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MOLECULAR STUDIES OF HUTCHINSON-GILFORD PROGERIA SYNDROME

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ABSTRACT

Hutchinson-Gilford progeria syndrome (HGPS) is a very rare genetic disease, with an incidence of 1 in 4-8 million live births, that causes segmental premature aging in children. The children look normal at birth but begin to develop symptoms of disease within the first years of life. The symptoms include growth retardation, scleroderma, osteoporosis and atherosclerosis of the coronary and cerebrovascular arteries. Myocardial infarction or stroke is the most common causes of death at a median age of 13 years. The aims of this work includes: to increase the understanding of the molecular mechanisms underlying progeria, to see if there is any possibility of disease reversal and to develop a specific method for analyzing *LMNA* transcripts during normal and *in vitro* aging. For these purposes, we developed an inducible tissue-specific transgenic mouse model system that included a minigene of human *LMNA* with either the wild-type sequence or the most common HGPS mutation, 1824C>T, and assays for absolute quantification of the *LMNA* transcripts in HGPS patient material and controls of different ages.

PAPER I: Animal models are crucial to increase understanding of the ongoing molecular process during disease, especially for rare and severe diseases like HGPS. To get a better understanding of the HGPS skin phenotype, we developed an inducible and tissue specific model system with keratin 5-targeted transgenic expression. Bitransgenic animals with the HGPS mutation have a progressive phenotype. The phenotype is first characterized by an intermediate stage with varying degrees of hyperplasia of the interfollicular epidermis, mis-expression of keratins 5 and 6 and increased proliferation. The end stage is seen later, with loss of subcutaneous fat and fibrosis of the dermis, similar abnormalities to those seen in the skin of HGPS patients. The severity of the disease phenotype correlates with the level of transgenic expression (higher expression gives more severe disease phenotype). Animals expressing the wild-type allele had a normal appearance of the skin.

PAPER II: To examine if expression of progerin affects the expression of lamin B or the progress of the hair cycle, we first characterized the normal expression of lamin A/C and B in mouse skin cell types during hair cycling. Immunohistochemical staining of the whole back skin of FVB/NCrl wild-type mice showed strong expression of lamin A/C and B in the basal layer of the epidermis, the outer root sheath of the hair follicle and the dermal papilla during all stages of the hair cycle. Lower expression was seen in the suprabasal cells of the epidermis, in the hypodermis and the bulb of catagen follicles. Analyzing the different phases of the first postnatal hair cycle and the expression of lamin B in our mouse model of HGPS did not reveal any shifts in the hair cycle or in the expression of lamin B.

PAPER III: To examine if progeria disease is reversible and learn more about the possibility of treatment for the children with progeria who are already manifesting the disease, we used our inducible transgenic mouse model of HGPS. After disease development, transgenic expression was suppressed and the animals were observed for reversal in disease phenotype. The external phenotype of hair loss and skin crusting improved after only 1 week of suppression and after 6 or 13 weeks the external skin phenotype looked completely normal in most of the animals. The lower weights of bitransgenic animals increased after transgenic suppression and followed the trend of the wild-type curve. The disease pathology seen in the skin of bitransgenic animals was

almost indistinguishable from wild-type after 6 and 13 weeks of suppressed transgenic expression. This shows that the expression of the HGPS mutation does not cause irreversible damage, at least in these tissues, which gives promise for future treatment for this disease.

PAPER IV: To characterize the expression levels of the *LMNA* locus transcripts in HGPS patients and age-matched and parental controls and during *in vitro* aging, we developed a method for absolute quantification using real-time RT-PCR. Lamin A, C and lamin AΔ150 transcripts were quantified in HGPS and normal cells of different ages. Our results showed that lamin C is the most highly expressed transcript from the *LMNA* locus. The lamin AΔ150 transcript was present in unaffected controls but at >160-fold lower levels than in HGPS patient cells. While the levels of lamin A and C remained unchanged during *in vitro* aging, the lamin AΔ150 transcript increased in late passage cells from both HGPS and parental controls, which suggests that similar mechanisms exist in HGPS and normal aging cells.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their roman numerals:

I. **Targeted transgenic expression of the mutation causing Hutchinson-Gilford progeria syndrome leads to proliferative and degenerative epidermal disease**
Sagelius H, Rosengardten Y, Hanif M, Erdos MR, Rozell B, Collins FS, and Eriksson M
Journal of Cell Science 2008; 121: 969-978

II. **Differential expression of A-type and B-type lamins during hair cycling**
Hanif M, Rosengardten Y*, Sagelius H*, Rozell B, and Eriksson M
PloS ONE 2009; 4(1): e4114
**** Rosengardten and Sagelius contributed equally to this work***

III. **Reversible phenotype in a mouse model of Hutchinson-Gilford progeria syndrome**
Sagelius H, Rosengardten Y, Schmidt E, Sonnabend C, Rozell B, and Eriksson M
Journal of Medical Genetics 2008; 45: 794-801

IV. **Increased expression of the Hutchinson-Gilford progeria syndrome truncated lamin A transcript during cell aging**
Rodriguez S, Coppedè F, Sagelius H, and Eriksson M
European Journal of Human Genetics 2009;Jan 28 [Epub ahead of print]

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LIST OF ABBREVIATIONS

aa	amino acid
bp	base pair
CMT2	Charcot-Marie tooth disease 2
DCA	dilated cardiomyopathy
dox	doxycycline
EDMD	Emery-Dreifuss muscular dystrophy
FPLD	familial partial lipodystrophy
FTI	farnesyl transferase inhibitor
HF	hair follicle
HGPS	Hutchinson-Gilford progeria syndrome
htx	haematoxylin
IF	immunofluorescence
IHC	immunohistochemistry
INM	inner nuclear membrane
K1-14	keratins 1-14
LA	lamin A
LAP	lamin associated protein
LBR	lamin B receptor
LC	lamin C
LGMD	Limb-Girdle muscular dystrophy
MAD	mandibuloacral dysplasia
ONM	outer nuclear membrane
PHH3	phosphohistone H3
RD	restrictive dermopathy
RNAi	RNA interference
RT	room temperature
rtTA	reverse tetracycline transactivator
tetop	tet-operator
tTA	tetracycline transactivator
Zmpste24	zinc metalloproteinase related to Ste24

1 INTRODUCTION

All people age, and age-related symptoms appear at very different time points in life for different reasons. This is probably due to various genetic and environmental factors. Aging is a very complex process, and its molecular basis is not fully understood. Some factors can be influenced by the individual to increase or reduce their life span e.g., whether the individual exercises, their diet and stress factors. There are also genetic factors that increase or shorten the life span that cannot be influenced by the individual. Mutations in single genes have been shown to increase the life spans of nematodes, yeast, fruit flies and mice. The most often affected pathways are those evolutionarily-conserved pathways that regulate growth, energy metabolism, nutrition sensing and/or reproduction [1]. Examples include genes encoding factors involved in the insulin/insulin-like growth factor 1 (IGF-I) signaling pathway [2], the target of rapamycin (TOR) pathway [3], and the mitochondrial electron transport chain [4]. Many pro-longevity mutations mimic dietary restriction (underfeeding without malnutrition), which has been shown to extend the life span of rodents [5], while mutations causing inactivation of autophagy reduce life span [6]. Many of the pathways that are affected during either increased or reduced life span are conserved throughout species: therefore, lower organisms such as nematodes, flies and mice can be used to study the mechanistic bases of human aging [1]. Some mutations are known to cause diseases of premature aging, mostly affecting the DNA repair system or the nuclear lamina. Mutations in the *LMNA* gene give rise to laminopathies, which are diseases affecting many different tissues, and some are classified as premature aging syndromes. There are unimodal progeroid syndromes (e.g., Alzheimer's disease), which only affect one tissue [7], and segmental progeroid syndromes that affect several tissues [8], (e.g., Down's syndrome, Werner's syndrome and Hutchinson-Gilford progeria syndrome).

1.1 HUTCHINSON-GILFORD PROGERIA SYNDROME

Hutchinson-Gilford progeria syndrome is a very rare genetic disease, which is classified as a segmental progeroid syndrome. The reported incidence is 1 in 4-8 million live births [9, 10].

1.1.1 Clinical features

Children born with HGPS usually appear normal at birth, but begin to show signs of disease within the first years of life. The symptoms include growth retardation with short stature and low weight, alopecia and pyriform chest. They have prominent scalp veins, prominent eyes, a small and beaked nose, micrognathia, delayed and abnormal dentition with hypodontia, crowding of teeth and oral soft tissue alterations. Progeria children also have thin lips, protruding ears lacking ear lobes, dystrophic fingernails, a high-pitched voice and they do not enter puberty, but they have normal intelligence [9, 11-25] (the GENEReviews database at www.geneclinics.org).

1.1.1.1 Skin changes

After failure to thrive, the skin phenotype, characterized by scleroderma and loss of subcutaneous fat, is normally the first symptom that is noticed. The skin becomes tight over the abdomen and thighs during the second or third year of life, but the children also have regions with wrinkled skin that becomes thin, dry and atrophic, sometimes with hyperkeratosis. Hyperpigmentation can be seen on the scalp and limbs. Several symptoms are seen in older patients, including a thin epidermis, fibrosis in the dermis with thickened and disorganized collagen fiber bundles, a reduced number of sweat glands and sebaceous glands, and atrophic subcutaneous adipose tissue. Between 6 months and 2 years of age the hair usually falls out and the children usually have complete alopecia between 2 and 3 years, except for some fine downy hairs [9, 11-17, 19, 20, 23, 25] (the GENEReviews database at www.geneclinics.org).

1.1.1.2 Bone changes

The bone phenotype appears as mild osteoporosis manifested as acro-osteolysis in the distal phalanges, clavicular resorption and later osteolysis in the long bones as well as generalized osteopenia. Progeria children also have coxa valga and joint contractures that lead to a horse-riding stance and difficulty moving the knees, elbows and fingers [9, 11, 13, 15-17, 19-23] (the GENEReviews database at www.geneclinics.org).

1.1.1.3 Cardiovascular changes

Initially the patients do not have any cardiovascular problems, but they develop shortness of breath with exertion as well as increased pulse rates and blood pressure. A relatively small diameter of the intima and media and extensive loss of smooth muscle

cells are found at autopsy and plaque formation is also found sometimes. Death is commonly due to complications arising from atherosclerosis of coronary and cerebrovascular arteries at a mean age of 13 years [26, 9, 27, 28, 25, 18, 29, 20, 23] (the GENEReviews database at www.geneclinics.org).

1.2 THE INNER NUCLEAR LAMINA AND DISEASE

1.2.1 The nuclear envelope

The nuclear envelope consists of the outer and inner nuclear membranes (ONM and INM respectively), nuclear pore complexes and an underlying network of filaments called the inner nuclear lamina, which are mainly composed of lamin proteins (see Fig. 1). The lamina is believed to give the nucleus its shape, structure and strength, to have a role in DNA replication, nuclear pore positioning and function, heterochromatin organization and to provide anchoring sites for chromatin domains, various proteins and transcription factors at the nuclear periphery [30-32]. The lamina is disassembled and reassembled by phosphorylation and dephosphorylation during mitosis, along with the rest of the nuclear envelope [33, 34].

The lamin filaments are polymers of nuclear-specific intermediate filament proteins. The lamins, like all intermediate filament proteins, consists of a central α-helical coil-coiled rod domain flanked by a small non-α-helical N-terminal globular domain and a larger C-terminal globular domain [33] (see Fig. 2). There are two types of lamins that form stable yet dynamic structures, A- and B-type. The *LMNA* gene encodes the four different A-type lamins: lamin A, C, AΔ10 and C_2 [30]. We have detected the expression of lamin AΔ10 in human fibroblasts [paper IV], which previously has been found in cells from human colon, placenta, leukocytes and carcinoma tumor cells [35]. Lamin C_2 is expressed in spermatocytes [36]. Lamin A is encoded by exon 1-12 and lamin C by exon 1-10. Lamin A and C are identical except lamin A has a unique 90 amino acid (aa) region at its C-terminus, whereas lamin C has a unique 6 aa sequence [33]. Two different genes encode the B-type lamins; *LMNB1* encodes lamin B_1 and *LMNB2* encodes lamin B_2 and B_3, which is only expressed in spermatocytes [37-39]. While B-type lamins (B_1 and B_2) seem to be expressed in all cells during development and in adult tissues, A-type lamins are mainly expressed in terminally-differentiated cells [40].

