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DEVELOPMENT OF A MOUSE MODEL FOR HUTCHINSON-GILFORD PROGERIA SYNDROME REVEAL DEFECTS IN ADULT STEM CELL MAINTENANCE

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ABSTRACT

Hutchinson-Gilford progeria syndrome (HGPS) is a very rare genetic disease that presents some features of accelerated aging. Children with the disease are born appearing healthy but start to develop signs of the disease within their first years of life. The disease affects multiple tissues, and the symptoms include growth retardation, alopecia, lack of subcutaneous fat, scleroderma-like skin changes, bone abnormalities and joint stiffness. Heart disease and other atherosclerotic complications are the most common cause of death, which occurs at a median age of 13 years. Ninety percent of HGPS cases are caused by a *de novo* point mutation in the *LMNA* gene (c.1824C>T, p.G608G). The *LMNA* gene encodes, by alternative splicing, the different isoforms of A-type lamins. These intermediate filaments are the main proteins of the nuclear lamina, a meshwork of proteins located underneath the inner nuclear membrane. The nuclear lamina serves important functions in determining the shape and size of the nucleus as well as being involved in fundamental cellular processes such as DNA replication and transcription.

This thesis is focused on gaining a deeper understanding of the cellular effects caused by the most common HGPS mutation. For this purpose, we developed an inducible mouse model that carries a human mini gene for lamin A with the c.1824C>T mutation. The mini gene caused over-expression of both wild-type human lamin A and human progerin, the mutated form of the lamin A. Targeting the expression of the transgene to keratin 5-expressing tissues resulted in a progressive skin phenotype. The phenotype evolved from an intermediate stage with hyperplasia of the interfollicular epidermis and increased proliferation to an end stage with hypoplastic epidermis, fibrotic dermis and loss of subcutaneous fat. The end stage of the condition resulted in mice that had similar abnormalities to those seen in the skin of HGPS patients. This mouse model was used to further investigate the molecular effects of the HGPS mutation in the skin.

To examine whether expression of the lamin A c.1824C>T mutation influences the hair cycle or the expression pattern of lamin B, we first characterized the normal hair cycle and expression patterns for lamin A/C and B. Immunohistochemical stainings of the dorsal skin of FVB/NCrl wild-type skin from animals of different ages showed strong expression of lamin A/C and B in the basal cells of the epidermis, the outer root sheath of the hair follicle and the dermal papilla in all phases of the hair cycle. Analyzing the progression of the phases of the first hair cycle and the expression of lamin B in our HGPS mouse model, we could not distinguish any effects of progerin expression.

The lamin A c.1824C>T expressing mutant mice were also used to study the effects of the expression of this mutant lamin during embryogenesis. Animals with embryonic expression of progerin developed a rapidly progressing phenotype. In summary, our inducible models will be useful for testing different treatments and could also be used for tissue-specific expression in other tissues affected in HGPS.

The work presented in this thesis provides insights into the molecular mechanism underlying the skin symptoms seen in patients with HGPS.

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, 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, Eriksson M.
PLoS ONE 2009; 4(1): e4114
* Rosengardten and Sagelius contributed equally to this work.

III. **Stem cell depletion in a mouse model of progeria**
Rosengardten Y, McKenna T, Grochová D, Eriksson M
Submitted manuscript

IV. **Embryonic expression of the HGPS mutation**
Rosengardten Y, McKenna T, Petzold A, Grochová D, Eriksson M
Manuscript

Related paper but not included in this thesis:
Reversible phenotype in a mouse model of Hutchinson-Gilford progeria syndrome
Sagelius H, Rosengardten Y, Schmidt E, Sonnabend C, Rozell B, Eriksson M.
Journal of Medical Genetics 2008; 45: 794-801

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LIST OF ABBREVIATIONS

BrdU	Bromodeoxyuridine (5-bromo-2-deoxyuridine)
CMT2	Charcot-Marie-Tooth disease type 2
d0	Day of birth
d21	postnatal day 21
DCM	Dilated cardiomyopathy
dox	doxycycline
DMD	Emery-Dreifuss Muscular Dystrophy
FACS	fluorescence-activated cell sorting
FPLD	Familial Partial Lipodystrophy of Dunnigan type
H&E	Hematoxylin and Eosin
HGPS	Hutchinson-Gilford progeria syndrome
htx	hematoxylin
IF	immunofluorescence
IHC	immunohistochemistry
K1	Keratin 1
K5	Keratin 5
K10	Keratin 10
K5tTA	Keratin 5 tetraycline transactivator
LGMD	Limb-Girdle muscular dystrophy
MAD	Mandibuloacral Dyslpasia
PFA	paraformaldehyde
RD	Restrictive dermopathy
rtTA	reverse tetracycline transactivator
tetop	pTRE element
tTA	tetracycline transactivator

1 INTRODUCTION

All people are destined to age. However, how and when the symptoms of aging appear is different for different individuals. Aging is a complex process, and the diverse development of symptoms is probably due to various genetic and environmental factors. The biological basis of aging is not fully known, but intensive research in the field increases the understanding of underlying mechanisms.

One way to study the features of aging is to study progeroid syndromes. Connections between these syndromes and physiological aging can shed light on fundamental mechanisms. Progeroid syndromes that only affect one tissue are called unimodal progeroid syndromes (Martin, 1982), while segmental progeroid syndromes affect several, but not all, tissues in the body (Martin, 1978).

Hutchinson-Gilford progeria syndrome (HGPS) is classified as a segmental progeroid syndrome. Children born with the disease display several symptoms of accelerated aging (DeBusk, 1972; Brown, 1992).

1.1 THE NUCLEAR ENVELOPE

The nucleus of a cell is bordered by an envelope, which separates the contents of the nucleus from the cytoplasm. The structure of the nuclear envelope and transport across it are highly regulated and organized. The nuclear envelope consists of two separate membranes, the outer membrane and the inner membrane. The outer membrane is directly connected to the rough endoplasmatic reticulum and is often coated with ribosomes. The inner membrane is connected to the filamentous proteins of the nuclear lamina. The nuclear pore complexes, which allow protein transport into and out of the nucleus, connect the two membranes. Located underneath, and also connected to, the inner membrane is the nuclear lamina (Evans *et al.* 2004). The nuclear lamina is an intermediate type filamentous network of primarily lamin proteins with both structural and regulatory properties. As well as determining the size and shape of the nucleus, the lamina is involved in DNA replication. Furthermore, the lamina determines the position and the function of the nuclear pore complexes, and it has also been shown to bind to other proteins attached to the nuclear envelope. The binding of heterochromatin and the presence of anchoring sites for chromatin domains and transcription factors to the nuclear periphery suggest that the lamina has an additional role in DNA transcription (Stuurman *et al.* 1998; Moir & Spann 2001; Burke & Stewart 2002).

The A-type lamins, one of the major components of the nuclear lamina, have been shown to bind a number of different proteins *in vitro*. These proteins include emerin (Clements *et al.* 2000; Lee *et al.* 2001), lamin associated protein (LAP 1 (Foisner & Gerace 1993), Lap2α (Dechat *et al.* 2000), nesprin 1 (Mislovw *et al.* 2002), nesprin 2 (Zhang *et al.* 2005), actin (Sasseville & Langelier 1998), pRb (Ozaki *et al.* 1994), sterol regulatory element-binding protein (SREBP) 1 (Lloyd *et al.* 2002), SUN1 (Crisp *et al.* 2006), SUN2 (Crisp *et al.* 2006), one or more components of RNA polymerase II dependent transcription complexes (Spann *et al.* 1997) and DNA replication complexes (Spann *et al.* 1997). Together with the SUN proteins and nesprin, lamin A and C participate in the LINC complex (LInk the Nucleoskeleton with the Cytoskeleton). In the LINK complex, nesprins in the outer nuclear membrane interact with the SUN proteins in the lumen of the nuclear envelope, which in turn interact with nesprins in the inner nuclear membrane and with lamins A and C. This protein complex thereby links the nucleoskeleton with the cytoskeleton (Crisp *et al.* 2006).

1.2 THE LAMIN FILAMENTS

Lamins are divided into types A and B according to their expression pattern, behavior during mitosis and biochemical properties. Two genes encode the B-type lamins: *LMNB1* encodes lamin B_1 and *LMNB2* encodes lamin B_2 and B_3. Lamin B_3 is only expressed in spermatocytes, whereas the other B-type lamins are expressed in all cells during development and adulthood. The different A-type lamins are encoded by the *LMNA* gene and are mainly expressed in terminally differentiated cells (Röber *et al.* 1989). The two main isoforms of the *LMNA* gene, lamin A and lamin C, are identical except in the C-terminal end where lamin A has a unique 90 amino acid region, while lamin C has a unique 6 amino acid sequence (Fisher *et al.* 1986). Lamin A is encoded by exons 1-12, while lamin C is encoded by exons 1-10.

The lamins are type V intermediate filaments and consist of a central α-helical rod domain flanked by a small globular domain at the N-terminus and a larger globular domain at the C-terminus. Through coil-coiled interactions between the rod domains, lamins A and B1 form homodimers that, in turn, form homopolymers by head to tail associations. (Fisher *et al.* 1986; Strelkov *et al.* 2004) (Fig. 1). These polymers are the building blocks for the nuclear lamina. The lamin A and B type lamins forms separate, but interacting, meshworks in the nuclear lamina (Shimi *et al*. 2008).

