainly of fibroblasts and collagen fibers and the hypodermis with glands and fat tissue. Taken from Lohani et al. (2014).

The epidermis is the upper skin layer. This layer of ectodermal origin, a stratified structure, consists mainly of keratinocytes, which undergo constant replacement maintained by stem cells of the most basal layer (Fuchs, 2007). The final differentiation step forms corneocytes out of the keratinocytes which are continuously replaced by shedding from the skin surface. Next to keratinocytes the epidermis also contains melanocytes, which influence the skin color and UV protection by melanin production (Junqueira, 2005; Hsu et al., 2014).

The second layer, the dermis, is tightly connected to the epidermis and to the lower tissue layers and provides the mechanical stability of the skin. It consists of connective tissue populated with fibroblasts and various immune cells (Hsu et al., 2014). An extracellular matrix

is formed by proteins produced from dermal fibroblasts. It consist of glycoproteins such as laminin and fibronectin, which build a network with the other components and fibers (Singh and Schwarzbauer, 2012). These fibers, mainly different forms of collagens and elastin account for the stability and the flexibility of the dermis. During aging the synthesis of collagen is reduced and the skin gets less elastic and more wrinkled (Montagna and Carlisle, 1979; Junqueira, 2005).

The hypodermis is a layer of loose connective tissue connecting the skin with organs underneath. Depending on the site of the body it consists of more or less fat tissue.

The skin is necessary to shield from injury, infections and environmental dangers such as heat or UV radiation. At the same time the skin balances our hydration and temperature status and takes part in immunological processes (McCullough and Kelly, 2006). This is why the maintenance of our skin is vitally important. The homeostasis of the skin is maintained by continual rejuvenation, which promotes the replacement of cells and the repair of tissue damage (Fuchs, 2007). For this reason the skin is highly dependent on the adult stem cell populations residing in the different layers and compartments of the skin.

These compartments, partly specialized microenvironments, are for example the basal layer of the epidermis. It consists of progenitors that replenish the keratinocytes in the shedding upper layers of the epidermis and therefore maintain the protective barrier (Fuchs, 2008; Mimeault and Batra, 2010). Another niche for adult stem cells is the hair follicle where hair follicle stem cells reside in the bulge and in the hair germ together with melanocyte stem cells. Besides new hair follicles, bulge stem cells are also able to form sebaceous glands and epidermis in the case of injuries (Fuchs, 2008). Another niche is the sebaceous gland itself which contains an adult stem cell population forming sebocytes (Fuchs, 2007). The dermal papilla, which is located directly beneath the hair follicle contains progenitor cells of mesenchymal origin which are involved in hair growth and have multipotent properties, allowing them to differentiate into different cell types (Fuchs, 2008; Driskell et al., 2011; Hsu et al., 2014). A similar population of multipotent adult stem cells was isolated from the dermis and is called skin-derived precursors (SKP) cells (Fernandes et al., 2004; Toma et al., 2005). First evidence suggested the follicle dermal papilla as niche for SKP cells. However, further studies revealed that there must exist several niches for SKP cells since they can be routinely isolated from human foreskin which contains no hair follicles (Toma et al., 2005; Fernandes

et al., 2008). Due to the possibility to isolate SKP cells from various skin sites they represent a large and easy accessible reservoir for multipotent adult stem cells.

2.3.2 Skin-derived precursor cells

While the epidermal and hair bulge adult stem cells have been extensively studied, dermal stem cells remained rather undescribed until recently. Within this thesis an adult stem cell population derived from dermis, the SKP cells are further analyzed.

SKP cells were isolated from the dermis of mammalian skin and described for the first time in 2001 but remained less known (Toma et al., 2001). The easy accessible dermal precursor cells were shown to grow as floating spheres. They express multipotency markers and are able to proliferate and differentiate into neural and mesodermal cell lines such as neurons, glia cells, smooth muscle cells and adipocytes and were therefore defined as multipotent adult stem cells of neural-crest origin (Toma et al., 2001). Experiments showed a striking differentiation potential since it was including cell types such as neurons and smooth muscle cells. It was shown that those SKP cells from rodent skin could be isolated starting from embryonic skin and were still persistent in lower number in adult rodent skin (Fernandes et al., 2004). In 2005 it was shown that SKP cells could also be isolated from human foreskin of children up to 12 years of age (Toma et al., 2005).

Further characterization revealed the following features: Human SKP cells can be expanded and grown in cultures, they are able to differentiate into multilineage progeny and they are present at all ages. Similar to neurospheres SKP cells express the neural crest marker and the intermediate filament protein nestin, which was shown to be expressed in adult stem cells, as well as the markers fibronectin and vimentin which are also expressed in dermal fibroblasts and NGFR (p75NTR) (Toma et al., 2005; Fernandes et al., 2008; Alvarez et al., 2012).

A number of studies in the last years showed that SKP cells can be isolated from various skin types such as foreskin, scalp, breast or arm from subjects of very young to very old age groups (Toma et al., 2001; Joannides et al., 2004; Toma et al., 2005; Gago et al., 2009).

Recent studies indicate that SKP cells can be passaged in vitro for more than a year (Toma et al., 2001) but show a decline in number during those long-term cultures (Gago et al., 2009). An additional loss of multipotency has been observed when the floating spheres were attaching to plastic in cultures (Gago et al., 2009).

Furthermore, it was speculated that the SKP cell number in skin diminishes with rising age of the donor (Gago et al., 2009). This was found to be the same for the stem cell function, similar to other adult stem cell populations (Pollina and Brunet, 2011). Recent studies show that in addition to the differentiation into neuronal cells, glia cells, adipocytes and smooth muscles cells, SKP cells are able to differentiate into chondrocytes and osteocytes (Toma et al., 2005; Hill et al., 2012).

SKP cells may not only reside in the dermis to support its rejuvenation but might as well be present in other fibroblast containing tissues and as such contribute in several regeneration processes in our body The aging process and the changes occurring in SKP cells are therefore an important topic to study.

3 Aim of the thesis

Stem cells as a tool for medical treatments and for use in regenerative medicine are expected to provide major improvements in personalized therapies, and are the focus of an increasing number of studies. The use of embryonic stem cells for these purposes, has been hotly debated, but their application bears huge ethic and legal burdens as well as high risks. Within the last years, adult stem cell research has brought new insights, shedding light on the possibilities for adult stem cells in basic science and translational medicine. However, thorough characterization of the different adult stem cell populations is urgently needed. This is necessary not only to understand adult stem cell related mechanisms underlying aging, but also to develop therapeutic avenues and guidelines for the use of adult stem cells in regenerative medicine, which might become a reality in the very near future.

Adult skin stem cells are one of the most easily accessible populations of adult stem cells. Skin stem cells can be obtained with minor surgery, low pain and low risk for the patient. It has been shown that skin-derived precursor (SKP) cells, can be isolated from dermis and can be differentiated into various cell lineages such as adipocytes, fibroblasts, smooth muscle cells or neurons. An age-dependent depletion of SKP cells was observed in previous studies. However, the cause for this depletion remains unclear and the stem cell properties of SKP cells were not well characterized, nor compared between differently aged populations, or followed in vitro during long-term cultures. In regard to the potential use of SKP cells for medical applications, further research is required.

In addition, it is known that abnormal lamin forms such as progerin are not only expressed in premature aging diseases, e.g. HGPS, but also in cells from healthy individuals during physiological aging. Recently, it has been shown that abnormal lamin variants can affect stem cell performance and that pathways which are involved in stem cell regeneration could be linked to A-type lamins and their binding partners. The impact of lamin and abnormal lamin expression on SKP cells is therefore an important factor in understanding how stem cells age in vivo and in evaluating their functionality in vitro.

It is hypothesized that the expression and/or accumulation of abnormal lamin A occurs in skin stem cells during aging, impacts their renewal potency and stem cell capacity, and results in a progressive decline in tissue function.

This work investigates the following aims.

(1) To define changes that occur in skin-derived precursor cells with age in culture as well as in vivo and that reflect the aging process. Therefore phenotypic changes and molecular alterations are analyzed.

(2) To determine the A-type lamin expression profile of skin-derived precursor cells including the expression of abnormal lamin A, to investigate whether abnormal lamin A is involved in triggering senescence with age and, to determine whether SKP cells are retained as functional skin stem cells throughout life.

(3) To characterize and to analyze SKP cells from patients affected by the premature aging disease HGPS and to determine the impact of the abnormal protein progerin on the stem cell function.

As experimental approach adult dermal stem cells are isolated from two different age groups and cultivated in long-term cultures. The cells are screened for typical stem cell markers at different culture stages. The expression and the status of lamins are analyzed correspondingly. Furthermore, the differentiation potential and the senescence state of the SKP cells are determined at all culture stages for both age groups. Similarly, SKP cells from HGPS patients are analyzed concerning their stem cell markers, lamin expression and differentiation potential.

The results will lead to a better understanding of the processes occurring in aging SKP cells and will help to develop methods to assess the quality of adult stem cells concerning their stem cell properties and their differentiation potential. The influence of abnormal lamins will be investigated. This is especially of importance in regard to a future use of SKP cells in regenerative medicine. The knowledge about molecular changes in adult stem cells during aging will open new ways to understand aging related diseases and open avenues for therapies.

4 Material and Methods

4.1 Material

The following items were used for experimental procedures and cell culture.

4.1.1 Equipment

Table 1: Technical equipment

Device	**Manufacturer**
Axio Imager D2	Zeiss
Axiovert 40CFL	Zeiss
Biofuge fresco	Heraeus
Chemi-Doc MP Imaging System	BioRad
Eppendorf research 10, 100, 1000	Eppendorf
iCycler	BioRad
Incubator	Binder
Microtome CM3050 S	Leica
Mini Gel cell	BioRad
Mini Trans-Blot cell	BioRad
Minispin plus	Eppendorf
MS2 Minishaker	IKA
Nano Drop Spectrometer ND-1000	PeqLab
Pipetus	Hirschmann Laborgeräte
Real time PCR MX3000P	Stratagene
Rocking platform	VWR
Sonifier 250	Branson
Thermo Scientific Multifuge 3S-R+	Heraeus

4.1.2 Consumables

Table 2: Consumables

Name	**Manufacturer**
10 cm tissue culture dish	Falcon
24-well tissue culture plate	Falcon
4–20 % Mini-PROTEAN TGX Gels	BioRad
Cover-glasses 12 mm Ø	VWR
Cryomold Biopsy 10x10x5 mm	Tissue Tek
Disposable scalpel #10	Swann-Morton
Microscope slides Superfrost plus	ThermoScientific
PAP Pen MKP-1	Kisker Biotech
Polypropylene conical tubes (15, 50 ml)	Falcon
Serological pipets (1, 2, 5, 10, 25 ml)	Sarstedt
Superslip Coverslips	Fisher Scientific
T12.5 tissue culture flask	Falcon
T25 non-treated culture flask	Falcon
Tip one pipet tips (10, 20, 200, 1000 µl)	Star Lab

4.1.3 Reagents

Table 3: Reagents

Reagent	**Manufacturer**
2-Mercaptoethanol	BioRad
Agarose ultrapure	Invitrogen
B27 supplement (50x)	Gibco
Collagenase XI	Sigma Aldrich
DEPC treated water	Invitrogen
Dispase II	Sigma Aldrich

DMEM (1x) GlutaMax 1 g/l D-glucose+pyruvate	Gibco
DMEM (1x) GlutaMax 4.5 g/l D-glucose+pyruvate	Gibco
Dulbecco's phosphate-buffered saline (DPBS)	Gibco
Ethanol absolute	VWR Chemicals
Ethidium bromide	Sigma
F-12 nutrient mixture (Ham)+L-glutamine	Gibco
Fetal bovine serum	Gibco
Fibroblast growth factor-basic (FGFb, FGF-2)	Invitrogen
Fungizone antimycotic (250 µg/ml)	Gibco
Gentamicin (10 mg/mL)	Gibco
Glutamine 200 mM	Gibco
Glycine	Sigma
Hank's balanced salt solution (HBSS)	Gibco
Laemmli Buffer	BioRad
Methanol	AppliChem Panreac
O.C.T. compound	Tissue-Tek
Oil Red O	Sigma Aldrich
Platelet derived growth factor-BB (PDGF)	Invitrogen
Penicillin-Streptomycin (5,000 U/mL)	Gibco
Phenylmethanesulfonylfluoride	Sigma
Phusion Hot Start II	Finnzymes
Precision Plus Protein Dual Color Standard	BioRad
Recombinant human epidermal growth factor (EGF)	Invitrogen
Sodium dodecyl sulfate	Merck
Sso Fast Eva Green Supermix	BioRad
TGF-β	Gibco
Triton X-100	Sigma
Trizma base	Sigma
Trypsin 0.25 % EDTA	Gibco
Tween 20	AppliChem Panreac
Vectashield mounting medium	Vector Inc.

4.1.4 Kits

Table 4: Commercially available kits

Name	Manufacturer
Clarity western ECL substrate	BioRad
Omniscript RT Kit	Qiagen
QIAshredder Kit	Qiagen
RNeasy Mini Kit	Qiagen
Senescence detection Kit	BioVision

4.1.5 Antibodies

Table 5: Primary antibodies

Antibody	Species	IF	WB	Supplier	Order Number
Anti-fibronectin	rabbit	1:600	-	Sigma	F3648
Anti-Ki67	mouse		-	BD	610968
Anti-lamin A	mouse	1:200	-	Abcam	ab133A2
Anti-lamin B1	goat	1:100	-	SantaCruz	SC-6216
Anti-lamin B1	goat	-	1:1000	Santa Cruz	SC-6217
Anti-lamin C	rabbit	1:500	-	Abcam	Ab8981
Anti-lamin A/C	rabbit	1:500	1:3000	-	Chaudhary/Courvalin, 1993*
Anti-lamin A/C	mouse	-	1:4000	Thermo	MA3-1000
Anti-Nanog	rabbit	1:400	-	Abcam	ab21624
Anti-nestin	mouse	1:200	-	Millipore	MAB5326
Anti-nestin	mouse	1:200	-	Abcam	ab6142
Anti-NFL 160	mouse		-	Sigma	N5264
Anti-NGFR	rabbit	1:500	-	Sigma	HPA004765
Anti-Oct4	rabbit	1:400	-	Abcam	ab19857
Anti-p16	mouse	1:50	-	GeneTex	GTX22419

Anti-p21	mouse	1:100	-	Millipore	MAB88058
Anti-P4HB	mouse	1:200	-	Acrys	AF0910-1
Anti-prelamin A	goat	1:400	1:250	Santa Cruz	SC-6214
Anti-progerin	rabbit	1:4	-	-	McClintock et al., 2007
Anti-progerin	rabbit	1:500	-	-	Mc Clintock et al. 2007
Anti-SMA	mouse	1:50	-	Dako	IS611
Anti-Sox2	rabbit	1:400	-	Abcam	ab97959
Anti-TG30	mouse	1:400	-	Millipore	MAB4427
Anti-vimentin	mouse	1:200	-	Chemicon	CBL202
Anti-β-actin	mouse	-	1:10000	Sigma	A1978
Anti-β-tubulin	mouse	-	1:1000	Sigma	T8328

*Anti-lamin A/C: kindly provided by Dr. N. Chaudhary

Table 6: Secondary antibodies

Name	**Species**	**Dilution**	**Supplier**	**Order Number**
α-mouse-Alexa Fluor 488	donkey	1:800	Invitrogen	A21202
α-rabbit- Alexa Fluor 488	donkey	1:800	Invitrogen	A21206
α-mouse- Alexa Fluor 555	donkey	1:500	Invitrogen	A31570
α-rabbit- Alexa Fluor 555	donkey	1:500	Invitrogen	A31572
α-goat- Alexa Fluor 555	donkey	1:500	Invitrogen	A11055
α-mouse-HRP	goat	1:5000	Jackson ImmunoResearch	115-035-003
α-goat-HRP	donkey	1:1000	Jackson ImmunoResearch	705-035-003
α-rabbit-HRP	goat	1:5000	Jackson ImmunoResearch	111-035-003

4.1.6 DNA Oligonucleotides

Table 7: Oligonucleotide sequences and sources

Name	Number	Sequence (5‘ - 3‘)	Source
GAPDH	NM_002046.3	CTCTGCTCCTCCTGTTCGAC TTAAAAGCAGCCCTGGTGAC	primerde-pot.nci.nih.gov
Nestin	NM_006617	AAGATGTCCCTCAGCCTGG GAGGGAAGTCTTGGAGCCAC	primerde-pot.nci.nih.gov
Oct4	NM_002701	GATGGCGTACTGTGGGCCC TGGGACTCCTCCGGGTTTTG	Liedtke, S. et al. 2007
Sox2	NM_003106	GGGAAATGGGAGGGGTGCAAAAGAGG TTGCGTGAGTGTGGATGGGATTGGTG	Takahashi, K. et al. 2007
Nanog	NM_024865	CTCCAACATCCTGAACCTCAGC CGTCACACCATTGCTATTCTTCG	Origene
LMN A/C	NM_170707	GCAAAGTGCGTGAGGAGTTT GAGTTCAGCAGAGCCTCCAG	primerde-pot.nci.nih.gov/
LMN A	NM_170707	CTGAGTACAACCTGCGCTCG GAGTGACCGTGACACTGGAG	NCBI Primer-Blast
LMN C	NM_005572	GTGGCCATGCGCAAGCTGG CTCAGCGGCGGCTACCAC	NCBI Primer-Blast
LMN A & Progerin	NM_170707	GCAACAAGTCCAATGAGGACCA CATGATGCTGCAG-TTCTGGGGGCTCTGGAC/T	Collins et al, 2011

α-smooth muscle actin	NM_001615	CTACAATGAGCTTCGTGTTGC ATGGCTGGGACATTGAAAG	Wenzel et.al. 2012
Myosin heavy chain	NM_001040113	CAGGAGTTCCGCCAACGCTA TCCCGTCCATGAAGCCTTTGG	Wenzel et.al. 2012
Smoothelin	NM_001207017	ACTGGTGTCGAGCCAAGACT GCCTCAGGGAAGAAGTTGTG	Wenzel et.al. 2012
β-actin	NM_001101	CCCAGCACAATGAAGATCAA GTGTAACGCAACTAAGTCAT	Mc Clintock et al. 2007
LMN A & Progerin	NM_170707	GTCACTGGAAAGGGGAGAGC ATGGGAAATGGGGAGGCAAG	NCBI Primer-Blast

4.1.7 Cell culture media

The following media were prepared sterile according to the recipes below and were stored at 4 °C.

Transport medium	
DMEM (1 g/l glucose)	500 ml
Penicillin/streptomycin (10000 U/ml)	5 ml
Fungizone (250 µg/ml)	5 ml

Fibroblast growth medium	
DMEM (4.5 g/l glucose)	500 ml
FBS	75 ml
Gentamicin (10 mg/ml)	1.25 ml
Penicillin/streptomycin (10000 U/ml)	5 ml
Glutamine (200 mM)	5 ml

1x SKP proliferation medium	
DMEM (1 g/l glucose)	9 ml
F-12	3 ml
B27 Supplement	960 µl
FGF2 (0.1 mg/ml)	5 µl
EGF (0.1 mg/ml)	2.5 µl
Gentamicin (10 mg/ml)	30 µl
Penicillin/streptomycin (10000 U/ml)	150 µl
Fungizone (250 µg/ml)	150 µl

2x SKP proliferation medium	
DMEM (1 g/l glucose)	9 ml
F-12	3 ml
B27 Supplement	960 µl
FGF2 (0.1 mg/ml)	10 µl
EGF (0.1 mg/ml)	5 µl
Gentamicin (10 mg/ml)	30 µl
Penicillin/Streptomycin (10000 U/ml)	150 µl
Fungizone (250 µg/ml)	150 µl

Freezing medium	
FBS	9 ml
DMSO	1 ml

Adherence medium	
DMEM (1 g/l glucose)	500 ml
FBS	25 ml
Penicillin/Streptomycin (10000 U/ml)	5 ml

SMC differentiation medium	
DMEM (1 g/l glucose)	4 ml
FBS	600 µl
TGF-β (10 µg/ml)	2.5 µl
PDGF (10 µg/ml)	10 µl
Penicillin/Streptomycin (10000 U/ml)	40 µl

4.1.8 Buffers

10x SDS gel running buffer	
1.92 M Glycine	144 g
248 mM Tris-Base	30 g
1 % SDS	10 g
H_2O	Ad 1 l

Western blot transfer buffer	
200 mM Tris-Base	2.42 g
150 mM Glycine	11.3 g
20 % MetOH	200 ml
0.1 % SDS	1 g
H_2O	Ad 1 l

Sample laemmli buffer	
Laemmli buffer (BioRad)	950 µl
2-Mercaptoethanol	50 µl
Proteinase inhibitor cocktail	10 µl
PMSF	5 µl

4.2 Methods

4.2.1 Human skin biopsies

Foreskin biopsies of healthy individuals from 0-12 years were obtained from post-surgery material at the dermatology department at TU München and in accordance with the guidelines of the Ethics Committee at TU München. All patients signed an informed consent form.

Adult skin samples were obtained from unaffected individuals aged 50 years and older. Post-surgery material was used in accordance with the Institutional Review Ethic Committee at TU München. All Patients signed informed consent form.

4.2.2 Isolation and cultivation of primary dermal fibroblast cultures derived from human infant foreskin and adult skin samples

Human skin samples were obtained from post-surgery material (see 4.2.1). All skin samples were stored and transported to the laboratory within 8 hours in transport medium. To prepare the cell isolation, all fat tissue was removed and the skin was washed in HBSS, sterilized in 70 % ethanol for 2 minutes and cut into pieces of 4-6 mm^2. Epidermis and dermis of the skin were separated by incubation in dispase II solution (1 U/ml) at 4 °C overnight, followed by 15 minutes at 37 °C in a water bath. After this, the epidermis was removed manually and the dermis was homogenized, followed by another digestion step in collagenase XI solution (0.05 mg/ml). The digested dermis was mechanically dissociated by pipetting up and down. Cells that dissociated from the tissue were collected with the supernatant in 4 washing and centrifugation steps. The supernatant was passed through a 70 mm cell strainer (BD Falcon) to remove any remaining tissue and was centrifuged at 1,200 rpm for 7 minutes.

After isolation, the cell pellet was resuspended in fibroblast growth medium and seeded in 10 cm culture dishes. Cells were passaged when they reached 80 % confluence by splitting with 0.25 % trypsin solution (Gibco). Cells were washed with PBS and incubated with 0.25% trypsin solution for 5 minutes at 37 °C. The cells were rinsed off the cell culture dish with fibroblast medium, collected by a centrifugation step at 1,200 rpm for 5 minutes, resuspended in fibroblast medium and seeded in new dishes at a 1:3 splitting ratio. After two passages, frozen aliquots were stored for further experiments.

4.2.3 Isolation and cultivation of SKP cells from human infant foreskin and adult skin samples

Human skin samples were obtained from post-surgery material in accordance with the Institutional Review Ethic Committee at TU München. Cells were isolated as described above for primary fibroblast cultures (4.2.2) and as shown below (Figure 9). The obtained cell pellet was resuspended, the cells were seeded in T12.5 low adherence culture flasks in 1xSKP proliferation medium as described previously (Toma et al., 2005) and incubated at 37 °C. Within the first 7-10 days sphere growth was observed. After the formation of spheres the culture was transferred into T25 flasks. The cells were cultured for over 250 days depending on their growth with a conditional medium change every three days keeping the concentration of the growth factors constant by using double concentrated medium (2x SKP proliferation medium).

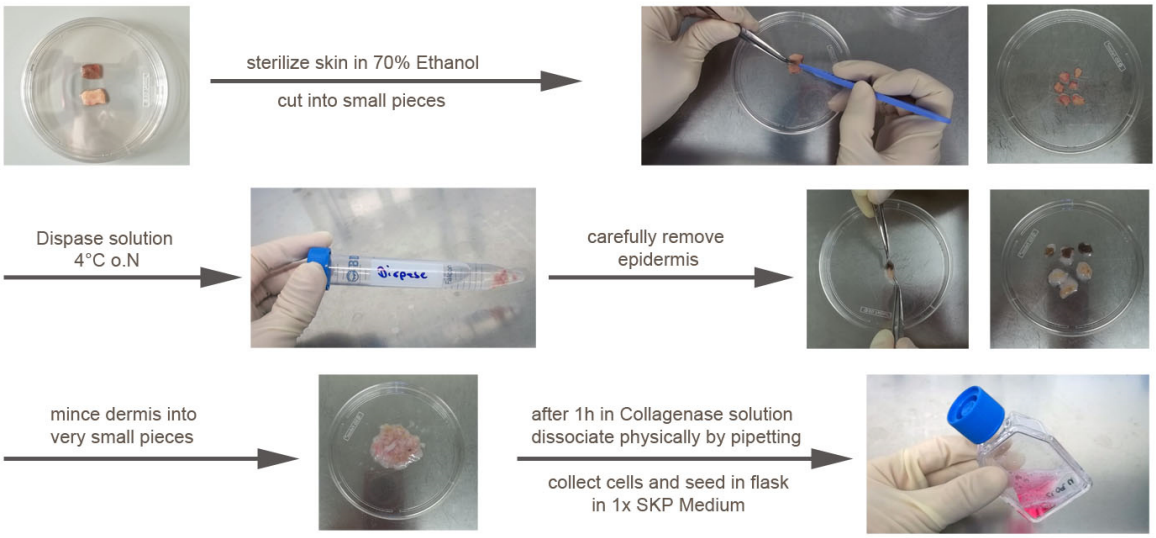

Figure 9: Direct isolation method for SKP cells from skin. The sterilized skin was cut into small pieces and treated with dispase solution over night at 4 °C. After removing the epidermis, the dermis was minced into very small pieces and treated with collagenase at 37 °C for 1 hour to loosen the connective tissue. Dissociated cells were washed out of the tissue, collected and seeded into 1x SKP proliferation medium.

The SKP cell cultures were shaken vigorously in the flasks every second day to prevent adherence. Cells were passaged into a new bottle every week and when starting to attach. At high sphere density, the cultures were split into two cell culture flasks. According to the

growth of the cells, distinct culture stages were defined. SKP cells from adult skin varied in growth, many cultures could be kept only for 60 to 90 days.