A-type lamins have been shown to bind to emerin [41, 42], lamin associated protein (LAP) 1 [43], LAP2α [44], nesprin 1 [45], nesprin 2 [46], actin [47], pRb [48], sterol regulatory element-binding protein (SREBP) 1 [49], SUN1 [50], SUN2 [50] and one or more components of RNA polymerase II dependent transcription complexes [51] and DNA replication complexes [52] *in vitro*. Lamin A and C participate in the LINC complex that, along with the nesprin and SUN proteins, LInk the Nucleoskeleton with the Cytoskeleton. Actin-binding nesprins in the ONM interact with SUN proteins in the lumen of the nuclear envelope, which in turn interact with nesprins in the INM as well as lamins A and C and thereby link the nucleoskeleton with the cytoskeleton [50] (see Fig. 1).

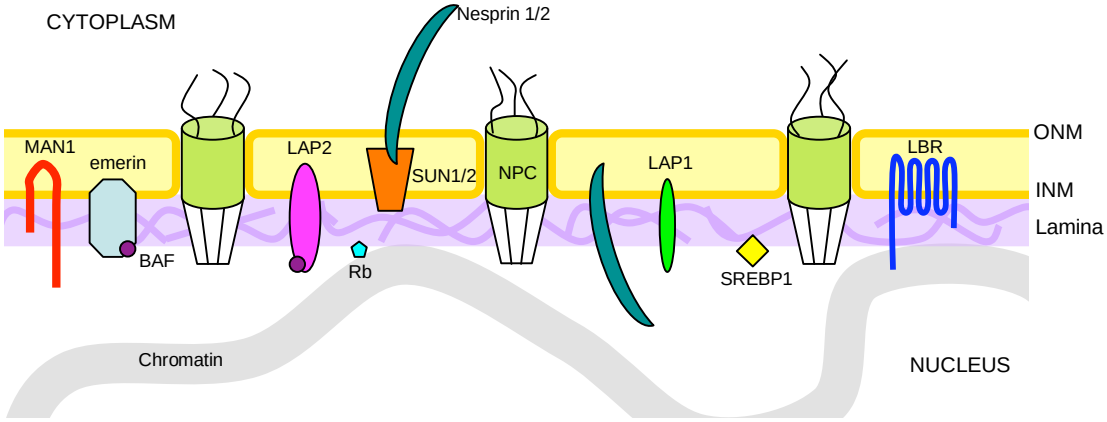

Figure 1. Schematic picture of the structure and function of the nuclear lamina. The inner nuclear lamina is the purple structure underneath INM, called lamina in the picture, representing lamin A/C and B.

1.2.2 Pre-lamin A processing

Lamin A is produced as a precursor protein, prelamin A, which undergoes posttranslational processing to become mature lamin A (see Fig. 3). The C-terminal end of prelamin A contains a CaaX motif (C, cysteine; a, any aliphatic amino acid; X, any amino acid), which is CSIM for prelamin A. This motif is modified by farnesylation of the C-terminal cysteine residue [53-55], followed by cleavage of the three N-terminal residues (-aaX) and carboxymethylation of the cysteine [56, 57]. The endopeptidase cleavage can be performed by Rce1 (Ras-converting enzyme 1) [58] or Zmpste24 (Zinc metalloprotease related to Ste24p) [59, 60] and the carboxymethylation is catalyzed by the enzyme Icmt (isoprenylcysteine carboxyl methyltransferase) [61]. Carboxymethylation results in the insertion of prelamin A into the INM [30]. The last step of lamin A posttranslational processing removes the C-terminal 15 residues of

prelamin A through proteolytic cleavage by Zmpste24 and yield mature lamin A [62, 60, 63]. Lamin C lacks a farnesylation site and therefore does not go through this processing [30]. The lamin B proteins also contain a CaaX motif [64] and go through all of the processing steps, except the final cleavage [65].

1.2.3 Nuclear envelopathies

Nuclear envelopathies are the group of diseases that are caused by mutations in genes encoding for nuclear envelope proteins. Disease causing mutations are currently reported for several different genes, e.g., *LMNA*, *FACE-1*, *LMNB1*, *LMNB2*, lamin B receptor (*LBR*), *MAN1* and *LAP2* [66, 67]. The nuclear envelopathies include the laminopathies, which usually are divided into primary and secondary laminopathies.

1.2.3.1 Primary laminopathies

Today more than 200 mutations have been identified in the *LMNA* gene (www.dmd.nl/lmna_seqvar.html). The *LMNA* gene is unique in that no other gene is known to cause as many different diseases when mutated [68]. There are at least ten different autosomal recessive and autosomal dominant genetic diseases linked to mutations in the *LMNA* gene, which are called the primary laminopathies [69] (see Fig. 2).

They are often divided into four different groups depending on the phenotype i.e., muscular dystrophies, lipodystrophies, neuropathies and segmental progeroid syndromes. The **muscular dystrophies** include Emery-Dreifuss Muscular Dystrophy (EDMD), Dilated Cardiomyopathy (DCM) and Limb-Girdle Muscular Dystrophy (LGMD). EDMD results in progressive wasting of specific muscles of the lower leg, upper arm and shoulder as well as cardiac conduction defects [70]. Patients with DCM have cardiac-specific muscular dystrophy that does not affect the skeletal muscle [71], while LGMD mainly cause muscle wasting in the proximal limbs [72]. The **lipodystrophies** include Familial Partial Lipodystrophy (FPLD), Generalized Lipodystrophy type 2 and Mandibuloacral Dysplasia (MAD) type A. FPLD is characterized by loss of subcutaneous white adipose tissue from the limbs, gluteal and trunkal regions and a simultaneous accumulation of white adipose tissue in the neck, face and abdominal areas [73]. Generalized Lipodystrophy type 2 is characterized by lipoatrophy from birth and severe insulin resistance associated with hyperpigmentation of the skin, muscular hypertrophy, hepatomegaly, glucose intolerance or diabetes, and

hypertriglyceridemia [74]. MAD type A is a disease with lipodystrophy, skeletal abnormalities, stiff joints and skin atrophy [75, 76]. The **neuropathic disorder** Charcot-Marie-Tooth disease type 2B1 (CMT2) is characterized by slightly reduced or unaffected nerve conduction velocities, motor neuron demyelination and axonal degeneration [77]. The **segmental progeroid syndromes** are classical HGPS (described previously), atypical HGPS, atypical Werner's syndrome and restrictive dermopathy (RD). Atypical HGPS patients have disease phenotypes similar to classical HGPS patients, but they have additional or lack distinct phenotypes seen in HGPS. Werner's syndrome is often called "progeria of the adult" and is characterized by growth retardation from the second decade. The classical form is due to a mutation in *WRN*, which encodes a RecQ helicase protein [78]. They also have short stature, cataracts, skin atrophy and alopecia, loss of adipose tissue, diabetes, osteoporosis, arteriosclerosis, hypogonadism and a predisposition to cancer. The cause of death is usually cardiovascular disease or neoplasia and the average life span is 47 years [79, 80]. RD is a neonatal disorder that is characterized by tight skin, prominent vessels, joint contractures, respiratory insufficiency and premature death during gestation [81].

Even though numerous mutations in the *LMNA* have been identified in various laminopathies, they are not distributed evenly across the gene (see Fig. 2). Those affecting striated muscles are spread throughout the *LMNA* gene and are thought to result in the misfolding of the coiled-coil rod domain or to affect the correct assembly of the proteins. Most of the lipodystrophies are located in the C-terminal domain of lamin A and are suggested to have gain of function mutations, thereby causing disease by increasing or creating binding to other proteins [82]. Premature aging syndromes are also mostly distributed in regions close to the C-terminus. When looking at progeroid syndromes, it seems that the severity of the clinical features increases with the size of the internal deletion. A patient with a mutation giving rise to a 35 aa deletion and the same clinical features as a HGPS patient, who have a 50 aa deletion (described further later), had a much later onset of disease and lived until 45 years of age [83], while a mutation causing RD had a 90 aa internal deletion in lamin A [81]. Interestingly, the three deletions totally overlap, but it is unclear how the different deletions affect lamin A function. It is not surprising that the larger deletions give rise to worse disease phenotypes, but this also highlights the significance of the different amino acids in the C-terminal region of lamin A and that the incomplete processing of the protein (described later) is not the only factor that affects disease development and severity.

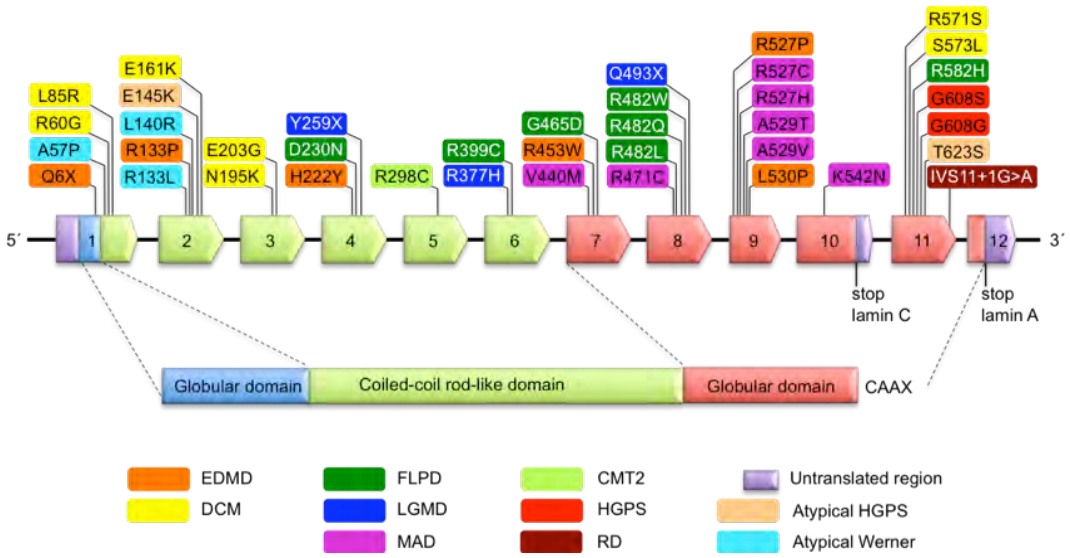

Figure 2. Schematic picture giving examples of different mutations in the *LMNA* gene that cause various laminopathies. Not to scale. (Picture was inspired by [82, 84, 85]).

1.2.3.2 Other nuclear envelopathies

Secondary laminopathies are caused by mutations in *FACE-1* (*ZMPSTE24* in mice) (e.g., MAD type B, RD) [69, 86, 85]. *LMNB1* and *LMNB2* alterations are also known to cause nuclear envelopathies. Duplication of *LMNB1* causes Autosomal Dominant Leukodystrophy, which is a neurodegenerative disease with progressive myelin loss in the central nervous system [87, 88], and mutations in *LMNB2* cause Acquired Partial Lipodystrophy, which is a sporadic form of progressive lipodystrophy [84, 89, 67]. Mutations in LBR cause the Pelger-Huet anomaly (PHA), an apparently harmless alteration in chromatin distribution and morphology of neutrophil nuclei [90], and Hydrops-ectopic calcification-"moth eaten" (HEM) skeletal dysplasia, an in utero lethal, short-limb skeletal dysplasia [91]. Mutations in *LEMD3*, the gene encoding MAN1, have been identified to cause Osteopoikilosis, Buschke-Ollendorff syndrome and melorheostosis, which are skeletal dysplasias, characterized by sclerosis or increased bone density [92]. Another envelopathy, DCM, is caused by a mutation in LAP2α [93].

1.3 MOLECULAR BASIS OF HGPS

90% of all HGPS cases are caused by a *de novo* heterozygote base substitution in exon 11 of the *LMNA* gene, G608G (1824C>T). Since the mutation is in exon 11 it will only affect the lamin A protein. The mutation increase the use of a cryptic splice site that leads to an internal deletion of 150 nucleotides in the end of exon 11. The reading frame is still intact and the final truncated protein, named progerin or lamin AΔ50, has

a 50 aa deletion near the C-terminal end [94, 95]. Progerin still retains the CaaX-box, but lacks the site for the final endoproteolytic cleavage that leads to a farnesylated and methylated protein product [96] (see Fig. 3). Progerin disrupts the structure of the nuclear lamina, intranuclear architecture and macromolecular interactions, which collectively could have a major impact on nuclear function. The activation of the cryptic splice site is only partial and therefore, normal protein is also produced by the mutant allele [94, 95].

