Epithelial Stem Cells In Hutchinson-Gilford Progeria Syndrome

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Thesis for Doctoral Degree (Ph.D.)

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In ómós do mo mháthair agus do m`athair,
mo bhean agus mo mhab.
Abstract

Hutchinson-Gilford progeria syndrome (HGPS) and restrictive dermopathy (RD) are two rare genetic disorders that affect children. Complications from cardiovascular disease, including atherosclerosis, are the most common cause of death in HGPS, which occurs at around 13 years of age. RD patients seldom live beyond their first few weeks of life, with their characteristically tight skin causing death by restricting respiration.

These diseases are caused by mutations that cause the accumulation of prelamin A, or one of its truncated forms, prelamin AΔ50 (progerin). The majority of HGPS cases are due to a de novo single point mutation in exon 11 of the LMNA gene, c.1824C>T (p.G608G). This gene encodes the A-type lamins, which are major proteins of the inner nuclear lamina. Most cases of RD are caused by loss of function mutations in the lamin A processing zmpste24 enzyme.

Inducible and tissue specific mouse models were used to examine the mechanistic effects of progerin. The mouse models carried an artificial minigene which encoded lamin A with the most common HGPS mutation. This minigene was targeted to the skin in Papers I and II by means of the keratin 5 (K5) promoter, and to the cardiovascular system in Paper III by means of the sm22α promoter.

In Paper I, the effects of progerin were examined with a focus on postnatal skin. Our results showed a reduced population of keratinocytes with stem cell properties. This was associated with downregulation of P63 (an epidermal stem cell maintenance protein) in the HGPS mice, an effect also found with increased ageing in HGPS patients cells.

In Paper II, to test the hypothesis that the more severe symptoms in RD, as compared with HGPS, are due to the higher levels of farnesylated lamin A produced in RD, the LMNA c.1824C>T, p.G608G was expressed in embryonic skin causing early postnatal death, as in the human condition. This was accompanied by increased inflammation, prolonged expression of the lamin B receptor gene, and arrested skin development.

In Paper III a model was designed to examine the effects of progerin in the cardiovascular system. However the expression of the reverse sm22α transactivator was barely detectable in arteries, and this low level of expression was not sufficient to induce expression of the target human lamin A gene.

This thesis offers novel findings about the intricate molecular disease mechanisms underlying HGPS and RD.
Publications

I. Stem cell depletion in Hutchinson-Gilford progeria syndrome

II. Embryonic expression of the common progeria lamin A splice mutation arrests postnatal skin development

III. Low levels of the reverse transactivator fail to induce target transgene expression in vascular smooth muscle cells

Related Publications

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Related Manuscripts

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• Global genome splicing analysis reveals an increased number of alternative spliced genes with aging
  Rodríguez S, Grochová D, McKenna T, Borate B, Trivedi N, Wolfsberg T, Baxevanis A, Erdos M, Eriksson M.

• Effects of lamin B receptor overexpression on heterochromatin localization and cellular differentiation
  Sola-Carvajal A, McKenna T, Wallén Arzt E, Eriksson M.
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1. Introduction

1.1. Hutchinson-Gilford Progeria Syndrome

Hutchinson-Gilford Progeria Syndrome (HGPS, Online inheritance in man 176670 (OMIM)), is a rare genetic disease causing features suggestive of premature ageing in children. First described in 1886 by Hutchinson, and in 1904 by Gilford,\(^1,2\) it was not until 2003 that the genetic cause of progeria was found,\(^3,4\) a \textit{de novo} mutation in exon 11 of the \textit{LMNA} gene, c.1824C>T. This silent mutation (G608G), causes no change in the resultant amino acid (which remains a Glycine), however it increases the use of an aberrant cryptic mRNA splice site, resulting in the production of a protein known as progerin.\(^3,4\) Although numerous mutations have now been discovered that cause HGPS (Figure 5), this mutation accounts for the vast majority of HGPS cases. It is the accumulation of progerin that is thought to cause the ageing-pathophysiology of HGPS. The disease occurs in both sexes with a slightly higher incidence in males (1.2:1), and is found across diverse biogeographical ancestries seemingly without bias.\(^5\)