4.2.4 Generation and cultivation of SKP cells derived from pre-existent fibroblast cultures

Fibroblast cultures were used as source for the isolation of SKP cells as described previously (Wenzel et al., 2013).

Briefly, primary fibroblast cultures or fibroblast cultures from tissue banks (HGPS-fibroblasts and matched controls, see 4.2.5) were used for isolations after several passages and freezing. The fibroblasts were cultivated in 10 cm tissue culture plates and processed for SKP sphere isolation when they reached 80 % confluence. The culture plate was washed with PBS and incubated with 2 ml 0.25 % trypsin solution (Gibco) for 1 hour at 37 °C. The cells were collected in PBS and incubated at 4 °C for 24 hours. Alternatively, the cells were incubated in trypsin for approximately 18 hours at 37 °C. After this treatment, the cells were collected by centrifugation. After a washing step the cell pellet was resuspended in SKP growth medium and seeded in a T12.5 flask in SKP proliferation medium as described previously (Toma et al., 2005) (Figure 10). Cultures were incubated at 37 °C. A conditional medium change with 2x SKP proliferation medium was performed as described above every three days.

The growth of spheres was observed within the first 10 days. Afterwards the cultures were transferred into T25 flasks and were passaged into a new bottle every week. Equal to SKP cells which were isolated directly from skin the cultures were split into two flasks at high sphere density. Cell cultures were maintained over 250 days.

Samples were taken at culture stages which were defined due to the SKP growth behaviour. Stages were defined as <30 days (‘early’), between 45 to 75 days (‘intermediate’) and after more than 90 days (‘late’) of culture.

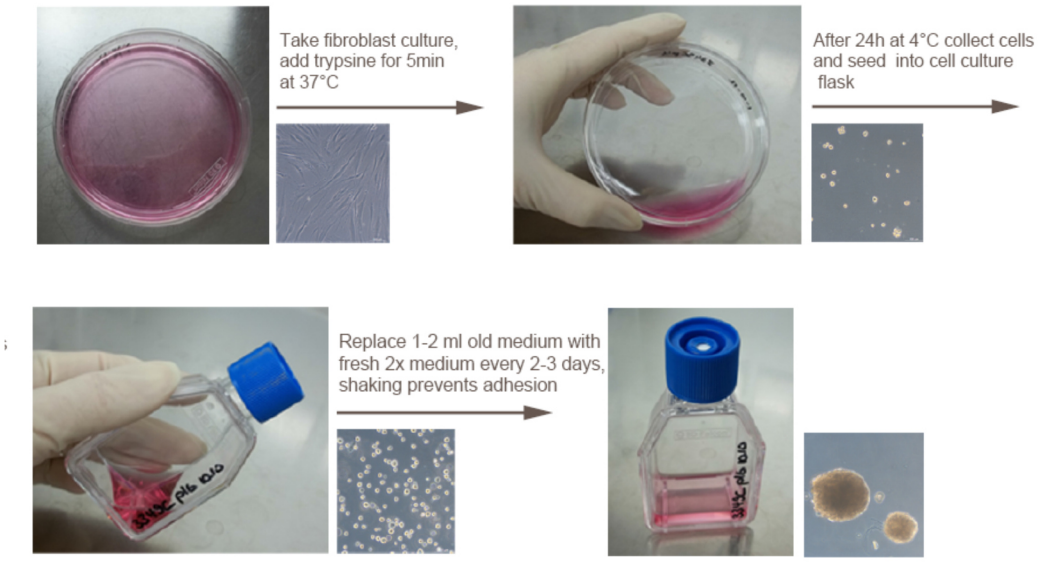

Figure 10: Isolation method for SKP cells from pre-existing fibroblast cultures. Fibroblast cultures were washed with PBS. The cells were treated with 0.25 % trypsin solution for 5 minutes at 37 °C and kept in 0.25 % trypsin solution for 24 hours at 4 °C. Cells were collected, centrifuged and resuspended in 1x SKP proliferation medium. Cultures were seeded into T12.5 flasks. Medium was replaced every 2-3 days and sphere formation was observed within 10 days. Adapted with permission from: Wenzel et al. (2013).

SKP cells derived from fibroblast cultures of individuals between 0-12 years denote 'SKP cells derived from young skin' in the experiments starting from results section 5.2, if not further specified in the experiment all SKP cells that were used were SKP cells derived from fibroblast cultures from young skin. SKP cells derived from fibroblast cultures of individuals between 50-94 years denote 'SKP cells derived from old skin' in experiments starting from results section 5.2. SKP cells derived from HGPS patients denote HGPS-SKP cells.

4.2.5 HGPS-fibroblast strains and control fibroblast strains

Dermal fibroblasts from subjects with HGPS were obtained from the Progeria Research Foundation Cell and Tissue Bank (www.progeriaresearch.org). The following fibroblast strains were used: HGADFN003 (M, age 2), HGADFN178 (F, age 7), HGADFN164 (F, age 4) and HGADFN188 (F, age 2).

Age-matched control fibroblasts were obtained from the Coriell Institute for Medical Research (Camden, NJ) and are derived from young skin. The following strains were used: GM01652C (F, age 11), GM02036A (F, age 11), GM03348E (M, age 10), GM08398A (M,

age 8). The human mesenchymal line, BMOI.55 was used as control (kindly provided by Toguchida T. and Aoyama T. at Kyoto University, (Okamoto et al., 2002)).

The Institutional Review Board at TU-Munich approved the use of human cells previously established from skin biopsies from HGPS patients and unaffected individuals.

4.2.6 SKP single cells and self-renewal assay

SKP spheres were washed with PBS and were dissociated by incubation with trypsin solution for 15 minutes. To dissociate the spheres the cells were pipetted up and down. The reaction was stopped and the cells were spun down. A PBS washing step followed. For single cell staining the cell solution was applied on glass coverslips and allowed to dry before performing immunocytochemistry (see 4.2.11).

For performance of a self-renewal assay the cells were seeded in 1x SKP proliferation medium in serial dilutions to obtain single cells. After sphere formation within approximately 30 days the spheres were stained for Ki67 to detect proliferation.

4.2.7 Directed differentiation of SKPs

Single spheres from ‘early’ and ‘late’ culture stage were plated on glass coverslips in 24-well culture dishes for 24 to 48 hours in adherence medium (see 4.1.7). After the spheres adherence was confirmed by microscope, the differentiation conditions were applied. Cells were washed with PBS and the medium was changed to differentiation medium.

To obtain fibroblasts, spheres were cultivated in fibroblast growth medium (see 4.1.7). Cells were passaged when they reached 80 % confluence and underwent repeated passaging and cryopreservation. Screening for a fibroblast marker was performed after several passages.

To obtain smooth muscle cells (SMC) the medium was replaced with SMC differentiation medium (see 4.1.7). The medium was changed every 2-3 days. In case of rapid growth the cells were split on new coverslips when reaching 80 % confluence. Smooth muscle markers were screened after 28-35 days in culture by immunofluorescence. Alternatively a medium consisting of high-glucose DMEM (Gibco) containing 5 % FBS, 5 ng/mL PDGF-BB (Invitrogen) and 2.5 ng/mL TGF-b1 (Invitrogen) as previously described (Hill et al., 2010) was used for HGPS-SKP differentiation.

4.2.8 HGPS skin sections

The Progeria Research Foundation Cell and Tissue Bank provided frozen skin sections derived from a skin biopsy of a 9-year-old donor with HGPS carrying *LMNA* G608G mutation (HGADFN143). Sections of the same skin biopsy were used previously to determine the in vivo pattern of expression of progerin in the HGPS skin (McClintock et al., 2006).

4.2.9 SKP sections

Spheres from SKP cultures at different stages (see 4.2.4) were harvested and washed in PBS. The spheres were embedded in OCT compound after removal of any remaining liquid, cryopreserved and stored at -80 °C if necessary. Serial 10 µm sections were prepared with a CM3050 S microtome (Leica) and stored at -80 °C.

4.2.10 Immunohistochemistry

Skin sections or SKP sphere sections were circled with a PAP pen on the slide. O.C.T. compound was removed to avoid unspecific spots. The cells were fixed by adding drops of ice-cold methanol and incubated at -20 °C for 10 minutes. The sections were washed in PBS afterwards. A permeabilization step was performed with 0.25 % Triton X-100 in PBS for 10 minutes. A blocking step was performed to prevent unspecific bindings by blocking with PBS containing 3 % bovine serum albumin for 60 minutes. Slides were incubated with primary antibody (see table 5) diluted in blocking buffer over night at 4 °C. The following day the slides were washed with PBS five times and incubated with the according AlexaFluor488-/AlexaFluor555-conjugated secondary antibodies (see table 6) for 1 hour at room temperature. After the incubation the samples were washed with PBS seven times, mounted with Vectashield mounting medium (Vector Inc.) and covered by a coverslip. Images were acquired on an Axioplan fluorescence microscope (Carl Zeiss, Germany).

4.2.11 Immunocytochemistry

Fibroblasts and differentiated SKP cells were grown adherent on coverslips. A washing step with PBS was performed to remove medium. In case of complete SKP sphere staining, SKP spheres were either spun down or settled on coverslips and dried. The cells were fixed in ice-cold methanol at -20 °C for 10 minutes and stored at -80 °C if necessary. The fixed

coverslips were permeabilized with 0.3 % Triton X-100, followed by a blocking step with 15 % FBS in PBS for 1 hour. According to immunohistochemistry (see 4.2.10) the cells were incubated in primary and secondary antibody solutions, separated by washing steps. Antibodies see table 5 and table 6. The samples were covered with Vectashield mounting medium (Vector Inc.) and images were acquired with an Axioplan fluorescence microscope (Carl Zeiss, Germany).

4.2.12 RNA Isolation

RNA was extracted from control fibroblasts, SKP cells at different culture stages and differentiated smooth muscle cells. Cell pellets were collected, washed with PBS and isolated with the RNeasy Minikit (Qiagen, Valencia, CA). The smallest possible volume was used for eluation with DEPC treated water. RNA purity was assessed by NanoDrop measurement of the 260 nm/280 nm ratio. Ratios between 1.9 and 2.1 were accepted for experiments.

4.2.13 cDNA preparation

cDNA was transcribed from total mRNA using the Omniscript Reverse Transcriptase (Qiagen). A total amount of 2 µg was synthesized per 20 µl reaction, assuming a final cDNA concentration of 50 ng/µl cDNA.

4.2.14 Oligos

The genes that were analyzed by real-time PCR and the corresponding oligo sequences can be found in table 7. Oligos were designed using the NCBI Primer-Blast, Primer3, were taken from literature or from primerdepot.nci.nih.gov as indicated in the table. All designed oligos were tested with the NCBI e-PCR tool and were adjusted to an annealing temperature of 60 °C.

4.2.15 PCR

For semi-quantitative analysis of progerin and lamin A, a PCR was performed. The used oligos are spanning the site of deletion so a shorter amplificate could be detected in the case of progerin.

A mix containing Phusion polymerase (Finnzymes), Phusion HF buffer, 1 mM dNTP mix (Invitrogen) and a final oligo concentration of 375 nM (oligo sequences see table 7) of lamin A/C and β-actin oligos in a total volume of 20 µl was used. 250 ng cDNA were added to the mix. Amplification was carried out using an iCycler (Bio-Rad). Initial denaturation was performed for 3 minutes at 95 °C followed by 37 cycles of 10 seconds at 98 °C, 30 seconds at 58 °C and 15 seconds at 72 °C. A final extension step at 72 °C for 10 minutes was added.

15 µl of the PCR product were analyzed on a 2 % agarose gel. If necessary the bands were quantified using a Bio-Rad scanner and Quantity One software according to the density of the band. The density of the β -actin band that was amplified in the same tube was used for normalization.

4.2.16 Real-time PCR

To perform real-time PCR analysis the Power SYBR Green PCR Mastermix (Applied Biosystems) was used. A final concentration of 375 nM of each primer and 50 ng of template cDNA was used in a 20 ml volume for each reaction. Real-time PCR was carried out using the Mx3000P Real-Time PCR Detection System (Stratagene) with the following amplification program: an initial denaturation step (95 °C for 2.5 minutes) followed by 40 cycles (95 °C for 5 seconds and 60 °C for 20 seconds). To assess the uniformity of the product a dissociation curve was measured (1 minute 95 °C, 30 seconds 60 °C, 30 seconds 95 °C). All samples were run in triplicates and multiple experiments were performed for each assay. GAPDH was used as an endogenous control. Changes in relative expression were calculated using the ΔΔCt method to evaluate the results (Livak and Schmittgen, 2001).

4.2.17 Western blot

Freshly harvested cell pellets of SKP cells and control fibroblasts were washed in chilled PBS, centrifuged at 4500 g for 5 minutes at 4 °C and extracted in laemmli sample buffer (Bio-Rad) containing 5 % β-mercaptoethanol and PMSF as protease inhibitor. The pellets were sonicated three times for 5 seconds and boiled for 5 minutes at 95 °C.

Protein concentrations were determined by dot blot quantification of 2 µl drops of samples and a diluted BSA standard on a membrane. 30 µg of total protein extract were loaded on a 4-20 % polyacrylamide gel. After separation by electrophoresis, proteins were transferred to

nitrocellulose membranes and incubated with blocking buffer containing 1 % milk powder and 0.3 % Tween20 as described previously (McClintock et al., 2006). Membranes were incubated with primary antibody (see table 5) over night, washed and then incubated with a corresponding secondary antibody conjugated with horseradish peroxidase (see table 6). Protein bands were visualized using the enhanced chemiluminescence detection system (Bio-Rad) and scanned with a Bio-Rad scanner. Relevant bands were analyzed and normalized to internal tubulin as well as to the controls by densitometry using Quantity One 1D analysis software (Bio-Rad) on the scanned images.

4.2.18 Statistical analysis

Results are presented as means ± SEM. Comparisons were performed with student's t-test. $P<0.05$ was defined as statistically significant (*), $P<0.01$ as highly statistically significant (**).

5 Results

5.1 Isolation methods for SKP cells

The direct isolation of SKP cells from human skin was described previously (Toma et al., 2005). Within this project, a new isolation method was developed, which allows the isolation of SKP cells from human primary fibroblast cultures. The isolation of SKP cells from young and old individuals could be performed with both methods. In order to evaluate the newly developed method, SKP cells isolated from skin and from primary fibroblast cultures were compared in regard to their morphology and specific marker expression.

5.1.1 Direct isolation and cultivation of SKP cells from skin of young and old donors

SKP cells could be isolated directly from young foreskin biopsies and from adult skin biopsies of various body sites such as belly, thigh, breast and thorax (Figure 11).

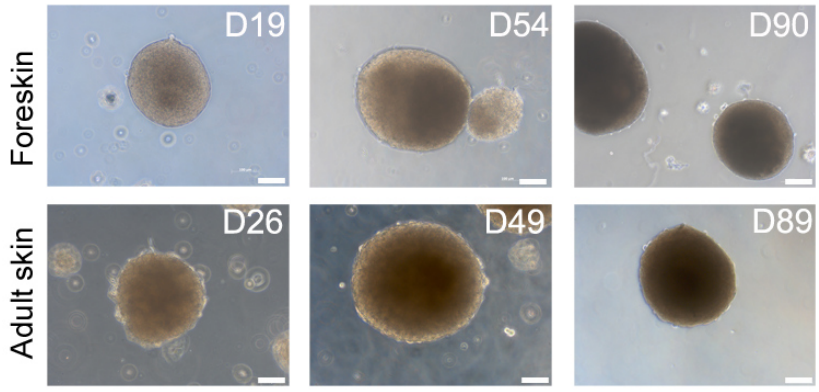

Figure 11: Long-term cultures of SKP cells isolated directly from young and old skin. SKP cells were isolated from skin biopsies derived from young and old probands. Upper row: Foreskin-derived SKP cells on day 19, day 54 and day 90. Lower row: SKP cells derived from adult skin on day 26, day 49 and day 89. The cultures derived from different body sites of old skin and showed growth deficiencies after around 90 days. Scale bar: 100 µm, scale bar old skin D89: 200 µm.

The cells formed floating spheres with a diameter of approximately 50 µm within the first 10 days of culture. During the culture period the sphere number decreased and the diameter of the SKP spheres increased up to a size of 1000 µm. A combination of small spheres with a diameter of 50 µm and larger spheres of more than 400 µm was observed within the first two months of culture while larger spheres were more prominent at later culture stages. SKP cells isolated directly from old skin showed decreased growth in cultures by day 90 and the sphere numbers dropped dramatically. The sphere numbers of SKP cells derived from young skin decreased as well but the cells could be maintained in cultures for a considerably longer period of time.

5.1.2 Isolation and cultivation of SKP cells from primary fibroblast cultures derived from young and old skin

During cell culture experiments with control fibroblast, it was observed that fibroblast cultures undergoing massive stress (e.g. temperature variations during transport) were able to recover and few cells could survive. Therefore, it was hypothesized that these cells were stress resistant and might be adult stem cells, sharing similar properties with those derived from the dermis directly. According to this observation, a new isolation method was developed based on the application of a stress trigger on fibroblast cultures. The stress trigger hereby consisted of a prolonged period of trypsin treatment and nutrient deprivation.

It could be shown that after this stress trigger, surviving cells from fibroblast cultures formed spheres that resembled the ones directly isolated from dissociated skin. Those fibroblast-derived SKP cells started to form small clusters in the first few days of the culture and developed into spheres of approximately 50 µm by day 10. These spheres shared all morphological features in size and shape with the conventionally isolated SKP cells (Figure 12).

Primary fibroblast cultures derived from young skin and as well from old skin could be used to isolate SKP cells. Even after 14-20 passages of the fibroblast cultures, similar sphere formation rates were obtained after isolation. Freezing, thawing and re-growing of the fibroblast cultures did not affect the SKP isolation process.

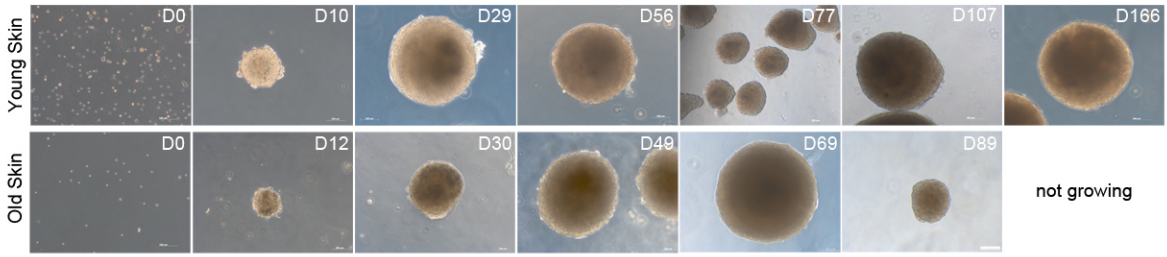

Figure 12: Long-term cultures of SKP cells derived from fibroblast cultures of young and old skin. SKP cells were isolated from fibroblast cultures derived from young and old skin. Upper panel: SKP cells derived from fibroblast cultures from young skin. Spheres were observed after approximately 10 days of culture. Cultures could be maintained up to 250 days. Lower panel: SKP cells derived from fibroblast cultures from old skin. Spheres were observed after 10 days of culture. Cultures could be maintained for approximately 90 days before sphere growth stopped. Scale bar: 100 µm.

Long-term cultures showed that SKP cells derived from fibroblast cultures from young skin could be maintained in cultures for up to 250 days. In contrast, SKP cells derived from fibroblast cultures from old skin showed a strong decline in sphere numbers after approximately 90 days of culture.

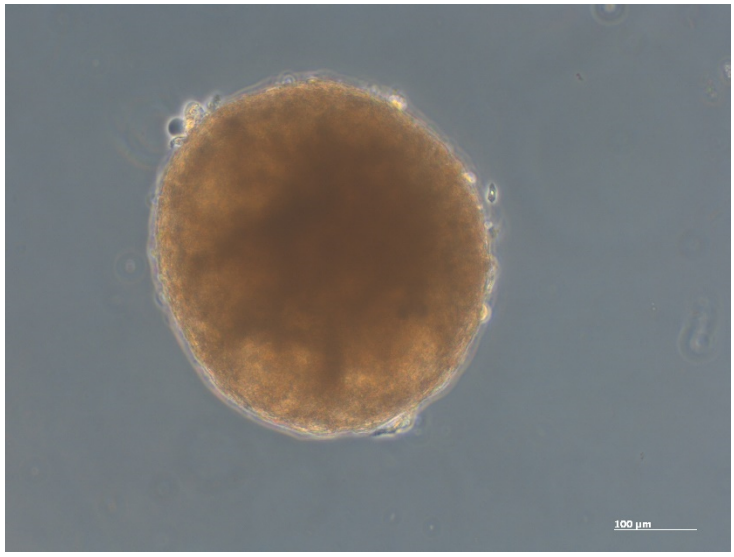

Figure 13: Size and morphology of a SKP sphere after 190 days in culture. SKP sphere derived from a fibroblast culture from young skin. Spheres grew to a diameter of up to 400-1000 µm, color and shape of the spheres were similar to the first weeks of culture. Scale bar 100 µm.

After long-term cultures, the shape of the SKP spheres still resembled the starting cultures but the diameter of most spheres increased over the culture period to a size of 400-1000 µm (Figure 13).

5.1.3 Comparison of SKP cells isolated from skin and from fibroblast cultures

Further characterization was performed to confirm that the cells isolated from primary fibroblast cultures were identical to those directly isolated from skin.

Despite the different isolation methods, a comparison showed that both approaches lead to a similar sphere formation capacity and that spheres formed in both cultures around day 10 (Figure 14). In addition, the morphology of the spheres was similar concerning their size and shape.

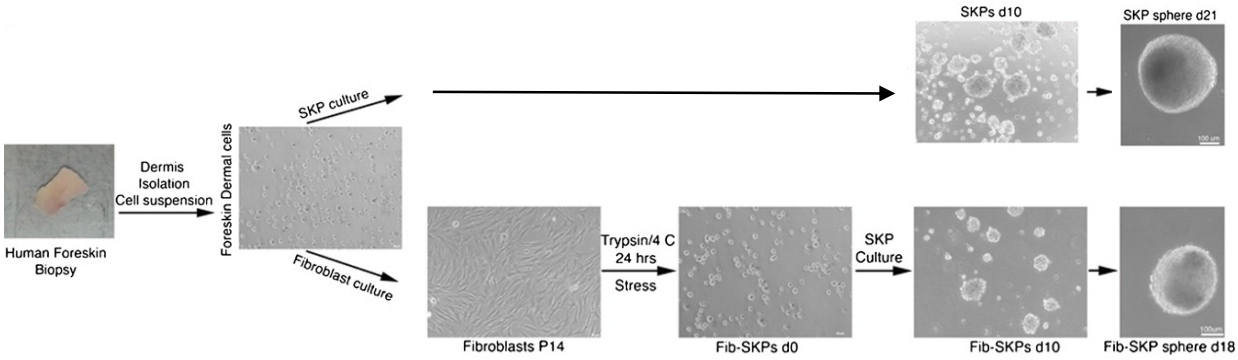

Figure 14: Generation of SKP spheres from human foreskin and from corresponding foreskin primary fibroblast cultures. The method for SKP sphere cultures is outlined starting from a typical skin biopsy (skin image), followed by the dermal cell suspension which was either used directly for SKP cultures or for the establishment of primary fibroblast cultures. After 14 passages the fibroblast cultures were used for SKP cell isolation. Adapted with permission from Wenzel et al. (2012).

After approximately three months of culture, similar patterns of halted growth were observed in both, SKP cells derived from old skin directly and SKP cells derived from primary fibroblast cultures from old skin.

All SKP cell characteristics could be observed even though the fibroblast cultures had been passaged several times or had been cryopreserved and re-thawed, indicating the persistence of adult stem cells in the primary fibroblast cultures.

Furthermore, it was shown that both, SKP cells derived from skin directly and SKP cells derived from primary fibroblast cultures express the same panel of markers, such as nestin, fibronectin and vimentin, that are characteristic for this precursor population (Toma et al., 2005). Additionally, the expression of the stem cell markers Oct4 and TG30 as well as the expression of lamin A/C could be detected at similar levels in SKP cells derived from both isolation methods (Figure 15).

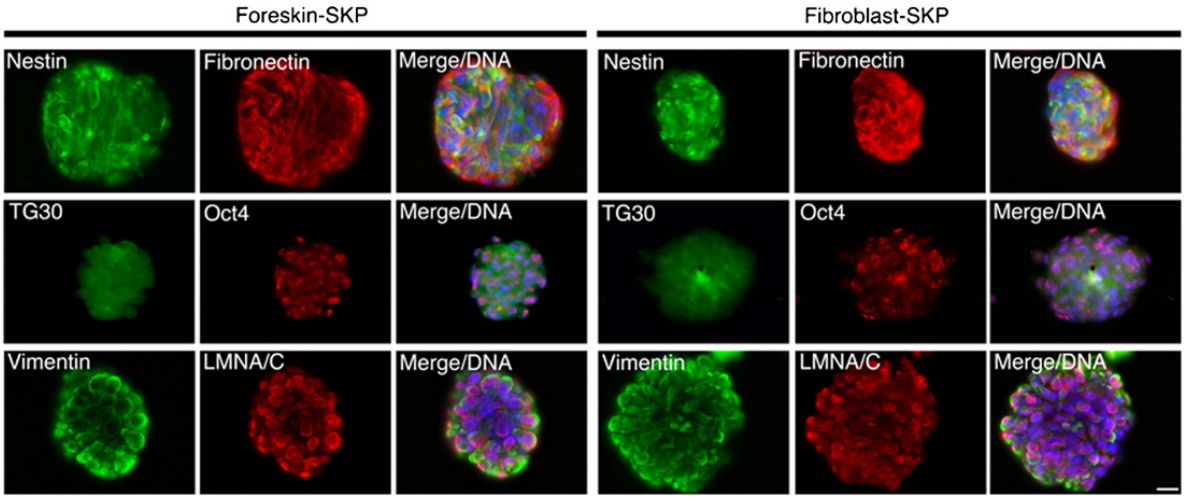

Figure 15: SKP spheres from human foreskin and from respective foreskin-derived primary fibroblast cultures show identical characteristics. SKP spheres between day 16 and day 18 of culture were used for characterization. Immunofluorescence staining of the SKP markers nestin, fibronectin and vimentin was performed. A staining for the multipotency markers Oct4 and TG30, as well as for lamin A/C was added for further characterization. Triple stainings are shown with DAPI-stained nuclei. Whole spheres were stained as described in the material and methods section. Foreskin-SKP denotes SKP cells derived from direct isolation from foreskin. Fibroblast-SKP denotes SKP cells derived from primary fibroblast cultures of young skin. Scale bar 20 µm. Adapted with permission from Wenzel et al. (2012).