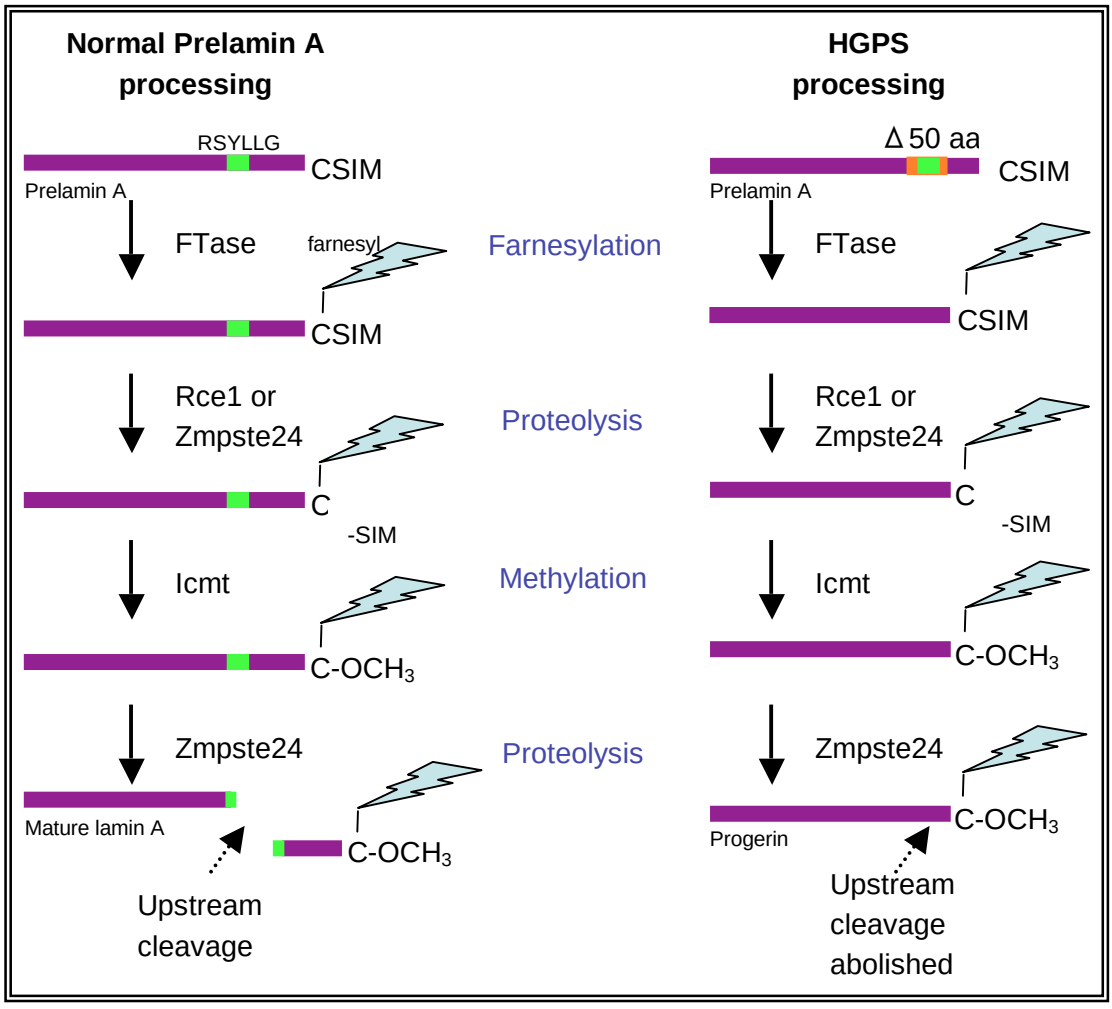

Figure 3. Schematic picture of normal and HGPS prelamin A processing, which differ only in the final proteolytic cleavage because the cleavage site is abolished in HGPS mutant prelamin A.

Primary fibroblasts from HGPS patients show severe changes in nuclear shape, including lobulation of the nuclear envelope, loss of peripheral heterochromatin, clustering of nuclear pores and a thickened lamina [95, 97]. These structural changes seem to get worse as HGPS cells age in culture, and their severity correlates with an increase in progerin [97]. The presence of progerin alters mitosis and leads to

chromosome missegregation and binucleation [98, 96], defective DNA repair [99], downregulation of several nuclear proteins and altered histone modification patterns [100]. Interestingly, progerin is also present in normal aging cells, which show nuclear aberrations similar to those seen in HGPS cells [101, 98, 102].

1.4 DISEASE HYPOTHESIS

There are several hypotheses for how disease progresses in HGPS and laminopathies. Different hypotheses can be true for different laminopathies, but a combination of two or more theories will most likely give a more accurate picture of the disease mechanisms.

The **mechanical stress** hypothesis implies that abnormalities in nuclear structure result in increased susceptibility to cellular damage by physical stress [32, 103-105, 66, 106, 68]. It has been shown that the lamina in HGPS cells has a significantly reduced ability to rearrange under mechanical stress [107]. Cells expressing progerin also have a reduced lamin response from shear stress and this is also seen in neighboring normal cells [108]. HGPS fibroblasts develop progressively stiffer nuclei with increasing passage number and are more sensitive to mechanical strain showing more apoptotic and necrotic cells upon repetitive strain as well as a significantly impaired cell cycle response and migration upon strain stimulation [109]. This theory suits laminopathies that affect muscle and other tissues exposed to mechanical stress, but is not suitable for e.g., lipodystrophies. This theory cannot explain the full phenotype seen in HGPS patients, since not all tissues affected are under mechanical stress, but it might explain the loss of vascular smooth muscle cells in the large arteries.

The **gene expression** model proposes that specific DNA transcription can be altered by mutations in lamins [32, 103-105, 66, 106, 68]. This might influence disease progression in all laminopathies since lamin A is believed to be involved in many cellular regulatory systems such as gene transcription and cell signaling. In HGPS, several genes have been showed to have altered expression levels [110-112, 100], and the largest functional category affected was transcription factors [112]. Loss of peripheral heterochromatin has been seen in late passage HGPS cells [97], and this might indicate a change in gene expression. This theory can for example be applied for laminopathies where fat distribution is affected. It has been shown that prelamin A accumulation at the nuclear envelope results in the sequestration of SREBP1 to the

nuclear envelope. This in turn has been proposed to reduce the pool available for the activation of PPARγ, thereby inhibiting adipogenesis [113].

The **stem cell** hypothesis proposes that mutations in *LMNA* impair the control of cell proliferation and regulation of the cell cycle and impose a senescent phenotype on adult stem cells that prevents their amplification and therefore, their capacity for regeneration [66, 106, 114]. It is proposed that tissues that require stem cells for regeneration and repair of ongoing damage, (e.g., those that undergo continuous mechanical stress or those that are required to support continuous growth) are the tissues that degenerate in HGPS patients, while the tissues not under mechanical strain do not develop a disease phenotype [114]. One thought is that HGPS cells undergo increased apoptosis due to more fragile nuclei and may in turn deplete stem cell pools [115, 114]. In adition, if the stem cells have a determined life span before they enter senescence, the regeneration capacity of the affected tissues may be exhausted due to the large number of cell cycles needed to repair the destroyed tissues [106]. This means that the tissue-specific stem cells, which are required to replenish the damaged tissues, cannot meet the repair needs and an accelerated process of tissue degeneration results [114]. Downstream targets of the Notch pathway, which is a major regulator of cell fate and stem cell differentiation, have been shown to be increased in stable cell lines expressing progerin fused to GFP [116], giving a direct link between the progeria nuclear defects and stem cell dysfunction. This theory can be applied to laminopathies involving tissues undergoing mechanical stress or that have continuous regeneration, such as heart, skin and adipose tissue.

Telomere length might be used as a biomarker for cellular aging and the telomeres in fibroblasts from HGPS patients are significantly shorter than young normal controls [117]. A change in lamina organization may cause accelerated telomere shortening and lead to rapid replicative senescence and progeroid phenotypes: this is seen in fibroblasts over-expressing mutant lamin A [118]. Telomere shortening has also been shown to suppress the proliferative capacity of stem cells *in vitro* [119]. This theory can be applied on progeroid laminopathies, such as HGPS or atypical Werner's syndrome, and on laminopathies that affect tissues under mechanical strain or with continuous regeneration.

Epigenetics has been defined as the inheritance of changes in gene function without any changes in the DNA sequence. Epigenetic patterns are now being found in aging tissues and cells, and a lot of research is focused on this field [120]. The best-known epigenetic mechanism is methylation, which gives rise to changes in

chromatin structure [121]. The chromatin changes are important for cellular regulation of processes such as transcription, replication and recombination. Histone methylation is regarded as a more long-term epigenetic mark with a relatively low turnover of the methyl group [122]. This theory can be applied to all laminopathies, and progerin expression has been shown to affect the epigenetic control of facultative and constitutive heterochromatin in HGPS. Cultured cells from HGPS patients and normal old individuals have reduced levels of lysine 9 trimethylation in histone H3 (H3K9me3) [100, 101], and the levels of lysine 27 trimethylation in histone H3 (H3K27me3), a marker of facultative heterochromatin, are also reduced in HGPS cell cultures [123]. Trimethylation of lysine 20 in histone H4 (H4K20me3), which is a marker of constitutive heterochromatin, is upregulated in both HGPS cells and old rats [124, 123]. This could possibly indicate a similar mechanism in normal aging and children with HGPS.

1.5 ANIMAL MODELS OF NUCLEAR ENVELOPATHIES

There are several published mouse models that are of special interest when working with nuclear envelopathies (see Table 1).

MOUSE MODELS OF NUCLEAR ENVELOPATHIES			
Mouse model	**Affected proteins**	**Phenotype**	**Refs**
Primary laminopathies			
Lmna$^{-/-}$	No expression of lamin A/C	Look normal at birth, but they later experience reduced growth, an abnormal gait, develop cardiac and skeletal myopathy and die at 8 weeks.	[125]
Lmna$^{LCO/LCO}$, *Lmna*$^{LCO/-}$	Two or one allele express(es) lamin C only	The mice are entirely healthy. No premature death.	[126]
BAC transgenic G608G	Over-expression of human lamin A/C and progerin	A BAC-transgenic mouse model carrying the G608G mutated human *LMNA* that shows no external phenotype of progeria, but demonstrates the progressive vascular abnormalities that closely resemble the most lethal aspect of the human phenotype. No premature death.	[127]
K14promotor - FLAG - progerin	Over-expression of progerin with FLAG tag	Transgenic mouse model with a K14 promoter has normal hair growth and wound healing. No premature death.	[128]