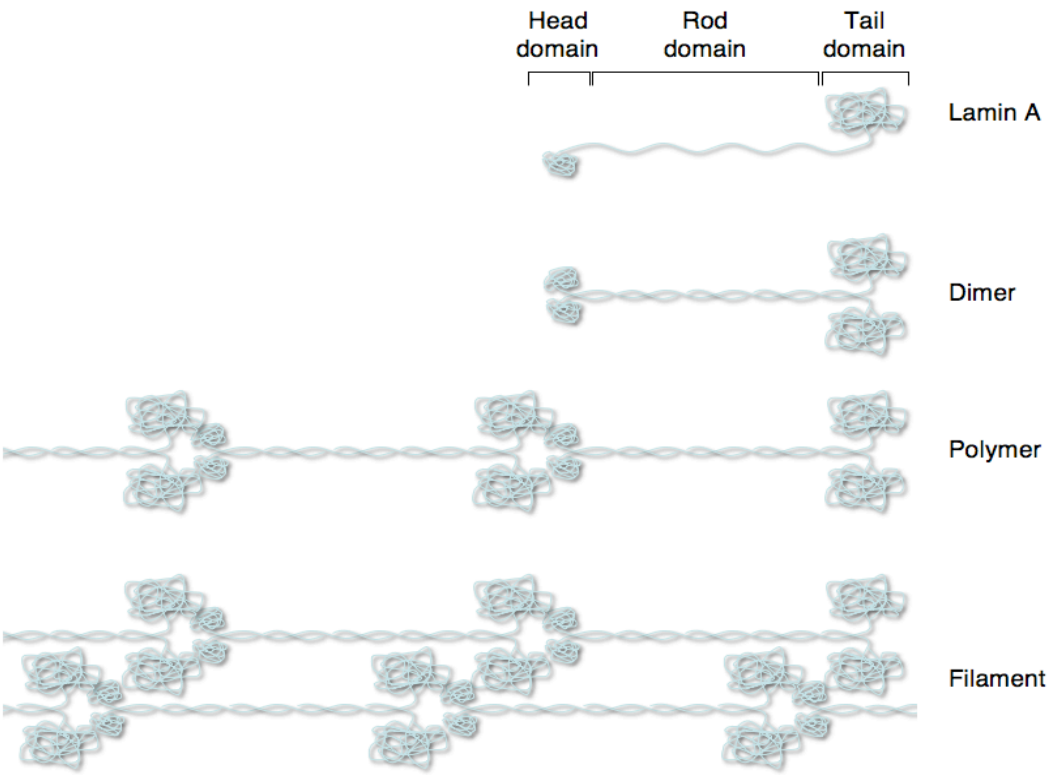

Figure 1. Schematic figure of the lamin filaments. The lamin filaments consist of a central rod domain flanked by small globular head domain at the N-terminal and a larger globular tail domain at the C-terminal. The lamins form dimers by coil-coiled interactions between the rod domains. Head to tail associations of the dimers form polymers, which form the lamin filaments.

1.3 LAMINOPATHIES

Diseases caused by mutations in genes encoding proteins associated with the nuclear envelope are collectively called nuclear envelopathies (Somech *et al*. 2005). The nuclear envelopathies include the primary and secondary laminopathies. Primary laminopathies are diseases caused by mutations in the *LMNA* gene, while secondary laminopathies are caused by mutations in the *LMNB1*, *LMNB2* or *FACE-1* (*Zmpste24* in mice) genes.

1.3.1 Primary laminopathies

Genetic studies have shown that mutations in the *LMNA* gene cause about a dozen distinct clinical disorders, often divided into four groups by phenotype. The four different groups are the following: muscular dystrophies, lipodystrophies, neuropathies and segmental premature aging disorders.

The muscular dystrophies include Emery-Dreifuss Muscular Dystrophy (EDMD), Dilated cardiomyopathy (DCM) and Limb-Girdle Muscular Dystrophy (LGMD). Patients with EDMD classically have early contractures of the elbows, rigidity of the spine and slowly progressive weakness in the upper arms and lower legs (Emery & Dreifuss 1966). In DCM, skeletal muscles are minimally affected or unaffected, while in LGMD patients, the skeletal muscles around the shoulders and hips are primarily affected. As in EDMD, both DCM and LGMD have a predominant dilated cardiomyopathy with an early onset ventricular conduction block (Fatkin *et al.* 1999; Muchir *et al.* 2000).

Lipodystrophies are a group of diseases characterized by the absence or reduction of subcutaneous adipose tissue. The lipodystrophies caused by *LMNA* mutations include Familial Partial Lipodystrophy of Dunnigan type (FPDL) and Mandibuloacral Dysplasia (MAD). Patients with FPDL are born with a normal distribution of fat but beginning at puberty exhibit a gradual loss of adipose tissue from the extremities, associated with insulin resistance and diabetes mellitus (Dunnigan *et al.* 1979). In MAD partial lipodystrophy, extreme insulin resistance and hypermetabolism have been observed. In addition, patients with MAD suffer from skeletal abnormalities, joint contractures and atrophic skin (Novelli *et al.* 2002, Simha *et al.* 2003), features also implicated in accelerated aging.

Patients with Charcot-Marie-Tooth disease type 2 (CMT2) have normal or slightly reduced motor nerve conduction velocities, loss of large myelinated fibers and axonal degeneration, classifying the disease as a neuropathy (Chaouch *et al.* 2003).

The segmental progeroid syndromes caused by mutations in the *LMNA* gene include HGPS, atypical HGPS, MAD, RD and atypical Werner syndrome. This thesis is focused on HGPS and RD, and these two diseases will be described below. Atypical HGPS has similar, but lack some or have additional, symptoms to classical HGPS. Werner syndrome is often called “progeria of the adult” and is classified by the premature appearance of features associated with normal aging and cancer. Individuals with Werner’s syndrome develop normally until the end of the first decade, and symptoms typically start to develop in the second or third decade of life. The main clinical features in Werner’s syndrome include scleroderma-like skin, short stature, premature arthrosclerosis, diabetes mellitus, osteoporosis and premature thinning and/or graying of scalp hair. The classical form of Werner’s syndrome is caused by mutations in the *WRN* gene, which encodes a RecQ helicase (Yu *et al.* 1996).

1.3.2 Secondary laminopathies

Some diseases classified as primary laminopathies are also classified as secondary laminopathies, given that the same disorder can be caused by mutations in different proteins. These diseases include EDMD, RD and MAD. EDMD was first shown to be inherited in an X-linked manner before autosomal inheritance was described (Emery and Dreifuss, 1966). The gene responsible for X-linked EDMD encodes emerin, a protein of the inner nuclear membrane (Bione *et al.* 1994; Manilal *et al.* 1996; Nagano *et al.* 1996). RD can be caused by mutations in either *LMNA* or in the *Zmpste24* (*FACE-1*) gene, the endoprotease responsible for the last step in the processing of prelamin A (described below). Compound heterozygous mutations in *Zmpste24* have also been described as causing MAD (Shackleton *et al.* 2005).

Other secondary laminopathies can be caused by alterations in the *LMNB1* or *LMNB2* genes. Duplication of the *LMNB1* gene causes Autosomal-dominant Leukodystrophy, a neurodegenerative disease that exhibits a progressive loss of myelin in the central nervous system (Schwankhaus *et al.* 1994; Padiath *et al.* 2006). Mutations in *LMNB2* cause Acquired Partial Lipodystrophy, a sporadic progressive lipodystrophy (Worman & Bonne 2007; Capell & Collins 2006; Hegele *et al.* 2006).

Nuclear envelopathies can also be caused by mutations in proteins associated with the nuclear envelope. Mutations causing disease have been reported in several different genes, e.g., *LBR*, *LEMD3*, *SYNE1*, *TMPO*, *TOR1A* and *LAP2α* (Worman *et al.* 2010).

Taken together, a number of human diseases have been described that are caused by mutations or alterations in genes encoding lamins or proteins associated with the nuclear envelope, and this number is likely to continue to grow over time.

1.4 POST-TRANSCRIPTIONAL PROCESSING OF LAMIN A

Mature lamin A, one of the major isoforms encoded by the *LMNA* gene, is produced after a series of rapid post-translational modifications. The C-terminal end of the lamin A precursor protein (prelamin A) contains a CaaX motif (C= cysteine, a= any aliphatic amino acid, X= any amino acid). In prelamin A, the CaaX motif is composed of the amino acids CSIM. The processing of prelamin A starts by farnesylation of the cysteine in the CaaX motif, followed by an endopeptidase cleavage step where the last three amino acids (-aaX) are removed (Beck *et al.* 1988; Holtz *et al.* 1989; Beck *et al.* 1990; Sinensiy *et al.* 1994; Zhang & Casey 1996). The cleavage step is followed by

carboxymethylation of the cysteine, which results in the insertion of the prelamin A into the inner nuclear membrane (Stuurman *et al.* 1998; Leung *et al.* 2001; Corrigan *et al.* 2005; Winter-Vann & Casey 2005). The last step involves removal of the last 15 amino acids by proteolytic cleavage, yielding mature lamin A (Kilic *et al.* 1997; Corrigan *et al.* 2005; Rusinol & Sinensky 2006). Figure 2, show a schematic picture of the processing, including the enzymes responsible for each step. Through different splicing at the C-terminal end lamin C lacks the CaaX motif and is not subjected to this processing, while the lamin B proteins go through the entire process except the final cleavage step (Fransworth *et al.* 1989; Stuurman *et al.* 1998; Kitten & Nigg 1991).

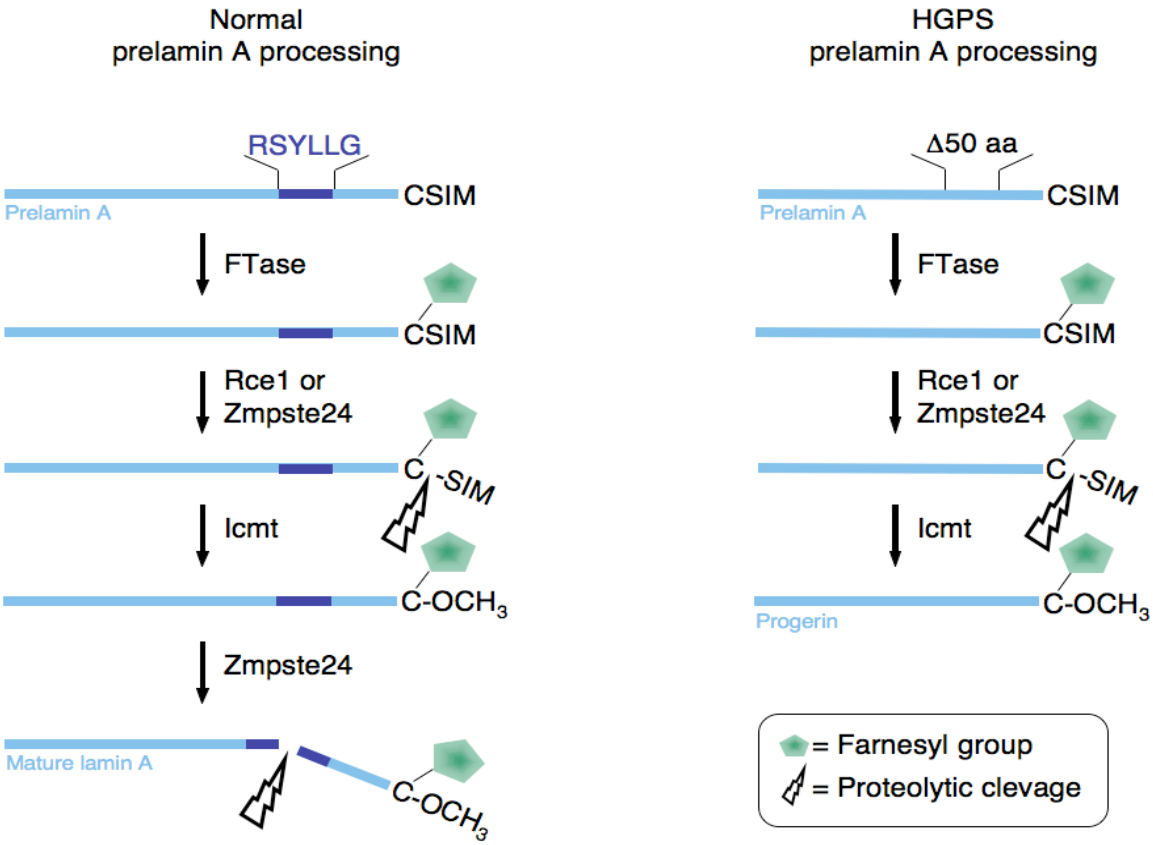

Figure 2. The post-transcriptional processing of normal lamin A and lamin A Δ50. Modification of the prelamin A to form mature lamin A involves several enzymes in a multi step processing. The only step that differs between normal lamin A and lamin A Δ50 is the last step, which is eliminated in the mutant lamin A due to the 50 amino acid deletion.
(FTase = farnesyltransferase)