The results prove that SKP cells can be isolated from pre-existent fibroblast cultures with the newly developed method. Due to the availability of fibroblast cultures from tissue banks and the uncomplicated expansion of SKP sphere cultures, this method creates huge advantages for basic science research and pharmacological applications.

For all of the following experiments, SKP cells derived from fibroblast cultures were used.

5.2 Characterization of SKP cells isolated from fibroblast cultures derived from young and old skin

The experiments described above compared SKP cells that derived directly from skin with SKP cells that derived from primary fibroblast cultures. These primary fibroblast cultures were established before from skin biopsies of young or old individuals. All following experiments of this thesis were performed using SKP cells isolated from fibroblast cultures. From this point on 'SKP cells/cultures/spheres derived from young/old skin' always refers to SKP cells that were isolated from corresponding fibroblast cultures.

In order to characterize SKP cells in the context of aging, SKP cells derived from young and old skin were analyzed with regard to their sphere forming capacity, their growth behavior and their stemcellness at different culture stages during long-term cultures. The culture stages 'early' (<D30), 'intermediate' (D45-75) and 'late' (>D90) were defined and samples were taken at these culture stages for analyses.

5.2.1 Average number of SKP spheres per culture

It was observed that the number of SKP spheres decreased during long-term cultures. Sphere counting in at least five independent SKP cultures derived from young and old skin showed a massive decrease in sphere numbers starting from day 21 in both age groups (Figure 16). SKP cells derived from young skin showed an average decrease in sphere numbers of 93 % from day 21 to day 91. Sphere numbers of SKP cells derived from old skin decreased similarly of about 95 % between day 21 and day 91.

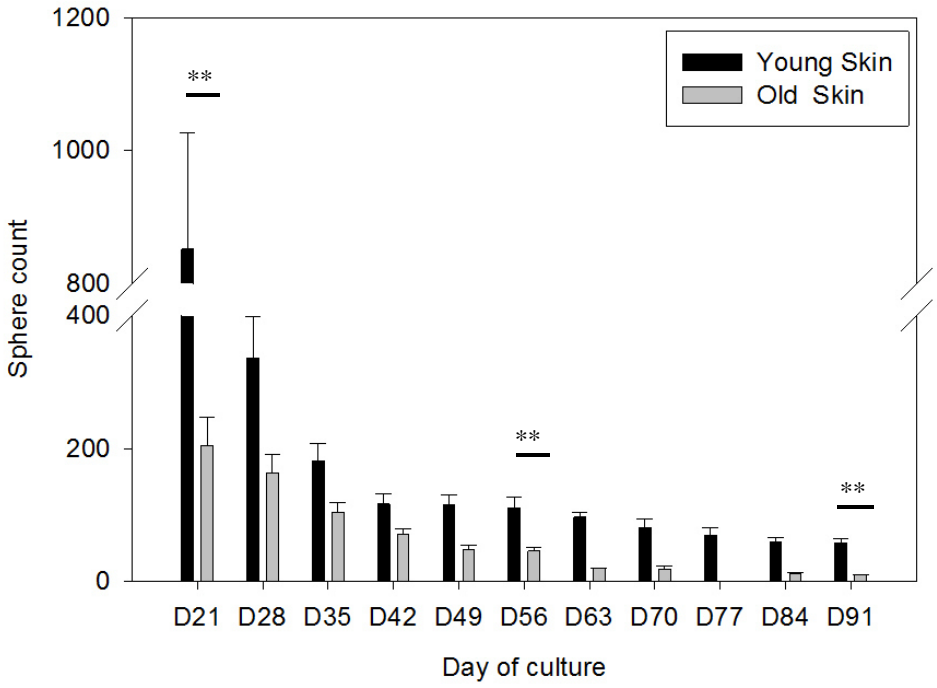

Figure 16: The number of SKP spheres decreases with increasing culture time. Comparison of SKP sphere numbers in cultures derived from young and old skin between day 21 and day 91. Spheres were counted per culture flask under the microscope. Cultures were started from 6 million fibroblast cells. Y-axis break ranges from 400 to 800 counts. n≥5 for all cultures except for SKP spheres derived from old skin from D70 to D91 (n=2) due to growth limitations, values are missing for D77 of SKP cultures derived from old skin. Data shown as means ± SEM. P values of $p<0.05$ were defined as statistically significant, $p<0.01$ as highly significant. Significance was tested for exemplary values of 'early', 'intermediate' and 'late' stage.

The sphere numbers were counted under the microscope with the help of a grid, dividing the cell culture flask in several sectors.

A highly significant difference in the sphere numbers was observed at day 21 of SKP cultures derived from young and from old skin. While SKP cultures derived from young skin had an average sphere count of 852 spheres, the SKP cultures derived from old skin only formed an average of 204 spheres. A significant difference in the number of spheres was observed during all culture stages e.g. at 'intermediate' stage day 56 and at 'late' stage day 91.

The average sphere formation rate at day 21 for SKP cultures derived from young skin was 0.014 % while the sphere formation rate for SKP cultures derived from old skin only averaged 0.003 % (Figure 17).

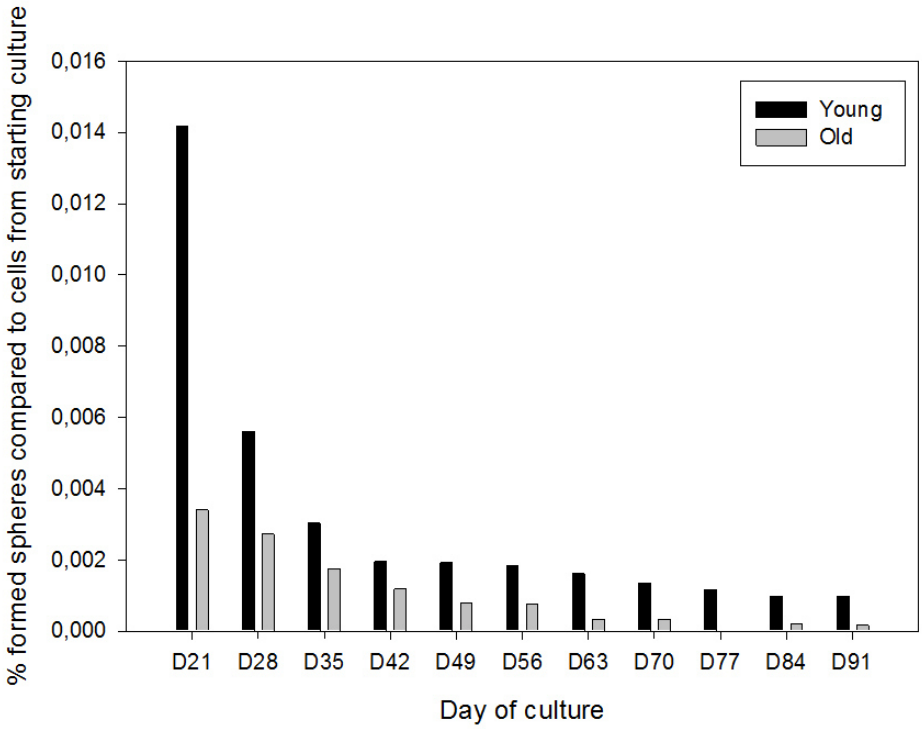

Figure 17: The percentage of formed spheres is higher in SKP cultures derived from young skin. Percentage of spheres derived from young and old skin calculated in relation to 6 million fibroblast cells of the starting culture. The average number of counted spheres (Figure 16) was used for the calculation. Values missing for D77 of SKP cultures derived from old skin due to growth limitations.

5.2.2 Average diameter of SKP spheres

The diameter of SKP spheres from young skin increased with time from an average of 200 µm on day 21 to 720 µm by day 91 (Figure 18). The average diameter of SKP cells derived from old skin was slightly larger with 290 µm on day 21 of the culture. However, the difference between young and old was not statistically significant at day 21 (p=0.06). During the culture period the diameter of the spheres in both age groups increased up to an average of

720 µm and 800 µm, respectively. The largest spheres exceeded 1000 µm of size. Comparison of sphere size between day 21 and day 91 showed a highly significant increase in size ($p<0.001$) both for SKP spheres derived from young and old skin.

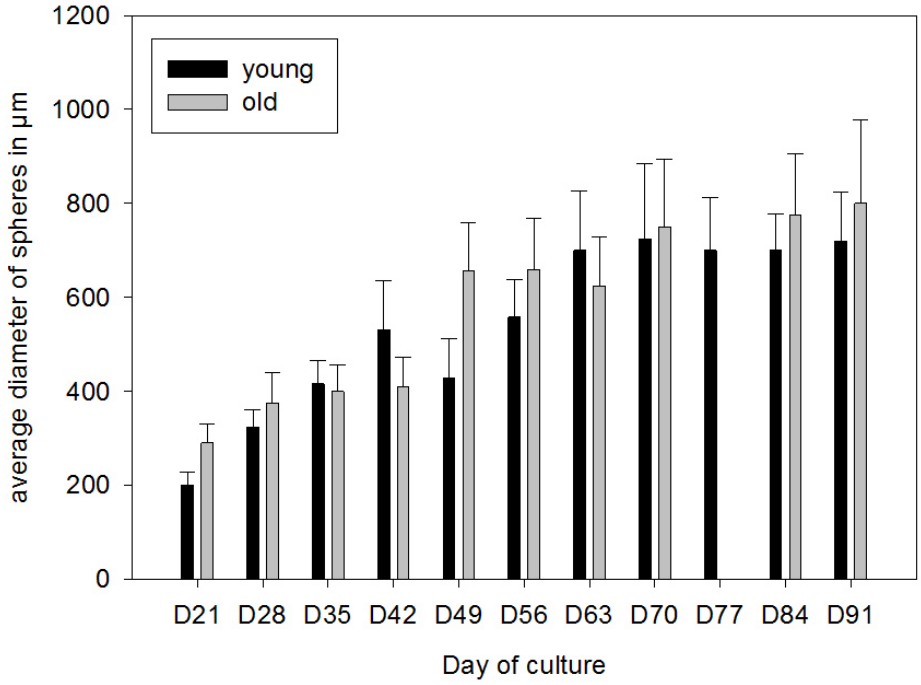

Figure 18: The average diameter of SKP spheres in long-term cultures increases. Four sphere diameters representing the range per culture were measured from each culture ($n\geq5$, old skin from day 70 n=2 due to growth limitations, values missing for D77 of SKP cultures derived from old skin). Data shown as means ±SEM. Comparison of D21 to D91 showed a highly significant increase in size ($p<0.001$) for both, SKP spheres derived from young and old skin.

5.2.3 Proliferation and self-renewal of SKP cells

By performing a dilution assay it could be shown that SKP cells which dissociated from a single SKP sphere are capable of self-renewal. Single cells derived from spheres formed small clusters after approximately 15 days and a typical sphere appeared after 30 days. Approximately 0.2 % of the cells from a single dissociated sphere were capable of forming new spheres. Proliferating cells were detected by Ki67 staining, indicating that the cells in the

newly formed spheres were able to proliferate (Figure 19). In addition, the observed expression of the stem cell marker nestin indicates that the SKP2 cells express the same stem cell markers and possess the same phenotype as the original SKP spheres.

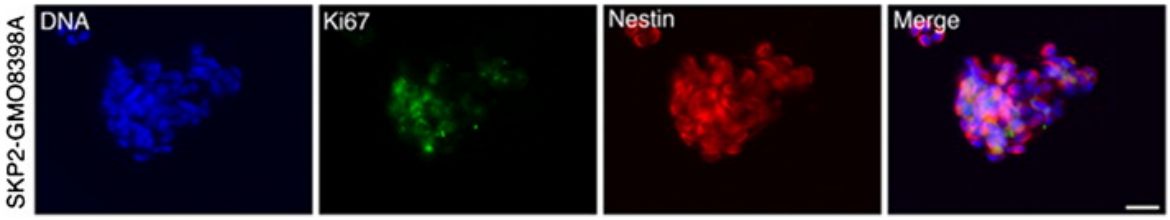

Figure 19: SKP cells are able to self-renew. SKP2 spheres resulting from single cells of SKP spheres derived from young skin. At day 25 to 30 of the culture the SKP2 spheres were double stained for the proliferation marker Ki67 and for the stem cell marker nestin. Nuclei are stained with DAPI. Scale bars: 20 µm. Adapted with permission from Wenzel et al. (2012).

5.2.4 Stem cell marker profiling in SKP cells derived from young and old skin at different culture stages

To compare the stem cell potential of SKP cells derived from young skin to the potential of SKP cells derived from old skin, immunofluorescence analysis and real-time PCR analysis of stem cell markers were performed. At the same time, the stem cell properties during long-term cultures were examined in both age groups. The experiments were performed at the culture stages ‘early’ (<30 days), ‘intermediate’ (45 to 75 days) and ‘late’ (>90 days).

5.2.4.1 Immunofluorescence detection of stem cell marker expression

It was shown that SKP cells from young skin expressed the stem cell markers nestin, Oct4, Sox2, TG30 and Nanog as well as the neural crest stem cell marker NGFR and fibronectin. These proteins were expressed at all cultures stages during the culture period of 90 days (Figure 20).

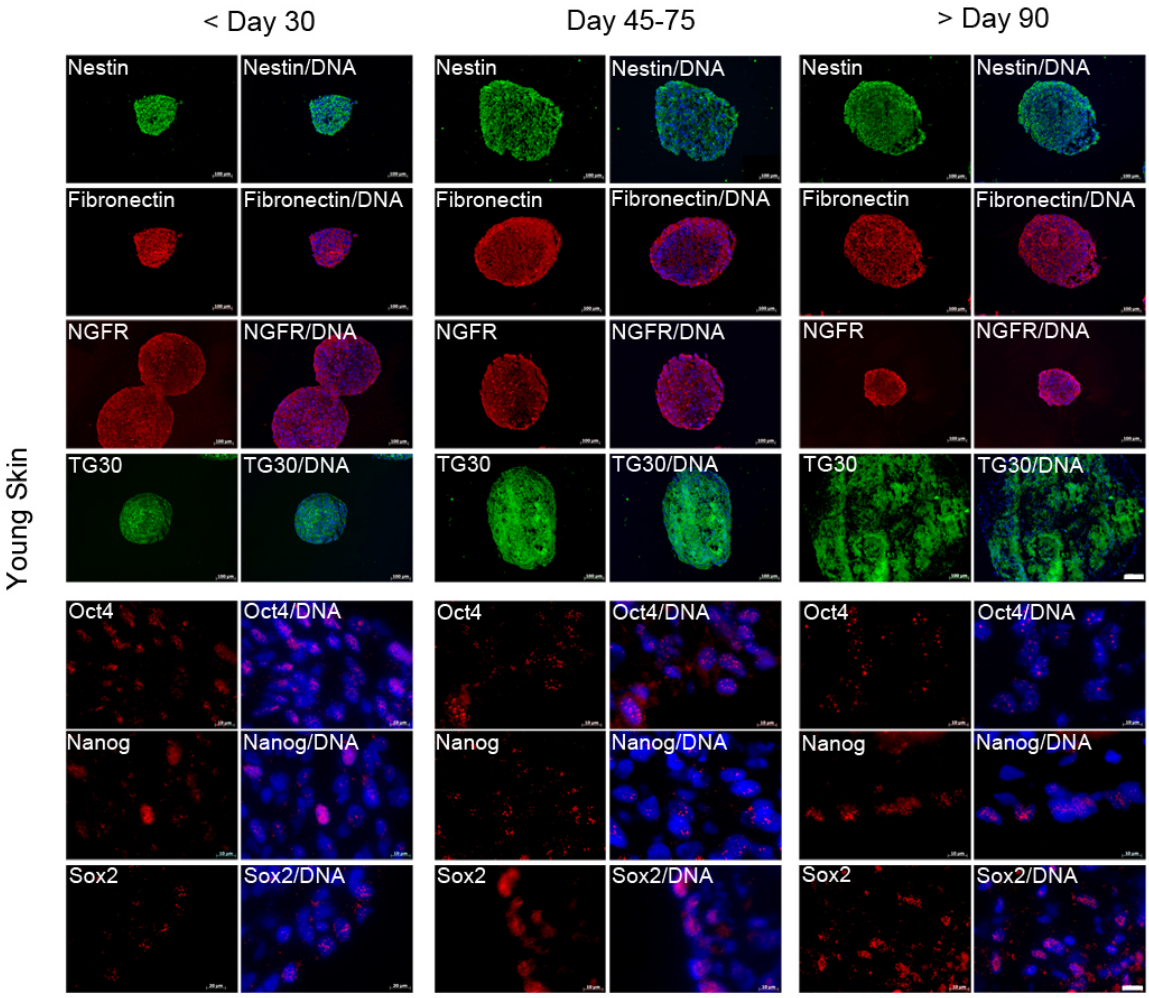

Figure 20: The protein levels of stem cell markers in SKP cells derived from young skin do not change with increasing culture time. Immunofluorescence staining of 10 µm cryo-sections of SKP cells derived from young skin (0-12 years). Spheres were collected from cultures at <30 days ('early'), 45 to 75 days ('intermediate') or >90 days ('late'). The sections were fixed with methanol and stained for nestin, fibronectin, NGFR, TG30 and as well for the multipotency transcription factors Oct4, Nanog and Sox2. Experiments were repeated three times. Scale bar upper section: 100 µm. Scale bar lower section: 10 µm.

Similar to the results observed in SKP cells derived from young skin, the immunofluorescence staining of SKP cells derived from old skin revealed that all stem cell markers were expressed in 'early', 'intermediate' and even in 'late' culture stages on a comparable level (Figure 21). Despite the observed growth limitations of SKP cells derived from old skin, the protein levels of the stem cell markers appeared stable.

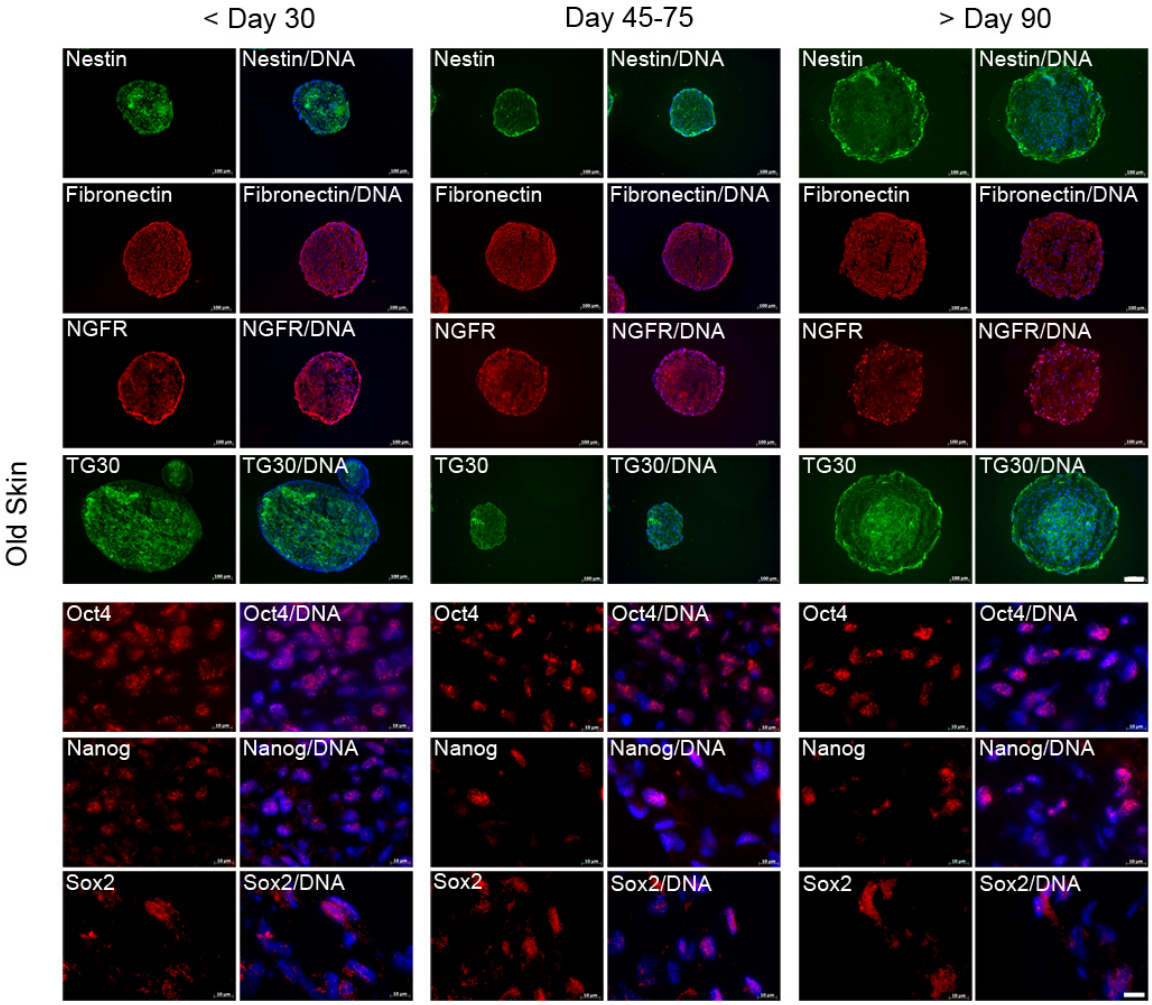

Figure 21: The protein levels of stem cell markers in SKP cells derived from old skin are maintained over the culture period until 'late' culture stages. Immunofluorescence staining of 10 µm cryosections of SKP cells derived from old skin (50-94 years). Spheres were collected from cultures at <30 days ('early'), 45 to 75 days ('intermediate') or >90 days ('late'). The sections were fixed with methanol and stained for nestin, fibronectin, NGFR, TG30 and as well for the multipotency transcription factors Oct4, Nanog and Sox2. Experiments were repeated three times. Scale bar upper section: 100 µm. Scale bar lower section: 10 µm.

5.2.4.2 Real-time PCR analysis of stem cell markers

Stem cell marker mRNA levels were studied to detect differences that could correlate to the observed variations in growth of SKP cells derived from young and from old skin. The expression of mRNA encoding the stem cell markers nestin, Oct4, Sox2 and Nanog was analyzed.

SKP cells derived from young skin did express increasing levels of nestin mRNA during the culture period. The nestin mRNA levels were significantly higher at 'intermediate' stage compared to 'early' stage (p=0.01). As shown in Figure 22 the nestin mRNA levels were 2.8 times higher at 'late' stage by comparison to 'early' stages of the cultures. This increase is statistically highly significant (p=0.0001).

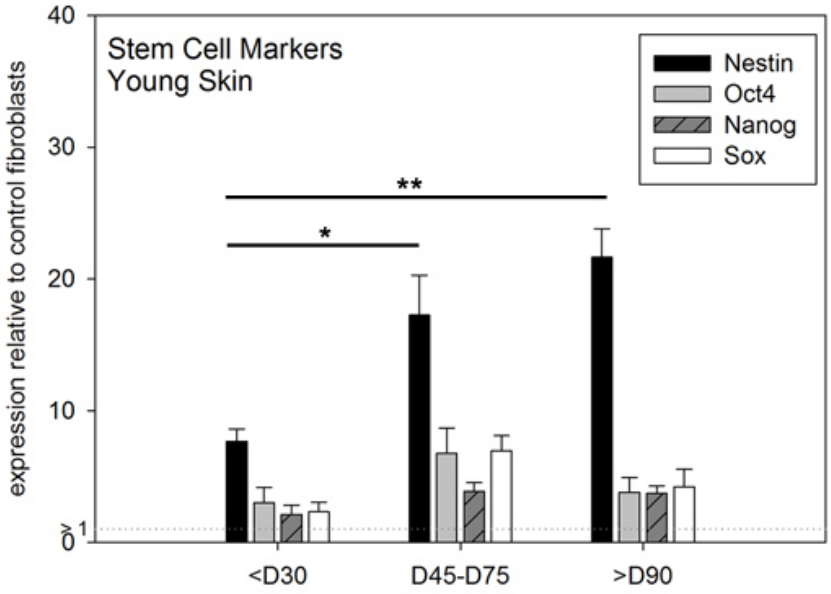

Figure 22: mRNA levels of stem cell markers remain constant or even increase in SKP cells derived from young skin during long-term cultures. Relative x-fold mRNA levels from SKP cells derived from young skin (0-12 years) in relation to mRNA levels of control fibroblasts. Analysis of the markers nestin, Oct4, Sox2 and Nanog. SKP cell cultures before day 30 ('early'); SKP cell cultures between day 45 and day 75 ('intermediate') and SKP cell cultures older than day 90 ('late') were used. ΔΔCt Values were calculated for analysis. $n \geq 4$, data shown as means ± SEM. P values of $p < 0.05$ were defined as statistically significant, $p < 0.01$ as highly significant.

In contrast, the mRNA levels for Oct4 and Nanog remained at comparable levels over the culture time and did not show statistically significant differences. Only Sox2 mRNA levels increased significantly from ‘early’ to ‘intermediate’ stage (p=0.02) but decreased again at the ‘late’ stage of the cultures.

SKP cells derived from old skin did not provide good RNA preparations in quality and amounts at ‘late’ culture stages. For this reason, real-time PCR analysis was only performed at ‘early’ and ‘intermediate’ stage (Figure 23).