Children with HGPS appear normal at birth, however during their first years of life the phenotype becomes apparent. The skin is where the symptoms first appear, and appear most strikingly, with a scleroderma-like appearance. The children also fail to thrive, showing severe growth retardation (Figure 1).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{child_hgps.png}
\caption{A child with HGPS. A child, aged seven years old in the picture on the left, and twelve on the right, shows the characteristic diminished stature and alopecia of HGPS.\(^2\)}
\end{figure}
These symptoms are followed by the development of a striking aged-like appearance revealing itself with symptoms such as alopecia (hair loss, affecting even the eyebrows), prominent forehead and scalp veins. A loss of subcutaneous fat causes a thin and tight appearance of the skin.\textsuperscript{6-10}

Not only do these children take on the appearance of an aged individual, but many ageing-associated health problems also affect them. The disease affects many organ systems of the body (such as bones, adipose, skin, hair, cardiovascular). Osteolysis, the loss of bone, occurs as the children develop, particularly affecting the distal phalanges and clavicles.\textsuperscript{6,10-13} Other bone abnormalities are seen, such as delayed and abnormal dentition and micrognathia (a small jaw). Joint stiffness leading to limited joint mobility also occurs. Some organs seem to be completely unaffected, such as the kidney, liver, lung, gastrointestinal tract, bone marrow and brain. The patients show normal intelligence and studies on brain reveal no significant disease pathology.\textsuperscript{6,11,12} This segmented tissue specificity of HGPS is one of the diseases most intriguing characteristics.

The children with HGPS die at an average age of approximately 13 years, with over 90\% of deaths caused by complications due to the progressive atherosclerosis of the coronary and cerebrovascular arteries.\textsuperscript{14,15}

### 1.2. Restrictive Dermopathy

Restrictive dermopathy (RD, OMIM 275210) is a rare, lethal autosomal congenital disorder. It is caused by dominant \textit{de novo} mutations in the \textit{LMNA} gene or, more commonly, recessive null \textit{ZMPSTE24} mutations. The earliest symptoms are intrauterine growth retardation and decreased foetal movement. After a usually premature birth the child displays a thin, translucent and tight skin, as well as joint contractures and respiratory insufficiency. Other symptoms include a small pinched nose, posteriorly rotated and low-set earlobes, lack of eyebrows and eyelashes, micrognathia and a mouth fixed in a characteristic ‘o’ shape. Death occurs within a few weeks of birth by respiratory failure due to the tight skin.\textsuperscript{16,17}

Histology of the skin revealed thin epidermal layers with regular structure, immature and poorly developed sebaceous glands and hair follicles, and a flat epidermal-dermal junction. The dermis displayed parallel collagen bundles and almost no elastic fibres.\textsuperscript{16,18} Mechanistically RD is thought to be similar to HGPS, with progerin or prelamin A accumulation causing the disease. It has been suggested that the more severe effects of RD compared to HGPS correspond with increased levels of progerin or prelamin A.\textsuperscript{16,19}

**Figure 2. The nuclear envelope (NE) (Opposite page).** The nuclear A- and B-type lamins lie under the nucleoplasmic side of the inner nuclear membrane (INM). They provide nuclear stability, an organisational binding platform for chromatin, and also facilitate localisation and binding of a large family of nuclear envelope proteins. Lamina-associated polypeptide 1 and 2, LAP1 and LAP2; Lamin B receptor, LBR; LEM domain-containing protein 3, MAN1; Outer nuclear membrane, ONM; Perinuclear space, PNS; Nuclear envelope spectrin repeat proteins 1 and 2, Nesprin 1 and Nesprin 2; Nuclear pore complex, NPC; SUN, Sad1p and UNC-84 homology.\textsuperscript{20}
1.3 The Nucleus, Nuclear Envelope, And The Lamina