tetop-LA^{wt}; K5tTA	Over-expression of human lamin A	Inducible transgenic mouse model with a K5 promoter is indistinguishable from wild-type controls except for partial hair thinning. No premature death.	Paper I
tetop-LA^{G608G}; K5tTA	Over-expression of human lamin A and progerin	Inducible transgenic mouse model with a K5 promoter that has growth retardation, hair thinning and premature death at a median age of 7 and 29 weeks of age when transgenic expression was turned on at birth or at day 21, respectively.	Paper I, III
tetop-LA^{progerin}; K5tTA	Over-expression of progerin	Inducible transgenic mouse model with a K5 promotor that shows no signs of disease, except for mild hair thinning in older mice. No premature death.	Unpublished data
Lmna$^{HG/+}$	One allele expressing progerin and the other lamin A/C	A knock-in mouse model exhibiting growth retardation, bone disease, micrognathia, loss of subcutaneous fat and premature death. They die at ~28 weeks of age.	[129, 130]
Lmna$^{HG/HG}$, *Lmna*$^{HG/-}$	Two or one alleles expressing only progerin and no lamin A/C	The homozygous animals are very small with complete absence of adipose tissue and have many spontaneous bone fractures. They die at 3-4 weeks of age with poorly mineralized bones, micrognathia, abnormal skull shape and open cranial sutures. The mice expressing only one allele with progerin have a milder phenotype than the homozygotes, but more severe than *Lmna*$^{HG/+}$. They get multiple rib fractures and die at 10-14 weeks of age.	[130]
Lmna$^{HG/LCO}$	One allele expressing progerin and the other allele expressing lamin C and no lamin A	When comparing with the *Lmna*$^{HG/+}$ mouse model, the animals have improved weight curves, reduced number of rib fractures and increased survival. They die around 30 weeks of age.	[131]
Lmna$^{nHG/nHG}$, *Lmna*$^{nHG/+}$	Both alleles expressing nonfarnesylated progerin or one allele expressing nonfarnesylated progerin and the other allele expressing WT lamin A/C	The homozygotes have the same, but more severe, phenotype as the *Lmna*$^{HG/+}$ and the heterozygote mice and die at 17 weeks of age. The heterozygotes have the same, but milder, phenotype as the *Lmna*$^{HG/+}$ mouse model and die around 42 weeks of age.	[132]
Lmna$^{ggHG/+}$	One allele expressing geranylgeranylated progerin and one allele expressing WT lamin A/C	The mouse model elicits a milder disease phenotype with later onset and better survival than *Lmna*$^{HG/+}$ mice and they die around 32 weeks of age.	[133]
Lmna$^{N195K/N195K}$, *Lmna*$^{N195K/+}$	Both alleles express lamin A/C with a point mutation or one allele expresses	Homozygote mice have postnatal growth retardation and die at 12-14 weeks of age due to conduction defects. Heterozygote	[134]

	lamin A/C with a point mutation and one allele expresses WT lamin A/C	animals are indistinguishable from wild-type controls.	
Lmna$^{H222P/H222P}$, *Lmna*$^{H222P/+}$	Both alleles express lamin A/C with a point mutation or one allele expresses lamin A/C with a point mutation and one allele expresses WT lamin A/C	Heterozygote animals are indistinguishable from wild-type controls. Homozygote mice have adult growth retardation and muscular dystrophy and die between 4-13 months of age due to dilated cardiomyopathy.	[135]
Secondary laminopathies			
Zmpste24$^{-/-}$	No expression of Zmpste24	Mice lacking *Zmpste24* are defective in prelamin A processing. They look normal at birth but exhibit post-natal growth retardation, skeletal abnormalities, spontaneous bone fractures, abnormal teething, muscle weakness and premature death. They die at 20-30 weeks of age.	[136, 137]
Zmpste24$^{-/-}$ *Lmna*$^{LCO/+}$, *Zmpste24*$^{-/-}$ *Lmna*$^{LCO/LCO}$	No expression of Zmpste24, one allele expresses lamin C and one allele lamin A/C or no expression of Zmpste24 and both alleles only express lamin C	The mice were indistinguishable from wild-type controls. No premature death.	[126]
Other nuclear envelopathies			
Lmnb1$^{\Delta/\Delta}$	Lamin B_1 with internal deletion	The mice have growth retardation, bone and lung abnormalities and die shortly after birth.	[138]
Lap2$\alpha^{-/-}$	No expression of Lap2α	The mice were viable and indistinguishable from their wild-type littermates externally. When looking histologically a thickness in the paw epidermis (basal to granular layers) was found. No premature death	[139]
Lmna$^{L530P/L530P}$	Lamin A with L530P mutation, lamin A Δ exon 9, lamin A with aa from intron 9 with a stop in exon 10	The mice are normal at birth, but develop severe growth retardation and die within 4-5 weeks. They have skin changes, skeletal abnormalities and osteoporosis.	[140]

Table 1. A summary of the available mouse models for HGPS as well as other laminopathies and envelopathies.

1.6 TREATMENT OPTIONS IN HGPS

There are currently no available cures for children with HGPS. Growth hormones have been used for the treatment of a few patients and resulted in increased body weight and height. However, this only improves part of the phenotype, and is not considered a cure; therefore, other treatments need to be found [23]. A clinical trial

using farnesyltransferase inhibitors (FTIs) was initiated in May 2007 [141]. FTIs have previously been used for anti-cancer therapy [142-144]. FTIs block the farnesylation of proteins, including prelamin A, and thereby inhibit the production of mature progerin (see Fig. 3). The use of FTIs on HGPS cells has resulted in improved morphology of the nuclei and redistribution of progerin from the nuclear envelope to the nucleoplasm or intranuclear foci [145-148, 129, 149]. Treatment with FTIs in mouse models of HGPS and laminopathies, *Zmpste24*$^{-/-}$ and *Lmna*$^{HG/+}$, has resulted in a milder disease phenotype. Both mouse models exhibit increased body weight, a reduced number of rib fractures and increased survival, when compared to untreated animals of the same genotype. It is hard to predict if FTI treatment in these mouse models would be as effective for an existing phenotype, since the treatment was initiated before a significant disease phenotype developed [150, 130, 151]. Administration of FTIs in the BAC-transgenic mouse model, which carries the G608G mutation, significantly prevents both the onset of the cardiovascular phenotype as well as the late progression of existing cardiovascular disease [152].

Recently, it has been found that alternative processing by geranylgeranylation of prelamin A occurs instead of farnesylation in the presence of FTIs. The alternative processing leads to production of progerin [153]. A new way of preventing the maturation of progerin is using a treatment combined of statins and aminobisphosphonates that inhibits both the production of farnesyltransferase and geranylgeranyltransferase [154]. This treatment has also been used for anticancer therapy and it results in a clear improvement in morphology when administered to HGPS cells. *Zmpste24*$^{-/-}$ mice treated with this combination therapy markedly improved their aging-like phenotypes, including growth retardation, loss of weight, hair loss, bone defects and life span [153]. One clinical study is planned using this therapy, and participants are currently being recruited: another study, which is currently active, combines statins and aminobisphosphonates with FTIs [http://clinicaltrials.gov/ct2/results?cond=%22Progeria%22].

Additional treatment strategies include RNA interference (RNAi), which has been shown to down-regulate progerin production and improve cellular morphology when administered to HGPS cells. This treatment is currently not an option due to the difficulty of delivering the RNAi to all tissues [155, 156]. Modified oligonucleotides, morpholinos, targeting the cryptic splice site induce a normal cellular morphology in HGPS cells, rescue the cellular levels of lamina-associated proteins and reestablish proper expression of several misregulated genes [100]. This type of morpholinos has

been successfully used for delivery in animals and humans, and may therefore be used as therapy for HGPS patients [157]. The morpholinos will probably not completely inhibit the adverse splicing and some production of progerin will still occur.

1.7 SKIN STRUCTURE AND KERATIN EXPRESSION

The skin is composed of three primary layers: the epidermis, dermis and hypodermis (Fig. 4) [158]. The epidermal layer is divided into several layers, and different proteins are expressed in the cells of the different layers [159]. The layers and expression patterns of a few proteins are showed in Fig. 4. The epidermis consists of keratinocytes, but only the basal layer divides. The basal cells lose their proliferative potential when they detach from the basement membrane and start moving towards the skin surface. When the basal cells enter the spinous layer, they strengthen their cytoskeletal and intercellular connections, gaining resilience to mechanical stress. The cells then enter the granular layer, where they constitute the epidermal barrier. Finally, the cells become metabolically inactive and are flattened scales at the skin surface. The scales of the stratum corneum eventually shed from the skin surface and are replaced continually by inner layer cells moving outward [159, 160]. The keratins are often expressed in pairs in the different skin layers [159]. Our construct is expressed under a keratin 5 (K5) promoter. K5 is normally expressed together with K14 and for example, is expressed in the basal layer of the epidermis in skin and the outer root sheath of the hair follicle (HF). Additionally K5 is also expressed in the myoepithelial cells of the salivary gland, the basal and suprabasal cells of the esophagus, stomach, tongue, nose cavity and trachea [161, 162]. K5 expression is also found in the ameloblasts in the teeth [163]. Keratin 6 is normally expressed in the inner root sheath of the HF, but is also seen in the spinous layer of hyperproliferative epidermis [164, 165].

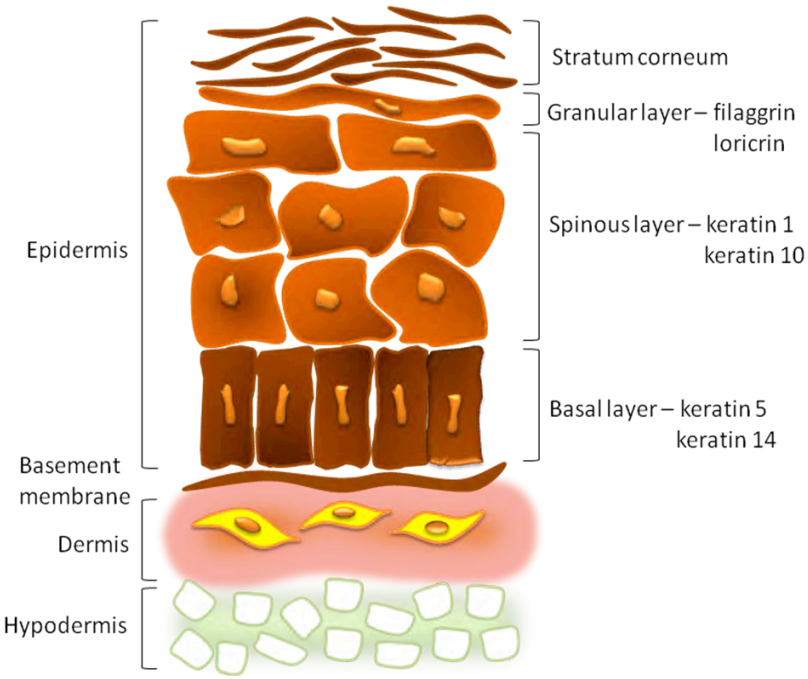

Figure 4. Schematic representation of the different layers of the skin and expression of various proteins. (Picture was inspired by [159, 165, 160]).

1.8 HAIR CYCLE

Mammalian hair growth is not continuous, but cyclic. To have constant hair growth the hair cycle goes through three stages: anagen (growing), catagen (regression) and telogen (resting). In anagen, there is rapid proliferation in the bulb, and new hair shafts are produced that grow and differentiate. In catagen, there is extensive apoptosis, and in telogen, there is no significant activity. The different phases are of different lengths. The follicle is contiguous with the epithelium and is separated from the dermis by a basement membrane. The HF varies in length during the different phases: in telogen, the resting HF is completely localized in the dermis; during anagen, the HF grows down into the hypodermis; and in catagen, it regresses back into the dermis. The total thickness of the skin varies during hair cycling and during the growing phase, the skin is thicker than when the HFs are resting. The hair cycle can be affected by many different factors including genetic background, sex, environmental factors and nutrition [166, 167].

1.9 TETRACYCLINE-CONTROLLED TRANSCRIPTIONAL REGULATION SYSTEMS

The tetracycline-controlled transcriptional regulation system can be utilized for spatial and temporal expression of a desired transgene. The expression can be regulated by adding or removing doxycycline (dox) to the system. It consists of two parts: (i) the regulatory part consisting of the transcriptional transactivator (tTA) or reverse

tetracycline transactivator (rtTA), which is constitutively expressed under a promoter of choice, and (ii) the gene of interest linked to tet-operator (tetop) binding sites for the tTA or rtTA. In the absence of dox, a tetracycline derivative, tTA, binds to the tetop and activates transcription of the downstream gene. In the presence of dox, tTA undergoes a conformational change and dissociates from the tetop, resulting in termination of transcription of the target gene. rtTA works in the opposite way i.e., gene expression is active in the presence of dox (see Fig. 5) [168, 169].