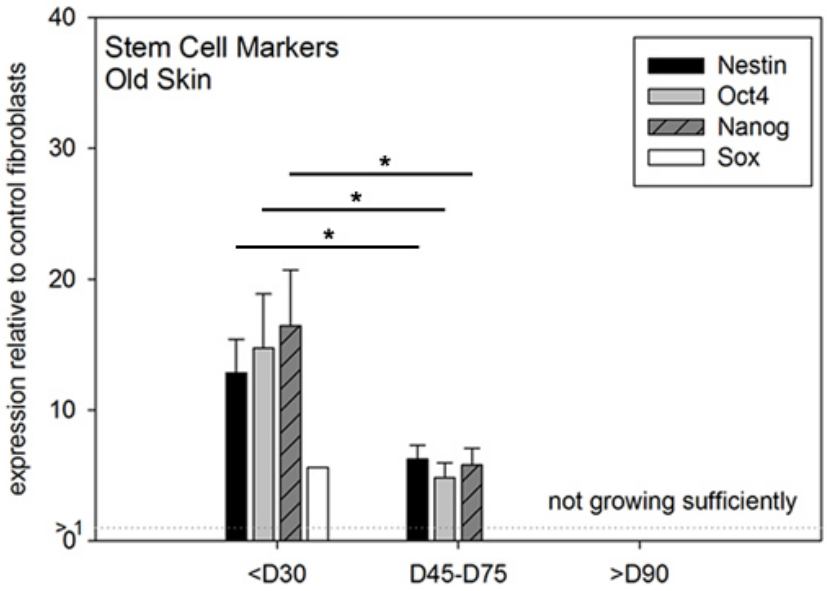

Figure 23: Stem cell marker mRNA levels decrease significantly during long-term cultures in SKP cells derived from old skin. Relative x-fold mRNA levels from SKP cells derived from old skin (50-94 years) compared to mRNA levels of control fibroblasts for nestin, Oct4, Sox2 and Nanog. SKP cell cultures before day 30 (‘early’) and SKP cell cultures between day 45 and day 75 (‘intermediate’) were used. SKP cells derived from old skin did not provide enough material to isolate mRNA of good quality after culture periods over 90 days (‘late’). ΔΔCt Values were calculated for analysis. (n≥ 5; Sox2 n=2, only analyzed in ‘early’ stage), data shown as means ± SEM. P values of p <0.05 were defined as statistically significant.

At ‘early’ stage of the SKP cultures, high levels for nestin, Oct4 and Nanog were detected (Figure 23). These levels decreased significantly from ‘early’ to ‘intermediate’ stage. Nestin mRNA levels decreased by half (p=0.03), while Oct4 and Nanog mRNA levels were significantly reduced to approximately one third of the ‘early’ stage levels (p=0.02; p=0.01). Notably, the levels of nestin were not increased at ‘intermediate’ stage compared to Oct4 and Nanog as observed in SKP cells derived from young skin (compare Figure 22).

In general, the mRNA levels of nestin, Oct4 and Nanog in SKP cells derived from old skin were significantly higher when being compared to the levels of SKP cells derived from young skin at ‘early’ culture stage (no graph shown: nestin p=0.045, Oct4 p=0.025, Nanog p=0.021). At ‘intermediate’ stage the mRNA levels of SKP cells derived from young and old skin were comparable besides for nestin mRNA levels, which were significantly higher in SKP cells derived from young skin (p=0.01). However, the rapid decrease of mRNA levels of the stem cell markers nestin, Oct4 and Nanog in SKP cells derived from old skin seems to correlate with the growth deficiencies that were observed in corresponding cultures that derived from old skin.

Taken together, it was shown that SKP cells can be isolated from fibroblast cultures derived from young and old skin. SKP cells derived from old skin showed growth limitations after 90 days, while SKP cells derived from young skin were able to grow in cultures for more than 200 days. Sphere formation was decreased in cultures derived from old skin. SKP cells derived from both age groups expressed stem cell markers during all culture stages of up to 90 days. However, the mRNA levels of stem cell markers decreased rapidly in SKP cells derived from old skin, which possibly relates to the observed growth limitations in those cultures.

5.3 Isolation and characterization of SKP cells from HGPS disease state fibroblasts

HGPS is caused by a mutation in the *LMNA* gene which leads to the expression of the truncated protein progerin. Progerin accumulates in distinct cells of the patients and affects the cellular function. Skin biopsies of HGPS patients cannot be obtained in higher numbers or from several patients due to the rare number of cases, the health condition and for ethical reasons. Therefore, the newly developed method for SKP isolation provides an easy and unique way to obtain and analyze SKP cells isolated from HGPS-patient fibroblast strains.

5.3.1 HGPS-fibroblast strain characterization

Four HGPS-fibroblast strains and four control fibroblast strains were characterized concerning their lamin status and progerin expression. The control fibroblasts strains derived from age-matched, young individuals.

As shown in Figure 24, the lamin A levels in HGPS-fibroblasts were comparable to the levels of control fibroblast strains. Immunofluorescence detection of lamin B1 showed a slightly lower expression of lamin B1 in HGPS-fibroblasts indicating a more senescent state as described previously (Shimi et al., 2011). Dysmorphic nuclei of HGPS-fibroblasts could be detected by bright lamin A staining. These dysmorphic nuclei showed strong progerin expression while no progerin positive cells were detected in control fibroblasts. The number of dysmorphic nuclei varied from 23.7 % to 33.0 % in HGPS-fibroblasts while only 3.2 % to 6.1 % of the control fibroblasts showed notable changes in nuclear shape.

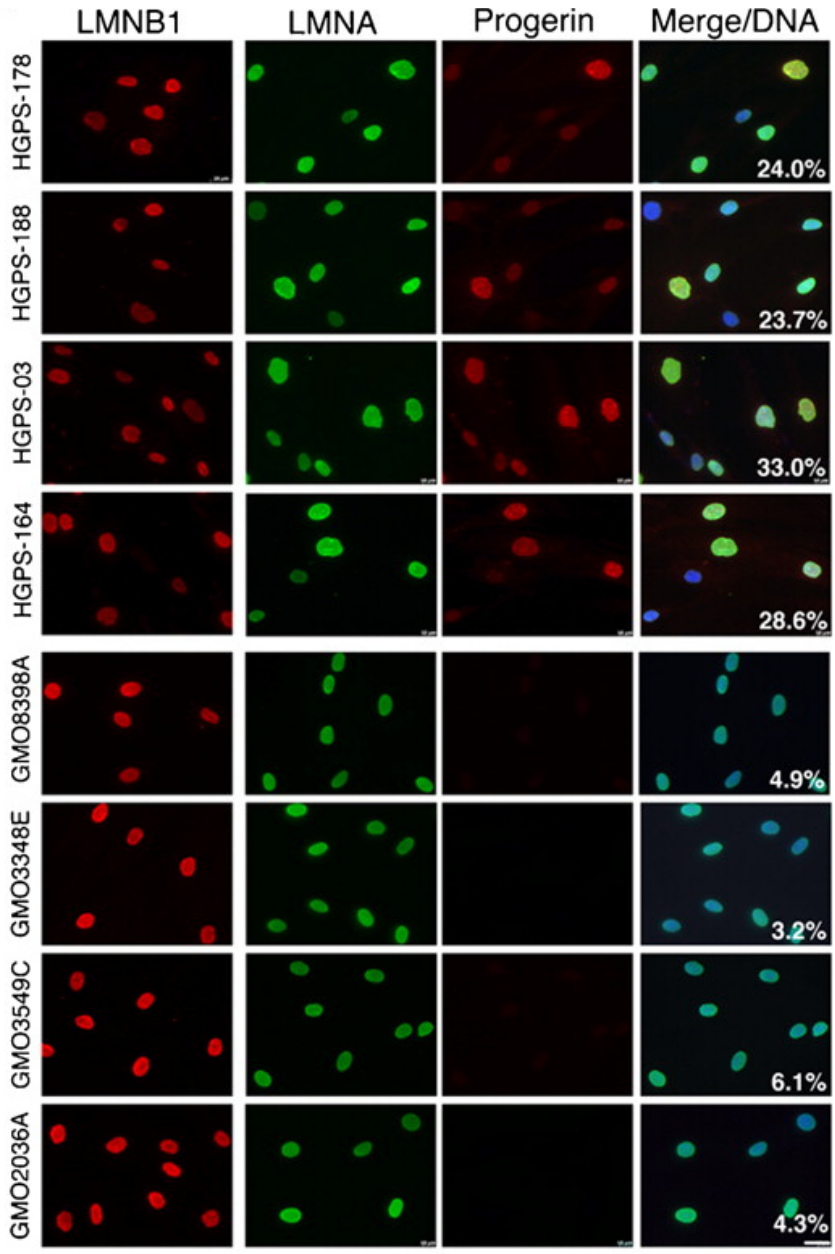

Figure 24: Characterization of HGPS-fibroblast strains and control fibroblast strains prior to SKP isolation shows progerin expression in HGPS-fibroblasts. Immunofluorescence detection of lamin B1, lamin A and progerin in four HGPS fibroblast strains (upper panel) and four healthy control fibroblast strains (lower panel) from PPD 20 to 35 are shown. Fibroblasts were grown on coverslips. Cells showing dysmorphic nuclei are indicated in percent per total count (n=3). Scale bar: 20 µm. Adapted with permission from Wenzel et al. (2012).

The expression of progerin could also be detected by western blot analyses. All four tested HGPS-fibroblast strains showed the progerin protein band migrating between the lamin A and the lamin C bands while no progerin band was detectable in the four fibroblast control strains (Figure 25).

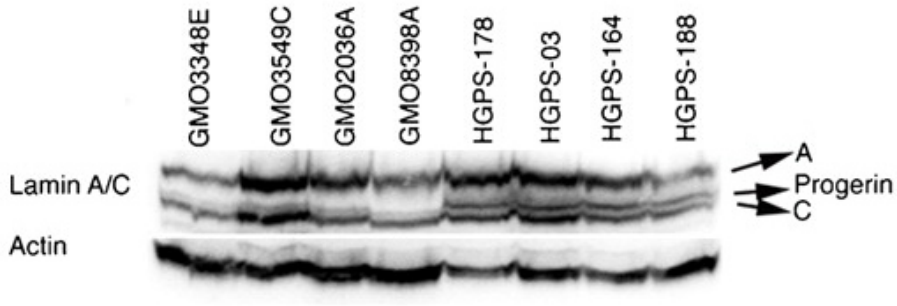

Figure 25: Western blot analysis of HGPS-fibroblast strains and control fibroblast strains shows progerin expression in HGPS-fibroblasts. Detection of lamin A/C and progerin in western blot analysis. 30 µg of total protein extracts in laemmli buffer were used. Actin was detected as housekeeper protein. The progerin band could be detected between the lamin A and lamin C band due to the 50 amino acid deletion which leads to a shorter protein length compared to lamin A. n=3. Adapted with permission from Wenzel et al. (2012).

5.3.2 Isolation and cultivation of HGPS-SKP cells

HGPS-SKP spheres could be isolated from four different HGPS-fibroblast strains. The HGPS-SKP spheres showed the same characteristics as control SKP sphere cultures. Within the first days of culture, small cell clusters were visible and by day 8 to day 10 spheres formed (Figure 26).

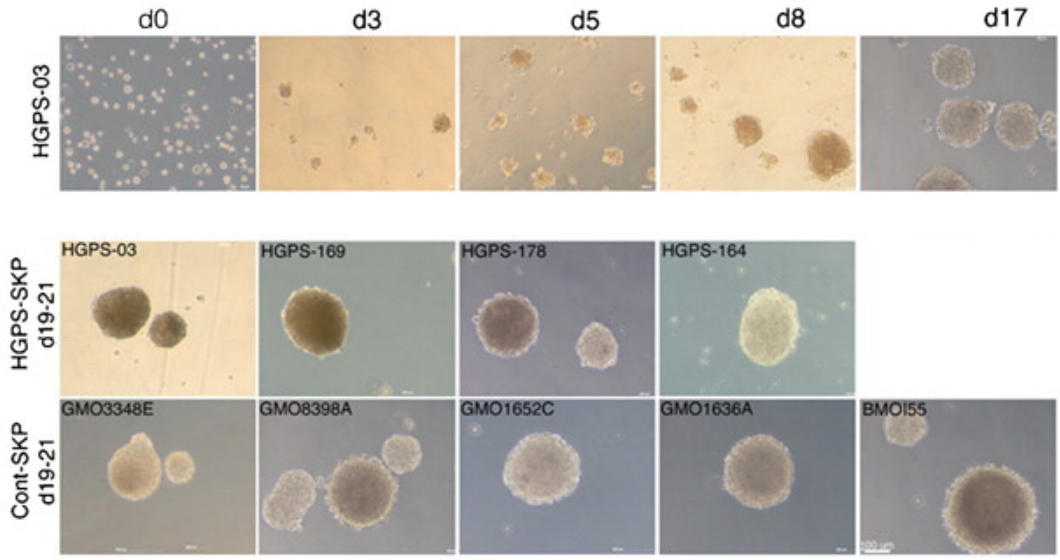

Figure 26: Typical SKP spheres form in the cultures after isolation from HGPS primary dermal fibroblast cultures. Upper panel: In vitro formation of SKP spheres derived from the HGPS fibroblast strain HGPS-003 (HGADFN003). Development of the spheres from day 0 to day 17 in culture. Lower panel: Typical 3D spheres derived from four HGPS and four normal fibroblast strains and a human mesenchymal stem cell line (BMOI.55). Spheres between days 19 to 21 of culture are shown. At least five SKP cell cultures have been established for each fibroblast strain. Scale bar: 100 µm. Adapted with permission from Wenzel et al. (2012).

By day 19 to day 21, all four HGPS-SKP sphere cultures contained spheres that were morphologically similar to the ones that formed in the control cultures. Spheres of the mesenchymal control BMOI.55 showed similar size and shape (Figure 26). SKP cells could be isolated from all cell lines with the newly developed isolation method. Sphere diameter, shape and the initial growth behavior of the sphere cultures were similar in all cell lines.

5.3.3 Proliferation and self-renewal of HGPS-SKP cells

HGPS-SKP spheres that were dissociated on day 18 of the culture and diluted into single cell cultures were able to self-renew and form new spheres from single cells. Small cell clusters formed around day 15 of the cultures and the newly formed SKP2 spheres were detectable around day 30 (Figure 27). The SKP2 spheres were similar in size and morphology compared to the SKP spheres that formed directly after the isolation from fibroblast

cultures. However, SKP2 spheres formed after a longer initial phase than normal SKP cultures.

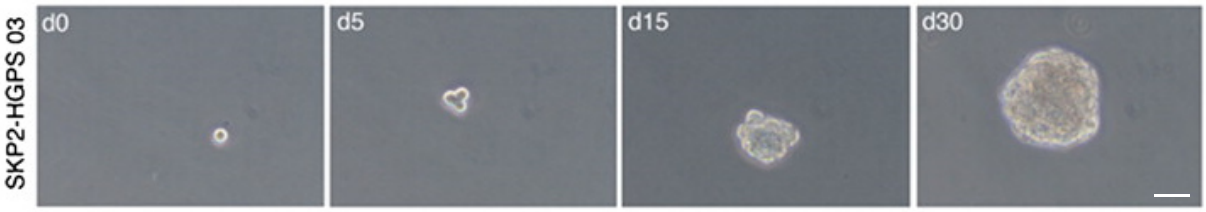

Figure 27: HGPS-SKP cells are able to self-renew and to form new spheres from one single cell. Limiting dilution assays showed that a single cell derived from a HGPS003-SKP sphere at day 18 of culture re-formed a sphere (SKP2-HGPS003). A typical SKP2 sphere appeared after approximately 30 days. Sphere size at day 30 was 50 µm. Scale bar: 20 µm. Adapted with permission from Wenzel et al. (2012).

SKP2 spheres from HGPS-fibroblasts were able to proliferate as shown by Ki67 staining at day 25 to 30 of the culture. Immunofluorescence detection of the stem cell marker nestin showed that SKP2 spheres still express stem cell markers (Figure 28).

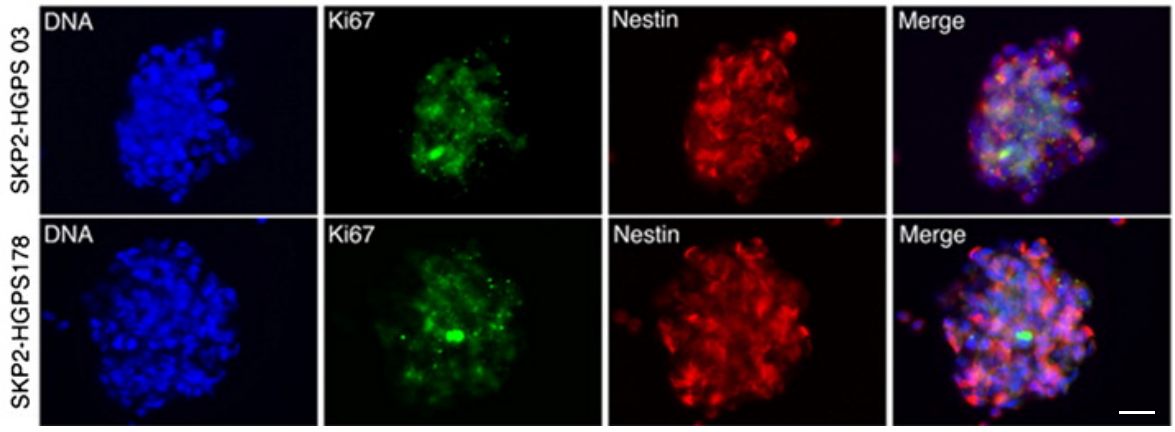

Figure 28: HGPS-SKP2 cells are able to proliferate and express the stem cell marker nestin equal to SKP cells from healthy skin. SKP2 spheres derived from HGPS-SKP cells at day 25 to 30 of the culture. The spheres were double stained with anti-Ki67 and anti-nestin antibodies. Nuclei are stained blue with DAPI. Whole spheres were stained as described in material and methods. Scale bar: 20 mm. Adapted with permission from Wenzel et al. (2012).

5.3.4 Stem cell marker expression in HGPS-SKP cells

HGPS-SKP cells and control SKP cells were stained for the stem cell markers nestin, Oct4, Sox2, Nanog, TG30 and for fibronectin. HGPS-SKP cells between day 18 and day 21 of culture expressed all tested stem cell markers with a similar intensity as the control SKP cells (Figure 29).

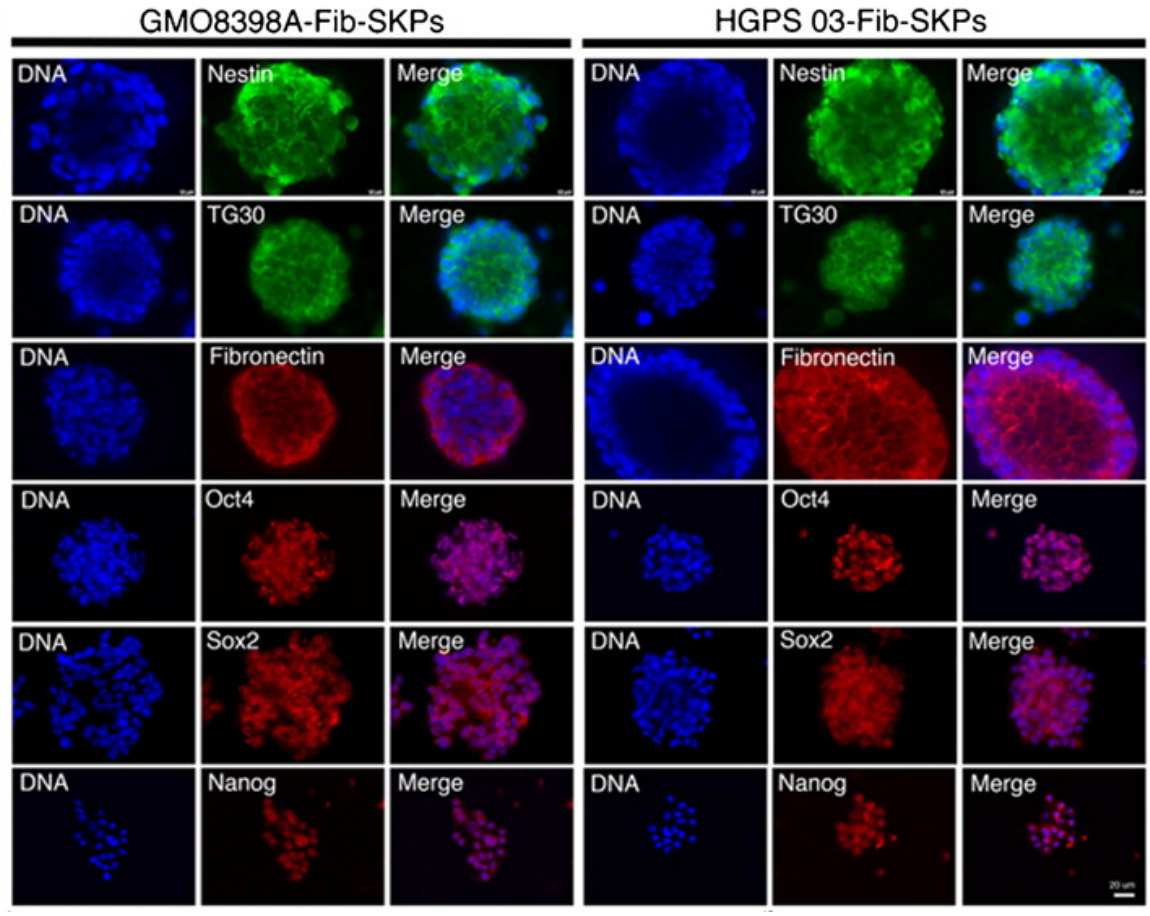

Figure 29: SKP spheres derived from HGPS-fibroblasts express stem cell markers comparable to control SKP spheres. SKP-spheres derived from control fibroblasts (left) and HGPS-fibroblasts (right) on day 18 to 21 of culture were immunostained for the stem cell markers nestin, TG30, Oct4, Sox2, Nanog and fibronectin (n=3; similar stainings were obtained in SKP-spheres derived from the other fibroblast strains). Whole spheres were stained as described in material and methods. Adapted with permission from Wenzel et al. (2012).

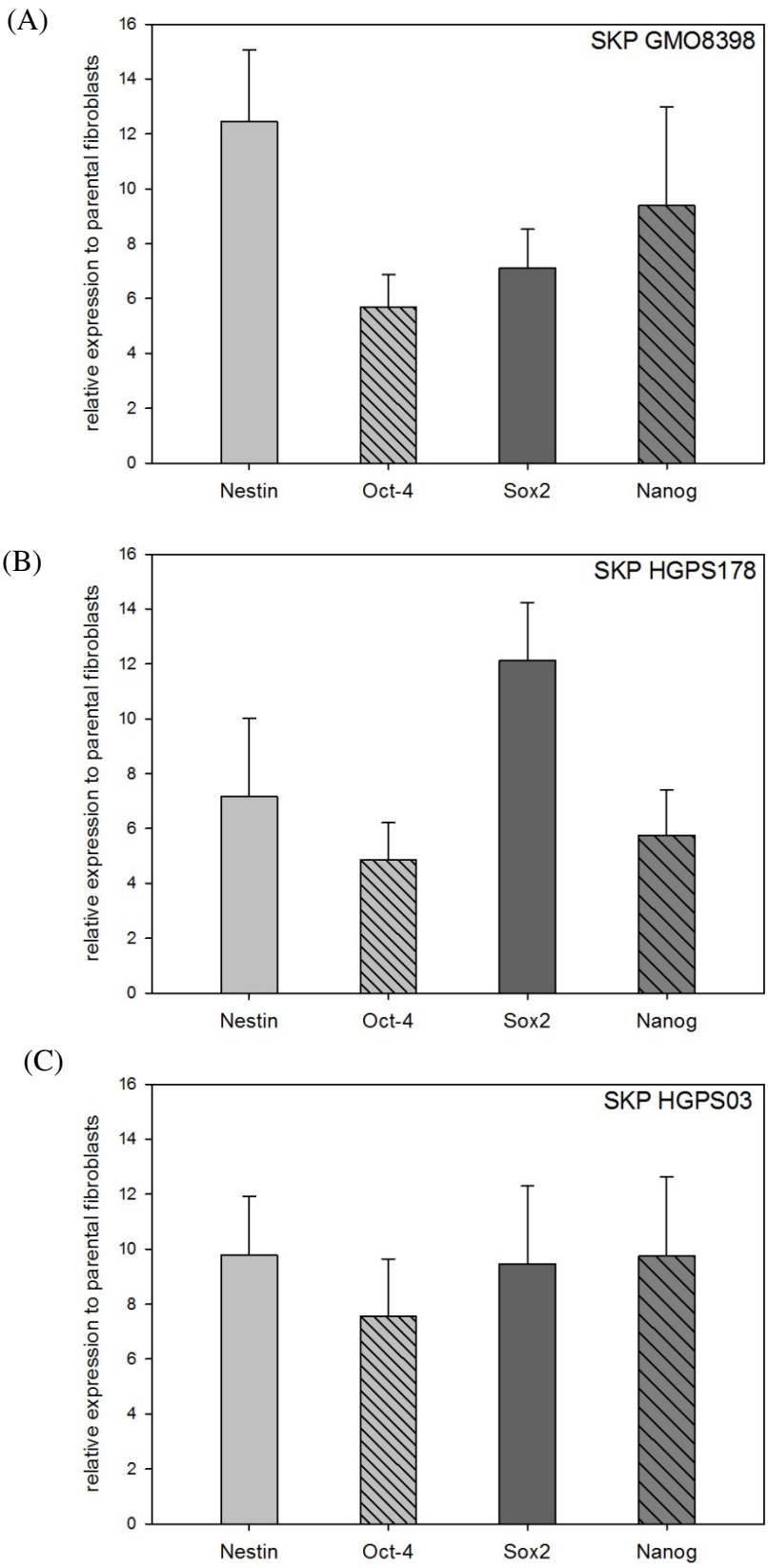

Figure 30: mRNA levels of stem cell markers are comparable in SKP cells derived from HGPS-fibroblasts and SKP cells from control fibroblasts. Real time PCR analysis of the stem cell markers nestin, Oct4, Sox2 and Nanog of HGPS-SKP cells and normal SKP cells relative to the parental fibroblasts. The following cell strains were used: GMO8398 (A),

HGPS178 (B) and HGPS03 (C). Cultures from day 21 to 24 were used for mRNA isolation. ΔΔCt values were calculated for analysis. n=4. Adapted with permission from Wenzel et al. (2012).