1.5 THE HUTCHINSON-GILFORD PROGERIA SYNDROME

Hutchinson-Gilford progeria syndrome (HGPS or progeria) is a very rare and fatal genetic disease. The name of the disease is derived from the Greek words progērōs meaning "prematurely old", and the disease is characterized by certain features of accelerated ageing. The reported incidence of progeria is about 1 in 4-8 million live births (DeBusk 1972; Brown 1992). It affects both sexes equally, and patients have been reported from all over the world. Children with progeria are born looking healthy but start to develop symptoms of disease within their first year of life.

1.5.1 Clinical features of HGPS

In general, the first signs of disease noticed in HGPS patients are a failure to thrive, along with short stature and low weight. After the growth retardation, the skin phenotype is normally the first symptom noted. During the second or third year of life, the skin becomes tight over the abdomen and thighs. The children also have wrinkly skin that develops into thin, dry and atrophic regions, sometimes accompanied by hyperkeratosis. Between 6 months and 2 years of age, most patients start to lose their hair. By the age of 3 years, the children usually have almost complete alopecia, except for some downy hairs. The skin phenotype is also characterized by scleroderma and loss of subcutaneous fat. In older patients several symptoms are seen, including a thin epidermis, fibrosis in dermis with thickened and disorganized collagen bundles, a reduced number of sweat and sebaceous glands and atrophic subcutaneous tissue (Gillar *et al.* 1991; Erdem *et al.* 1994; Stables & Morley 1994; Rodriguez *et al.* 1999; Jansen & Romiti 2000; Sarkar & Shinton 2001; Hennekam 2006; Merideth *et al.* 2008; Ackerman & Gilbert-Barness 2002).

The symptoms of disease also include hyperpigmentation in the skin of the scalp and the limbs and prominent scalp veins. The patients also display a pyriform chest, prominent eyes, a small and beaked nose, micrognathia, thin lips, protruding ears lacking earlobes, dystrophic fingernails and a high-pitched voice. In addition, they have alterations in the oral soft tissue, abnormal dentition with hypodontia, crowding of the present teeth with double rows and delayed dental eruption. Children with HGPS have normal intelligence, but they do not enter puberty (DeBusk 1972; Nair *et al.*2004; Gordon *et al.* 2007; Mazereeuw-Hautier *et al.* 2007; Merideth *et al.* 2008; Domingo *et al.* 2009)

The phenotype also includes coxa valga and joint contractures resulting in a horse-riding posture and difficulty in moving the knees, elbows and fingers. Furthermore, the bone phenotype includes osteoporosis (Stables & Morley 1994; de Paula Rodrigues *et al.* 2002; Sevenants *et al.* 2005).

Death generally occurs due to cardiovascular complications at a mean age of 13 years. Initially, the patients do not demonstrate any cardiovascular problems, but with age the cardiovascular function is reduced. After exercise, patients develop short breath, increased pulse and increased blood pressure. At autopsy, the intima and media are found to have a relatively small diameter, but an increased tunica thickness and occasionally plaque formations are found. Additionally, the media also show extensive loss of vascular smooth muscle cells (Makous *et al.* 1962; Nair *et al.* 2004; Merideth *et al.* 2008; Stehbens *et al.* 1999; Stehbens *et al.* 2001; Gordon *et al.* 2005).

1.5.2 Molecular basis of HGPS

HGPS is a genetic disease, and in 90% of cases, it is caused by a *de novo* single nucleotide mutation in exon 11 of the *LMNA* gene (c.1824C>T, p.G608G) (Table 1). The mutation is a heterozygous base substitution, and, because it localizes to exon 11, it only affects the lamin A protein. The substitution does not affect the amino acid sequence, but it increases the use of a cryptic splice site in exon 11. This splice site activation leads to the production of an incorrectly spliced mRNA with an internal deletion of 150 nucleotides at the end of exon 11. The reading frame is kept intact, but the final protein has a 50 amino acid deletion close to the C-terminal end (lamin A Δ50 or progerin) (De Sandre-Giovannoli *et al*. 2003; Eriksson *et al*. 2003).

Prelamin A Δ50 still retains the CaaX-box, but the internal deletion contains the recognition site for zmpste24, the enzyme responsible for the last step in the processing of prelamin A to mature lamin A. The initial steps still take place, but with the last step abolished the result is a truncated prelamin A protein that still carries the incorporated farnesyl and methyl groups (Dechat *et al*. 2007) (Fig. 2). This farnesylated and methylated truncated protein is referred to as progerin or lamin AΔ50 (De Sandre-Giovannoli *et al.* 2003; Eriksson *et al.* 2003).

1.5.3 Cellular phenotype of HGPS

Progerin acts in a dominant negative way by disrupting the normal function of lamin A. Because progerin stays farnesylated, it has been suggested that it permanently binds to

the nuclear membrane and thereby disrupts the normal function of the lamina. This disruption results in a nucleus with visible blebs or lobulations of the nuclear envelope, disrupted heterochromatin structure, loss of peripheral heterochromatin and a thickened lamina. All of these structural changes could lead to extensive transcriptional abnormalities. In addition, HGPS cells show mislocation and clustering of the nuclear pore complexes, which might impair normal trafficking of proteins and mRNA in and out of the nucleus (Eriksson *et al.* 2002; Goldman *et al.* 2004). Cells from HGPS patients have been shown to have a transcriptional misregulation and a large number of genes with altered expression (Csoka *et al.* 2004). Studies have also shown a defect in genomic stability with increased double-strand breaks as a consequence (Liu *et al*. 2005; Liu *et al.* 2006). Cells from HGPS patients accumulate progerin in an age-dependent manner when they are grown in culture (Goldman *et al*. 2004; McClintock *et al.* 2006). This accumulation correlates with more severe structural changes and a reduced growth rate (Goldman *et al*. 2004).

1.6 RESTRICTIVE DERMOPATHY

Restrictive Dermopathy (RD) is a rare and lethal congenital disorder. The disease is considered to be similar to HGPS but with a more acute progression (Rodríguez & Eriksson, 2010).

1.6.1 Clinical features of RD

Patients with RD display very characteristic symptoms, including intrauterine growth retardation and decreased fetal movement due to a thin and taut skin. The patients are often born prematurely and show typical facial dysmorphisms with a small, pinched nose, posteriorly rotated and low-set earlobes, lack of eyebrows and eyelashes, micrognathia and a mouth fixed in an “o” position. The tautness of the skin causes akinesia or hypokinesia, and patients usually die within the first week of life due to reduced respiratory movements (Mok *et al*. 1989, Smigel *et al*. 2009). Histological studies of the skin from RD patients have revealed skin pathology, including thin epidermal layers with regular structure and a flat epidermal-dermal junction, as well as immature and poorly developed sebaceous glands and hair follicles. The pathology also affects the dermis with parallel collagen bundles and a nearly complete absence of elastic fibers. (Khanna *et al.* 2008; Smigiel *et al.* 2009).

1.6.2 Molecular basis of RD

RD can be caused by mutations in either the *LMNA* gene or in the gene coding for *ZMPSTE24*, the enzyme catalyzing the last cleavage step in the processing of prelamin A. Common among the different mutations is the cellular accumulation of prelamin A. Mutations in *ZMPSTE24* can be homozygous or compound heterozygous. All known cases of mutations in the enzyme result in the complete loss of both zmpste24 and mature lamin A, which is followed by prelamin A accumulation. The disease can also be caused by heterozygous mutations in the *LMNA* gene. These mutations result in the partial or complete loss of exon 11, including the recognition site for zmpste24, with a corresponding accumulation of truncated prelamin A as a result (Table 1) (Navarro *et al.* 2004).

The similar molecular genetics between HGPS and RD, together with similarities in the clinical symptoms, indicate a common disease mechanism with a correlation between the cellular levels of progerin/prelamin A and the severity of the phenotype.

Table 1. Mutations causing HGPS and RD.