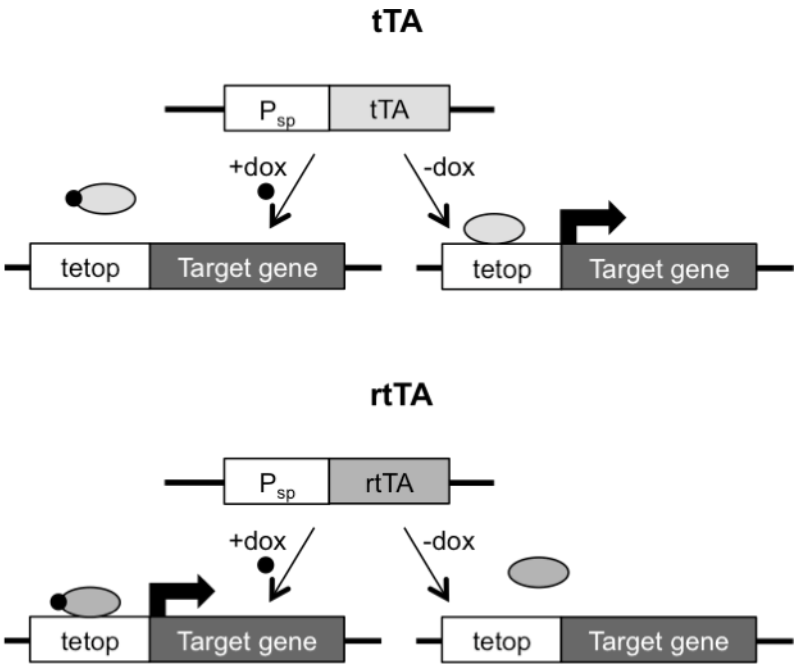

Figure 5. Schematic of the regulation of the tet-off and tet-on system. P_{sp} = specific promoter.

2 METHODOLOGY

2.1 LABORATORY ANIMALS

Animals were used in accordance with the guidelines for care and use of experimental animals approved by Stockholms Södra Djurförsöksetiska Nämd. Mice had access to water and were supplied with a standard diet *ad libitum*. Bitransgenic animals and controls received dissolved pellets on the cage floor and/or dox in their drinking water (100 µg/ml, 2.5% sucrose), which was changed every third day and wrapped in foil. Intercross breedings received dox water, which was removed at birth (d0) or at weaning at postnatal day 21 (d21).

2.2 SCREENING OF FOUNDER LINES

Three different minigene constructs (see Fig. 6) were generated and injected in embryos to create different founder lines. Bitransgenic animals from the intercrossed founder lines were analyzed by Western blot and immunofluorescence (IF) for transgenic expression in biopsies from both dorsal skin and the tail.

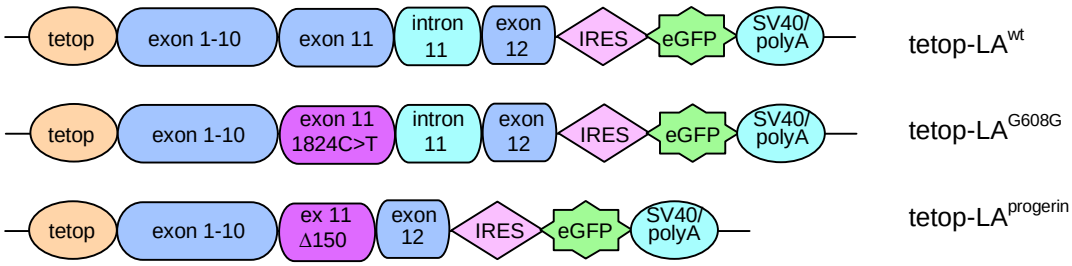

Figure 6. Schematic of the minigene constructs injected into the mice. The top construct, tetop-LA^{wt}, over-expresses human wild-type lamin A; the middle construct, tetop-LA^{G608G}, over-expresses human wild-type lamin A and progerin; and the bottom construct, tetop-LA^{progerin}, over-expresses progerin. All of the constructs contain a tet-operator (binding site for the transactivator), an IRES (internal ribosomal entry site) that allows translation initiation in the middle of the mRNA, eGFP (enhanced green fluorescent protein) and a polyA tail, which is important for the nuclear export, translation and stability of mRNA.

2.3 PCR-GENOTYPING

DNA was extracted from mouse-tail biopsies using standard phenol-chloroform protocol [170]. Genotyping was performed with PCR for the lamin A (LA) minigenes (tetop-LA^{G608G}, tetop-LA^{wt} and tetop-LA^{progerin}) [95] and K5tTA [162]. PCR with primers for Myc was used as a positive control for quality and presence of genomic DNA. See Table 2 for primers and size of PCR products. See Table 3 and 4 for protocol of PCR mixes and run programme.

PCR GENOTYPING			
	Forward primer (F)	**Reverse primer (R)**	**Product size**
LA	5´-GCAACAAGTCCAATGAGGACCA-3´	5´-GTCCCAGATTACATGATGC-3´	961/ 489 bp
K5tTA	5´-CTCGCCCAGAAGCTAGGTGT-3´	5´-CCATCGCGATGACTTAGTAA-3´	199 bp
Myc	5´-CTGAATTGGAAAACAACGAAAAG-3´	5´-AAGTCCTTTTCAGAGGTGAGCTT-3´	106 bp

Table 2. The primers used for genotyping of transgenic mice and expected size of the PCR fragment. bp = base pair. PCR on animals from tetop-LA^{wt} and tetop-LA^{G608G} gives a fragment of 961 bp, while PCR on tetop-LA^{progerin} animals gives a fragment size of 489 bp.

PCR MIX (1 reaction)			
	Myc (µl)	**LA** (µl)	**K5** (µl)
S-H_2O	7.2	7.1	10.1
10x PCR buffer	2	2	2
5x Q-buffer	4	4	1
25 mM $MgCl_2$	1.2	1.2	1.2
Myc F (10 pmol/µl)	1	-	-
Myc R (10 pmol/µl)	1	-	-
LA F (10 pmol/µl)	-	1	-
LA R (10 pmol/µl)	-	1	-
K5 F (10 pmol/µl)	-	-	1
K5 R (10 pmol/µl)	-	-	1
10 mM dNTP	0.4	0.5	0.5
Amplitaq Gold enzyme (5 U/µl)	0.2	0.2	0.2
DNA	3	3	3

Table 3. PCR reaction mixes for genotyping.

PCR PROGRAM		
Temp	**Time**	**Cycles**
94 °C	15 min	1
94 °C	45 sec	35
57 °C	45 sec	
72 °C	1 min	
72 °C	10 min	1
4 °C	∞	1

Table 4. PCR run program for genotyping.

2.4 SOUTHERN BLOT AND COPY NUMBER QUANTIFICATION

A Southern blot was performed on the different founder lines to analyze the copy number of the integrated minigenes. First, 10 µg of genomic DNA was digested with *Sac*I in a 200 µl reaction at 37 °C overnight, and the enzyme was then heat inactivated at 65 °C for 20 min. The samples were concentrated to 30 µl using a vacuum centrifuge. Before loading the samples to the gel, they were heated to 56 °C for 2-3 min, spun down in a microcentrifuge and loading dye was added to the sample. The samples were loaded on a 1% agarose gel and run at 40 V overnight or for 1 hour and then at 60 V for 6 hours. The gel was soaked in running buffer containing EtBr and a picture was taken with a ruler. The gel edges were trimmed off and the size of the gel was measured. The gel was denatured (1.5 M NaCl, 0.5 M NaOH) 2×20 min and then neutralized (0.5 M Tris-HCl, 1 M NaCl) 2×20 min on a rocking table with fresh solution. The gel was then rinsed in MQ-water and transferred (0.4 M NaOH, 0.7 M NaCl) to a Hybond N+ membrane according to standard procedure [170]. After transfer, the wells of the gel were marked on the nitrocellulose membrane and it was soaked in 5×SSC for 30 sec and then pre-hybed at 65 °C for 30-45 min. The probe was prepared using Ready-To-Go labeling beads (dCTP, Amersham) and incubated with the membrane at 65 °C overnight. The filter was then washed with 2×SSC, 0.1% SDS for 25 min at 65 °C and with 1×SSC, 0.1% SDS for 10 min at 65 °C. The filter was then put in a cassette and was subjected to film and phosphoimaging. The probe was created using PCR of human *LMNA* (exon 11, intron 11 and exon 12 to the stop codon) with primers 5´-ACCCCGCTGAGTACAACCT-3´ and 5´-ACATGATGCTGCAGTTCTGG-3´. The PCR product was TA cloned (TOPO TA-cloning kit, Invitrogen) and digested with *Eco*RI, to release a fragment of 609 bp that was gel purified (Wizard SV Gel and PCR Clean-Up System, Promega) and used as a probe for the LA minigenes. Depending on the number of minigenes integrated, different sizes were detected on the filters. For single transgenic integration the probe hybridized to a fragment larger than 3,387 bp, with a size dependant on the location of next *Sac*I site following the integration site. For multiple minigene integrations in tandem, the probe hybridized to an additional fragment of 3,690 bp (see Fig. 7). All filters contained human genomic DNA digested with *Sac*I where the probe bound to a fragment of 4,449 bp. To calculate the copy number of the transgenes, a comparison was made to the *Sac*I digests of non-transgenic genomic DNA that were spiked with different amounts of plasmid (1, 5, 10 and 20 copies estimated per genome), which

contained the tetop-LA^{G608G} transgenic construct. The probe hybridized to a fragment of 7,034 bp in the spiked DNA. Calculations for the estimation of transgenic copy number were in accordance with recommendations from the Gene Targeting and Transgenic Facility (University of Virginia Health System).

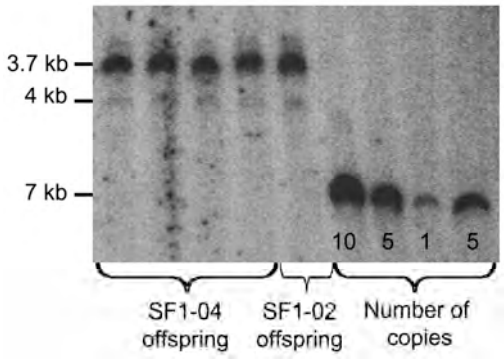

Figure 7. Picture of a Southern blot with DNA from two different mouse strains with four copies of the transgene integrated and genomic DNA spiked with plasmid.

2.5 ANIMAL TISSUE COLLECTION AND PROCESSING

Animals were sacrificed by an overdose of isoflurane and cervical dislocation. The tissues were frozen in liquid nitrogen or fixed in 4% paraformaldehyde (pH 7.4) at 4 °C overnight (or 4 hours at room temperature [RT] for biopsies). The tissues were then dehydrated in 70% ethanol and bigger tissues, e.g., the skin (see Fig. 8), were divided into sections. The skin, gastrointestinal system, liver, pancreas, spleen, thymus, mammary glands, salivary glands, brown fat, tear glands, kidneys, adrenal glands, reproductive organs, skull, tongue, brain, the respiratory mucosa of the nasal cavity, trachea and lungs were analyzed for pathology. The tissues were further processed in a vacuum infiltration processor (V.I.P., Sakura Tissue Tek) before they were embedded in paraffin. The embedded tissues were sectioned using a microtome (Microm HM 355 S, Thermo Scientific) to 4- to 5- µm sections and dried at 59 °C on Superfrost plus gold slides (Menzel-Gläser) for IHC and IF and at 37 °C on Superfrost slides (Menzel-Gläser) for haematoxylin (htx), overnight.

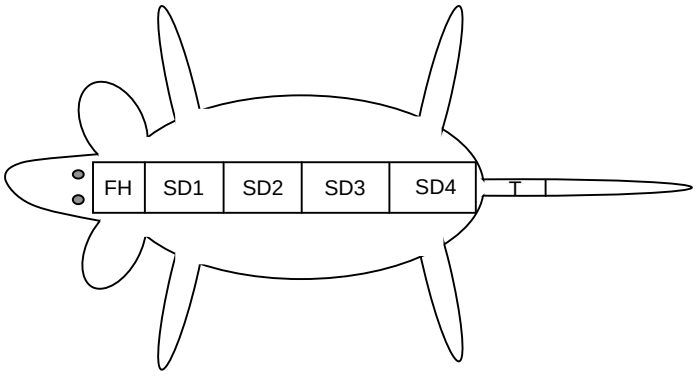

Figure 8. Schematic of how the dorsal skin of the mice was divided into different anatomical regions.

2.6 IMMUNOHISTOCHEMISTRY

In this thesis, the term immunofluorescence (IF) is used for all immunohistochemistry (IHC) done with antibodies tagged with fluorophores, while the term IHC is used for the other forms of immunochemical staining.

IHC/IF was used to analyze the expression of certain proteins in different cells and in different layers of the skin and other tissues/organs; negative controls (only secondary antibody) and positive controls (tissues of known immunoreactivity) were used.