Further analysis with real-time PCR showed that mRNA levels of SKP cells derived from control fibroblast cultures (GMO8398) and HGPS-SKP cells (HGPS178, HGPS03) were comparable and did not show significant differences in the expression of the stem cell markers nestin, Oct4, Sox2 or Nanog (compare Figure 30).

In general, HGPS-SKP cells did show the same growth behavior within 'early' stage cultures as SKP cells derived from control fibroblasts. HGPS-SKP cells were able to proliferate and to self-renew and did express all stem cell markers at a comparable levels concerning mRNA and protein in relation to SKP cells derived from healthy young skin.

5.4 Lamin status in SKP cells

The lamin status of SKP cells derived from healthy skin at young and old age and of SKP cells derived from HGPS patients was analyzed in order to examine if the lamin status correlated with the stem cell function of adult stem cells. In particular, the expression levels of different A-type lamins were investigated.

5.4.1 Lamin status in SKP cells derived from young and old skin at different culture stages

Since some lamin forms are known to have an influence on cellular aging in premature aging diseases, it was investigated whether the lamin status changed during the aging of SKP cells derived from healthy individuals.

To analyze the A-type lamin protein status in SKP cells derived from young and old skin, immunohistochemistry and western blots were performed. mRNA levels of A-type lamins were analyzed by real-time PCR.

5.4.1.1 Immunofluorescence detection of A-type lamins in SKP cells

Immunofluorescence detection of lamin A/C, lamin A and lamin C of SKP cells derived from young skin showed the expression of all A-type lamin forms from 'early' to 'late' stage in vitro (Figure 31). No obvious expression changes during the culture period could be observed at the protein level by immunohistochemistry.

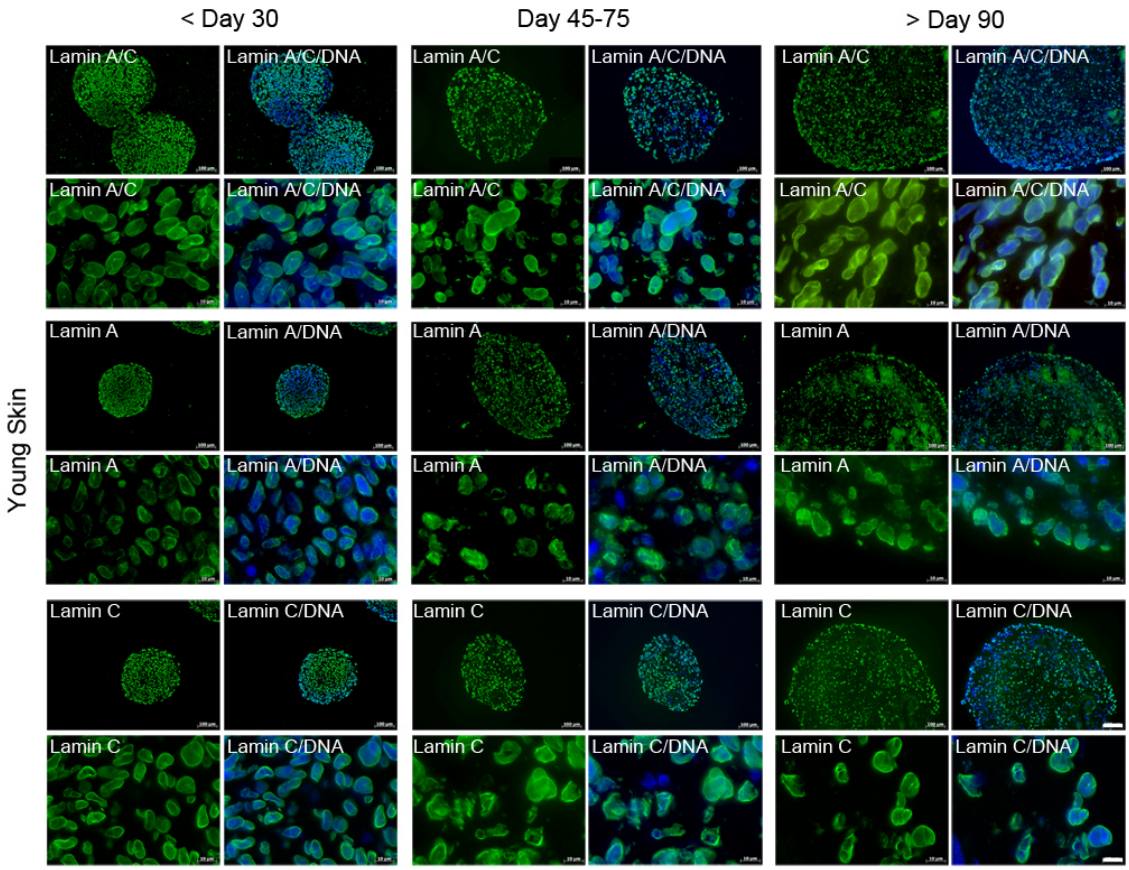

Figure 31: A-type lamin proteins are expressed in SKP cells derived from young skin starting from 'early' stage and during long-term cultures on a comparable level. Immunofluorescence staining of SKP cells derived from young skin (0-12 years). 10 µm cryosections of SKP cells were fixed with methanol and stained for lamin A/C, specific lamin A and specific lamin C. Spheres were collected from culture at <30 days ('early'), 45 to 75 days ('intermediate') or >90 days ('late'). Single stainings and the merged images of DAPI and specific staining are shown. Stainings representing at least three different experiments. Different panels: upper rows show overview, scale bar: 100 µm; lower rows show close-up, scale bar: 10 µm.

Immunofluorescence staining of SKP cells derived from old skin showed a positive signal for all A-type lamins during the entire period of cultures. A stronger signal was observed in SKP cells from 'late' stage for lamin A/C, lamin A and lamin C (Figure 32). In particular, the stronger signal could be observed in the nuclei at high magnification (Figure 32, close-up).

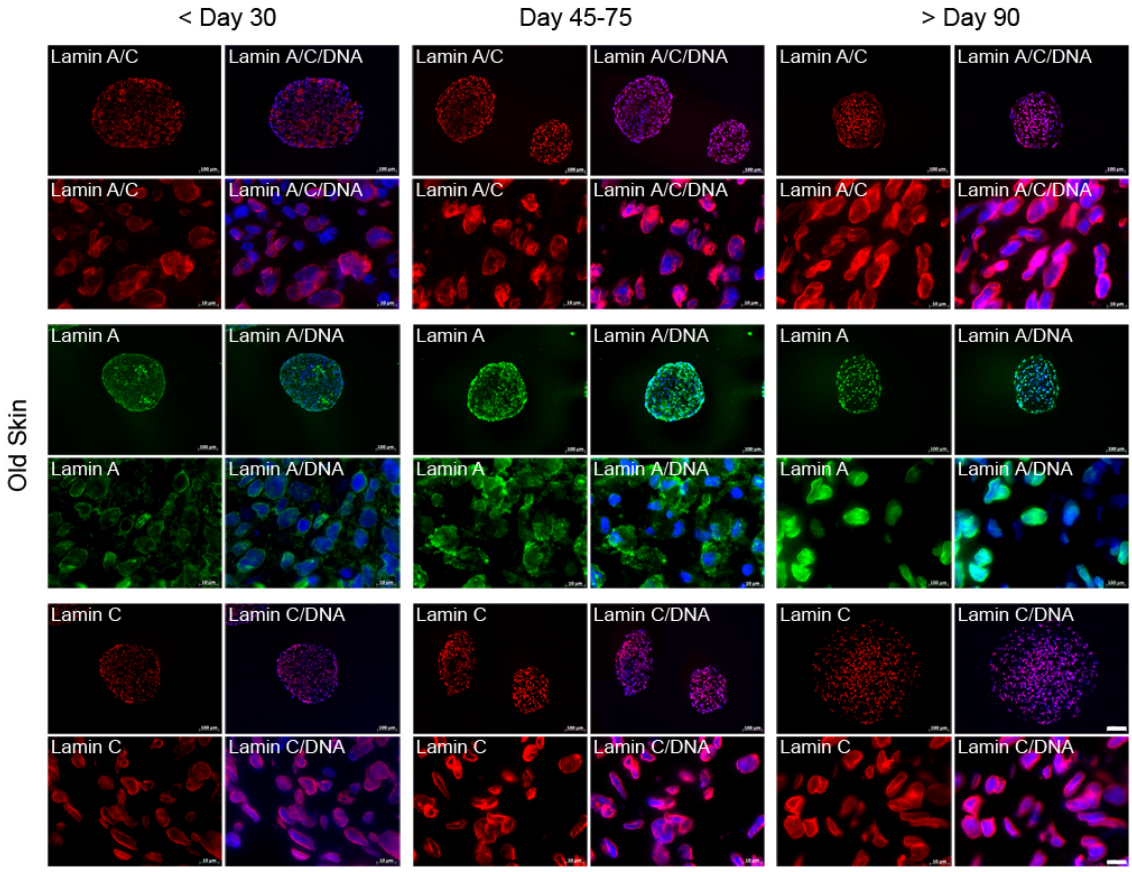

Figure 32: A-type lamin protein levels increase in SKP cells derived from old skin during long-term cultures. Immunofluorescence staining of SKP cells derived from old skin (50-94 years). 10 µm cryosections of SKP cells were fixed with methanol and stained for lamin A/C, specific lamin A and specific lamin C. Spheres were collected from culture at <30 days ('early'), 45 to 75 days ('intermediate') or >90 days ('late'). Single staining and the merged images of DAPI and specific staining are shown. Stainings represent at least three different experiments. Different panels: upper rows show overview, scale bar: 100 µm; lower rows show close-up, scale bar: 10 µm.

5.4.1.2 Western blot analysis of A-type lamins in SKP cells

To further quantify the levels of lamin proteins in SKP cells derived from young and old skin western blot analyses of different cultures were performed.

Western blot analyses of SKP cells derived from young skin showed increasing levels of A-type lamins concomitantly with increasing period of culture (Figure 33). However, the lamin A and lamin C levels of the fully differentiated control fibroblasts were still higher than the levels of SKP cells derived from young skin at 'late' culture stage.

Lamin C protein levels did increase significantly by factor 3 from 'early' to 'intermediate' stage (p=0.04) but were constant from 'intermediate' to 'late' stage. In addition, no further increase of lamin C was observable after culture times of more than 200 days (Figure 33).

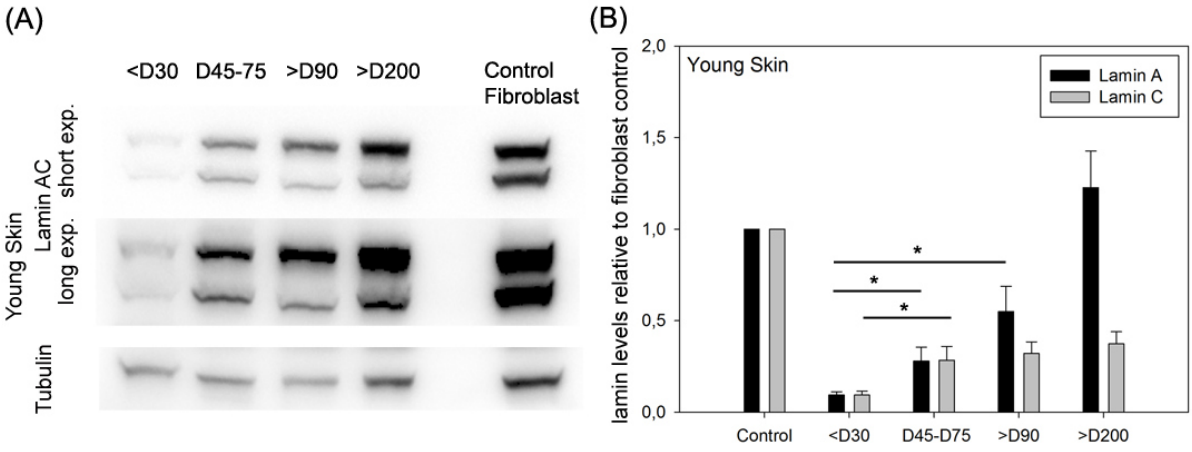

Figure 33: Lamin A protein levels in SKP cells derived from young skin increase significantly during long-term cultures. (A) Western blot analysis detecting lamin A and C in total protein extracts of SKP cells derived from young skin (0-12 years). Spheres from culture points at <30 days ('early'), 45 to 75 days ('intermediate'), >90 days ('late') and >200 days were used. 30 µg of full protein extract were loaded on the gels. Tubulin was used as housekeeping gene and loading control. Lamin bands at short exposure times, which were used for quantification and at longer exposure times are shown. (B) The volumes of the lamin bands were normalized according to each tubulin band and relative values to control fibroblasts were calculated. Quantification was performed using the software Image Lab. The graph shows the x-fold levels of lamin A and C relative to the lamin levels of the fibroblast control for n=5 experiments (>200 days: n=2). Data shown as means ±SEM. P values of $p < 0.05$ were defined as statistically significant, $p < 0.01$ as highly significant.

In contrast, lamin A protein levels increased from stage to stage. A 3-fold increase was observed from ‘early’ to ‘intermediate’ stage and an additional 2-fold increase from ‘intermediate’ to ‘late’ stage. Both increases were statistically significant when compared to the ‘early’ stage levels ($p=0.04$, $p=0.01$). The average lamin A protein expression level of two analyzed samples of SKP cells derived from young skin at more than 200 days of culture increased by factor 13 compared to ‘early’ stage and was even comparable to the control fibroblast level (Figure 33).

Due to the densely connected spheres at ‘intermediate’ and ‘late’ stage the protein isolation from SKP cells derived from old was difficult and yielded very low protein amounts at the end of the extraction. Only ‘early’ and ‘late’ stage samples of SKP cells derived from old skin were analyzed (Figure 34). At the same time, the analysis of western blots of SKP cells derived from old skin was technically challenging due to the difficult resolution of lamin bands on SDS PAGE and diffuse bands, which made the protein detection with anti-lamin A/C antibodies and the quantification problematic.

Lamin C protein levels in SKP cells derived from old skin increased slightly from ‘early’ to ‘late’ stage as similarly observed in SKP cells derived from young skin (Figure 34). However, considerably higher lamin C levels could be observed in SKP cells derived from old skin compared to SKP cell derived from young skin.

Lamin A protein levels of SKP cells derived from old skin increased significantly from ‘early’ to ‘late’ culture stage by factor 7.4 ($p=0.036$). At ‘late’ culture stage the levels were already comparable or even higher than the levels of control fibroblasts (Figure 34). This strong increase of lamin A protein levels in SKP cells derived from old skin was similar to the observed increase in SKP cells derived from young skin (5.8-fold average increase) from ‘early’ to ‘late’ stage.

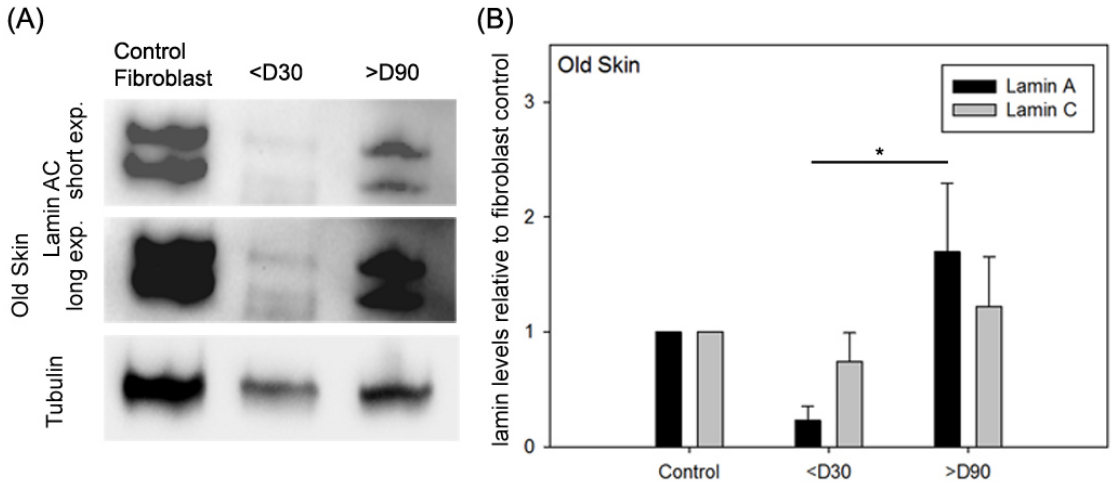

Figure 34: Lamin A protein levels in SKP cells derived from old skin increase significantly during long-term cultures. (A) Western blot detecting lamin A and C in total protein extracts of SKP cells from old skin (50-94 years). Spheres from culture points at <30 days ('early') and >90 days ('late') were used due to difficult protein extractions and limited protein yield. 30 µg of full protein extract were loaded on the gels. Due to limited protein yield from SKP cells derived from old skin only 'early' and 'late' stage cultures were used. Tubulin was used as housekeeping gene and loading control. Lamin bands at normal exposure times, which were used for quantification and at longer exposure times are shown. (B) The volumes of the lamin bands were normalized to according tubulin bands and relative values to control fibroblasts were calculated. Quantification was performed using the software Image Lab. The graph shows the x-fold levels of lamin A and C relative to the lamin levels of the fibroblast control for n=4 experiments. Data shown as means ±SEM. P values of $p<0.05$ were defined as statistically significant, $p<0.01$ as highly significant.

5.4.1.3 Real-time PCR analysis of A-type lamins in SKP cells

Real-time PCR analysis was performed to quantify the expression of the A-type lamin forms at mRNA level. Similarly to the western blot results, it could been shown that the mRNA levels of lamin A/C increased significantly from 'early' to 'late' stage in SKP cells derived from young skin ($p=0.03$). In SKP cells derived from old skin the total lamin A/C mRNA levels increased slightly in average but not significantly due to high variations (Figure 35A). RNA quality and recovery from preparations of SKP cells derived from old skin at 'late' stage was not sufficient and did not allow any analysis.

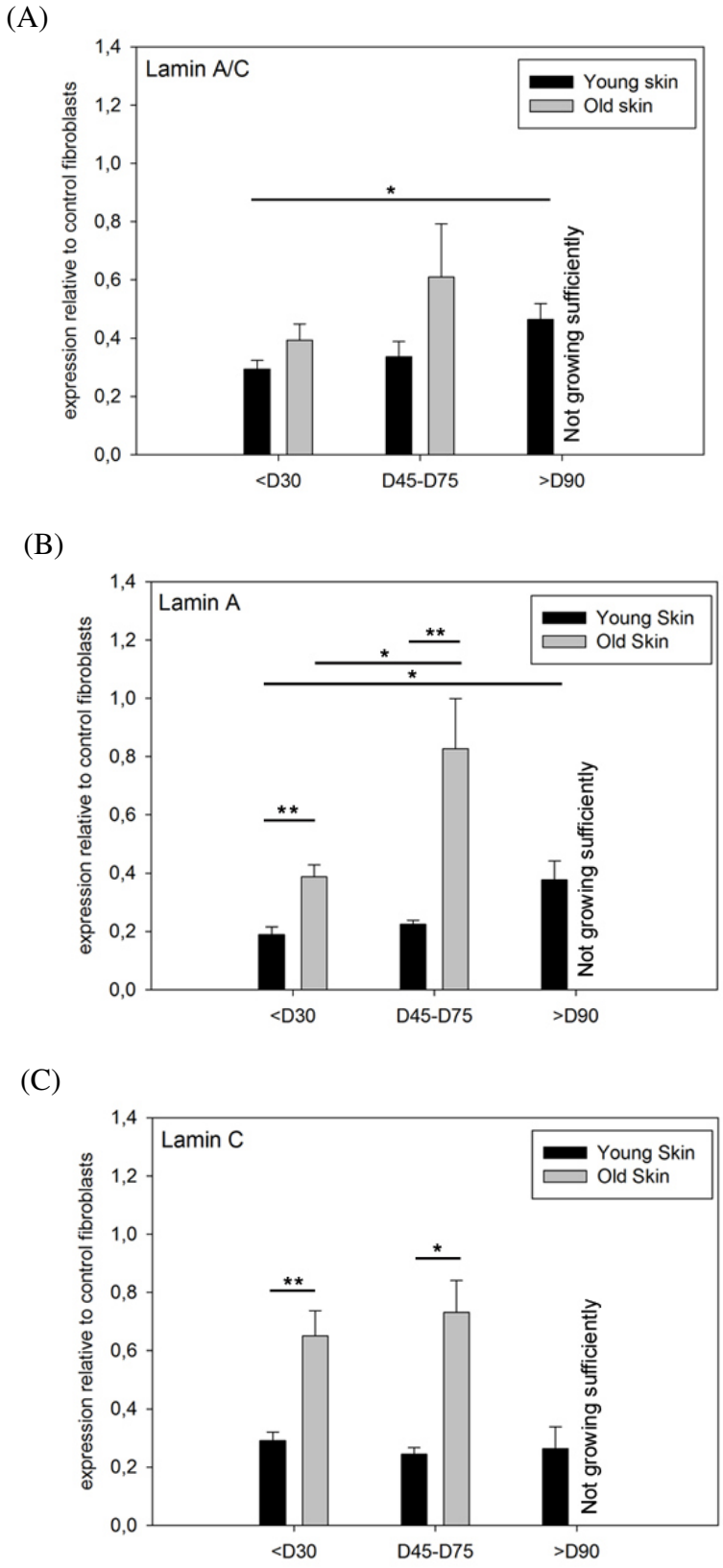

Figure 35: Lamin A mRNA levels increase during long-term cultures in all SKP cells and are in general higher in SKP cells derived from old skin. Lamin status of SKP cells during long-term cultures of SKP cells derived from young (0-12 years) and old skin (50-94 years). (A) Lamin A/C mRNA levels of SKP cells derived from young and old skin relative

to control fibroblasts levels. Oligos amplifying all isoforms and splice variants of the *LMNA* gene were used. SKP cell cultures before day 30 ('early'); between day 45 and day 75 ('intermediate') and after day 90 ('late') were used. SKP cells that derived from old skin did not provide enough material to isolate mRNA of good quality at 'late' culture stages. n≥ 5, data shown as means ±SEM. P values of $p < 0.05$ were defined as statistically significant, $p < 0.01$ as highly significant. (B) Lamin A mRNA levels of SKP cells derived from young and old skin relative to control fibroblasts levels. Oligos amplifying only the lamin A splice variants including progerin were used. Conditions as in (A), n≥3. (C) Lamin C mRNA levels of SKP cells derived from young and old skin relative to control fibroblasts levels. Oligos amplifying only the lamin C splice variant were used. Conditions as in (A), n≥3.

Lamin A mRNA levels were shown to be constant from 'early' to 'intermediate' stage of the culture, but to increase significantly from 'early' to 'late' stage in SKP cells derived from young skin ($p=0.03$). SKP cells derived from old skin showed a 2-fold increase of the lamin A mRNA levels from 'early' to 'intermediate' stage, which was statistically significant ($p=0.027$) (Figure 35B). The mRNA levels of lamin A were more than 2-fold higher in SKP cells derived from old skin compared to SKP cells derived from young skin at 'early' and 'intermediate' culture stage. This increase was shown to be significant ($p=0.004$; $p=0.005$).

Lamin C mRNA levels were shown to remain constant from 'early' to 'late' culture stages of SKP cells derived from young skin. Similarly, the mRNA levels of lamin C in SKP cells derived from old skin remained constant over the culture period (Figure 35C). However, lamin C levels in SKP cells derived from old skin were significantly higher than the levels in SKP cells derived from young skin at 'early' ($p=0.006$) and 'intermediate' stage ($p=0.019$) of the culture.

In conclusion it was shown that A-type lamin levels increased during long-term cultures of SKP cells. This could be observed at the mRNA and at the protein level in both, SKP cells derived from young and from old skin. However, lamin A levels increased strongly over the whole culture period while lamin C levels remained on a constant level after an initial increase. In general, SKP cells derived from old skin showed higher A-type lamin levels compared to SKP cells derived from young skin.

5.4.2 Lamin status and progerin expression in HGPS-SKP cells

The involvement of the abnormal A-type lamin progerin in the premature aging disease HGPS is known and the accumulation of progerin has been shown in various differentiated HGPS-cells. The newly developed isolation method allows to screen SKP cells from HGPS patients concerning their lamin status. The lamin status and the expression of abnormal lamins such as progerin was analyzed in SKP cells from HGPS disease states in comparison to SKP cells derived from healthy skin to determine abnormalities in the lamin status and to investigate if progerin was expressed in adult stem cells.

5.4.2.1 Immunofluorescence detection of A-type lamins in HGPS-SKP cells

Immunofluorescence detection was used to determine the lamin status and to investigate whether progerin was expressed in naïve HGPS-SKP cells. The detection of A-type lamins in HGPS-SKP cells showed that lamin A/C and, in particular, lamin A had a similar signal intensity in HGPS-SKP cell compared to SKP cells derived from control fibroblasts (Figure 36).

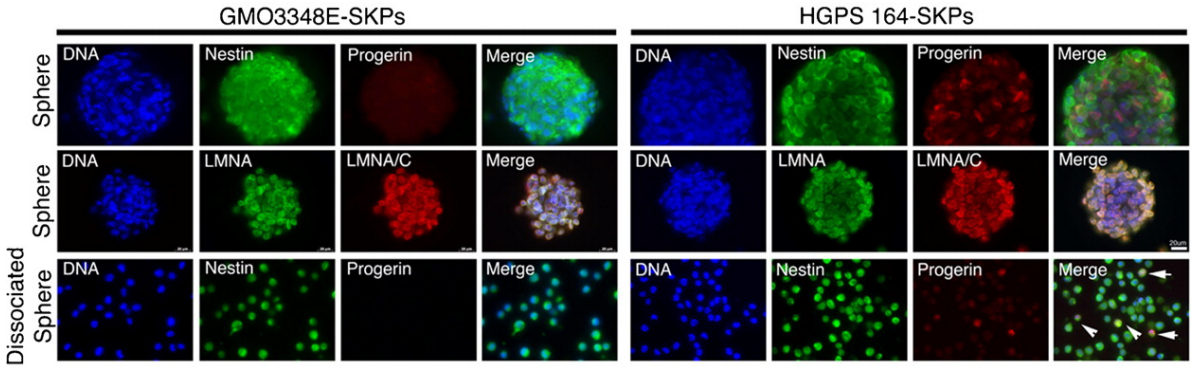

Figure 36: Progerin is expressed in HGPS-SKP cells in vitro. HGPS-SKP cells (HGPS164) and control SKP cells (GMO3348E) were stained for progerin and nestin or lamin A and lamin A/C. Nuclei were counterstained with DAPI. Whole spheres were stained as described in the material and methods section. Lower panel: immunofluorescence analysis of dissociated SKP spheres derived from a control fibroblast strain (GMO3348E) and a HGPS fibroblast strain (HGPS164) at day 18 of the culture. Anti-progerin and anti-nestin staining as indicated. Arrows in the merge image indicate progerin and nestin double-positive cells. Scale bar: 20 µm. n=4. Adapted with permission from Wenzel et al. (2012).