Disorder	Gene	Mutation	Effect on zmpste24	Effect on lamin A/C	Reference
HGPS	*LMNA*	p.G608G		progerin	De Sandre-Giovannoli *et al.* 2003 Eriksson *et al.* 2003
	LMNA	p.G608S		progerin	Eriksson *et al.* 2003 Cao & Hegele 2003
RD	*LMNA*	IVS11+1G>A		prelamin A Δ 90 deletion exon 11	Navarro *et al.* 2004
	ZMPSTE24	p.I19Y fsX28 homozygous	premature termination codon	prelamin A accumulation	Moulson *et al.* 2005
	ZMPSTE24	p.L91_L209del / p.L362 fsX18	premature termination codon	prelamin A accumulation	Navarro *et al.* 2004 Navarro *et al.* 2005
	ZMPSTE24	p.P99L fsX38 / p.L362 fsX18	premature termination codon	prelamin A accumulation	Navarro *et al.* 2005
	ZMPSTE24	p.I198Y fsX20 / p.L362F fsX19	premature termination codon	prelamin A accumulation	Moulson *et al.* 2005
	ZMPSTE24	c.627 + 1G>C	partial loss deletion exon 5	prelamin A accumulation	Sander *et al.* 2008
	ZMPSTE24	p.E239X homozygous	premature termination codon	prelamin A accumulation	Chen *et al.* 2009
	ZMPSTE24	p.L362F fsX19 homozygous	premature termination codon	prelamin A accumulation	Moulson *et al.* 2005
	ZMPSTE24	p.L362 fsX18 homozygous	premature termination codon	prelamin A accumulation	Navarro *et al.* 2004 Navarro *et al.* 2005
	ZMPSTE24	p.L362 fsX18 / p.Q417X	premature termination codon	prelamin A accumulation	Navarro *et al.* 2005
	ZMPSTE24	p.I198Y fsX20 homozygous	premature termination codon	prelamin A accumulation	Moulson *et al.* 2005
Intermediate syndromes	*LMNA*	p.G608G		progerin	Navarro *et al.* 2004
	LMNA	p.V607V		progerin	Moulson *et al.* 2007
	LMNA	c.1303C>T homozygous		Mutant lamin A p. Arg435Cys	Youn *et al.* 2010

1.7 LAMINS AND AGING

Physiological aging is a complex process most likely involving many different molecular mechanisms. A link between nuclear morphology and age has been shown in the nematode *C. elegans,* in which age was accompanied by major changes in the nuclear architecture. Age-related changes included progressive alterations of the nuclear shape with a loss of heterochromatin and an abnormal distribution of the nuclear envelope proteins in wild-type animals (Haithcock *et al*. 2005)

The first indication of a link between lamins and physiological aging came from a study of fibroblasts. The study showed that small amounts of the same mutant lamin A protein seen in HGPS patients were present in healthy individuals. However, neither the amount of mutated protein nor the amount of mRNA encoding it increased with the age of the donor (Scaffidi & Misteli, 2006). Other, more recent studies have shown that when fibroblasts from healthy donors age in culture, the amount of mutated protein or mRNA increases with the number of passages (Cao *et al.* 2007; Rodriguez *et al*. 2009). *In vivo* studies have confirmed that progerin levels in skin increase with age, and it seems to be expressed in cells that have reached terminal differentiation or senescence (McClintock *et al*. 2007).

1.8 SKIN STRUCTURE

The skin is the largest organ in the body and has four major functions: i) Protection. The skin protects the body against ultraviolet light and mechanical or chemical insults. It also provides a barrier for microorganisms and prevents dehydration. ii) Sensation. The skin contains various receptors for touch, pressure, pain and temperature. iii) Thermoregulation. The skin protects the body from heat loss by the presence of hair and subcutaneous adipose tissue. Evaporation of sweat and increased blood flow facilitates heat loss. iv) Metabolism. The epidermis synthesizes vitamin D, and the subcutaneous adipose tissue constitutes a major store of energy (Wheater's Functional Histology A Text and Colour Atlas).

The basic structure of the skin can be divided into three different layers: the epidermis, the dermis and the hypodermis (Fig. 3). The epidermal layer is the outermost layer and is in turn divided into multiple layers. The thick dermal layer, composed of fibro-elastic tissue, supports the epidermis, contains many sensory receptors, and is highly vascular. Located underneath the dermis is the hypodermis, which mainly contains adipose tissue.

The interfollicular epidermis of the skin is a multilayered stratified squamous epithelium, which maintains homeostasis by proliferation of adult stem cells in the basal layer (Fig. 3). As basal cells detach from the basement membrane, they leave the cell cycle, initiate a multi-step program of terminal differentiation and gradually move upwards to the skin surface. The gradual differentiation of the keratinocytes creates the different layers of the epidermis (Fuchs & Byrne 1994; Alonso & Fuchs 2003).

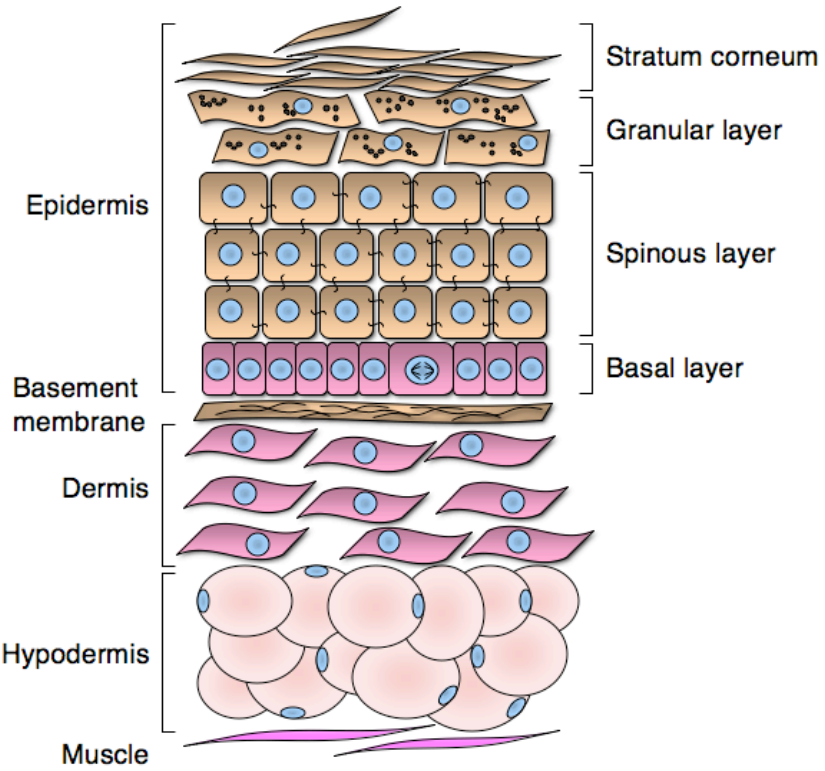

Figure 3. Graphic illustration of the different layers of the skin. The stem cells responsible for the constant renewal of the epidermis are located in the basal layer. In the spinous layer tight-junctions between the cells are important for the function of the epidermal barrier.

1.9 THE HAIR CYCLE

The hair coat, which keeps most mammals warm and dry, requires a steady supply of new hairs throughout the animal's lifetime. To produce new hairs, existing follicles in the skin undergo cycles of degeneration and regression throughout life. Hair follicles are continuous with the interfollicular epidermis and are composed of a number of cell layers. The outer root sheath, the outermost epithelial layer, maintains contact with the basement membrane separating the follicle from the dermal cells (Fuchs, 2007).

The hair cycle is divided into three phases: anagen, catagen and telogen. The phases of the hair cycle begin in a wave from the top of the head and spread caudally towards the tail and laterally down the sides of the animal. The anagen phase is characterized by growth of the hair follicle and proliferation of the cells in the bulb. During each anagen, the hair follicle produces an entire hair shaft, and the length of the phase determines the length of the hair. Each anagen is followed by a catagen phase, characterized by hair follicle regression. During catagen, the lower "cycling" portion of the follicle regresses in a process that includes reduction of the epithelial cells of the bulb and outer root sheath by apoptosis. The hair cycle is completed by entrance to the telogen phase, a resting phase in which the follicles are inactive (Müller-Röver *et al.* 2001; Alonso & Fuchs, 2006).

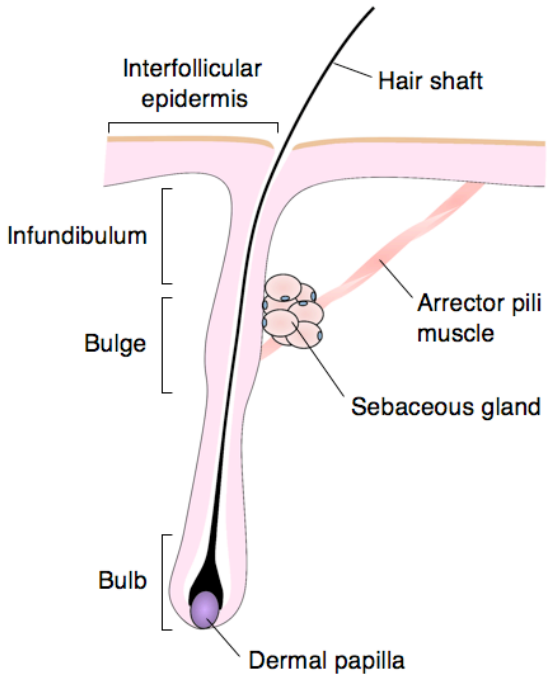

Figure 4. Graphic illustration of a hair follicle in late anagen phase. The lower part of the follicle, under the bulge, is the cycling portion that will regress in the catagen phase.

1.10 THE ADULT STEM CELLS IN THE SKIN

The cyclic growth of hair follicles and the constant renewal of the epidermis and sebaceous glands are maintained by adult stem cells. These three different compartments of the skin are believed to contain their own stem cells (Fig 4) (Fuchs & Horsley 2008).

The stem cells located in the lowest permanent part of the hair follicle, called the bulge region, are activated at the onset of anagen to generate a new hair follicle (Cotsarelis *et al.* 1990). During the transition to anagen, the growing phase of the hair follicle, most bulge cells remain quiescent, while a small number of cells are activated (Tumbar *et al.* 2004). The stem cells from the bulge migrate along the outer root sheath to the base of the follicle, where they become activated (Oshima *et al.* 2001). Association with the dermal papilla at the base of the follicle is important to trigger cell proliferation. When the dermal papilla moves away from the bulge, the epidermal stem cells in this region return to quiescence, while the cells connected to the base of the follicle remain highly proliferative. Through local differences in the microenvironment, the proliferative cells progress to produce the hair and the inner root sheath (Oshima *et al*. 2001). In addition to the bulge stem cells, a population of $Lgr5^+$ cells located in the bulge and secondary germ of telogen hair follicles and in the lower outer root sheath of anagen hair follicles have been shown to have stem cell properties (Jaks *et al.* 2008).

The interfollicular epidermis contains stem cells that maintain the constant renewal of the skin. The endothelial cells of the epidermis are organized in columns where basal cells occasionally divide to produce cells with a more transient proliferative capacity (Mackenzie, 1970). Both symmetric and asymmetric cellular divisions have been observed in the epidermis. They are both believed to supply new cells through the formation of layers. Symmetric division relies on detachment of the cells from the basement membrane for differentiation (Mackenzie, 1970; Smart, 1970; Lechler & Fuchs, 2005), while in asymmetric division basal cells orient their spindle asymmetrically towards the basement membrane. Such division leads to one proliferative basal daughter and one suprabasal daughter detached from the basement membrane (Lechler & Fuchs, 2005).

Stem cells of the sebaceous gland are believed be unipotent and only contribute to homeostasis of the sebaceous glands (Horsley *et al*. 2006).