Several different methods have been tested for antigen retrieval for IHC and IF. To obtain even staining, we microwaved sections in citrate buffer for IHC and used a pressure cooker with EDTA for IF. In general, we learned that it is very hard to get good epitope exposure of the lamin proteins, especially the JOL2 antibody against lamin A/C.

PRIMARY ANTIBODIES USED FOR IHC AND IF				
Antibody	**Host**	**Clone or cat. no.**	**Company**	**Paper**
monoclonal anti-human lamin A+C	mouse	JOL2	Chemicon	I, III
polyclonal anti-keratin 5	rabbit	PRB-160P	Covance	I, III
polyclonal anti-keratin 6	rabbit	PRB-169P	Covance	I, III
polyclonal anti-phosphohistone H3	rabbit	06-570	Upstate cell signaling solutions	I, III
monoclonal anti-cleaved caspase 3	rabbit	5A1	Cell Signalling	I
polyclonal anti-adipophilin	guinea pig	GP40	Progen	I, III
polyclonal anti-loricrin	rabbit	AF 62	Covance	I, III
polyclonal anti-filaggrin	rabbit	PRB-417P	Covance	I
polyclonal anti-keratin 1	rabbit	AF 109	Covance	I
polyclonal anti-keratin 10	rabbit	PRB-159P	Covance	I, III
polyclonal anti-lamin A/C	rabbit	2032	Cell Signalling	II
polyclonal anti-lamin B	goat	M-20	Santa Cruz Biotechnology	II

Table 5. Summary of primary antibodies used for IHC and IF in the different papers.

After antigen retrieval, the IHC continued with elimination of endogenous peroxidase activity in methanol (containing hydrogen peroxide) and then blocking with goat serum in PBS. Thereafter, the slides were incubated with the primary antibody (see Table 4 for antibodies used) diluted with BSA in PBS for 1 hour at RT or 4 °C overnight. After washing, a biotinylated secondary antibody diluted with goat serum in PBS was added. The Vectastain ABC kit (Vector Laboratories) and DAB solution (Dakocytomation, Dako) were used for peroxidase visualization according to the manufacturers recommendations. Nuclear counterstaining was done using htx and the slides were mounted with PERTEX (Histolab) (see Fig. 9).

The IF continued with a blocking step in normal goat serum followed by a mouse-to-mouse blocking reagent (SCYTEK Laboratories, if the primary antibody was generated in mouse) and antigen retrieval. The primary antibody (see Table 5 for antibodies used) was incubated at 4 °C overnight and the fluorescent secondary antibody was added after the sections were washed. Following an incubation at RT, the slides were mounted with Vectashield mounting medium, which contains dapi and is good for maintaining fluorescent staining by preventing photo bleaching (Vector Laboratories) (see Fig. 9).

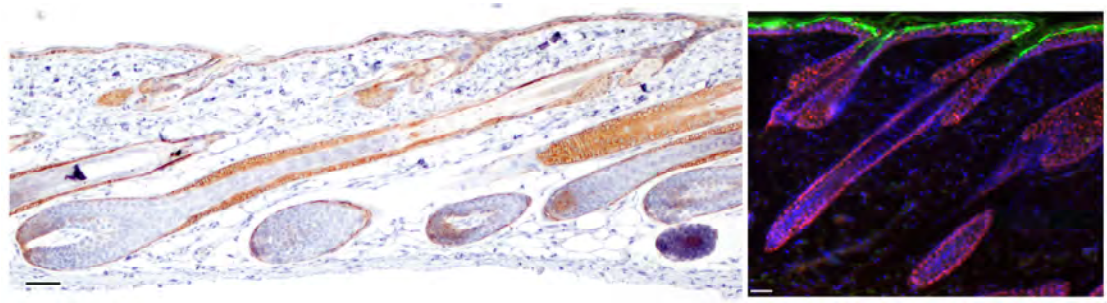

Figure 9. The left picture shows IHC of dorsal skin in a wild-type mouse with the K5 antibody (brown), counterstained with htx. The right picture shows IF staining of lamin A/C (red) and loricrin (green) in dorsal skin of a bitransgenic mouse with the G608G mutation, counterstained with dapi. Scale bar = 50 µm.

2.7 HAIR FOLLICLE DENSITY

4- µm skin sections were processed for htx and eosin staining and by looking in a light microscope the number of HFs per mm section was counted in sections from 4-6 different anatomical regions of dorsal skin; within forehead (FH) and dorsal skin 1 and 2 (SD1, SD2) (see Fig. 8) in 3 bitransgenic animals and 3 wild-type controls.

2.8 RNA EXTRACTION

RNA was extracted from mouse tissues using Trizol (~100 mg tissue) or a Micro-FastTrack 2.0 mRNA isolation kit (3 mm^2 biopsies from dorsal skin) (Invitrogen). The tissues were cut into smaller pieces on dry ice, homogenized in 1 ml Trizol using Lysing Matrix D and Fastprep 220A (setting 6, 40 sec) (Qbiogene) and then put on ice for 10 min. After an additional step of Fastprep 220A, the samples were put on ice again for 10 min and centrifuged for 10 min at 12,000×g at 4 °C. The supernatants were transferred to new tubes and incubated 5 min at RT before 0.2 ml chloroform was added. After vortexing, the tubes were incubated at RT for 2-3 min and then centrifuged for 15 min at 12,000×g at 4 °C. The see-through supernatants were transferred to new tubes containing 0.5 ml isopropanol, vortexed, incubated at RT for 10 min and centrifuged for 10 min at 12,000×g at 4 °C. The supernatants were removed and the RNA pellets were washed once with 75% ethanol. The tubes were again centrifuged for 5 min at 7,500×g at 4 °C. The pellets were dried on ice and dissolved in 100 µl RNase-free water and then incubated for 10 min between 55 °C and 60 °C. RNA was extracted from cell cultures using Trizol (Invitrogen) according to the manufacturer's standard procedure. The RNA concentration was quantified using a spectrophotometer (BioPhotometer, Eppendorf), and 20 µg RNA was treated with DNaseI at 37 °C for 30 min. A stop solution was then added and the samples were incubated at 65 °C for 10 min (RQ1 RNase-Free DNase, Promega). DNase was removed using RNeasy mini columns (Qiagen), and the RNA concentration was measured again.

2.9 RT-PCR

Total RNA of 800 ng was reverse transcribed with 50 ng random hexamers using the Superscript cDNA Synthesis Kit (Invitrogen). Transgenic expression in animal tissues was analyzed using PCR with primers against human lamin A and LAΔ150 (see Table 6). PCR with primers against the housekeeping gene TBP (see Table 6) was used on all samples. See Tables 7, 8 and 9 for PCR mixes and run programs.

cDNA PCR			
	Forward primer (F)	**Reverse primer (R)**	**Product size**
LA + progerin	5´-ACTGCAGCAGCTCGGGG-3´	5´-AGTTCTGGGGGCTCTGGGT-3´, 5´-TCTGGGGGCTCTGGGC-3	276/ 123 bp
TBP	5´-TGGGCTTCCCAGCTAAGTTC-3´	5´-GGAAATAATTCTGGCTCATA GCTACTG-3´	136 bp

Table 6. Transgenic expression of human lamin A and progerin was detected using the listed primers on cDNA.

PCR MIX (1 reaction)		
	Progerin + LA (µl)	**TBP (µl)**
S-H_2O	To 50 µl	To 50 µl
10x PCR buffer	5	5
5x Q-buffer	10	10
LA R (10 pmol/µl)	0.5	-
progerin F (10 pmol/µl)	1	-
progerin R (10 pmol/µl)	2	-
TBP F (10 pmol/µl)	-	1
TBP R (10 pmol/µl)	-	1
10 mM dNTP	0.5	0.5
Hot Star Taq enzyme (5 U/µl)	0.3	0.3
cDNA	X	Y

Table 7. PCR reaction mixes for detection of human lamin A and progerin on cDNA.

PCR PROGRAM LA + progerin		
Temp	**Time**	**Cycles**
94 °C	15 min	1
94 °C	30 sec	40
60 °C	30 sec	
72 °C	30 sec	
72 °C	7 min	1
4 °C	∞	1

Table 8. PCR run program for LA + progerin.

PCR PROGRAM TBP		
Temp	**Time**	**Cycles**
94 °C	15 min	1
94 °C	30 sec	40
58 °C	30 sec	
72 °C	1 min	
72 °C	10 min	1
4 °C	∞	1

Table 9. PCR run program for TBP.

2.10 QUANTITATIVE RT-PCR (TAQMAN©)

Taqman assays measure PCR product accumulation using a dual-labeled fluorogenic probe. One fluorescent dye serves as a reporter (5´- 6FAM, Applied Biosystems), and its emission spectra is quenched by the second dye [3´- nonfluorescent quencher dye (NFQ, Applied Biosystems)]. A sequence detector continuously measures the of fluorescent spectra of a 96-well plate in the thermal cycler during the PCR amplification, resulting in the real-time monitoring of the reactions. We used amplicon-specific primer pairs (see Table 10) and the housekeeping genes ß-glucoronidase and ribosomal protein large PO as controls with VIC and NFQ (Applied Biosystems) as the reporter and quencher dyes, respectively. The absolute copy numbers could be determined for the samples using a cloned standard curve of individual amplicons [171]. All samples and non-sample controls were run in triplicate and one primer per assay was designed to cover an exon-exon border to avoid amplification of genomic DNA. Each assay was optimized for primer and probe concentrations (see Table 10).

cDNA PCR					
	Forward primer (F)	**Primer conc.**	**Reverse primer (R)**	**Primer conc.**	**Product size**
LA	5´-TCTTCTGCCTC CAGTGTCACG-3´	300 nM	5´-AGTTCTGGGGG CTCTGGGT-3´	900 nM	139 bp
LC	5´-CAACTCCACTG GGGAAGAAGTG-3´	300 nM	5´-CGGCGGCTACC ACTCAC-3´	900 nM	123 bp
LAΔ150	5´-ACTGCAGCAGC TCGGGG-3	50 nM	5´-TCTGGGGGCTC TGGGC-3	300 nM	123 bp
Tagman probes				**Probe concentration**	
LA	5´-6FAM-ACTCGCAGCTACCG-MGMNFQ-3			200 nM	
LC	5´-6FAM-ATGCGCAAGCTGGTG-MGMNFQ-3			200 nM	
LAΔ150	5´-6FAM-CGCTGAGTACAACCT-MGMNFQ-3			200 nM	

Table 10. Primers and probes used for Taqman and expected product sizes.

2.11 PROTEIN EXTRACTION

Protein was extracted from mouse tissues using RIPA (including proteinase inhibitors, Roche), where the tissues were ground with needles, or with 8 M UREA, 5% RIPA (including proteinase inhibitors, Roche) for better extraction of the sticky lamin proteins. Samples were homogenized with Lysing Matrix D and Fastprep 220A

(Qbiogene). The tissues were first cut into smaller pieces on dry ice, homogenized in 1 ml 8 M UREA using Lysing Matrix D and Fastprep 220A (setting 6, 40 sec) (Qbiogene), put on ice for 10 min and after a repeated step of Fastprep 220A, the samples were put on ice again for 10 min and centrifuged for 20 min at 12,000×g at 4 °C. The supernatants were transferred to new tubes and stored at -80 °C. Protein was extracted from cells using RIPA (including proteinase inhibitors, Roche). The cells were trypsinized, centrifuged for 10 min at 100×g at 4 °C and washed twice with 5 ml of PBS, centrifuging between the steps as before. After the final centrifugation, the PBS was removed and the pellets were resuspended in 70 µl RIPA (for a T25 flask) and transferred to eppendorf tubes. The tubes were vortexed and incubated on ice for 20 min (with occasional vortexing). After centrifugation for 10 min at maximum speed at 4 °C, the supernatants were transferred to new tubes and stored at -80 °C.