Progerin expression could be detected in HGPS-SKP cells in varying levels but not in SKP cells derived from control fibroblasts. Nevertheless, the HGPS-SKP cells showed stem cell properties as indicated by the presence of a positive signal for the stem cell marker nestin. Progerin was detected in single cells from dissociated spheres at an average of 14-26 % of the HGPS-SKP cells (Figure 36, lower panel).

5.4.2.2 Western blot analysis of A-type lamins in HGPS-SKP cells

Western blot analysis confirmed the expression of progerin in SKP cells derived from HGPS-fibroblasts. The lamin levels in HGPS-SKP cells were in the average intensity comparable to the lamin signals of the SKP cells derived from control fibroblast cultures (Figure 37).

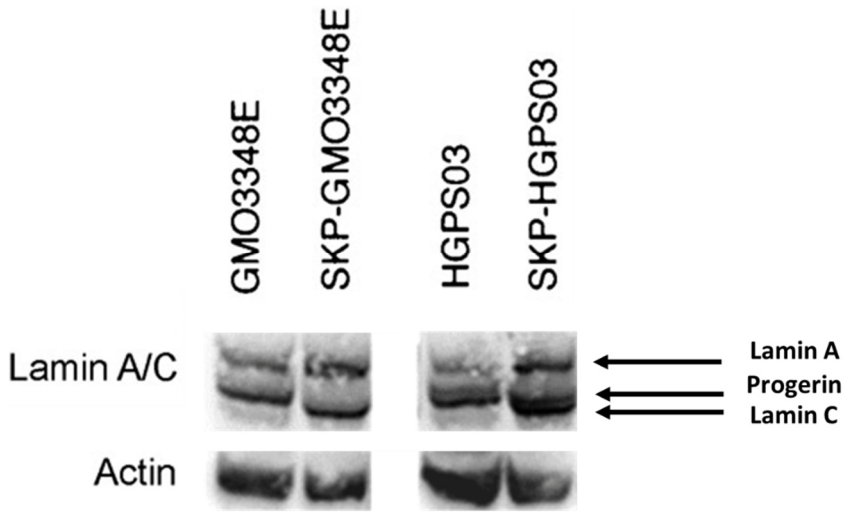

Figure 37: Progerin can be detected in Western blot analysis of HGPS-SKP cells. Western blot analysis detecting lamin A/C and progerin in total protein extracts of HGPS-SKP cells. One healthy control fibroblast strain and the according SKP cells that derived from the control fibroblasts (GMO3348E) and one HGPS-fibroblast strain and the according HGPS-SKP cells that derived from the HGPS-fibroblasts (HGPS03) were used. Spheres from day 21 were collected and used for protein extraction. 30 µg of full protein extract were loaded on the gels. Actin was used as housekeeping gene and loading control. Adapted with permission from Wenzel et al. (2012).

5.4.2.3 Real-time PCR analysis of A-type lamins in HGPS-SKP cells

Real-time PCR analysis confirmed that the levels of mRNA encoding all lamin A/C forms in HGPS-SKP cells were similar to the levels of SKP cells derived from control fibroblasts (Figure 38). SKP cells derived from two different control fibroblast strains (GMO8398, GMO3348) and HGPS-SKP cells derived from two different HGPS-fibroblast strains (HGPS178, HGPS03) were tested.

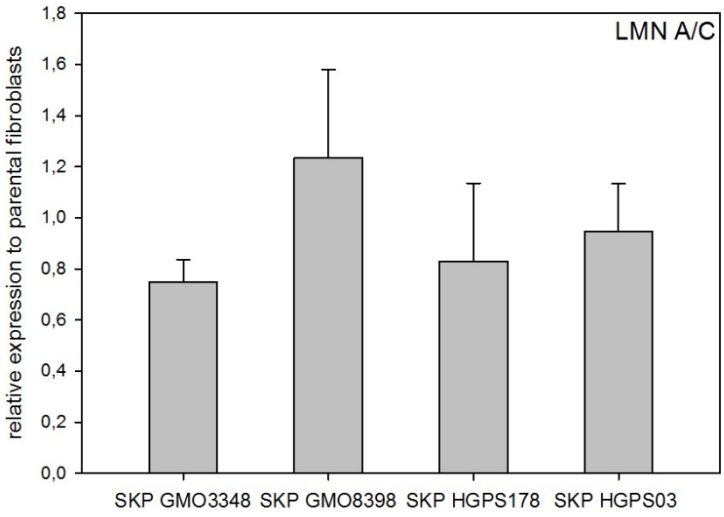

Figure 38: HGPS-SKP cells and control SKP cells show comparable mRNA levels of lamins. Relative expression of mRNA levels for *LMNA* gene transcripts in SKP cells compared to the according parental fibroblast levels. Oligos amplifying all isoforms and splice variants of the *LMNA* gene were used. Two SKP cell cultures derived from control fibroblasts (GMO3348E, GMO8398) and two HGPS-SKP cultures derived from HGPS-fibroblasts (HGPS178, HGPS03) were tested. Spheres were collected at day 21 of the culture for mRNA isolation. n=4, data shown as means ±SEM. Adapted with permission from Wenzel et al. (2012).

5.4.2.4 Semi-quantitative PCR analysis of A-type lamins in HGPS-SKP cells

Since it was not possible to perform real-time PCR analysis for progerin without amplifying non-specific products, a semi-quantitative PCR was performed to detect mRNA of progerin in HGPS-SKP cells (Figure 39).

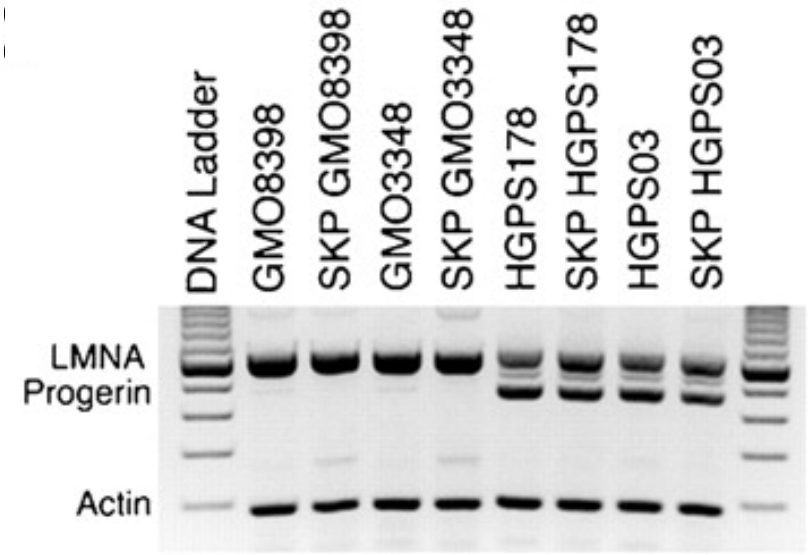

Figure 39: mRNA encoding for progerin can be detected in HGPS-SKP cells. Semi-quantitative PCR analysis of lamin A and progerin transcripts in fibroblasts and in SKP cells derived from according fibroblasts as indicated. Oligos for *LMNA* transcripts and actin were amplified in the same tube and separated on a 2 % agarose gel. The progerin amplificate with the deletion can be detected 150 bp under the amplificate of lamin A. n=3. Adapted with permission from Wenzel et al. (2012).

mRNA encoding progerin, which is shorter than lamin A mRNA due to the 150 bp deletion caused by alternative splicing, was equally expressed in all HGPS-SKP cells. No progerin mRNA was detected in normal SKP cells under these experimental conditions. The band for lamin A mRNA was slightly decreased in HGPS-SKP cells compared to SKP cells derived from control fibroblasts. This decrease occurred in HGPS cells due to the presence of the truncated mRNA species which encodes progerin as a result of the alternative splicing, activated by the *LMNA* mutation.

Taken together the analyses of the A-type lamin levels in HGPS-SKP cells indicated that progerin is expressed at the mRNA and protein levels in SKP cells derived from HGPS-fibroblast cultures. Lamin A mRNA levels were decreased in HGPS-SKP cells while immunofluorescence and western blot experiments showed that the lamin A/C protein levels remained comparable to SKP cells derived from healthy fibroblast cultures.

5.5 Immunofluorescence detection of progerin in SKP cells of HGPS-skin sections

To confirm the in vitro results showing that HGPS-SKP cells express progerin, sections of skin from a HGPS patient (HGADFN143) and normal healthy foreskin sections were screened for nestin positive cells and for progerin by immunofluorescence staining. Nestin was expressed throughout the dermis and sometimes in linear patches (Figure 40). No progerin positive cells could be observed in normal healthy foreskin sections. Progerin was detected in the dermis and in the upper layer of the epidermis in the sections of the HGPS patient. In addition, few nestin positive cells could be detected in the dermis of the HGPS section. Further examination showed that some of these cells were double positive, expressing nestin and low levels of progerin. This shows that SKP cells from HGPS patients have the same phenotype in vivo as in vitro and that a subset of the SKP cells already expresses progerin in their naïve state (Figure 40, close-up).

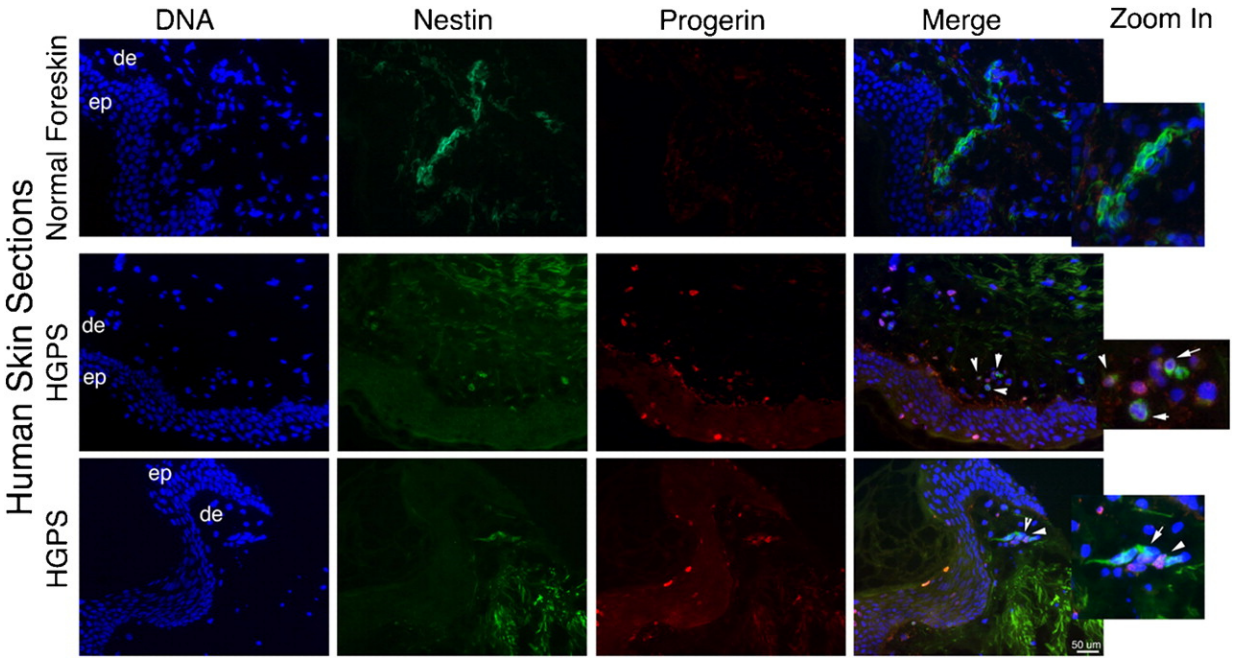

Figure 40: Progerin is expressed in naïve SKP cells in HGPS skin sections. In situ localization of progerin and nestin on healthy human foreskin sections and on skin sections derived from a subject with HGPS (HGADFN143). Nuclei were stained with DAPI (DNA). Morphologic entities are indicated: epidermis (ep), dermis (de). Arrows indicate progerin- and nestin-positive cells on merged images. Scale bar: 50 µm. Zoom in by a factor of 4. Adapted with permission from Wenzel et al. (2012).

5.6 Differentiation of SKP cells

The differentiation potential of SKP cells was analyzed to confirm aging related changes in the stem cell potential. SKP cells derived from young and old skin and SKP cells derived from HGPS disease states were differentiated into fibroblasts and smooth muscle cells.

5.6.1 Differentiation of control SKP cells and HGPS-SKP cells into fibroblasts

SKP cells derived from young controls and HGPS-SKP cells were differentiated into fibroblasts. After four passages of differentiation the cells showed a positive immunofluorescence signal for the fibroblast marker P4HD (Figure 41).

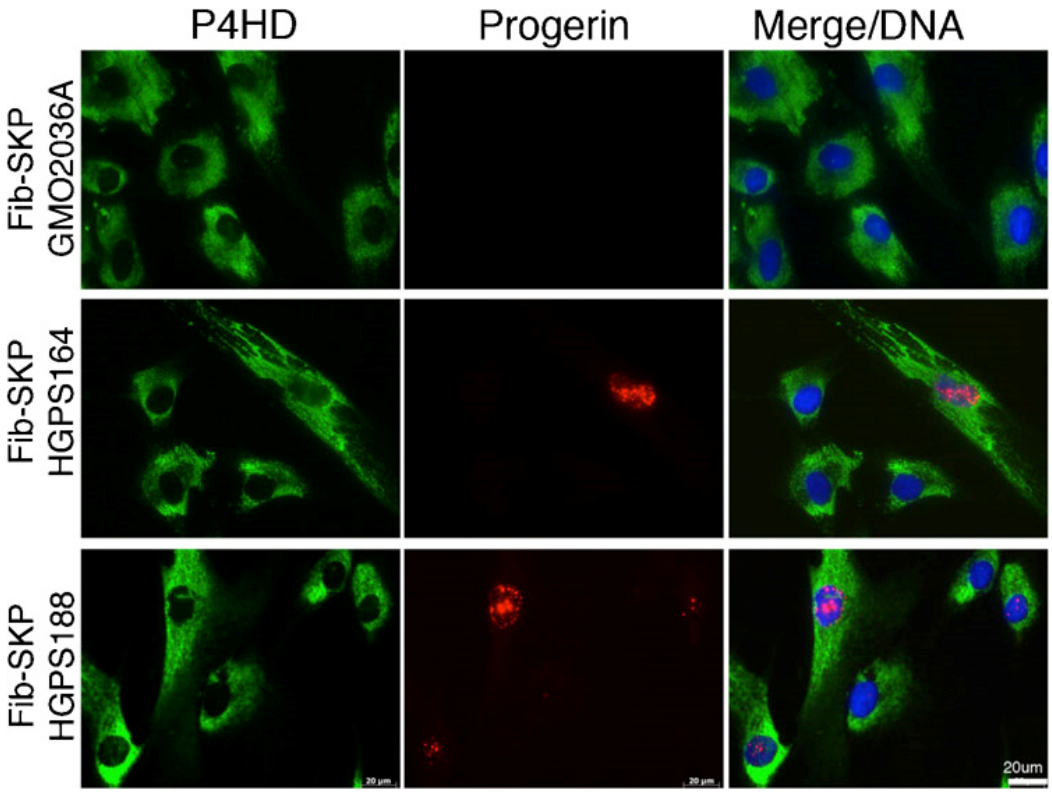

Figure 41: HGPS-SKP cells can differentiate into fibroblasts similar to control SKP cells. Directed differentiation of normal SKP cells and HGPS-SKP-cells into fibroblasts. Differentiation was started at 'early' stage of the SKP sphere cultures. Immunofluorescence analysis of the fibroblast-specific marker P4HD together with progerin after passage 4 of the differentiation. Scale bars: 20 µm. Adapted with permission from Wenzel et al. (2012).

Similar to control SKP cells, HGPS-SKP cells were able to differentiate into fibroblasts. However, progerin could be detected in the nuclei of the fibroblasts at early passages.

5.6.2 Differentiation of SKP cells derived from young and old skin at different culture stages into smooth muscle cells

SKP cells derived from young and from old skin were differentiated into smooth muscle cells at different culture stages. The morphology change of the cells and the expression of specific smooth muscle markers was investigated.

5.6.2.1 Morphology of smooth muscle cell differentiations

SKP cells derived from young skin were differentiated into smooth muscle cells to test their differentiation potential. The differentiation was started with SKP cells at 'early' culture stage, which were differentiated for three weeks (Figure 42).

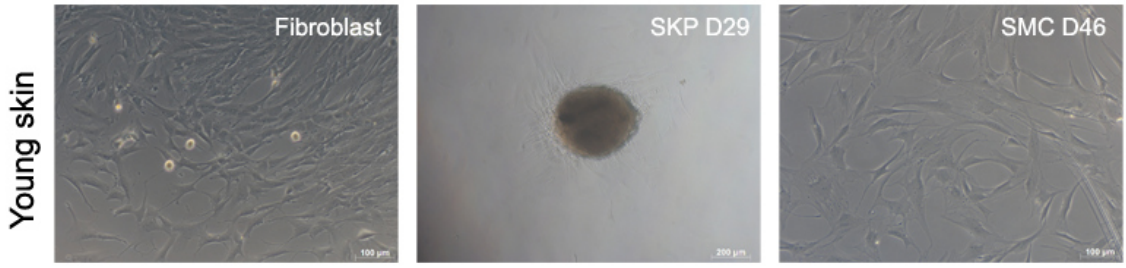

Figure 42: Morphology of fibroblast cultures, SKP spheres and smooth muscle cells. SKP cells were isolated from fibroblast cultures of young skin and were differentiated into smooth muscle cells (SMCs). Phase contrast images show the different culture steps: the initial fibroblast culture, the 'early' stage SKP sphere which was used for SMC differentiation and day 46 of the SMC differentiation with differentiated smooth muscle cells. Scale bar: as indicated.

A change in morphology was observed in phase contrast microscopy, indicating the differentiation (Figure 43). The cells flattened and enlarged during the differentiation process.

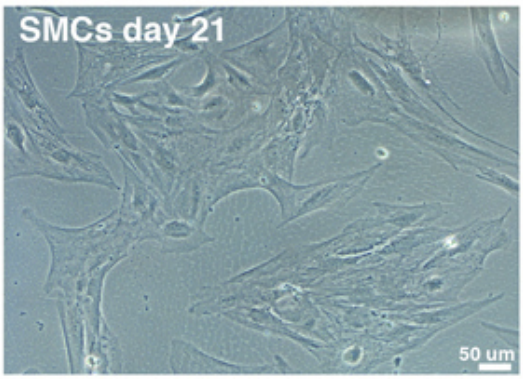

Figure 43: SKP cells derived from young skin can differentiate into smooth muscle cells and show a distinct morphology. SKP-spheres derived from young skin (0-12 years) were differentiated into smooth muscle cells (SMCs). SKP cells were collected for differentiation at 'early' culture stage (<30 days). Typical morphology of smooth muscle cells could be observed after 21 days of differentiation by phase contrast microscopy. Scale bar: 50 µm. Adapted with permission from Wenzel et al. (2013).

5.6.2.2 Immunofluorescence analysis of smooth muscle cell differentiations

Immunofluorescence analysis of the differentiated SKP cells derived from young skin was performed after three weeks of differentiation.

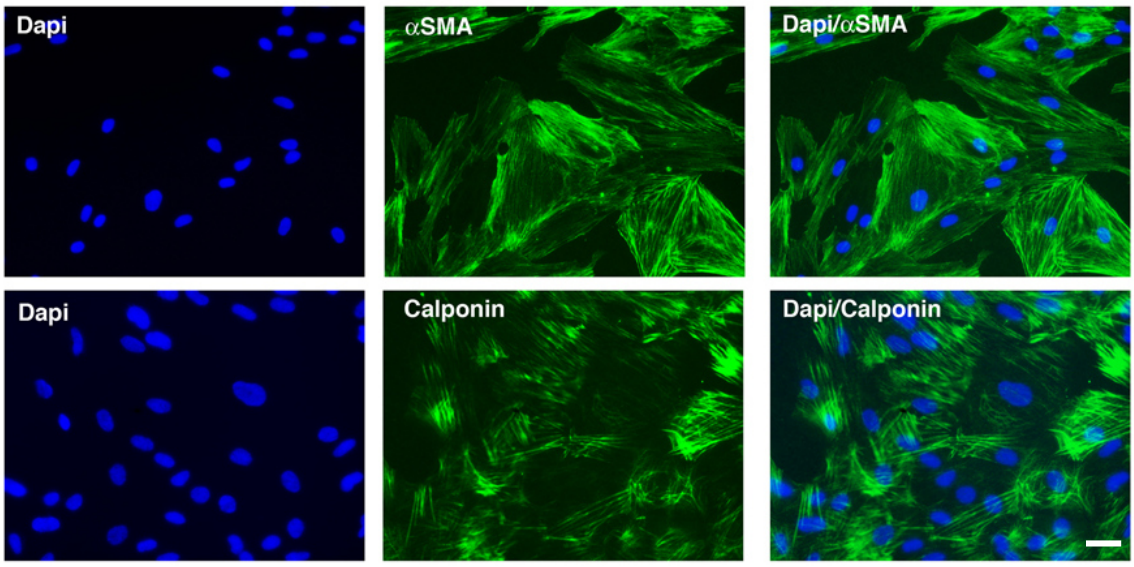

Figure 44: SKP cells derived from young skin differentiate into smooth muscle cells and express according protein markers. Smooth muscle cells were obtained after differentia-

tion of SKP cells derived from young skin (0-12 years). SKP cells were collected for differentiation at 'early' stage of the cultures. After three weeks of differentiation the SMCs were stained for the smooth muscle markers smooth muscle actin and calponin as well as for DAPI (DNA). Scale bar: 50 µm. Adapted with permission from Wenzel et al. (2013).

The differentiated cells expressed the smooth muscle cell markers smooth muscle actin and calponin (Figure 44). An average of 79 % of the differentiated cells were positive for smooth muscle actin (compare 5.6.3.1, Figure 46).

Further experiments were performed to test the differentiation potential of SKP cells from all culture stages and from both age groups. A differentiation into fibroblasts was performed as control for the smooth muscle marker staining (Figure 45).

SKP cells derived from young skin showed a good differentiation efficiency into smooth muscle cells at 'early' culture stage as shown before. A diminished efficiency was already observed at the 'intermediate' culture stage of SKP cells derived from young skin. While the cellular morphology still changed under the differentiation conditions, the detection of smooth muscle cell markers could not be observed in all cells after differentiation. Differentiations of 'late' stage cultures of SKP cells derived from young skin showed a very limited differentiation capacity. Only a small percentage of the initial cells survived and expressed smooth muscle cell markers. In contrast, the fibroblast control differentiations of SKP cells derived from young skin from all culture stages grew fast and showed signs of differentiation (Figure 45, upper panel).

SKP cells derived from old skin showed a very low differentiation efficiency into smooth muscle cells even at 'early' culture stage. Very few growing cells could be observed during differentiation. Nevertheless, immunofluorescence staining revealed that nearly all of the surviving cells did express smooth muscle actin (Figure 45, lower panel). Control differentiation into fibroblasts could be performed with a good efficiency. SKP cells derived from old skin at 'intermediate' stage showed a similar result when differentiated into smooth muscle cells; only very few cells survived during the differentiation process. These cells still showed smooth muscle cell morphology as well as weak smooth muscle actin staining. In contrast the fibroblast differentiation still showed moderate growth (Figure 45, lower panel). No differentiated cells could be found at the 'late' culture stage of SKP cells derived from old skin and very few cells grew at all. The fibroblast differentiation showed very slow growth of some few cells (data not shown).