In adult hair follicles, a primitive epidermal stem cell pool has been localized to a region above the follicle bulge. These cells express the marker *Lgr6* but none of the known markers for bulge stem cells. Prenatal $Lgr6^+$ cells have been shown to establish the hair follicle, sebaceous gland, and interfollicular epidermis. In adult tissue, $Lgr6^+$ cells contribute to the generation of the sebaceous gland and interfollicular epidermis, while their role in follicle lineages reduces with age (Snippert *et al.* 2010).

In normal skin homeostasis, the stem cells from the bulge and germlayer do not contribute to the renewal of the interfollicular epidermis and vice versa. However,

when homeostasis is disrupted, cells from all compartments aid in tissue repair and contribute to the production of all skin structures (Ito *et al.* 2005; Levy *et al.* 2007; Fuchs & Horsley, 2008).

1.11 ANIMAL MODELS TO STUDY HUMAN DISEASE

For studies of rare diseases, such as HGPS and RD, animal models are essential. The most common animal used for studying human genetic diseases is *mus musculus*, also known as the house mouse. A number of different methods can be used to induce a human disease in a mouse and thereby create a model organism. The methods can be divided into two major approaches: the non-directed and mutation driven and the directed and disease driven (Simmons, 2008).

The non-directed approach attempts to randomly induce mutations in the animals by exposure to mutagenic doses of radiation or other chemical compounds. Screening of the animals is made in an attempt to find individuals with phenotypes that resemble symptoms of human diseases (Hardouin & Nagy, 2000).

There are a number of directed approaches to induce a mutation in a mouse model. The method chosen depends on the type of mutation involved and for what purpose the model is created.

Transgenic models are generated by adding foreign genetic information to the nucleus of embryonic cells, thereby inducing gene expression. The transgene will be inserted randomly into the genome and, depending on the site of insertion, will be expressed at different levels. To decrease the effects of insertion, yeast or bacterial artificial chromosomes (YAC or BAC, respectively) are used. Their larger size makes them less susceptible to genomic positional effects and more likely to contain regulatory sequences necessary for normal gene regulation (Schedl, 1993; Lamb & Gearhardt, 1995).

The knock-out and knock-in models are useful when a single gene or a specific mutation is known to be the primary cause of a disease. Knock-out models are also used to investigate the function of a specific gene. Both approaches target a mutation to an endogenous gene by homologous recombination. Knock-out models carry a gene that has been inactivated, resulting in reduced expression of the gene, while knock-in models carry a transgene inserted into an exact location in the genome where it is expressed (Simmons, 2008).

Some genes are critical early in development, and therefore knock-out experiments are not useful due to early embryonic death. The Cre/*loxP* models are one way to overcome this problem because they activate conditional knock-out mutations. In Cre/*loxP* models, the Cre recombinase mediates recombination between two *loxP* sequences. To target a gene for a conditional knock-out mutation, an exon, for example, or a larger region of the endogenous gene can be replaced with a homologous gene segment flanked by *loxP* sequences. Mice carrying this targeted mutation are later mated with a strain carrying a *Cre* transgene under the control of a specific promoter. Offspring of this cross containing both transgenes will express Cre in tissues where the specific promoter is active. When Cre is expressed, it induces recombination at the *loxP* sites, deleting the contained sequence. By choosing a specific promoter to control the expression of Cre, it is thereby possible to direct the knock-out of a gene to a specific tissue or at a specific stage of development (Strachan & Read, 2004).

A way to overcome the problem of transgenic products that might be toxic when expressed during development or have negative effects on reproduction is through the use of inducible systems. In these systems an inducible promoter, which can be switched on or off by controlling the supply of a chemical ligand, is used to control the expression of a transgene (Strachan & Read, 2004). One of these systems is the tetracycline-regulated model, used in this thesis and explained in more detail below.

1.12 ANIMAL MODELS FOR HGPS AND RD

Several different approaches have been used to produce mouse models for laminopathies (Stewart *et al.* 2007). Three relevant models for studying HGPS and RD are the BAC transgenic model (Varga *et al.* 2006), the *Zmpste24*$^{-/-}$ knock-out model (Bergö *et al.* 2002; Pendas *et al.* 2002) and the *Lmna*$^{HG/+}$ model (Yang *et al.* 2006)

The BAC transgenic mouse model carries the human *LMNA* p.G608G mutation and over-expresses human lamin A/C and progerin in all tissues. The mice show no external phenotype but display a progressive loss of vascular smooth muscle cells in the media of the large arteries. This phenotype closely reassembles the most lethal aspect of the phenotype seen in patients with HGPS, but the animals do not die prematurely (Varga *et al*. 2006).

The *Zmpste24*$^{-/-}$ is a complete knock-out model without any expression of the zmpste24 enzyme, resulting in defective prelamin A processing. These animals look normal at birth but develop skeletal abnormalities with spontaneous bone fractures. In

addition, they exhibit progressive hair loss, abnormal teething, muscle weakness and premature death at 20-30 weeks of age (Bergö *et al.* 2002; Pendas *et al.* 2002).

The *Lmna*$^{HG/+}$ is a progerin knock-in model, in which one of the *Lmna* alleles only express progerin, and not lamin A or C. These animals are small in size and exhibit loss of subcutaneous fat, progressive hair loss, skeletal abnormalities with spontaneous rib fractures and reduced survival. Homozygous animals *Lmna*$^{HG/HG}$ display a more severe phenotype and die at 3-4 weeks of age with poorly mineralized bones, micrognathia and craniofacial abnormalities (Yang *et al.* 2005; Yang *et al.* 2006).

1.13 TETRACYCLINE REGULATED ANIMAL MODELS

Mouse models with tetracycline controlled gene expression are model systems with the ability to induce transgenic expression both temporally, by adding or removing doxycycline, and spatially, by using a tissue specific promoter. It is a binary transgenic system, which consists of two critical components: the regulatory part and a response element (pTRE element) targeting the expression of a gene of interest. The regulatory part is composed of the regulatory protein tTA or rtTA (the Tet-Off or Tet-On system, respectively) under the control of a tissue-specific promoter. The response element consists of a pTRE element (tetop) linked to the gene of interest. When the regulatory part and the response element co-exist in the same cell, the gene of interest is expressed upon binding of tTA or rtTA to the pTRE element. Both the Tet-On system and the Tet-Off system are regulated by tetracycline or its derivate doxycycline. In the Tet-Off system, the tTA binds to the pTRE element and activates transcription in the absence of doxycycline, while the Tet-On system works the opposite way, with rtTA binding to the pTRE element in presence of doxycycline (Gossen & Bujard, 1992; Zhu *et al.* 2002) (Fig. 5).

The Tet-On/Off systems have the advantages of regulating a specific gene both spatially and temporary *in vitro* and *in vivo*. This regulation is convenient when a gene product is believed to have toxic effects during embryogenesis, or when it can have negative effects during reproduction. It also makes it possible to study the primary effects of a gene product in one specific cell type in a tissue. Additionally, these systems make it possible to study the reversal of an existing phenotype by inhibiting the transgenic expression after the phenotype has developed.

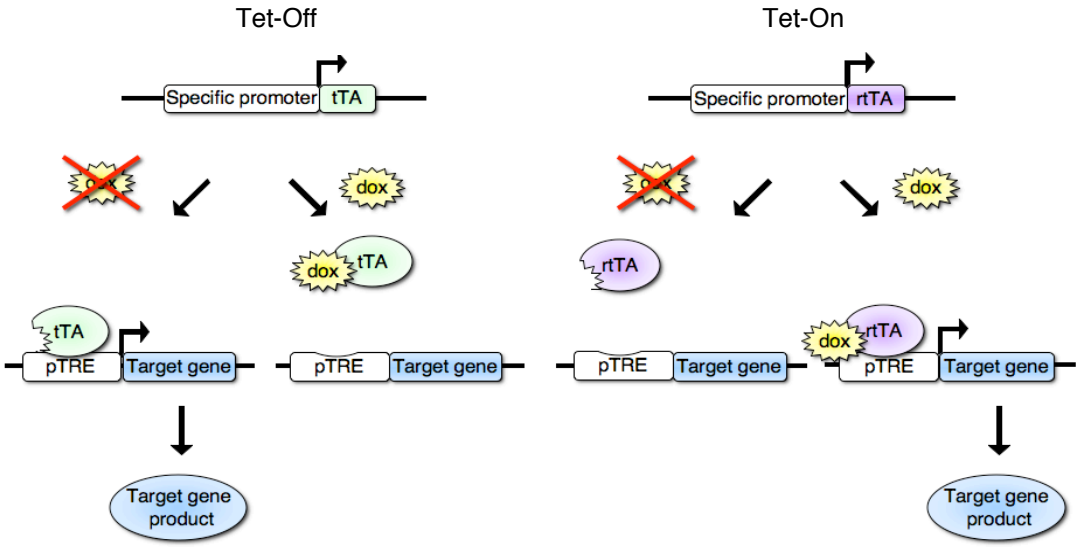

Figure 5. Schematic figure of the regulation of the Tet-Off and Tet-On systems. In this thesis I have been using the Tet-Off system.

1.14 TREATMENT POSSIBILITIES OF HGPS

Today there is no available cure for HGPS. A few patients have been treated with growth hormones, which resulted in increased body height and weight (Merideth *et al*. 2008). However, this approach only aims to improve part of the phenotype, and other treatments need to be found and evaluated.

One possible target for treatment is the post-transcriptional processing of prelamin A to become mature lamin A. In May 2007, a clinical trial using farnesyltransferase inhibitors (FTIs) was initiated. FTIs blocks farnesylation of proteins, including the processing of lamin A, and thereby inhibits the production of progerin. FTIs had previously been developed for anti-cancer therapies, but their use on HGPS cells resulted in the prevention and even the reversal of the misshapen nuclear morphology (Capell *et al.* 2005; Glynn *et al.* 2005; Mallampalli *et al.* 2005; Toth *et al.* 2005; Yang *et al.* 2005; Basso *et al.* 2006). Treatment of the two HGPS mouse models *Zmpste24*$^{-/-}$ (which have no functional zmpste24 enzyme) and *Lmna*$^{HG/+}$ (where one allele of *Lmna* was engineered to produce progerin only) resulted in a milder phenotype (Fong *et al.* 2006; Yang *et al.* 2006). In addition, treatment of the BAC-transgenic model (carrying the *LMNA* p.G608G mutation) with FTIs resulted in the prevention of both the onset as well as the late progression of the cardiovascular phenotype normally seen in these animals (Capell *et al.* 2008).