2.12 WESTERN BLOT

Mini-gel Western blots were done using precast 8% Tris-Glycine mini gels (Invitrogen) in an XCell *SureLock* Mini-Cell (Invitrogen). To enhance protein separation between the bands, as the lamin proteins are very close in sizes, we used a large Western blot system (PROTEAN II xi Cell, Bio-Rad). We used 1 mm thick, 20 cm long 4%/7.5% discontinuous Laemmli slab gels. The gels were run for 12 hours at 25 mA using a cooling system at 2 °C, according to the manufacturer's instructions. Protein transfer to a nitrocellulose membrane (Hybond-C +, Amersham Biosciences) with blot pads (Bio-Rad) on both sides of the gel and membrane was made using a Semidry Transfer Cell (Bio-Rad) at 24 V for 40 min in 200 ml tris/glycine transfer solution (Bio-Rad). After transfer, the filters were blocked in TBS-T, 5% milk for at least 1 hour at RT and then incubated with primary antibody (see Table 11) diluted in blocking at 4 °C overnight. After washing with TBS-T, the filters were incubated with secondary antibody diluted in TBS-T, 5% milk for 45 min at RT. All of the washing steps and antibody incubations were done on a rocking table. Following another washing step with TBS-T, ECL plus (Amersham Biosciences) was added to the filters for 5 min at RT. Directly following this step, the filters were exposed to film for different lengths of time.

PRIMARY ANTIBODIES USED FOR WESTERN BLOT				
Antibody	**Host**	**Clone or cat. no.**	**Company**	**Paper**
monoclonal anti-human lamin A+C	mouse	JOL2	Chemicon	I, III, IV
polyclonal anti-prelamin A	goat	C-20	Santa Cruz Biotechnology	I
polyclonal anti-lamin A/C	goat	N-18	Santa Cruz Biotechnology	I, IV
polyclonal anti-gfp	rabbit	ab290	Abcam Ltd	I
monoclonal anti-gfp	mouse	B-2	Santa Cruz Biotechnology	I
monoclonal anti-ß-actin	mouse	AC-15	Sigma	I, III, IV
monoclonal anti-lamin A	mouse	133A2	Abcam Ltd	IV
anti-lamin C	rabbit	BP4505	Acris antibodies	IV

Table 11. Summary of primary antibodies used for Western blot.

2.13 DENSITOMETRY

Protein quantification was performed on Western filters using the Versa Doc Imaging systems (Bio-Rad) and analyzed using the Quantity One software (Bio-Rad). Relative band intensities were normalized to the housekeeping gene β-actin in the same lane. Only samples on the same filter were compared with each other. Densitometry was made on films that were not overexposed.

3 AIMS OF THE THESIS

The overall aim of this thesis was to gain a better understanding of the molecular mechanisms of HGPS and to investigate connections between *LMNA* expression and normal aging.

The specific aims were:

I.
- To develop an inducible mouse model for HGPS that allows temporal and spatial regulation of the expression of progerin.
- To get a better understanding of the ongoing molecular process during the development of the HGPS skin phenotype.

II.
- To establish the normal expression pattern of lamin A/C and B in mouse skin and during hair cycling.
- To see if progerin expression in mouse skin alters the expression of lamin B or the progress of the hair cycle.

III.
- To test if progerin causes irreversible disease or if it is possible to reverse an existing phenotype in HGPS mice if the progerin production is eliminated.

IV.
- To analyze differences in the expression of *LMNA* locus transcripts in samples from progeria patients and controls.
- To quantify changes in expression levels of *LMNA* locus transcripts during *in vitro* aging in samples from progeria patients and controls.

4 RESULTS AND DISCUSSION

4.1 PAPER I

The aim of this study was to to gain a better understanding of the molecular mechanisms causing the HGPS disease phenotype by generating a progeria mouse model with spatial and temporal regulation of the transgene. Since the first phenotype after failure to thrive in children is seen in the skin, we decided to direct our transgenic expression to the skin.

We generated two transgenic mouse lines with minigenes of human lamin A, one carrying the human wild-type lamin A and the other the HGPS point mutation (G608G). We used a tetracycline-regulated system and a mouse strain with keratin 5 as a promoter. An obvious connection between the level of transgenic expression and disease phenotype was identified during the screening of several founder lines of the G608G strains. We did not find any pathologic changes, even up to two years of age in founder lines with low expression. When the transgenic expression was initiated at postnatal day 21 in the bitransgenic mice of the G608G strain with high expression, there was first an intermediate hyperproliferative stage with regions of milder or more severe epidermal hyperplasia associated with hypergranulosis and hyperkeratosis, which was evident after 6 weeks of transgenic expression. There were dystrophic changes in the HFs as well as enlarged and displaced sebaceous glands. Inflammatory cells and, in more severe cases, fibrosis were present in the dermis. The disease developed further into an end stage with loss of the hypodermis, fibrosis of the dermis and hypoplastic sebaceous glands within 17 weeks of transgenic expression.

Bitransgenic mice with the G608G mutation had an external phenotype of hair thinning and reduced weight. They also died prematurely. The median age of death was 14 weeks for bitransgenic G608G mice when transgenic expression was turned on at postnatal day 21 (d21) and 7 weeks when the transgenic expression was turned on at the day of birth (d0). When adding dissolved food on the cage floor the median survival increased to 29 weeks for animals with transgenic expression from d0. There were no changes in weight or survival in the bitransgenic animals expressing only the wild-type lamin A.

IHC with antibodies against keratins 5 and 6 on sections with severe epidermal hyperplasia showed the mislocalization of these proteins, which indicates hyperproliferation, and antibodies directed against phosphohistone H3 (PHH3)

showed increased proliferation. Analysis of cleaved caspase 3 showed no change in the amount of apoptosis. When looking at the differentiation markers keratin 1 (K1), keratin 10 (K10), filaggrin and loricrin, we found normal expression of K1 and K10 in the spinous layers, as in wild-type controls, except for a few cells seen in the basal layer. Loricrin and filaggrin were located in the granular layer where they are normally found, but filaggrin expression was also seen in the dermis of bitransgenic skin. This indicated that there was normal differentiation of mouse skin with epidermal hyperplasia, but the granular and spinous layers were thicker than normal.

When performing histopathology of organs other than skin, the only significant changes that were found included possible mild hyperplasia of the stomach as well as changes in the lower incisors and the surrounding tissue. The latter consisted of embedding and food in the pulp as well as acute inflammatory reactions and necrosis.

We have developed a tissue-specific transgenic mouse model for HGPS in which transgenic expression can be regulated temporally with dox in the drinking water. By over-expressing progerin in the skin, this mouse model develops a disease phenotype similar to that seen in HGPS patients. Therefore, this model should be very useful for understanding the disease progression seen in the skin in children with progeria as well as for testing possible treatments.

4.2 PAPER II

Various skin changes are seen in MAD, HGPS and RD, and progeria patients also experience alopecia. To get a better understanding the pathological mechanisms behind the skin phenotypes seen in these laminopathies, it is important to increase our understanding of normal lamin A/C and B expression in different cells of the skin during hair cycling. We also see hair thinning in our bitransgenic animal model of HGPS and decided to examine if there are any alterations in the progression of the hair cycle or changes in lamin B expression. Lamin B is believed to interact with lamin A in the nuclear lamina and prior *in vitro* studies of HGPS cells have shown alterations in lamin B expression. To establish any changes, we first had to classify the different stages of the hair cycle and the expression of lamin A/C and B in different skin compartments in wild-type mice during the different phases of the hair cycle. Therefore, the dorsal skin of wild-type FVB/NCrl mice was collected at different time points, from the day of birth (d0) to postnatal day 70 (d70).

The midline dorsal skin was divided into equal portions, starting from the ears

and caudally towards the tail (see Fig. 8). The different stages and substages of the hair cycle were defined using previously published morphology guidelines. d0-d12.5 mice showed developing skin with various stages of anagen (growing) HFs. The first postnatal hair cycle started at d15 when the first catagen (regressing) HFs were seen, and was followed by telogen (resting) HFs in d19 and d20 animals. Anagen follicles were again seen in d21 animals. The second post-natal hair cycle was initiated at d35 with catagen, the telogen phase was seen at d42-d70, but anagen HFs were also seen in regions SD1-3 of the dorsal skin at d70. On the same days, different HFs could be seen in different regions of the dorsal skin. Although HF morphogenesis and the first postnatal catagen, telogen and anagen follow a strict timeline, there are a number of variables that influence these processes (e.g., gender, strain background, and nutritional and environmental factors). We did not see any significant differences in the hair cycle between mice of the same age or different sexes, since all other conditions were kept constant during our study.

IHC with antibodies directed against lamins A/C and B (the antibody hybridized to lamin B_1, with minor cross-reactivity with lamin B_2 and B_3) showed strong expression in the basal cells of the epidermis, outer root sheath and the dermal papilla in all stages of the hair cycle. There was a constantly lower expression of lamins A/C and B in the suprabasal cells compared to the basal cells, which contradicts previous studies of human skin where lamins A and C were predominantly found in the suprabasal cells of the epidermis or at similar levels in all layers of the epidermis. The expression of lamin B has been described at high levels in basal cells and lower expression in the suprabasal cells, as in our study, or a similar expression pattern throughout the different layers or at higher levels in the suprabasal cells of the human epidermis. The variance may be due to different experimental procedures or differences of expression in human and mouse skin. The hypodermal cells only weakly stained for lamins A/C and B during all stages of the hair cycle. The sebaceous glands had even staining of lamins A/C and B in catagen and telogen, while there was a higher expression of lamin B in anagen. There was medium staining of lamins A/C and B during catagen. It is interesting that there was high expression of lamins A/C and B in the dermal papilla and outer root sheath during all stages of the hair cycle. The dermal papilla is believed to control the size of the HF, and may also be the regulator of the hair cycle. The outer root sheath contains cells that are especially important for regeneration of the epidermis after injury.

When looking in our inducible animal model for HGPS at postnatal days 15, 19

and 21, we saw no differences in hair cycle progression or expression of lamin B compared to wild-type controls bred on or off dox. This shows that expression of the progeria mutation does not lead to immediate alterations in lamin B expression or hair cycle progression.

4.3 PAPER III

The aim of this study was to see if it was possible to revert and inhibit the progression of an existing progeria disease phenotype, and thereby get an idea if there is any hope of curing children with HGPS. We took advantage of the inducible transgenic mouse model with the G608G mutation driven by the K5 promoter. After a severe disease phenotype developed at 7 weeks of age, we administered dox in the drinking water to turn off transgenic expression. Transgenic suppression was initiated at week 7 and animals were collected at 7, 13 and 20 weeks of age to see if there was a reversal of the phenotype.

Using RT-PCR and Western blot we saw that the lamin A and progerin expression (RNA and protein) was strong at 7 weeks of age in bitransgenic animals, before turning off the transgenic expression, as expected. Strong expression of human lamin A, following the K5 expression, was seen in bitransgenic animals in IF skin sections. After 6 and 13 weeks of transgenic suppression, lamin A RNA expression was almost completely turned off, and no expression was seen on the protein level using Western blot. Only a few positive cells in a few HFs were seen using IF on mouse skin. This indicates that the dox treatment efficiently turns off the transgenic expression.

At 7 weeks of age, bitransgenic animals had lower weight, growth retardation, hair thinning and skin crusting. Improvement in hair thinning and skin crusting was seen within only one week of dox treatment, and the external skin looked completely normal in most of the animals after 6 and 13 weeks of transgenic suppression. A small increase in weight was seen at dox initiation, following the trend of the wild-type curve, but the average weight still remained lower than wild-type controls. However, the animals have already passed the major growth phase at this point, and transgenic suppression may therefore not have any profound effect on body weight. We also saw a complete overlap with bitransgenic animals, in which the transgene was not suppressed, indicating that the transgenic suppression had little, if any, effect on weight.

Analysis of skin pathology in bitransgenic animals at 7 weeks of age showed varying degrees of epidermal hyperplasia, hyperkeratosis, hypergranulosis and enlarged

and displaced sebaceous glands. The presence of inflammatory cells and fibrosis were seen in the dermis. After 6 and 13 weeks of transgenic suppression, the skin pathology was close to normal and only a few regions with mild epidermal hyperplasia, hyperorthokeratosis, fibrosis and presence of inflammatory cells were found. This might indicate ongoing or previous wound healing where some degree of inflammation is needed to reverse established and progressive fibrosis. Established fibrosis may be hard to reverse and can lead to the presence of permanent fibrotic scars.