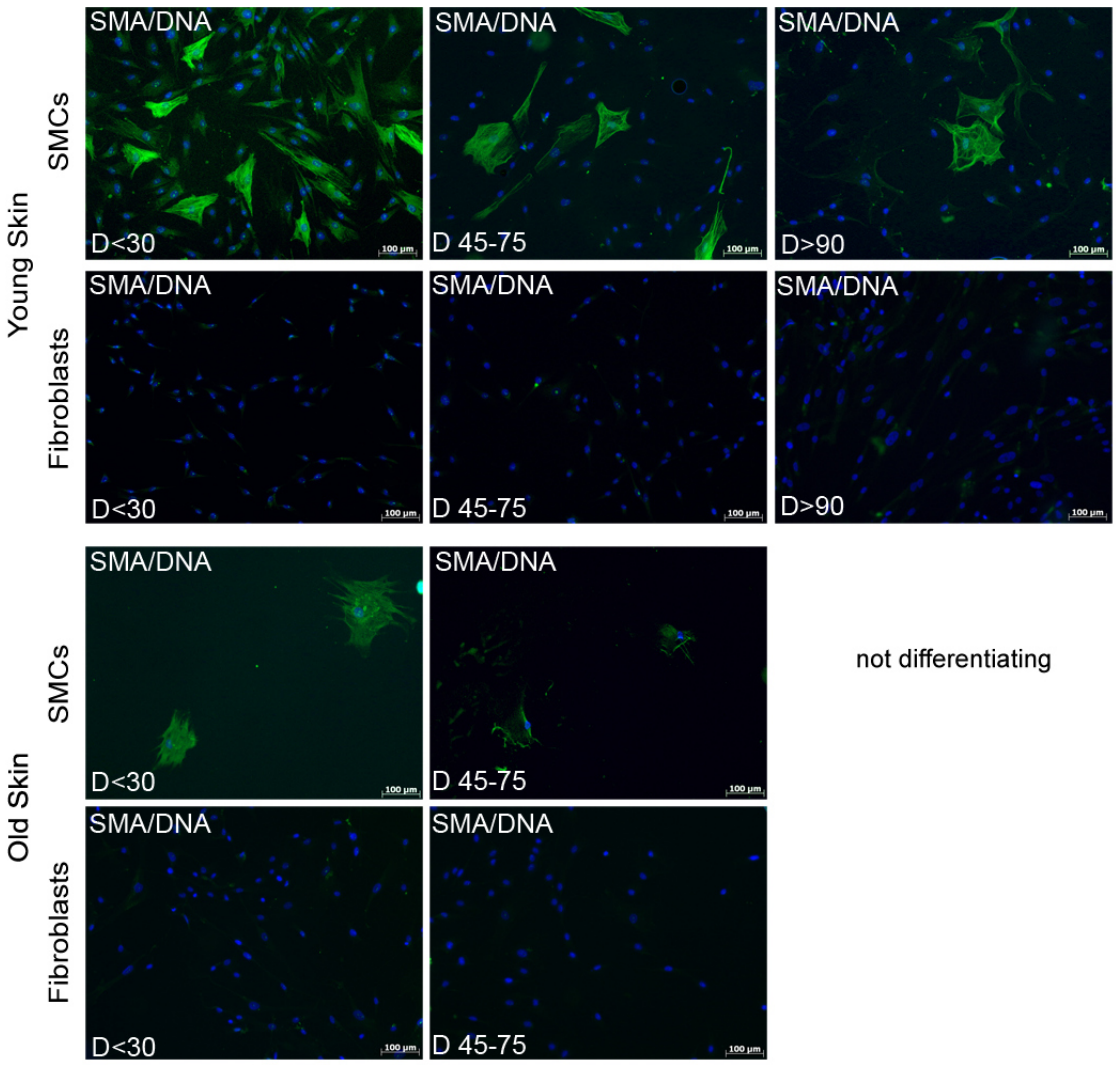

Figure 45: SKP cells derived from young skin differentiate into smooth muscle cells with a high efficiency which decreases dramatically with increasing culture time and in SKP cells derived from old skin. SKP-spheres derived from young skin (0-12 years) and old skin (50-94 years) were differentiated into smooth muscle cells (SMCs). SKP cells derived from young and old skin were collected for differentiation at the culture stages <30 days ('early'), 45 to 75 days ('intermediate') or >90 days ('late'). After 36 days of differentiation, the SMCs were stained for smooth muscle actin and DAPI. Differentiation into fibroblasts was performed and stained for smooth muscle actin as control for both age groups and all culture stages. SKP cells derived from old skin at 'late' culture stages did rarely attach and did not grow, therefore no differentiation could be verified by immunofluorescence staining. Scale bar: 100 µm.

It was shown that SKP cells derived from young skin were highly proliferative at 'early' culture stages and differentiated with a high efficiency but were less susceptible to differentiation at later culture stages. SKP cells derived from old skin exhibited a poor potency to differentiate into smooth muscle cells when subjected to smooth muscle differentiation medium. The few surviving cells that expressed smooth muscle markers might have originated from rare competent SKP cells that survived and possibly reflect the portion of competent stem cells in aged SKP spheres.

5.6.3 Differentiation of HGPS-SKP cells into smooth muscle cells

To further analyze the differentiation potential of HGPS-SKP cells, smooth muscle cell differentiations were performed. Progerin levels were analyzed to find possible connections between progerin accumulation and the differentiation potential.

5.6.3.1 Immunofluorescence analysis of differentiated HGPS-SKP cells

HGPS-SKP cell cultures were differentiated into smooth muscle cells. After three to four weeks under differentiation conditions a change in morphology was observed, indicating the development of flattened and enlarged smooth muscle cells (Figure 46, upper panel).

After two passages, immunofluorescence detection of smooth muscle actin and progerin was performed (Figure 46, lower panel). All tested HGPS-SKP cell cultures did differentiate into smooth muscle cells and showed similar differentiation efficiencies as SKP cells derived from healthy control fibroblasts. A percentage of 82 % and 75 % of SMA-positive cells were detected in HGPS-SKP differentiations versus 79 % SMA-positive cells in the control differentiation. While the differentiated control SKP cells did not show any progerin expression, 38 % and 39 % of the differentiated HGPS-SKPs expressed progerin. Most of the progerin positive cells did as well express smooth muscle actin (31 % and 32 %) indicating the portion of progerin positive HGPS-SKP cells that were able to differentiate. However, the cells that showed a strong progerin signal appeared to be negative for smooth muscle actin (Figure 46).

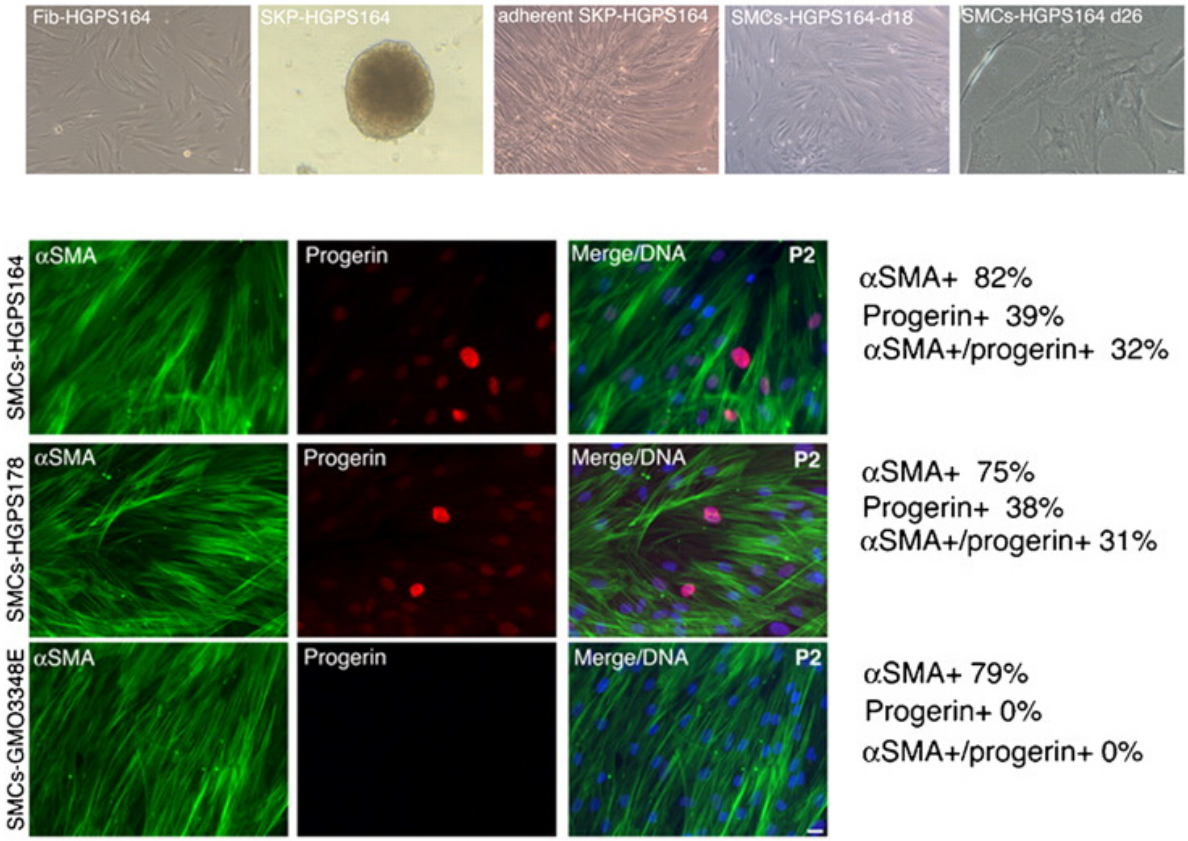

Figure 46: HGPS-SKP cells are able to differentiate into smooth muscle cells. SKP-spheres derived from HGPS fibroblasts and SKP cells derived from control fibroblasts were directed to differentiate into smooth muscle cells (SMCs). Four SMC differentiation experiments were performed using SKP cells derived from two control fibroblast strains (GMO3348E, GMO8398A (not shown)) and HGPS-SKP cells derived from three HGPS-fibroblast strains (HGADFN03 (not shown), HGADFN164, HGADFN178). Upper panel: Phase contrast images show the different culture steps from fibroblast culture to the SKP sphere formation and to the SMCs differentiation (HGPS164). Lower panel: SMCs from passage 2 (P2) of the differentiation were stained for smooth muscle actin and progerin. The percentages of SMA-positive- and progerin-positive cells are indicated (n=3). Scale bar: 20 µm. Adapted with permission from Wenzel et al. (2012).

Further immunofluorescence analyses were performed, using cells from passage 5 of the SMC differentiation. The results revealed that the extent of progerin positive cells did increase to 80-90 % of the total cell number, indicating an accumulation of progerin during the differentiation process (Figure 47). Most of the progerin positive cells did still express the smooth muscle markers calponin and myosin heavy chain in comparable levels to the control differentiation of SKP cells derived from healthy skin.

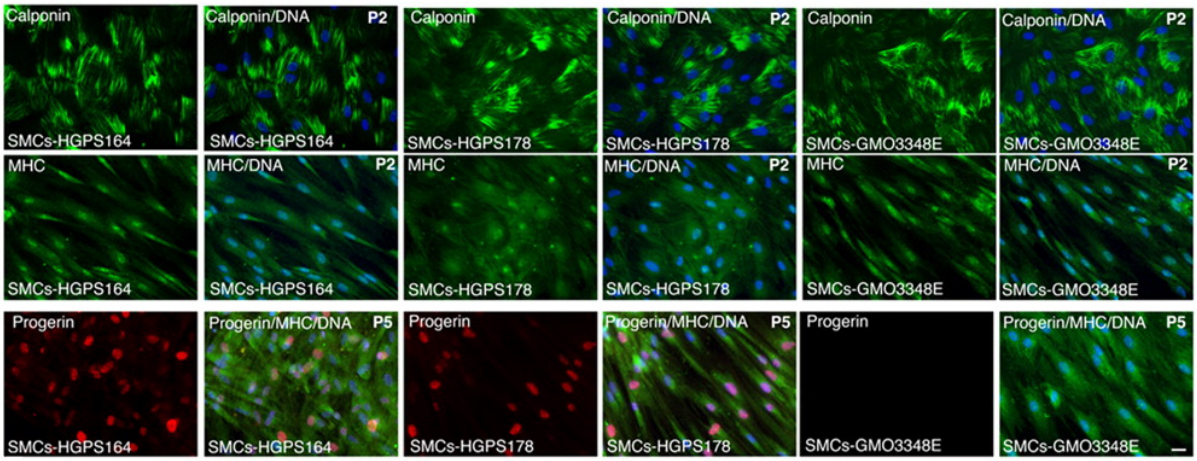

Figure 47: Progerin accumulates in HGPS-SMCs with higher passage number during differentiation. SKP cells derived from two HGPS-fibroblast strains (HGADFN164, HGADFN178) and SKP cells derived from a control fibroblast strain (GMO3348E) were differentiated into smooth muscle cells (SMCs). Smooth muscle cells were passaged and differentiated further until passage 5 (P5). SMCs were stained for calponin, myosin heavy chain and progerin. Merged pictures with DAPI stained nuclei. Scale bar: 20 µm. Adapted with permission from Wenzel et al. (2012).

5.6.3.2 Real-time PCR analysis of differentiated HGPS-SKP cells

Real-time PCR analysis of smooth muscle markers of HGPS-SKP cells did show robust mRNA levels, confirming the differentiation into smooth muscle cells after two passages (Figure 48).

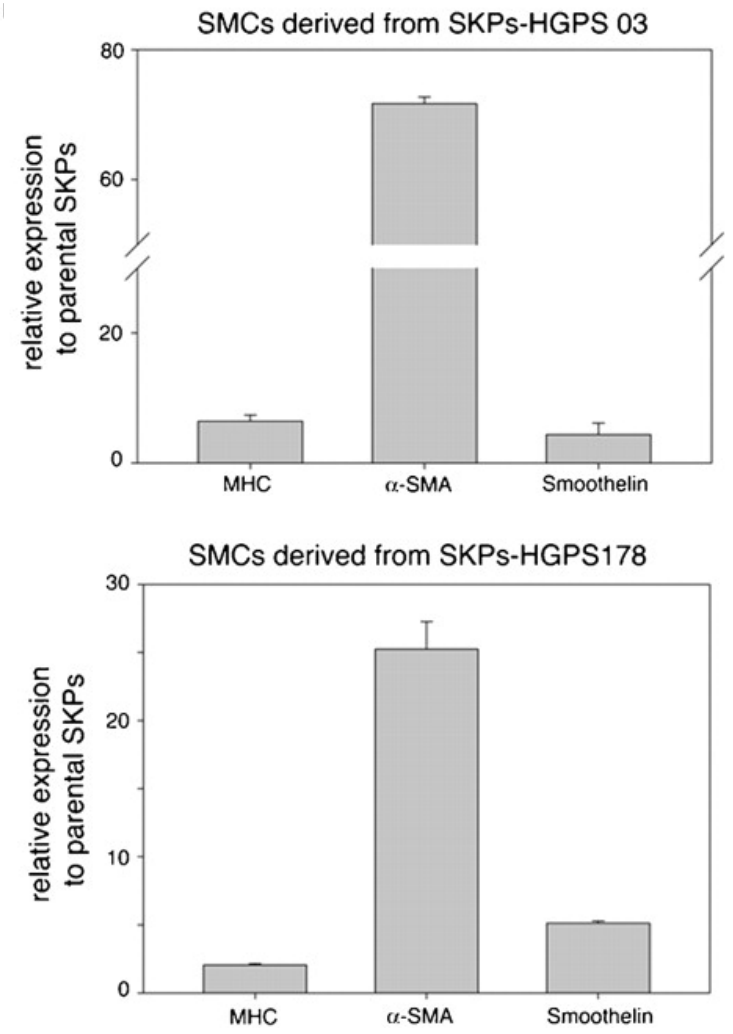

Figure 48: Real-time PCR analysis of the smooth muscle markers in HGPS-SMCs. mRNA levels of myosin heavy chain (MHC), α-smooth muscle actin (αSMA) and smoothelin as indicated in relation to the levels of the parental HGPS SKP-sphere cultures. SMCs were collected after 28 days of differentiation for mRNA isolation (n=3). Data shown as means ±SEM. Adapted with permission from Wenzel et al. (2012).

To sum up, it was shown that HGPS-SKP cells were able to differentiate into smooth muscle cells. The differentiated cells expressed smooth muscle markers at the protein and the mRNA levels. However, the differentiated cells did also express progerin similar to the naïve HGPS-SKP cells. Progerin started to accumulate during differentiation, which might reflect processes of the premature aging disease HGPS in vitro.

5.7 Senescence and prelamin A expression in SKP cells derived from young and old skin

SKP cells from young and old skin were analyzed for senescence markers and for the expression of prelamin A, since abnormal lamin forms were shown to be involved in defective stem cell function and premature aging.

5.7.1 β-galactosidase associated senescence assay of SKP cells at different culture stages

Cryosections of SKP spheres derived from young and old skin were prepared at the culture stages 'early', 'intermediate' and 'late'. The senescence-specific marker β-galactosidase was detected and analyzed by phase contrast microscopy.

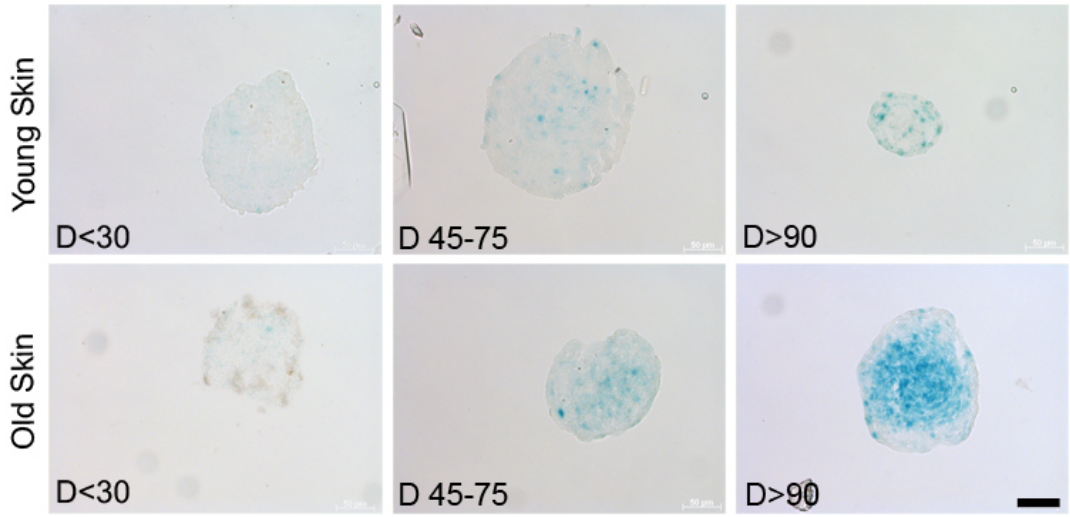

Figure 49: SKP cells derived from old skin show earlier and stronger signs of senescence in long-term cultures. A senescence associated β-galactosidase staining assay was performed on SKP spheres derived from young skin (0-12 years) and SKP spheres derived from old skin (50-94 years). Blue staining indicates senescent cells. 10 µm cryosections from spheres before day 30 ('early'), between day 45 and day 75 ('intermediate') and after day 90 ('late') of culture were used. At least three different cultures were tested (n≥3). Scale bar: 50 µm.

SKP cells from young skin did show none or very few β-galactosidase positive cells at 'early' culture stages. At 'intermediate' stage some cells were found to be senescent. Similarly, only some senescent cells were observed at 'late' culture stages of SKP cells derived from young skin.

SKP cells derived from old skin were found to have few senescent cells in average at 'early' stage, only in some cases higher numbers could be observed. SKP cells derived from old skin at 'intermediate' stage showed a stronger β-galactosidase staining, indicating a higher portion of senescent cells, while 'late' stage cells without exception showed a very strong staining (Figure 49).

Overall, SKP cells derived from old skin showed earlier and stronger signs of senescence in long-term cultures compared to SKP cells derived from young skin.

5.7.2 p16 immunofluorescence analysis

Another marker for cells undergoing senescence, p16, was analyzed by immunofluorescence staining. Both, SKP cells from young and from old skin did not show a positive signal for p16 at 'early' culture stage. In contrast, p16-positive cells were found in both age groups at a comparable level at the 'late' culture stage (Figure 50).

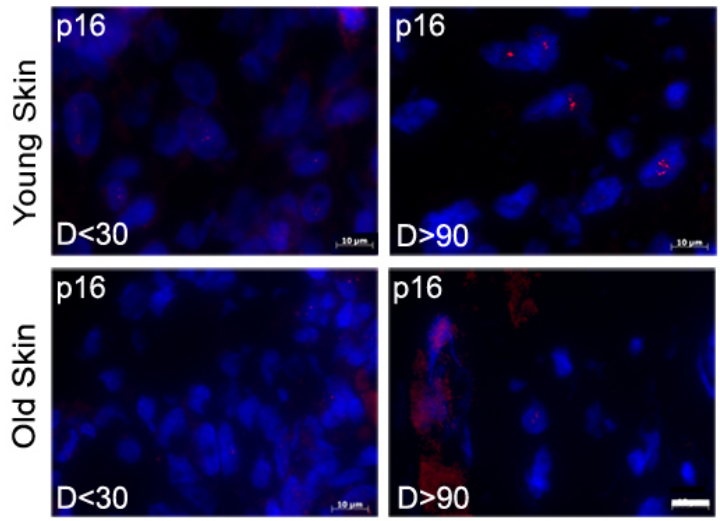

Figure 50: SKP cells at 'late' stage express the senescence marker p16. Anti-p16 immunofluorescence staining performed on cryosections of SKP spheres derived from young skin (0-12years) and from old skin (50-94 years) at culture stages 'early' (<D30) and 'late' (>D90). Merged images of p16 staining and DAPI nuclei staining. Scale bar: 10 µm.

5.7.3 p21 immunofluorescence analysis

A further senescence marker, p21, was analyzed as well by immunofluorescence staining of sphere sections of SKP cells derived from young and old skin. The culture stages ‘early’ and ‘late’ were screened.

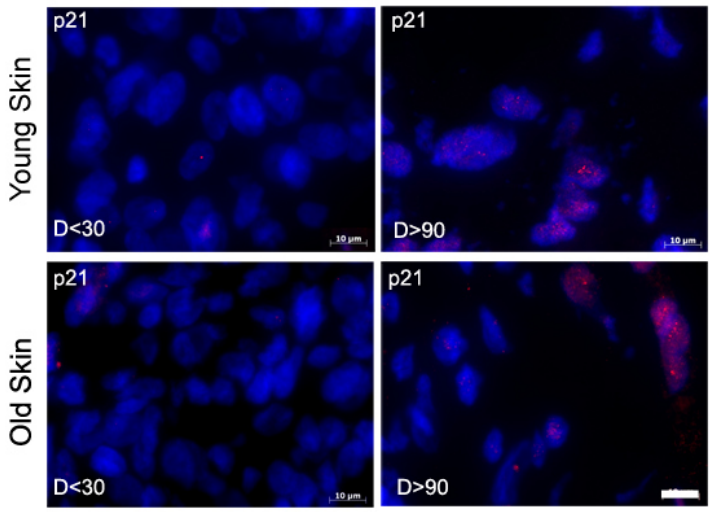

Figure 51: SKP cells at ‘late’ stage express the senescence marker p21. Anti-p21 immunofluorescence staining performed on cryosections of SKP spheres derived from young (0-12 years) and from old skin (50-94) at culture stages ‘early’ (<D30) and ‘late’ (>D90). Merged images of p21 staining and DAPI nuclei staining. n=3, scale bar: 10 µm.

No p21-positive cells were detected in ‘early’ stage SKP cells derived from young or from old skin. However, at ‘late’ stage of the cultures both, SKP cells from young and from old skin did express p21. A stronger p21 signal, which indicates a higher degree of senescence, was observed in SKP cells derived from old skin after long-term culture (Figure 51).

5.7.4 Prelamin A expression in SKP cells at different culture stages

Since abnormal lamins were correlated to cellular aging, the expression of prelamin A, a farnesylated and not finally processed lamin form, was analyzed in sections from all culture stages of SKP cells derived from young and old skin.

Prelamin A could be detected starting from ‘intermediate’ culture stage in both age groups at low levels. At ‘late’ stage a positive prelamin A staining could be observed in SKP cells from young and from old skin. A strong staining of most nuclei indicated a high expression

of prelamin A in SKP cells derived from old skin after long-term culture. In contrast, fewer nuclei of SKP cells derived from young skin showed a strong signal for prelamin A (Figure 52).

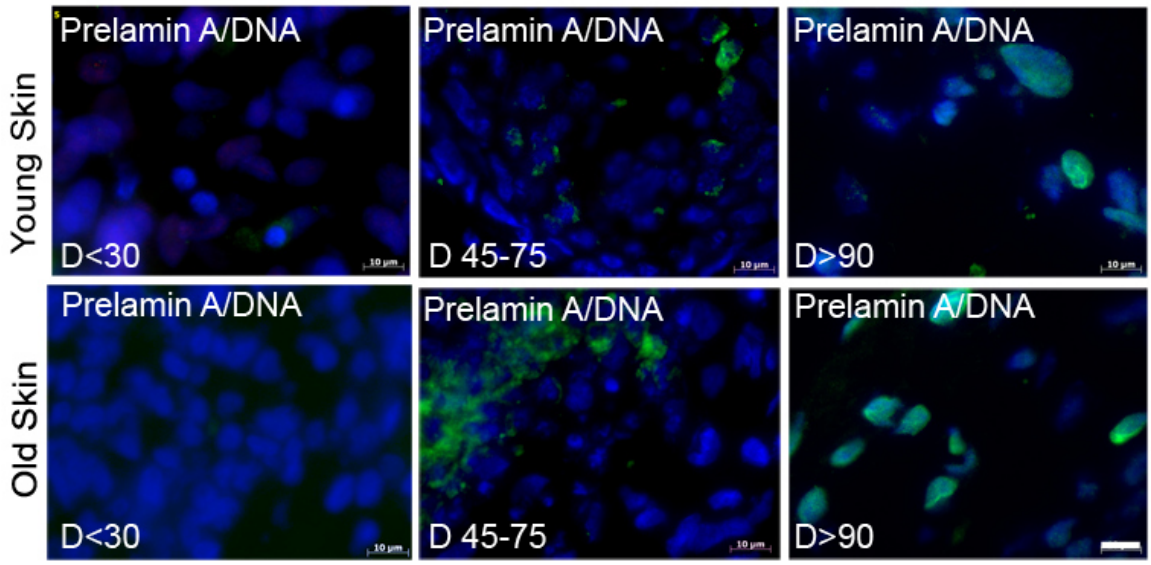

Figure 52: Prelamin A starts to accumulate in SKP cells derived from young and old skin during long-term cultures. Anti-prelamin A immunofluorescence staining performed on cryosections of SKP cells derived from young skin (0-12 years) and old skin (50-94 years) at culture stages 'early' (<D30), 'intermediate' (D45-D75) and 'late' (>D90). Merged images of prelamin A staining and DAPI (DNA). Scale bar: 10 µm.

These results correlate with the finding that more senescent cells were present in SKP spheres derived from old skin at later culture stages and show that a higher portion of abnormal lamin expression can be found in the context of senescence.

To analyze the role of prelamin A in senescence further, double immunofluorescence stainings for p21 and prelamin A were performed. Cryosections of SKP spheres derived from young and old skin were analyzed at 'early' and 'late' culture stages to investigate a correlation between prelamin A and cellular senescence (Figure 53).