In the presence of FTIs, an alternative processing of lamin A can occur. In this processing, the farnesylation is substituted with an alternative prenylation by geranylgeranyltransferase, nevertheless resulting in production of progerin. To prevent this, treatment of combined statins and aminobisphosphonates has been shown to inhibit both geranylgeranylation and farnesylation of progerin and prelamin A (Fig 6.). This combination has also been used for cancer treatments and has been shown to improve the phenotype of *Zmpste24*$^{-/-}$ mice and HGPS cells (Varela *et al*. 2008). An ongoing clinical trial involving 45 children with HGPS combines FTIs, statins and aminobisphosphonates (http://www.progeriaresearch.org/clinical_trial.html). Because other laminopathies also show an accumulation of progerin or un-processed prelamin A, the approach of blocking farnesylation might represent a therapeutic strategy suitable for other diseases as well as HGPS.

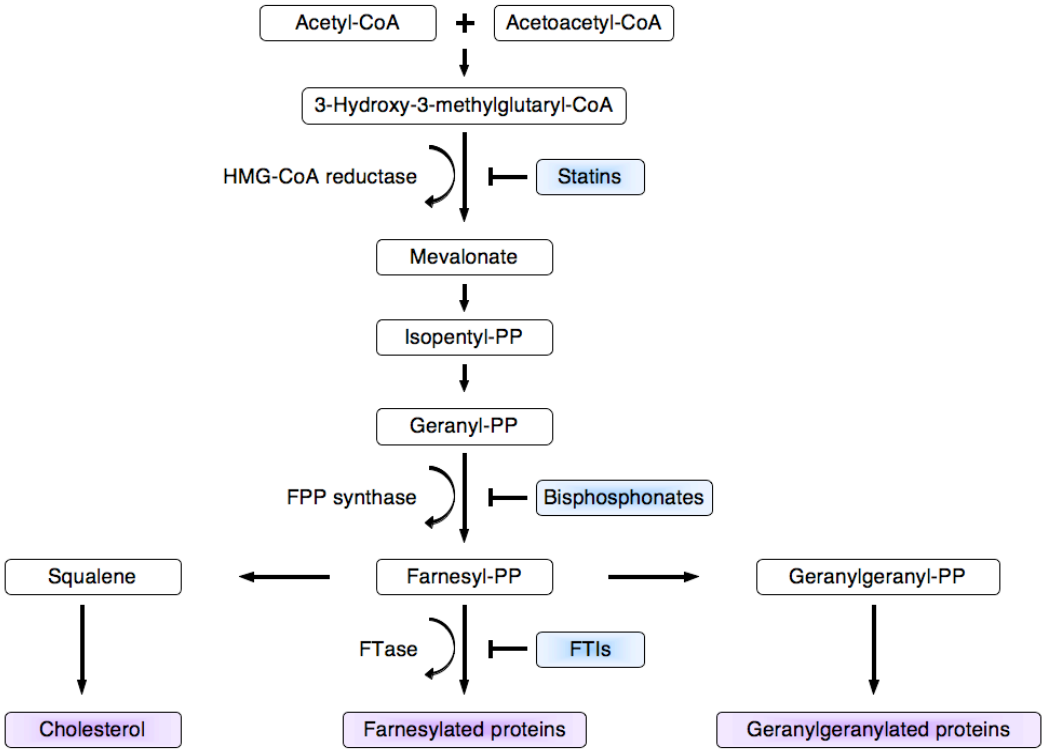

Figure 6. Schematic figure of the isoprenylation pathway and possibilities for treatments. (FPP = farnesyl-pyrophosphate, FTase = farnesyltransferase, FTI = Farnesyltransferase inhibitors, HMG-CoA = hydroxymeythylglutaryl co-enzyme A, PP = pyrophosphatase)

Additional targets for treatment could include different strategies to prevent the production of mutant forms of lamin A. This could be achieved through the use of modified oligonucleotides, so called morpholinos, or RNA interference (RNAi). RNAi treatments, with a short hairpin RNA (shRNA) designed to specifically target the mutant mRNA, have been shown to reduce the expression of progerin in HGPS fibroblasts and reduce the cellular phenotype. Maintaining expression of the shRNA and administration to targeted cells are current limitations of this strategy (Huang *et al.* 2005). Morpholinos that target the cryptic splice site is another strategy to prevent the production of progerin. This approach has been used to restore the nuclear morphology in HGPS cells as well as to rescue the normal nuclear localization and the cellular levels of lamina-associated proteins. The treatment could also reestablish normal expression of several misregulated genes (Saffidi & Misteli, 2005). Morpholinos have been delivered successfully over a continuous period of time in animal models and in humans, thereby overcoming one of the limitations of RNAi-based treatments (Sazini & Kole 2003).

2 METHODOLOGY AND MATERIALS

2.1 LABORATORY ANIMALS

Two different minigene constructs under the control of a tet-operator were generated to create the different founder lines (Fig. 7). One carrying the wild-type sequence of human lamin A (tetop-LA^{wt}) and the other carrying the lamin A c.1824C>T, p.G608G mutation (tetop-LA^{G608G}). Mice carrying these minigenes were intercrossed with mice carrying the keratin 5 transactivator (K5tTA) (Diamond *et al.* 2000) (paper I). In paper II-IV only the minigene with the lamin A c.1824C>T, p.G608G mutation was intercrossed with the K5tTA to get bitransgenic animals. Mice were intercrossed on doxycycline (100 μg/ml, 2.5% sucrose), which was removed at the day of birth (postnatal day 0, d0) or at weaning on d21 (paper I-III). The drinking bottles containing doxycycline were changed every third day and wrapped in foil in order to protect the light-sensitive antibiotic. In paper IV embryonic expression of the transgene was studied and mice were intercrossed on regular water (without doxycycline).

Animals were used in accordance with the guidelines for care and use of experimental animals at the Karolinska Institutet. All animal work was approved by Stockholms Södra Djurförsöksetiska Nämnd. The mice were housed within the animal facilities at the Karolinska Institutet, Huddinge, Sweden. A standard diet and water was supplied *ad libitum*. Bitransgenic animals and controls were supplied with dissolved pellets on the floor from d21.

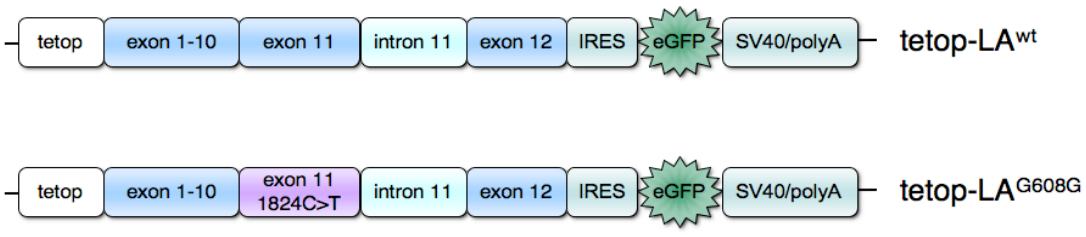

Figure 7. Schematic figure of the minigene constructs. The top construct, tetop-LA^{wt} , overexpresses human wild-type lamin A. The bottom construct, tetop-LA^{G608G}, overexpresses human wild-type lamin A and human progerin.
(tetop = pTRE element, IRES = internal ribosomal entry site, eGFP = enhanced green fluorescent protein, polyA = polyadenylation sequence)

2.2 PCR GENOTYPING

A small piece of the tip of the tail of each pup was clipped for DNA isolation. The DNA was extracted using standard phenol:chloroform method and precipitated by isopropanol. Presence or absence of the transgenes was confirmed by PCR with primers for the lamin A minigenes (tetop-LA^{G608G} and tetop-LA^{wt}) and K5tTA. Primers for *myc* were used as a positive control to analyze the quality of the DNA and the amount of genomic DNA in the extraction.

2.3 ANIMAL TISSUE COLLECTION AND PROCESSING

Animals for tissue collection were sacrificed by an overdose of isoflurane and cervical dislocation, after which the skin was harvested. The tissue was frozen in liquid nitrogen and stored at -80°C for further RNA or protein preparation. Tissues were also collected for β-galactosidase staining; see below. The tissues used for immunohistochemistry, immunofluorescence and histopathology staining were fixed in 4% paraformaldehyde (PFA) overnight at 4°C. After the overnight incubation, the tissues were dehydrated in 70% ethanol and further processed in a vacuum infiltration processor (V.I.P., Huddinge hospital). After the dehydration process, the tissues were embedded in paraffin and sectioned using a microtome. Four or five micrometer thick sections were dried and mounted onto glass slides. The slides were then subjected to the different staining methods.

In addition to skin, collection of the gastrointestinal system, teeth, lower jaws, liver, pancreas, spleen, thymus, mammary glands, salivary glands, brown fat, tear glands, kidneys, adrenal glands, reproductive organs, skull, tongue, brain, trachea, lungs and the respiratory mucosa of the nasal cavity was collected for pathological analysis (paper I).

2.4 STAININGS FOR HISTOPATHOLOGY

To be able to analyze distinctive histological structures, different staining methods were used. Sections of tissue were attached to Superfrost slides (Menzel-Gläzer).

The hematoxylin and eosin (H&E) stain was used in most cases for examination of pathologies. H&E is a common staining method where the hematoxylin component stains the nucleus blue/black, while the eosin stains cell cytoplasm and most connective tissue fibers in varying shades and intensities of red, pink and orange (Wilson and Gamble, 2002).

In the wound healing study in paper III, Masson's trichrome technique was used to distinguish collagen fibers in the wounded area. In Masson's trichrome staining, the nucleus is stained blue/black and the cellular cytoplasm, muscle and erythrocytes are stained red, while collagen is stained blue, and a darker blue corresponds to older/more dense collagen (Jones, 2002).

To distinguish collagen in young animals with transgenic expression during embryogenesis in paper IV, the van Gieson technique was used. In the van Gieson technique, the nuclei is stained blue/black and collagen is stained red, while other tissues are stained yellow (Jones, 2002).

All histological stainings were done according to standard procedures.