The nucleus is known as the control centre of the cell, and is the defining characteristic organelle of the eukaryotic cell. It contains the majority of the cells genetic material, and is segregated from the cellular cytoplasm by the bilayer nuclear envelope (NE) (Figure 2). The NE consists of concentric inner and outer nuclear membranes (the INM, and ONM). Between the INM and ONM lies the perinuclear space (PNS). The ONM is continuous with the rough endoplasmic reticulum (ER), and like the ER it is studded with protein-producing ribosomes. Large, complex and heterogeneous protein structures known as nuclear pore complexes (NPCs) connect the INM and ONM, and act as selective channels through the NE.\(^{21,22}\)

Small molecules under approximately 40 kDa are passively transported through the NPCs. Larger molecules such as mRNAs, tRNAs, ribosomes, proteins, lipids, carbohydrates or signalling molecules can be actively transported both into and out of the nucleus.\(^{23,24}\) The INM lies directly over, and is connected to, the filamentous proteins of the nuclear lamina.\(^{25}\) This thin (30-100nm) structure is a densely woven mesh made primarily of A-type lamin proteins, which are encoded by the \textit{LMNA} gene, as well as B-type lamins, and lamin associated proteins. It plays a major role in both structure, such as determining the size and shape of the nucleus, but also in cellular processes such as DNA replication and transcription.\(^{26-29}\)

The lamina also play a key role in anchoring proteins to the NE, such as the NPCs, as well as binding heterochromatin, chromatin and transcription factors to the nuclear periphery.\(^{27,30,31}\) The A and B type lamins form distinct but interacting lattices in the nuclear lamina, and lamins A and C segregate \textit{in vivo}, which suggests that lamins form distinct homodimers,\(^{32}\) although they have been found to form heterodimers \textit{in vitro}.\(^{26}\)

The cytoskeleton is a dynamic network of protein fibres which lies in the cytoplasm, and is linked to the nucleus by the NE. It is made up of microtubules, microfilaments and intermediate filaments. This structure has a multitude of functions, such as giving the cell shape and resistance to mechanical stress, aiding in cell division and intracellular transport, and acting as a scaffold to organise cellular contents (Figure 3).\(^{33}\)
1.4 Lamins And The \textit{LMNA} Gene

The \textit{LMNA} gene (OMIM: 150330) is composed of 12 exons located on chromosome 1q22 (Figure 5). By means of alternative splicing within exon 10 it encodes the major isoforms of A-type lamins, lamin A and lamin C, as well as the minor isoforms \(\Delta10\) and C2. Lamin A/C proteins form the majority of the nuclear lamina, and are mainly expressed in terminally differentiated cells, suggesting that they have a role in stabilising differential gene expression.\(^{34}\) Lamins A and C are identical for the first 566 amino acids, thereafter the C-terminal of lamin A has 98 unique amino acids and ends in a CAAX motif. Lamin C ends instead with a unique sequence of 6 amino acids. The second family of lamins consists of B-type lamins, lamin B1 encoded by the \textit{LMNB1} gene, and lamin B2 and B3 encoded by the \textit{LMNB2} gene. As with lamin A, the B-type lamins have a CAAX motif. However they are constitutively farnesylated, whereas lamin A loses its farnesyl group once transported to the lamina (Figure 6).\(^{35}\)
Lamin filaments have a central rod domain, with a small globular head domain at the N-terminal, and a large globular tail domain at the C-terminal. Dimers are formed by coil-coiled interactions between the rod domains, polymers are then formed by head to tail associations, which then in turn associate laterally to form lamin protofilaments.