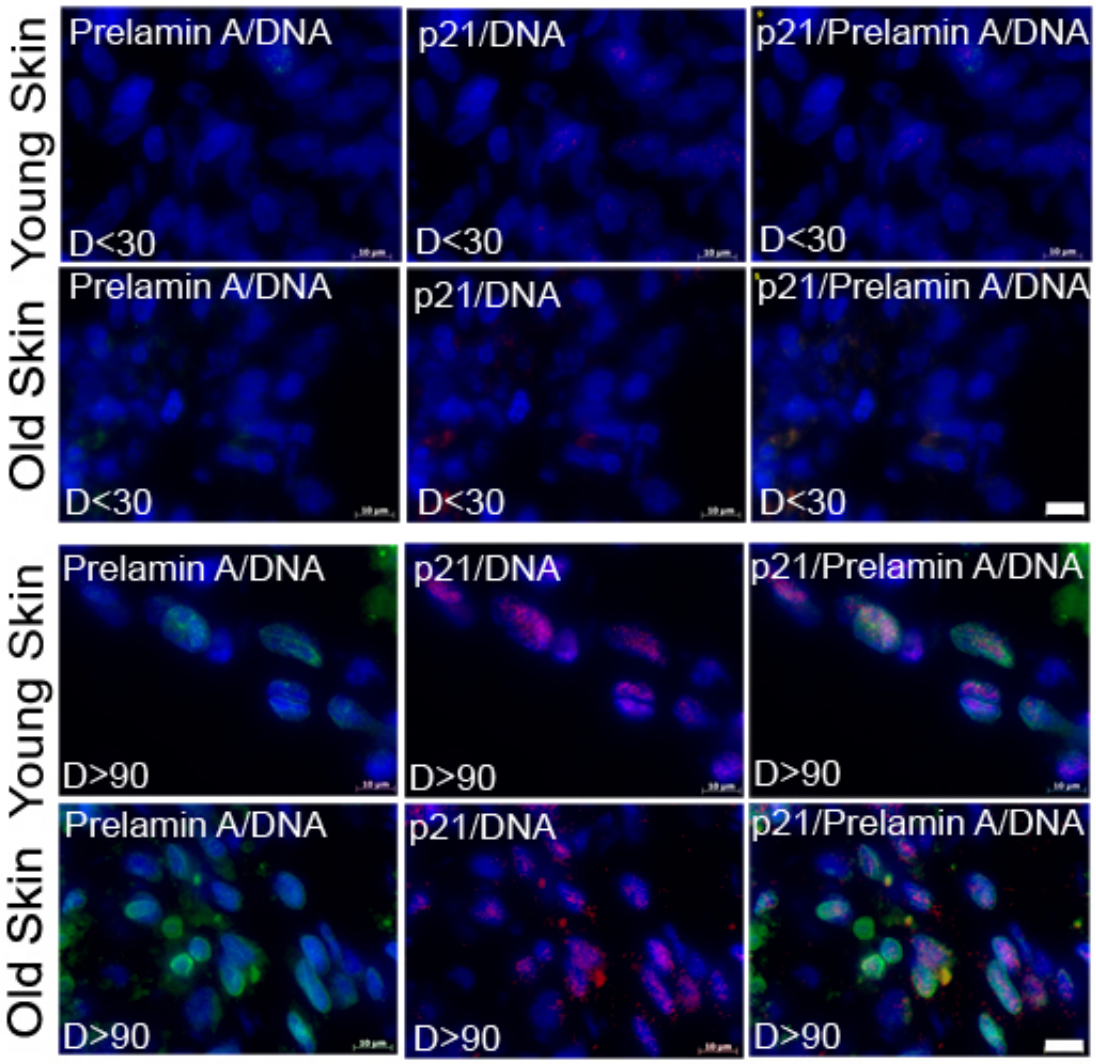

Figure 53: Prelamin A accumulates in p21 positive SKP cells derived from young and old skin at 'late' culture stages. Anti-prelamin A and anti-p21 immunofluorescence staining was performed on cryosections of SKP cells derived from young (0-12 years) and old skin (50-94 years). Upper panel: merged images of prelamin A/DNA, p21/DNA and prelamin A/p21/DNA at culture stage 'early' (<D30) for SKP cells derived from young and old skin. Lower panel: merged images as described above at culture stage 'late' (>D90) for both age groups. n=3. Scale bar: 10 µm.

As in the previous experiments, prelamin A was detected in SKP cells derived from young and old skin at 'late' culture stage. However, a higher portion of nuclei from SKP cells derived from old skin showed a positive signal. In addition, both age groups showed p21 expression at 'late' stage as observed before.

The immunofluorescence analyses showed that double positive cells were present at ‘late’ culture stages. Not all p21 positive cells were also positive for prelamin A. However, prelamin A positive cells were always positive for p21 (Figure 53).

In conclusion, it was shown that SKP cells derived from old skin showed earlier and stronger signs of senescence in long-term cultures compared to SKP cells derived from young skin. Prelamin A expression could be observed only in the context of cellular senescence and the expression of according markers. Consequently, a higher portion of prelamin A positive cells was observed in SKP cells derived from old skin. Double staining of p21 and prelamin A revealed that senescent cells seem to be a prerequisite for prelamin A expression since all prelamin A positive cells expressed p21 as well. This result indicates a correlation of prelamin A with stem cell aging and stem cell function.

6 Discussion

SKP cells can be isolated from pre-existing dermal fibroblasts

In this thesis a new method for the isolation of skin-derived precursor (SKP) cells was established. It was shown that SKP cells can be isolated from pre-existing primary fibroblast cultures and that they share the same phenotype with SKP cells isolated from skin directly, such as the expression of the neural crest marker nestin and the multipotency markers Oct4, Sox2, TG30 and Nanog (Wenzel et al., 2012; Wenzel et al., 2013).

Previous publications reported that SKP cells can be isolated from the dermis of mammalian skin. SKP cells were shown to grow in culture conditions that were originally used to culture stem cells of the central nervous system and to share some similarities with neuronal precursors (Toma et al., 2001; Toma et al., 2005). However, SKP cells are a distinct population of adult stem cells (Fernandes et al., 2004; Fernandes et al., 2008). Based on the isolation protocol described by Toma et al., SKP cells could be isolated from skin biopsies of young and elderly probands and kept in culture under specific culture conditions.

Since primary fibroblast cultures derive from dermis it might be possible that stem cells from the dermis are still existent within these cultures. This theory is supported by the observation that some cells in fibroblast cultures were resistant to stress (e.g. temperature variations during transport) and that the cultures were able to recover from few surviving cells. It was hypothesized that a stress-resistant subset of cells in fibroblast cultures might represent a stem cell population from the dermis. Within this project a method was developed which supports this hypothesis and takes advantage of the easy accessibility of primary fibroblast cultures. The hereby used approach for the isolation of adult stem cells is based on the application of a massive stress trigger and selects for stress-resistant cells in the culture. Surviving cells are cultivated with SKP culture conditions. In this setting SKP sphere growth was observed after approximately 10 days similar to SKP cultures isolated directly from skin biopsies. Comparable sphere formation times have been described previously for SKP cultures (Gago et al., 2009). Growth, morphology, culture time and the expression of stem cell markers were comparable for both isolation methods and consistent with the results of earlier studies (Toma et al., 2005). Therefore, all SKP cells that were used in further experiments of this thesis were isolated from primary fibroblast cultures derived from young or old skin and referred to as ‘SKP cells derived from young/old skin’.

The established method offers new possibilities for the characterization and for the use of SKP cells. The greatest benefit, however, is the use of fibroblast cultures as an easy accessible source for isolations in contrast to the conventional protocol with a continual need for skin biopsies. Established primary fibroblast cultures from small skin biopsies were shown to contain SKP cells even after expanding them for up to twenty passages. This allows the consecutive isolation of SKP cells from a fibroblast culture for different experiments. Even cryopreserved fibroblast cultures could be used for isolating SKP cells. Additionally, the isolation approach offers a remarkable alternative for the isolation of skin stem cells from disease states, where fibroblast cultures can be obtained but skin biopsies are not or only rarely available for research. This advantage was used in this project to isolate and characterize skin stem cells from HGPS patients. The mutant protein progerin, which causes HGPS, was shown to be expressed in the naïve state of HGPS-SKP cells, which leads to new insights in the development of the disease and the involvement of adult stem cells (Wenzel et al., 2012). Until now the only feasible way for analyzing stem cells of HGPS patients was the generation of induced pluripotent stem cells from HGPS-fibroblasts (Liu et al., 2011; Zhang et al., 2011). However, reprogrammed cells might not reflect the original state of adult stem cells in tissues of HGPS patients as discussed later, which emphasizes the importance of the newly developed method for the analysis of adult stem cells.

The approach of isolating dermal stem cells from fibroblast cultures of disease states and the possibilities resulting from systematic analysis might be also used in different other diseases such as Alzheimer's disease or muscular disorders. Hereby, new knowledge can be gained especially by differentiating SKP cells into cell types that are involved in the development of the disease.

SKP cell numbers decrease in long-term culture and are lower in cultures derived from old skin

It was shown that SKP cells can survive in long-term cultures for more than one year (Toma et al., 2001; Toma et al., 2005). This result could be confirmed in this thesis using SKP cells isolated from young skin-derived fibroblast cultures. The SKP cells could be kept in cultures for more than eight months. However, the sphere numbers decreased within this culture period while the diameter of the spheres was increasing. This result is in contrast to earlier studies, which showed slow proliferation rates during long-term cultures (Toma et al., 2005).

Differences might be caused by varying splitting procedures, such as more rigid mechanical sphere dissociation, which might lead to new sphere formations instead of increasing sphere sizes. This hypothesis is supported by the observation that dissociated single cells derived from spheres were able to self-renew and form new spheres. A mechanism which inhibits the proliferation of the SKP cells in spheres might be possible since the massive expansion of naïve stem cells would not be beneficial in vivo. Unlimited proliferation into high numbers of naïve stem cells could be interpreted as the phenotype of cancer stem cells, which seem to express similar stem cell markers (e.g. Oct4) and have been identified in several tumors (Zeineddine et al., 2014). It can be speculated that the balance and control of proliferation might be the distinguishing factor between adult stem cells and cancer stem cells.

SKP cells derived from old skin, in contrast to SKP cells derived from young skin, could not be maintained over culture periods exceeding three months. By this time the sphere numbers were decreased to an extent which made further analyses (requiring higher sphere numbers) difficult or not possible. A similar observation was made before by Gago et al. showing that SKP cells derived from adult skin underwent a selective loss during culture time (Gago et al., 2009).

By comparing the sphere building capacities between young and old skin it was shown that SKP cultures derived from old skin had a lower SKP sphere formation rate. This could point to either a decreased number of adult stem cells in old skin or to a decrease in the stress resistance of the cells. A diminished sphere building capacity has been discussed in previous studies. Some studies similarly found a correlation between SKP sphere formation rates and the age of the donor (Fernandes et al., 2004; Hunt et al., 2008; Gago et al., 2009). Others observed no correlation between sphere forming capacity and the age of adult skin (Hill et al., 2012). While not observed or investigated within the limits of this project, a recent study observed a decline in the sphere forming capacity with increasing passage number of the fibroblast cultures that were used for isolation (Hill et al., 2012). This observation might indicate a possible loss due to dilution of the stem cells during passaging.

mRNA levels of stem cells markers decrease in SKP cells derived from old skin during long-term cultures

Due to the observed growth limitations of SKP cells derived from old skin, a possibly related decline of the stem cell markers was analyzed. While SKP cells derived from young skin

expressed multipotency stem cell markers during the entire period of culture and did not show growth limitations within several months, SKP cells derived from old skin showed a strong decrease in stem cell markers at mRNA levels. This decline possibly reflects the halted growth of SKP cells derived from old skin at later culture stages. However, no decrease of the multipotency markers could be shown at the protein level in SKP cells derived from young and from old skin. This was reported similarly before regarding the expression of nestin, fibronectin and NGFR, which was shown to remain constant in SKP cells derived from young foreskin after one year in culture (Toma et al., 2005). However, the stable expression of other stem cell markers has not been investigated in long-term cultures in former studies. In addition, no mRNA levels of stem cell markers have been tested before.

Within this thesis, the mRNA levels of stem cell markers were shown to decrease over the culture period in SKP cells derived from old skin. At 'early' culture stage, however, the mRNA levels were higher when compared to SKP cells derived from young skin. This result needs to be interpreted with caution since the ex vivo isolation and cultivation of cells from elderly skin might select for cells that are least damaged and that still express high levels of the multipotency markers in contrast to a more heterogeneous but in average fitter cell population derived from young skin. After several weeks of culture the mRNA levels of stem cell markers in SKP cells derived from old skin decreased strongly, which mirrored the fast exhaustion in the proliferation of the cultures. The increased donor age might therefore cause a more rapid loss of stem cell markers in SKP cells and lead to growth limitations.

Lamin A levels are increased in SKP cells derived from old skin and after long-term cultures

A-type lamins have been shown to be involved in processes required for the differentiation of adult and embryonic stem cells (Hutchison and Worman, 2004; Constantinescu et al., 2006). Most differentiated cells were reported to express A-type lamins, while the absence or reduction of A-type lamin expression was shown in non-differentiated cells (Rober et al., 1989; Broers et al., 1997). Consequently, stem cells were considered to express no or very low levels of lamin A and C. Additionally, it was reported that high lamin A expression can negatively influence the ability of somatic cells to be reprogrammed into induced pluripotent (iPS) cells (Zuo et al., 2012).

In this study SKP cells derived from young skin showed increasing lamin A levels during culture but also considerable lamin A levels at the beginning of the cultures. However, the levels were lower than those of fully differentiated fibroblast. The results were comparable for both, protein and mRNA levels. Compared to SKP cells derived from young skin, the SKP cells derived from old skin expressed higher levels of lamin A starting from early culture stages on. When taken into consideration that these cells already showed signs of aging in their growth behavior and their stem cell marker expression during culture, the starting levels and the strong increase of lamin A expression during culture seems coherent to the former results and indicates a lower plasticity of the cells. Lamin A protein and mRNA levels increased similarly. The tested lamin A mRNA levels include abnormal lamin variants, such as progerin, that might be present in the cell. Therefore the decrease of multipotency markers and the later observed decrease in the differentiation potential might be due to the accumulation of different lamin A variants. Notably, progerin has already been shown to interfere with the function of stem cells and to influence the differentiation potential (Scaffidi and Misteli, 2008).

Lamin C expression levels did not increase much over the culture period but were significantly higher in SKP cells derived from old skin. Therefore, a correlation to aging seems possible but less significant compared to the increase of lamin A variants.

However, all SKP cells showed signs of multipotency despite expressing lamin A and C. Lamin A and C expression consequently does not seem to exclude stemcellness and multipotency in adult stem cells. Similarly, a study showed that pluripotent embryonic stem cells from mice do express low levels of lamin A/C (Eckersley-Maslin et al., 2013). The expression of lamin A therefore might be not completely limiting for the potential of stem cells but indicates a degree of maturation, which is typical for adult stem cells. As a consequence, the loss of stem cell properties or plasticity might be achieved faster and more inevitably during cellular aging compared to embryonic stem cells.

SKP cells can be isolated from HGPS patients and express progerin which accumulates during differentiation

The clinical picture of the premature aging disease HGPS shows fast aging and a rapid decline in tissue function. These symptoms point to the involvement of adult stem cells in the

disease, which might exhaust prematurely and therefore cause the fatal consequences. However, studying adult stem cells of HGPS patients was difficult since biopsies are hardly available and not ethically justifiable. Therefore, the opportunity to study SKP cells from HGPS was a chance to determine if those cells were involved in the disease and expressed progerin.

It was shown that SKP cells from HGPS showed the same morphology and growth characteristics, and expressed the same multipotency markers at comparable levels as SKP cells from non-affected probands. The A-type lamin levels of HGPS-SKP cells were comparable to the ones of SKP cells from age-matched controls. However, already the naïve state of HGPS-SKP cells expressed progerin indicating a role of progerin in stem cell dysfunction in HGPS. In contrast, the only available subject to study stem cells in HGPS, iPS cell from HGPS-fibroblasts, show no lamin A/C or progerin expression in their undifferentiated state after reprogramming (Liu et al., 2011; Zhang et al., 2011). Therefore, a potential impairment of the stem cells would not have been possible to detect with the iPS cell models. Furthermore, this thesis could demonstrate that HGPS patient skin sections contain a rare number of nestin positive cells, which are presumably SKP cells. A subset of these cells was shown to express progerin. In previous studies of HGPS skin sections it was shown that progerin was expressed in the dermis, in blood vessels, arrector pili muscle and keratinocytes (McClintock et al., 2006). With the in situ observation of adult stem cells expressing progerin in HGPS, the hypothesis is strengthened that the accelerated aging of HGPS patients might be the result of stem cell depletion and the loss of tissue function (Scaffidi and Misteli, 2008).

HGPS-SKP cells were able to differentiate into fibroblasts and into smooth muscle cells with similar efficiencies as HGPS-iPS cells (Liu et al., 2011; Zhang et al., 2011). Progerin accumulated in the smooth muscle cells during differentiation. This was similarly reported for iPS cells. iPS cells, that were differentiated into various cell types showed that progerin levels were highest in vascular smooth muscle cells , mesenchymal stem cells and in fibroblasts and that these cells showed defects, similar to those that had been reported in primary fibroblast cultures of HGPS cells (Zhang et al., 2011).When HGPS-iPS cells were differentiated into smooth muscle cells, nuclear abnormalities and premature senescence were observed upon progerin accumulation in the differentiated cells (Liu et al., 2011; Zhang et al., 2011). It seems that some cell types are more susceptible to progerin accumulations than others although lamin A/C is expressed in most differentiated cells. Notably, HGPS appears to affect only certain tissues such as skin, bone, vasculature and adipose tissue, for reasons that remain unclear up to now (Gordon et al., 2007). The accumulation of progerin during the

differentiation into smooth muscle cells might therefore reflect the consequences of the disease in vitro. This is supported by the finding that progerin was mainly accumulated in smooth muscle cells of the vascular system in tissue sections of HGPS patients (McClintock et al., 2006; Olive et al., 2010). A high degree of depletion of smooth muscle cells in blood vessels was similarly observed in autopsies from two HGPS patients (Stehbens et al., 2001). These parallels show that an in vitro model of differentiated SKP cells from HGPS patients can provide insight into the mechanisms of progerin expression in smooth muscle cells and the resulting effects. The results help to explain the vascular problems which mainly lead to the premature death of HGPS patients.

In general, the option to obtain SKP cells from pre-established fibroblast cultures of HGPS patient will improve the understanding of this disease and will create new possibilities to develop and to test therapies for HGPS.

The differentiation potential of SKP cells decreases during aging

Based on the knowledge about progerin expression in differentiating HGPS-SKP cells and the consequences of the disease, the question was raised how aging and increasing lamin levels can impact the differentiation potential of SKP cells from healthy skin of young and old probands. The phenotype of aged skin shows remarkable similarities to the skin of HGPS patients and the cardiovascular pathology of HGPS patients is similar to those of elderly people (Olive et al., 2010). It was shown before that progerin is also present in the nuclei of healthy aged probands but only in very small amounts (McClintock et al., 2007). Although the amounts of progerin were very low compared to HGPS patients it was shown that progerin levels increase in late passages of control fibroblasts (Rodriguez et al., 2009). Since the accumulation of progerin in SKP cells of HGPS patients occurred during differentiation, a similar mechanism could be thinkable for SKP cells from healthy donors. Due to the low levels of progerin that were shown to be expressed in healthy cells the involvement of other abnormal lamin variants could be possible. This thesis demonstrates that high rates of SKP cells derived from young skin differentiated into smooth muscle cells. With increasing culture time these rates were diminished but several cells were still able to differentiate into smooth muscle cells. Similarly, it was shown before that after one year in culture SKP cells derived from young foreskin were still able to differentiate into different cell types (Toma et al., 2005). A decreasing differentiation frequency, was observed within this thesis, but was

not mentioned within the study. However, SKP cells derived from old skin showed very poor differentiation potential even at very early culture stages and no differentiation into smooth muscle cells at later culture stages. An age-dependent decrease of the differentiation potential was also reported previously by Gago et al. (Gago et al., 2009). Since SKP cells derived from old skin maintained their stem cell markers over the culture period, the influencing factor is most likely the involvement of lamins. The increasing levels of lamin A expression during culture time, which were highest in SKP cells derived from old skin, could be responsible for this reduction of the differentiation potential. Additionally, the analysis of abnormal lamin expression and the senescence status of SKP cells derived from old skin showed a strong correlation to the differentiation potential as further discussed below.

SKP cells from old skin show fast signs of senescence

The analyzed senescent markers β-galactosidase, p16 and p21 showed that 'late' culture stages of both age groups were senescent, but that SKP cells derived from old skin showed earlier and stronger signs of senescence. P16 has been suggested as a marker for physiological aging previously due to its expression in large numbers of aging tissues and as well in aging stem cells (Kim and Sharpless, 2006; Campisi and d'Adda di Fagagna, 2007). p16 expression was shown to suppress stem-cell proliferation and tissue regeneration most probably due to inducing senescence. A causal function of p16 in the decline of the stem cell function has been discussed as well (Janzen et al., 2006). The physiological role of this mechanism could be part of the balance between aging and cancer. For example, mice that did not express p16 showed a retarded decline in stem cell function during aging but developed cancer prematurely (Campisi and d'Adda di Fagagna, 2007). The expression of p16 could serve as tumor suppression mechanism, marking the difference between stem cells and possible cancer stem cells that self-renew inappropriately (Clarke and Fuller, 2006). The observed increase in p16 expression in SKP cells during aging might therefore be a natural mechanism and might prevent the proliferation of aged stem cells, which are likely to bear more risks of DNA damage and consequently cancer development. However, the stem cell potential is diminished by this mechanism at the same time. One way or the other, the resulting development leads to fatal consequences.

The expression of p21, which increased during aging as well, is connected to the p53 antitumor response and such as p16 involved in triggering senescence. Together with the β-

galactosidase staining the expression of p21 and p16 indicate the senescent state at 'late' culture stages and especially in SKP cells derived from old skin, which possibly accounts for the functional decline of SKP cells derived from old skin.

Prelamin A can be used as a biomarker for stem cell aging

Senescence in aging SKP cells might cause processes leading to the decrease of the differentiation potential and the stem cell function. A potential cause was identified in this thesis, by the increasing levels of lamin A. Since the expression of lamin A can include abnormal variants, especially in aging cells, a correlation between these variants and the processes leading to the diminished stem cell function was analyzed. It was shown that prelamin A, a farnesylated precursor of the mature lamin A protein, accumulates in SKP cells during aging. An increased prelamin A expression was observable in SKP cells derived from old skin. This result correlates with the decrease of the differentiation potential into smooth muscle cells. Previous studies showed that prelamin A accumulates with senescence in aging cells e.g. in vascular smooth muscle cells, that prelamin A indicates senescence and that it is a marker for specific vascular aging (Ragnauth et al., 2010). Furthermore, prelamin A was shown to interfere with the heterochromatin organization which possibly affects the cell function (Lattanzi et al., 2007).

These mechanisms are similar to the effects caused by the expression of the mutant prelamin A form progerin, which triggers premature aging. Both proteins are farnesylated and might therefore lead to similar toxic effects when existent in the cell. While the expression of progerin is caused by a mutation, prelamin A might accumulate during cellular aging due to an impaired protein processing in the cell. A study showed that prelamin A accumulates in vascular smooth muscle cells before senescence and might be causal for the process (Ragnauth et al., 2010). In contrast, this thesis demonstrates that the cells were at first positive for the senescence marker p21 and that prelamin A was only expressed in those p21 positive cells. Therefore, a counter balance of the processes might be more likely.

However, the accumulation of abnormal lamins, especially when farnesylated, seems to influence the senescence process and the fate of the adult stem cells, as shown as well for progerin in the case of HGPS. Since progerin is only expressed at very small levels in healthy aging individuals, prelamin A might be the corresponding factor in the physiological aging process. Therefore, prelamin A could be used as a biomarker for the aging status of SKP

cells when being in the need to assess the cells for a proper stem cell function. This includes the evaluation of the multilineage differentiation potential, which might not be reflected only by the expression of multipotency markers as shown in this thesis. An assessment of SKP cells or other adult stem cells would be especially important in regard to a possible use of those cells for regenerative medicine.

7 Conclusion and Outlook

Taken together, it is shown within this thesis that SKP cells can be isolated from pre-existing fibroblast cultures. This finding gives rise to a variety of possible applications, starting with research applications where skin biopsies are not necessarily needed anymore to analyze dermal adult stem cells. Furthermore, the easy accessibility leads to the possibility of gaining and analyzing SKP cells from disease states such as HGPS but also any other disease where the involvement of adult stem cells needs to be examined. The multilineage differentiation potential of SKP cells offers not only the chance to study skin related stem cell problems but also muscular, vascular and adipose tissue related diseases. With the analysis of HGPS-SKP cells it could be shown that SKP cells can serve as an in vitro model to dissect and reconstruct disease development and causes. This opportunity could significantly simplify the development of new treatments and the assessment of new therapies for many diseases.

In regard to the differences between young and old skin it was shown that SKP cells can also be obtained from elderly skin, which offers new opportunities for regenerative approaches. The isolated SKP cells from elderly skin could be shown to be still functional. Nevertheless, it was shown that a thorough assessment of these cells would be necessary to discriminate between fully functional adult stem cells and impaired or aged stem cells. In regenerative medicine those cells would diminish the positive outcome of any therapy. Nowadays, clinical projects are testing the injection of different stem cells into various organs for therapeutic indications. While embryonic and iPS cells are connected to risks such as cancer development or immunologic reactions, SKP cells could be used as a low-risk autologous source of stem cells. In these cases it would be beneficial to test and select the cells for their stem cell potential before using them for treatment.

Within this context, the knowledge about lamins and abnormal lamins influencing the aging process of adult stem cell gains in importance. This thesis showed that increasing lamin A levels including abnormal lamin forms, such as prelamin A, can be detected during the aging process of adult skin-derived stem cells. In correlation, a decreasing differentiation potential and increasing senescence of the cells could be shown. Therefore, prelamin A might induce similar defects as progerin in normal physiological aging, and as such, could be used as a biomarker for evaluating the fitness of adult stem cells. Future usage of SKP cells for regenerative purposes could benefit from the determination of prelamin A levels within a preselection process.

The isolation and assessment of SKP cells therefore provides a variety of possibilities for therapeutic approaches and regenerative medicine and might help to treat or ameliorate disease states without the need for embryonic or iPS cells.

Within future research the results of this thesis need to be confirmed for different disease states and aging models. Knowledge about the functional mechanisms is essential in order to apply SKP cells in future treatments or to use them for new techniques in regenerative medicine. Moreover, further research is necessary to advance in a safe, reliable and responsible use of adult stem cells in medicine.

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