2.5 IMMUNOFLUORESCENCE STAINING

To analyze the expression of certain proteins in different cells and in different compartments of the skin, immunofluorescence staining was used. The use of secondary antibodies conjugated with different fluorophores made it possible to analyze more than one protein in the same sample. This makes it possible to search for co-expression of two proteins within the same cell. For immunofluorescent staining, 4-μm thick sections were re-hydrated in a series of incubations in decreasing concentrations of ethanol. To expose the epitope in the sections, a subsequent step of antigen retrieval was necessary. Several different methods for antigen retrieval have been tested for even and accurate stainings by different antibodies. The antigen retrieval methods use either a microwave oven to heat the sections in citrate buffer or a pressure cooker with EDTA. After antigen retrieval, the sections were blocked in normal goat serum followed by mouse-to-mouse blocking reagent (SCYTEC Laboratories, this step was only necessary if the primary antibody was raised in mouse). The primary antibody was incubated overnight at 4°C. On the following day, the sections were washed in PBS and incubated with the secondary fluorophore-conjugated antibody. Following a 1 hour incubation in the dark at room temperature, the slides were washed and mounted with Vectashield mounting medium containing DAPI (Vector laboratories). DAPI binds to DNA, and when excited it emits a blue light, thereby staining all cell nuclei in a tissue section blue. The sections were analyzed with a direct fluorescence microscope (Axioplan 2, Zeiss) or a laser inverted confocal microscope (LSM 510, Zeiss).

Immunofluorescent stainings were also made on primary keratinocytes that had been isolated from mice of different ages. Using a Shandon Cytospin 4 (Thermo

Electron Corporation), 100,000 cells were centrifuged onto a glass-slide. Cells were dried and fixed by an incubation in 4% PFA for 30 minutes. Using 1% NP-40 in PBS (Surfact-Amps NP-40, Pierce Biotechnology), cells were permeabilized. After permeabilization, the cells were blocked in 5% normal goat serum and 0.1% Birj (Surfact-Amps 58; Birj, Pierce Biotechnology), followed by incubation with the primary antibody at 4°C overnight. The samples were incubated with the secondary fluorophore-conjugated antibody at room temperature in the dark before mounting and analysis in a fluorescence microscope.

2.6 IMMUNOHISTOCHEMISTRY

Immunohistochemistry is an alternative method to analyze expression of proteins in a tissue section. By this method it is easier to see histological structures in the tissue and it is also possible to estimate expression levels of a protein. To achieve uniform staining a microwave oven was used for antigen retrieval to heat the tissue in citrate buffer. After the antigen retrieval endogenous peroxidase activity was eliminated by incubation in methanol containing hydrogen peroxide. Before incubation with the primary antibody the sections were blocked by normal goat serum in PBS. The primary antibody was diluted in BSA and incubated at 4°C overnight. After washing the secondary biotinylated antibody was added. The Vectastain ABC kit (Vector laboratories) was used to visualize the peroxidase according to manufacturer's recommendations. Nuclear counterstaining was accomplished by haematoxyline (htx) staining and sections were analyzed in a bright-field microscope.

2.7 BRDU LABELING AND LABEL-RETAINING CELL ANALYSIS

Bromodioxyuridine (BrdU) is an analogue to thymidine that, when present, incorporate into newly synthesized DNA during cell division. To assay the number of stem cells in the skin four day old bitransgenic and wild-type mice received five BrdU injections at 24-hour intervals (paper III). After the multiple injections almost all of the cells in the skin were labeled. During every cell division the BrdU label gets diluted, and after a chase period of 70 days, only the cells that have rarely divided kept their labels. Label retention is consequently a marker of the proliferation history of a cell. Because stem cells are slow cycling by definition, these label-retaining cells were considered stem cells (Braun *et al.* 2004). Animals were sacrificed 70 days after the last injection and

dorsal skin was collected. BrdU incorporation was detected by immunofluorescence staining on 4μm thick sections counterstained with K5.

2.8 WOUND HEALING

One important function of the epidermal stem cells is to actively contribute to wound repair (Ito *et al.* 2005). After a wounding of the skin the stem cells proliferate and their daughter cells migrate in order to close the open wound. To study the wound healing in our animals we introduced a 3mm wound on the backs of bitransgenic and wild-type animals (paper III). The wound healing was analyzed after four and seven days of recovery. After the recovery period the animals were sacrificed by an overdose of isoflurane and cervical dislocation, and the wounds with surrounding skin were excised. To mark proliferating cells BrdU was injected one-hour prior to sacrifice. BrdU is incorporated into DNA during DNA synthesis in dividing cells and with a short-term chase period only proliferating cells are marked. After the wounds had been excised they were processed for histological analysis with H&E and Masson's trichrome staining. BrdU incorporation was detected by immunofluorescent staining on 4μm thick sections counterstained with K5.

2.9 KERATINOCYTE ISOLATION

To analyze the properties of the cells in the epidermis keratinocytes were isolated from bitransgenic animals and wild-type littermates (paper III). The animals were sacrificed by an overdose of isoflurane and cervical dislocation, and the fur was trimmed with an animal hair clipper. The skin was removed and placed into Ca^{2+} free PBS. A scalpel was used to remove subcutaneous tissue and to divide the skin into stripes. The stripes were floated on trypsin with the epidermis upwards in a Petri dish for two hours at 32°C. After incubation the epidermis was scraped into S-minimal essential medium (S-MEM) supplemented with soybean trypsin inhibitor and BSA. The mixture was gently stirred with a magnetic stirrer for 20 minutes at room temperature. To get a single cell suspension the mixture was filtered through a 70 μm cell strainer and viable cells were counted after staining with tryphan blue.

2.10 FLUORESCENCE-ACTIVATED CELL SORTING

To study the population of stem cells in the skin the isolated keratinocytes were analyzed by fluorescence-activated cell sorting (FACS) (paper III). This method allows

measuring the expression of two different proteins within the same cell by the use of specific antibodies. In keratinocytes the co-expression of the two proteins α6-integrin and CD34 mark cells with stem or progenitor cell characteristics (Trempus *et al.* 2003, Tumbar *et al.* 2004, Silva-Vargas *et al.* 2005). For the FACS analysis single cell suspension of isolated keratinocytes were blocked before incubation with PE-Cy5-conjugated anti-α6 integrin (CD45f, BD biosciences) and PE-conjugated anti-CD34 (RAM34, eBiosciences) antibodies. The antibodies were diluted in 2% FBS (Chelex treated to remove Ca^{2+}) and incubated for two hours at 4°C. From each sample a minimum of 32,000 events were analyzed with a FACScalibur flow cytometer (BD biosciences).

2.11 COLONY FORMATION

When stem cells are cultivated *in vitro* they form large self-renewing colonies while their non-stem cell daughters divide only a few times before undergoing terminal differentiation (Barrandon and Greeen, 1987). To analyze the ability of primary keratinocytes to form colonies *in vitro* 30,000 isolated primary keratinocytes were plated onto collagen IV pre-coated six-well plates (paper III). The plates were pre-seeded with a feeder layer of growth arrested, by γ-irradiation (30 Gy), NIH-3T3 cells. The cells were grown in epidermal keratinocyte medium (CnT-02, CELLnTEC) for 16 days before they were rinsed with PBS and fixed in 4% PFA. To visualize the colonies the cells were stained with Rhodamine B (Sigma-Aldrich). Size of colonies was measured using a ruler and the amount of colonies of different sizes was recorded.

2.12 EPIDERMAL PERMEABILITY ASSAY

One of the most important functions of the skin is to serve as a barrier between the body and the environment. The epidermal permeability barrier is developed between embryonic day 16 and 17 (Hardman *et al.* 1998). To investigate if the formation of the epidermal barrier was delayed in bitransgenic animals in paper IV, where they had transgenic expression during embryogenesis, an epidermal permeability assay was performed. To know the age of the embryos, females in breeding were checked for a mating plug. The morning a mating plug was noted was considered embryonic day 0.5. At embryonic day 17.5 the female was sacrificed by cervical dislocation and the embryos were harvested. The embryos were sacrificed by an overdose of isoflurane and washed in PBS. After washing, the skin was dehydrated in methanol before being

rehydrated in water. After rehydration the animals were soaked in Toluidine Blue (0.1%) and washed in PBS before being photographed with a digital camera. Toluidine Blue is a dye that will penetrate skin with a poor epidermal barrier, staining it blue, while skin with a functional epidermal barrier will remain unstained.

2.13 WESTERN BLOT

To quantify protein levels in animals at different ages western blots were performed (paper I and IV). Protein was extracted from mouse skin in 8M urea supplemented with 5% RIPA buffer and homogenized with Lysing Martrix D (Qbiogene) and Fastprep 220A (Qbiogene). To achieve enhanced protein separation a large western blot system was used consisiting of 1mm thick and 20 cm long 4%/7.5% discontinuous Laemmli slab gel. The gel was run for 11 hours at 20 mA using a cooling system to achieve well-separated bands. Proteins were transferred to a nitrocellulose membrane (Hybond-C+, Amersham Biosciences) according to standard procedure using a Semidry Transfer Cell (BIORAD). After transfer the membrane was blocked in 5% milk in TBS-T before overnight incubation at 4°C with the primary antibody. Primary antibodies used for western blot were: anti-lamin A/C (N-18, Santa Cruz Biotechnology) and anti-β-actin (AC-15, Sigma). After washing the membranes were incubated with the HRP conjugated secondary antibody, diluted in 5% milk in TBS-T. After washing ECL plus (Amersham Biosciences) was added to the membranes for 5 minutes at room temperature. Directly following this step the membranes were exposed to film for different lengths of time. Quantification of the proteins was performed on western films by the use of Versa Doc Imaging systems (Bio-Rad) and analyzed using the Quantity One software (Bio-Rad). The relative band intensities were normalized to the housekeeping gene β-actin from the same lane and only bands from the same film were compared to each other. Care was taken to avoid quantification on films that had longer exposure times.

3 AIMS OF THE THESIS

This thesis is focused on gaining a deeper understanding of the effects caused by the expression of the most common HGPS mutation (*LMNA* c.1824C>T, p.G608G), thereby improving our understanding of the molecular mechanisms behind HGPS. A deeper knowledge of the *LMNA* c.1824C>T mutation would not only be important for patients with progeria and their families but also would allow us to gain insight into the role the *LMNA* gene might play in physiological aging.

The first aim of this thesis was to develop a transgenic mice model that exhibited inducible and tissue specific expression of the lamin A c.1824C>T mutation in order to produce a good model for the study of HGPS (Paper I).

The second aim was to investigate the effects of progerin on the progress of the hair cycle and to determine if expression of progerin influences the expression of lamin B, thereby broadening our understanding of the skin phenotype seen in the lamin A c.1824C>T expressing mice. To accomplish the second aim, we first characterized the normal hair cycle and expression patterns for lamin A/C and B (Paper II).