1.5 Laminopathies

Laminopathies are a diverse group of diseases that are caused by mutations affecting the nuclear lamina. There are primary laminopathies, with mutations in the *LMNA* gene. At present, 464 different mutations from 2,251 individuals have been found in the *LMNA* gene. There are also secondary laminopathies, with mutations in genes encoding prelamin A processing proteins (such as *ZMPSTE24*), B-type lamins (*LMNB1* and *LMNB2*), or lamin-binding proteins (such as *EMD*, *LAP2a*, *TMPO*, *LBR* and *LEMD3*). The mutations can be *de novo* (usually dominant), or heritable (both dominant and recessive), with either a gain- or loss-of-function effect. These diseases can also range in severity from minor arrhythmia to the neonatally lethal tight skin condition, RD. Lamin A is expressed in all differentiated cells, and so the segmental phenotypes of laminopathies is difficult to explain, however there are several hypotheses. Allele specific mutations of *LMNA* can have different effects, the C-allele (of a single nucleotide variant in exon 10) has been shown to be more frequently expressed. It accounts for ~70% of lamin A and C transcripts. An analysis of samples from six HGPS patients showed that the p.G608G mutation can be located on either allele. This could account for the variability in HGPS phenotype, and a similar phenomenon may be responsible for the variable phenotype in other laminopathies.
The location of the mutation on the LMNA gene can also cause a variation in effects, mutations in the central conserved α-helical domain cause different effects from mutations that cluster at site of the NLS, affecting nuclear import (Figure 5). Additionally if a mutation lies on exon 11, it can only affect lamin A. However mutations on exon 10 affect both lamin A and C, such as in the case of type-A mandibuloacral dysplasia (MAD, OMIM 248370), a lipodystrophy with some aspects of progeria syndromes.

The mutations causing laminopathies are pleiotropic, meaning that although they affect only one gene, they cause multiple, apparently unrelated phenotypic changes. For example, the mutation affecting amino acid 527 of LMNA, can be mutated to R527H and R527C resulting in MAD. Although one family had two siblings with homozygous instances of R527C that caused HGPS. Additionally, the mutation R527P has been found to cause Emery–Dreifuss muscular dystrophy (EDMD) (Figure 5). Mutations at the LMNA R25 locus have been found to cause EDMD, early-onset limb girdle muscular dystrophy, type 1B (LGMD1B), dilated cardiomyopathy, type 1A (CDM1A) or Dunnigan familial partial lipodystrophy (FPLD2) (Figure 5).

Different laminopathies can also be caused by the same missense mutation occurring at the same locus. For example, S573L in exon 11 of LMNA in one family gave rise to CDM1A, and in another family caused FPLD2. The E358K mutation in LMNA has been identified in patients with EDMD, LGMD1B, and congenital muscular dystrophy (MDC) (Figure 5). The same mutation can also cause different diseases, even when having occurred in the same family. The single nucleotide deletion at position 959, in exon 6 of LMNA, has been identified as causing one case of DCM, one of EDMD and two of LGMD1B (Figure 5). This disease heterogeneity indicates that there are other disease modifiers and interacting elements that have yet to be discovered in the genomes of patients with laminopathies.

Laminopathies can be divided into four categories based on their phenotypes: muscular dystrophies (affecting striated muscle), lipodystrophies (affecting adipose tissue), neuropathies (affecting peripheral nerves) and segmental progeria syndromes (causing several tissues to prematurely age) (Figure 5). Overlapping syndromes also occur, where symptoms from multiple categories are found. Segmental progeria syndromes include HGPS and RD, along with MAD, and atypical Werner syndrome (AWS, OMIM 277700). Werner syndrome is characterised by features of premature ageing and cancer, and is known as “progeria of the adult”. The main clinical features in Werner’s syndrome appear in the second or third decades of life, and include scleroderma-like skin, diminished stature, premature joint-stiffness and osteoporosis, diabetes and premature thinning of the hair. Classical Werner’s syndrome is caused by mutations in the WRN gene, a member of the RECQ family of DNA helicases, which are involved in DNA repair.

Figure 5. The lamin A gene (Opposite page). Lamin C is encoded by exons 1-9 and a section of exon 10. Lamin A is a result of alternative splicing, using exons 1 to 12, but without the lamin C specific part of exon 10 (lamin C specific amino acids are marked in green). The conserved α-helical regions of the central rod domain are marked with coil 1a, coil 1b, and coil 2. The numbers refer to primary sequence locations. Most (>90%) HGPS patients carry the de novo c. 1824C>T, p.G608G mutation, which is highlighted with a red asterisk, this is the mutation that was expressed in our mouse models (Paper I-III). AWS, atypical Werner syndrome; CDM1A, dilated cardiomyopathy, type 1A; CMT2B1, Charcot–Marie–Tooth disorder, type 2B1; EDMD, Emery–Dreifuss muscular dystrophy; FPLD, Dunnigan familial partial lipodystrophy; HGPS, Hutchinson–Gilford progeria syndrome; LGMD1B, limb girdle muscular dystrophy, type 1B; MAD, mandibuloacral dysplasia; MDC, Muscular dystrophy, congenital; MLF, Malouf Syndrome.
1.6 Post-Translational Lamin A Processing