Analysis of jaw pathology showed a non-mineralized enamel matrix, embedded hairs and regions with acute inflammatory reactions in the lower jaws of 7-week-old bitransgenic animals. After 6 weeks of transgenic suppression, the overall phenotype was milder, but the lower incisors still contained regions of inflammation and a few embedded hairs. After 13 weeks of transgenic suppression, the teeth had regenerated with only low-grade inflammation in a few areas, indicating functional tooth regeneration. We speculate that the tooth phenotype may be due to progerin inhibiting ameloblasts to secrete proteins important for bone mineralization, leading to a softened enamel matrix. This in turn enables hairs to enter the incisor and cause inflammation. Since there is continuous growth and regeneration of the incisors, transgenic suppression can improve the pathology.

Dorsal skin with epidermal hyperplasia from 7-week-old bitransgenic mice showed mislocalized expression of keratins 5 and 6 together with increased proliferation (seen using an antibody directed against PHH3). K10 and loricrin were expressed normally in the thickened spinous and granular layers. Most of the bitransgenic animals showed normal expression of K5 and K6 as well as proliferation after transgenic suppression for 6 and 13 weeks. The spinous and granular layers of the epidermis now had a thickness similar to that seen in the wild-type animals.

We have managed to almost completely turn off the expression of human lamin A and progerin after disease phenotype development by suppressing the transgene in an inducible mouse model of HGPS. Once the transgenic expression was turned off, there was a reversal of the phenotype in skin and teeth. Additional studies will be needed to see if it is possible to reverse the disease phenotype of other tissues affected by progeria. This study gives hope for the future development of treatments for children with progeria.

4.4 PAPER IV

In this study, we wanted to develop a method for absolute quantification of the *LMNA* locus transcript expression levels and to analyze the expression during cellular aging. This could also possibly show a connection between normal aging and what is seen in HGPS patients. RNA and protein was extracted from different passages of the primary dermal fibroblasts from progeria patients and age-matched and parental controls to examine the expression levels of lamin A, AΔ150 and C at the RNA level and lamin A, progerin and lamin C at the protein level.

We studied the *LMNA* locus transcript expression in HGPS patients and age-matched and parental controls by absolute quantification. The highest expression was of lamin C in all sample groups. Most mutations in *LMNA* affect lamin C as well as lamin A, but mutations causing progeroid diseases only affect lamin A. The function of lamin C has received very little attention compared to lamin A, but it has been shown that mice expressing only lamin C are completely healthy. This, together with our results, indicates that lamin C might be more important than previously thought. In HGPS samples lamin A and lamin AΔ150 were equally expressed, but the lamin A transcript were >240-fold higher than the lamin AΔ150 transcript in age-matched controls and parents. The lamin AΔ150 transcript was >160-fold higher in the HGPS group than the control groups, with only minor activation of the cryptic splice site in normal controls. It is surprising that the level of lamin A was not affected by the high expression of lamin AΔ150 in HGPS cells. The combined amounts of lamin A and lamin AΔ150 was approximately double the amount of lamin A in the control groups. This may be due to differences in mRNA stability and accumulation of the lamin AΔ150 transcript, or increased lamin A gene expression in HGPS cells; perhaps progerin is not recognized as a lamin A transcript and therefore, requires more lamin A production. More lamin C transcripts were detected in the parental control group than in the HGPS samples, but no significant differences were seen between the HGPS patients and the age-matched control group or between the two control groups. Since lamin A and C are considered to be regulated by the same promoter, this might indicate an increased splicing efficiency for the lamin C transcript or they are more stable.

To study the *LMNA* locus transcript expression in *in vitro* cellular aging, we looked at different passages of HGPS patient cell cultures as well as age-matched and parental control cultures. The lamin AΔ150 transcript was significantly increased during *in vitro* cell aging in HGPS cells and parental controls when comparing early and late passages.

This may indicate that there is a similar mechanism in progeria and normal aging, with a reduced stringency of the splicing machinery with aging. No other significant difference was seen in the *LMNA* locus transcript expression between early and late passages in the different sample groups.

Quantification of protein using densitometry on Western blots that had been hybridized with an antibody directed against human lamin A/C showed varied results within the sample groups. Some groups had increased expression, while others had reduced expression. Taken together, there was no significant difference in lamin A, progerin or lamin C expression within or between the groups during *in vitro* cell aging.

By developing a method for the absolute quantification of the *LMNA* locus transcripts we showed the highest expression from the *LMNA* locus was lamin C in all groups and that there is expression of the progerin transcript in normal controls, but at much lower levels than in HGPS patients. Progerin transcript expression increases during *in vitro* cellular aging in HGPS cells and parental controls.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

PAPER I

- We have developed an inducible mouse strain that carries the human minigene for lamin A with the mutation causing HGPS.
- When targeting transgenic expression to K5-expressing tissues we saw that the different mouse strains showed various levels of transgenic expression, and the level of transgenic expression was directly linked to phenotype severity.
- Expression of progerin in K5-expressing tissues results in a progressive skin phenotype. At first there was an intermediate stage with hyperplasia and hyperproliferation in the epidermis together with changes in keratin 5 and 6 expression and later an end stage with fibrosis and loss of subcutaneous fat. The end stage had several similarities to what is seen in the progeria children. This model is very useful for studying disease progression in HGPS.

PAPER II

- The normal expression pattern of lamins A/C and B during the hair cycle was determined.
- The expression of progerin in mouse skin does not seem to influence the progression of the first postnatal hair cycle or have any immediate effect on lamin B expression.

PAPER III

- We have shown that a developed disease phenotype in bitransgenic animals of HGPS is reversible. This gives hope for the future treatment of progeria children.

PAPER IV

- We have developed a method that enables the absolute quantification of the *LMNA* locus transcripts.
- We showed that lamin C was the transcript with the highest expression levels from the *LMNA* locus. In addition, we demonstrated that the lamin AΔ150 transcript is present in normal cells, but at >160-fold lower levels

than that found in progeria patients.

- We saw an increase of lamin AΔ150 transcript during *in vitro* cell aging in HGPS cells and parental controls, which indicates that there are similar mechanisms of aging in HGPS and normal cells.

Our work provides a system for studying different organs separately to examine whether the disease is reversible and to test different therapy methods. Our mouse model can provide a further understanding of the HGPS disease mechanisms in skin and in other organs with the use of different promoters. Systems of interest for HGPS and laminopathies are the studies of bone remodeling, skin biology, lipid metabolism, peripheral nerve development, cardiovascular disease and aging.

The method we developed for the analysis of *LMNA* transcript expression levels can be used on patient material from other laminopathies. Further studies of *LMNA* transcript expression in unaffected individuals of e.g., 90-100 years of age would be very interesting and important to get a better understanding of how the different transcripts affect and are affected in normal aging.

Progerin splicing can be studied in our mouse model with the HGPS mutation, but it would be interesting to examine it in a mouse model where the more general use of alternative splicing can be studied. Different treatments, including those directed against the splicing machinery, are of interest for studies in these mice and in our progeria mouse model. This work highlights the necessity of the combination of basic and clinical research to gain novel insights into disease pathophysiology and the development of new treatments.

The use of FTIs or statins and aminobisphosphonates for HGPS treatment is based on the belief that the farnesylgroup on progerin causes the aberrant function of the protein. The question is how does unfarnesylated prelamin A function and what happens to the other proteins that need farnesylation for their processing. FTI treatment has not resulted in evident side effects in mouse models or in cancer treatments, but these might have been short-term treatments and it is not known what happens when long-term treatment is needed. It has been shown with a mouse model that unfarnesylated progerin also gives rise to disease phenotype. Gene therapy with morpholinos is probably the most likely treatment in the near future for the inhibition of progerin production, but the search for novel treatments needs to continue.

New mutations in *LMNA* and associated proteins are found continuously and will add to the list of nuclear envelopathies. The study of different laminopathies and

envelopathies will increase the understanding of the many different functions of lamins in the cell. This might also lead to possibilities of treatment directed to downstream factors.

The recent theories about stem cell depletion and function in progeria and aging are very interesting and need to be studied in more detail. The results from our mouse studies might indicate that there is stem cell depletion during the transition from the intermediate stage with epidermal hyperplasia to the end stage with thin epidermis, which is seen in the skin. This work highlights the possibility of using premature aging diseases, such as HGPS, to look for genes and mechanisms involved in normal aging.

6 SVENSK SAMMANFATTNING

Hutchinson-Gilford progeria syndrome (HGPS) är en väldigt ovanlig genetisk sjukdom som orsakar en del symtom på förtidigt åldrande hos barn. Sjukdomen drabbar 1 på 4-8 miljoner och barnen ser vanligtvis normala ut när de föds, men börjar utveckla sjukdomssymtom under de första levnadsåren. Några typiska symtom är allvarlig tillväxthämning, håravfall och tunn hud (speciellt på huvudet) vilket gör att kärlen lyser igenom. Efter tillväxthämningen är hudfenotypen, som är karaktäriserad av sklerodermi på bålen och avsaknad av underhudsfett, det som noteras först. De får även mild osteoporos och har ledkontrakturer. Barnen avlider i tidiga tonåren, vanligtvis av hjärtinfarkt eller stroke.

Sjukdomen orsakas av en mutation i en gen som producerar ett protein som heter lamin A. Lamin A tillsammans med lamin B och C är huvudkomponenterna av laminan vilken befinner sig precis innanför kärnmembranet i cellen. HGPS mutationen leder till ett kortare lamin A protein som inte processas som det ska. Detta i sin tur leder till att proteinet förlorar en del av sina funktioner och får en del nya funktioner. Det mutanta proteinet kallas progerin. Man vet dock inte exakt hur det mutanta proteinet fungerar och interagerar med andra faktorer i cellen.

Målet med min avhandling var att få en bättre förståelse för de molekylära mekanismerna bakom HGPS och att studera uttrycket från lamin A lokuset i normalt åldrande.

I **artikel I** har vi gjort en musmodell som uttrycker HGPS mutationen i hud. Modellen är unik i sitt slag i.o.m. att vi kan reglera när vi uttrycker proteinet genom att tillsätta doxycyklin i dricksvattnet. När vi uttrycker progerin i huden hos mössen ser vi att de får en progressiv sjukdom. Först ser vi en förtjockad epidermis (det yttersta lagret i huden) med ökad celldelning och feluttryck av keratin 5 och 6, men det övergår sedan till en tunn epidermis med fibrotiskt dermis (lagret under epidermis i huden) och avsaknad av underhudsfett. Mössen har tillväxthämning och är tunnhåriga. Djuren utvecklar sjukdomen snabbare ju tidigare HGPS mutationen uttrycks postnatalt och de dör förtidigt. Den sena sjukdomsfenotypen liknar det man ser hos progeriabarnen vilket innebär att denna modell fyller en viktig funktion vid studier av HGPS.

Progeriabarnen har påverkan av huden och även håravfall så i **artikel II** har vi studerat det normala uttrycket av lamin A och lamin B och den första hårcykeln och dess faser. När vi sedan jämförde uttrycket av lamin B och faserna i den första

hårcykeln med våra progeria möss såg vi ingen skillnad. Detta innebär att uttrycket av progerin inte direkt påverkar hårcykeln eller uttrycket av lamin B i tidigt skede.

Eftersom de flesta barnen som lider av HGPS har en fullt utvecklad sjukdom när de får sin diagnos har vi i **artikel III** studerat om sjukdomsförloppet går att hindra eller t.o.m. bota. Vi använde oss av våra möss med progeria i huden och stängde av uttrycket av proteinet när mössen redan utvecklat sjukdom. Vi fann att de flesta mössen blev nästan helt återställda inom 13 veckor vilket ger hopp för framtida behandling och tillfrisknande av barn med progeria (i.a.f. av symtomen som ses i hud).

I **artikel IV** har vi tittat på uttrycksnivåerna av de olika proteinerna som kommer från lamin A lokuset i progeria patienter och i kontroller av samma ålder och föräldrar. Vi fann att lamin C är det transkript som uttrycks högst i alla grupper. Vi fann att progerin uttrycks lika mycket som lamin A i progeria patienter, men det fanns även ett svagt uttryck i normala kontroller. När vi tittade på vad som händer när man låter cellerna åldras i cellkultur fann vi att uttrycket av progerin ökade i progeria patienter och i celler från föräldrarna. Detta indikerar att det kan finnas liknande mekanismer i normalt åldrande och HGPS.

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