The third aim was to increase our understanding of the molecular mechanisms in HGPS by studying the effects of over-expression of the lamin A c.1824C>T mutation on the epidermal stem cells. In addition, we sought the molecular mechanisms behind the stem cell effects and the connection between progerin expression and senescence (Paper III).

The fourth aim was to investigate the effects from over-expression of the lamin A c.1824C>T mutation during embryogenesis. In addition, we wanted to test if over-expression of the HGPS mutation during embryogenesis would result in a more severe HGPS phenotype that would present with symptoms similar to RD (Paper IV).

4 RESULTS AND DISCUSSION

4.1 PAPER I

HGPS is a very rare disease with devastating symptoms for the patients. To gain insight into the molecular basis of the disease and to investigate possible interventions for the patients, animal models are essential. In this study, we generated two transgenic mouse lines. One of the lines expresses a minigene of human wild-type lamin A, and the other line expresses a minigene of human lamin A carrying the most common HGPS mutation (*LMNA* c.1824C>T, p.G608G). The two minigene constructs were under the control of an inducible promoter. By using the tet-off system, expression of the minigenes was limited to a specific tissue and was temporally regulated.

The clinical picture in HGPS involves multiple organs. Skin is one of the first organs to display a typical disease phenotype, including scleroderma, loss of subcutaneous fat and alopecia. Based on this, we decided to use the tetracycline-inducible transactivator line K5tTA (Diamond *et al.* 2000) to direct the expression of our minigenes to the basal cells of the interfollicular epidermis and the hair follicle.

Analysis of RNA from skin samples of bitransgenic animals with the lamin A c.1824C>T, p.G608G mutation showed the appearance of progerin RNA, indicating that the mouse splicing machinery recognized the human HGPS mutation as a splice site. At the protein level, the appearance of human progerin was determined by western blot in bitransgenic animals. The expression pattern of the transgene was evaluated by immunofluorescence staining with an antibody specific for human lamin A/C and counterstained with an antibody for K5. A connection between expression levels of the minigene and phenotype progression was noted. Founder lines with low expression showed no pathological changes in animals as old as two years. Even and high expression of the minigene was detected in bitransgenic animals from both lamin A wild-type- and lamin A c.1824C>T-carrying mouse lines in K5-expressing cells in the skin analyzed by immunofluorescent staining. The immunofluorescent staining was confirmed by western blot, with accumulation of human progerin and human prelamin A present in the skin of bitransgenic animals.

Bitransgenic animals with high expression of the lamin A c.1824C>T mutation had an external phenotype with thinned hair, growth retardation and premature death. By giving the animals soft food made from dissolved pellets, the median age was increased from 7 weeks to 29 weeks in bitransgenic animals, when doxycycline was

removed from the day of birth (d0). When doxycycline was removed at weaning (d21), the median survival age was 14 weeks. Bitransgenic animals with the wild-type minigene did not show any growth retardation or premature death.

Histological analysis of lamin A c.1824C>T-expressing bitransgenic animals revealed a progressive phenotype. When the expression of the transgene was induced at d21, the animals first entered an intermediate hyperproliferative stage with regions of milder or more severe epidermal hyperplasia. The hyperplasia was associated with hypergranulosis and hyperkeratosis, evident after 6 weeks of transgenic expression. The hair follicles showed dystrophic changes as well as hyperplastic and irregularly matured sebaceous glands. The phenotype also included inflammatory cells in the dermis and, in more severe regions, dermal fibrosis. The disease evolved further, and within 17 weeks of transgenic expression the phenotype developed into an end stage. In this stage, the skin was characterized by epidermal hypoplasia, hypoplastic sebaceous glands, absence of hypodermis and a well-developed fibrosis in the dermis.

To study the reason for the epidermal hyperplasia seen at the intermediate stage, immunohistochemistry stainings were performed with antibodies against keratins 5 and 6 (K5 and K6). Mislocalization of keratin 6 indicated hyperproliferation, and further staining with an antibody against phosphohistone H3 confirmed increased proliferation. Skin samples with hyperplasia from the intermediate stage were also examined to test if normal epidermal differentiation was altered. Immunofluorescent stainings with the differentiation markers keratin 1 and 10 (K1 and K10), loricrin and filaggrin indicated normal differentiation. However, both the granular and spinous layers were thicker compared to wild-type mice.

When other K5-expressing organs than the skin were analyzed, the only histopathological changes included possible mild hyperplasia in the stomach as well as more extensive changes in the lower incisors and surrounding tissue. The changes in the lower incisors consisted of food and other foreign material in the pulp, with acute inflammation and necrosis as a response.

In this study, we estblished a mouse model that expresses progerin in the skin. The induction of lamin A c.1824C>T expression in the basal cells of the epidermis leads to a skin phenotype affecting multiple layers of the skin. Even if the dermis and the hypodermis do not express the transgene severe effects are seen in these skin compartments, suggesting secondary effects of the transgene expression.

In summary, we have developed an inducible and tissue-specific mouse model, which expresses progerin in the skin. The expression of progerin induces a progressive

phenotype, beginning with hyperproliferation and ending with a phenotype resembling many of the clinical features of the skin reported in children with HGPS. Therefore, this model should be useful in studies contributing to extending the understanding of this disease as well as to examine possible treatments.

4.2 PAPER II

Major skin phenotypes are seen in patients with at least three different diseases caused by mutations affecting lamin A and C. These disorders include the premature ageing disorders HGPS and MAD as well as RD. To increase the understanding of the mechanisms behind these phenotypes, there was a need to improve our knowledge of normal lamin A/C and B expression in different cells of the skin during the hair cycle. One of the phenotypes seen in our bitransgenic animals expressing the lamin A c.1824C>T mutation is hair thinning. In light of this, we decided to examine if progerin expression alters the hair cycle or affects the expression of lamin B. To examine if there were any changes, we first classified the different stages in the normal hair cycle and the expression of lamin A/C and B.

To classify the normal hair cycle, skin from wild-type FVB/NCrl mice was collected at different time points, from the day of birth (d0) to postnatal day 70 (d70). The longitudinal midline of the dorsal skin was divided into equally sized portions, beginning between the ears and reaching gradually to the base of the tail. The skin was sectioned and stained with H&E and analyzed in a bright field microscope. The classification of the different stages and sub-stages of the hair cycle were performed according to previously published morphology guidelines (Müller-Röver *et al.* 2001). Analysis of skin from d0 to d12.5 mice demonstrated a developing skin with various stages of anagen hair follicles. The first postnatal hair cycle was indicated by growing follicles and entrance into the first catagen phase, which started at d15. The first catagen phase was followed by the first telogen phase, which was seen in 19- and 20-day-old animals. At d21, anagen follicles were already seen. This anagen phase lasted until d35, when the second hair cycle was initiated with follicles in the catagen phase. Catagen follicles were still present at d42 and d70. At d70, anagen follicles were first noted.

To analyze the normal expression of lamin A/C and B in wildtype mice, we performed immunohistochemistry, using an antibody directed against lamin A/C and an antibody directed against B_1 with minor cross-reactivity to B_2 and B_3. Strong

expression of lamin A/C and B was seen in the basal cells of the epidermis, the outer root-sheath and the dermal papilla in all stages of the hair cycle. When compared to the basal cells of the epidermis, the supra basal cells constantly showed lower expression of both lamin A/C and B. The hypodermal cells stained only weakly for lamin A/C and B in all phases, while the sebaceous gland had even staining of lamin A/C and B in catagen and telogen but a stronger staining of lamin B in the anagen phase.

The strong expression of the lamins in the basal cells of the epidermis and the outer root-sheath supports the importance of these proteins in these compartments of the skin. This is of great interest when we look at our inducible transgenic mouse model for HGPS, because the transactivator K5tTA directs the expression of our transgene to these skin compartments. When these animals were investigated, we could not observe any difference in the hair cycle progression or the lamin B expression at d15, d19 and d21 when compared to wild-type controls. Expression of the lamin A c.1824 C>T mutation consequently neither leads to any immediate alterations of the first hair cycle nor has a great effect on lamin B expression.

A number of factors can influence the progress of the hair cycle *i.e.* gender, strain background and nutritional and environmental factors (Paus & Cotsarelis 1999). Due to these factors it is not possible to make a guideline applicable for all conditions. In our study we kept these factors constant, regardless of gender. We did not see any significant differences in the hair cycle between mice of the same age or of different sexes. Although hair follicle morphogenesis and the first postnatal catagen, telogen and anagen phases followed a strict timeline we could see differently phased hair follicles in different regions of the skin at the same age.

In summary, in this study, we have classified the normal hair cycle in FVB/NCrl wild-type mice and analyzed the normal expression patterns of lamin A/C and B in the different phases of the hair cycle. Additionally, we investigated the hair cycle and the expression patterns of lamin B in mice expressing the lamin A c.1824 C>T mutation and did not find any obvious differences when compared to wild-type controls.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

We have developed an inducible mouse model that carries a human minigene for lamin A containing the most common causal mutation for HGPS (*LMNA* c.1824C>T, p.G608G). The minigene causes over-expression of both wild type human lamin A and progerin. Targeting the transgene expression to K5-expressing tissues resulted in a progressive skin phenotype. The phenotype evolved from an intermediate stage with hyperplasia and hyperproliferation of the epidermis to an end stage with hypoplastic epidermis, a fibrotic dermis and loss of subcutaneous tissue. The end-stage phenotype in our mice corresponds to several clinical features of the skin of patients with HGPS. This mouse model was used to further investigate the molecular effects of expression of the lamin A c.1824C>T mutation in the skin.

Using our model, we could conclude that progerin expression appears to not influence the normal progression of the first hair cycle or the expression of endogenous lamin B. To study the influence of the lamin A c.1824C>T mutation, we first determined the normal expression patterns of lamin A/C and B in the phases of the hair cycle as well as the normal hair cycle progression. The mouse model was also used to study the effect of the lamin A c.1824C>T expression on stem cells in the skin.

The work presented in this thesis provides insights to the molecular mechanisms behind HGPS. However, additional research is still needed to understand the disease mechanisms behind HGPS. Future studies would involve continued investigation of the phenotype seen in mice with embryonic expression of the *LMNA* c.1824C>T mutation.

Even if skin is one of the first tissues to present with a disease phenotype in patients with HGPS and remains a good system for studying the effects of stem cells, it is not certain that the results from these experiments can be extended to other tissues. To further understand the mechanisms behind HGPS and to obtain a more complete picture of the disease, it is important to study other affected tissues. Our mouse model provides a system to study individual organs separately by using different tissue-specific promoters. Other subjects of interest could be bone remodeling, lipid metabolism, brain function and effects on the cardiovascular system.

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