Mature lamin A is formed from a precursor protein called prelamin A. This protein must go through several post-translational steps in order to become mature lamin A (Figure 6). Lamin B1 and B2 also go through this process, however lamin C lacks a farnesylation site and therefore does not.\(^{30,46}\)

Prelamin A contains a CAAX box on exon 12 at the carboxyl terminus (Figure 5), this is a sequence of amino acids which act as a target for a process known as farnesylation. The CAAX sequence is; C: Cysteine, A: an aliphatic amino acid, and X: any amino acid. In prelamin A the CAAX sequence is cysteine, serine, isoleucine and methionine (CSIM).

This CSIM motif is identified by farnesyltransferase and geranylgeranyltransferase-I and is modified and removed during lamin A maturation.\(^{49}\)

Figure 6. Lamin A processing. The maturation of prelamin A to mature lamin A involves several steps, which in HGPS and RD are interrupted, resulting in the production of progerin in HGPS and prelamin A in RD.\(^{58}\) Notably the accumulation of progerin or other truncated prelamin A isoforms has also been found in RD.\(^{56}\)
The posttranslational steps are outlined below;

i. **Farnesylation**

The CSIM-cysteine is farnesylated by farnesyltransferase (FTase).

ii. **Initial cleavage**

The last three CSIM amino acids, SIM, are removed by zmste24 or RCE1.

iii. **Methylation**

The farnesylated cysteine is methylated by isoprenylcysteine carboxylmethyltransferase (ICMT), resulting in the localisation of prelamin A to the INM.\(^{30,50-52}\)

iv. **Terminal cleavage**

The terminal 15 amino acids are cleaved by zmste24, producing mature lamin A.

### 1.7 Post-Translational Lamin A Processing In HGPS And RD

The aberrant processing of prelamin A in HGPS and RD is shown in figure 6. In HGPS, a cryptic splice site is activated, resulting in the loss of 150 nucleotides from prelamin A mRNA. This results in a translated protein that lacks the 50 amino acids containing the recognition site for zmste24 (which is the RSYLLG motif). As a result, the terminal cleavage step fails to occur and the farnesylated, carboxymethylated C-terminus is retained. The resultant mutant protein is found attached to the INM and is known as farnesylated prelamin A \(\Delta\)50, lamin A \(\Delta\)50 or simply, progerin.\(^{3,30,53-55}\)

RD is caused most often by loss of function mutations in \(\text{ZMPSTE24}\) (which encodes zmste24, the enzyme that catalyses the final cleavage step in prelamin A processing), causing prelamin A accumulation (Figure 6).\(^{56}\) The disease can also be caused by heterozygous mutations in the \(\text{LMNA}\) gene (c.1824C>T) or c.1968+1G>A. These mutations resulted in the partial or complete loss of exon 11, which holds the recognition site for zmste24, also leading to prelamin A accumulation.\(^{56,57}\) The increased severity of RD cases where \(\text{LMNA}\) is mutated might be caused by increased levels of progerin compared to HGPS.\(^{59}\) The very severe symptoms in RD patients might also suggest that the zmste24 enzyme has other targets in addition to lamin A. Proteins with the CAAX motif are involved in many cellular functions such as proliferation, differentiation, and carcinogenesis.

Cultured HGPS patient cells have been shown to accumulate progerin in an age-dependent manner,\(^{60,61}\) correlating with more severe structural changes and a reduced growth rate.\(^{60}\) Studies have been performed to examine if progerin is also connected to normal ageing, and these studies revealed that not only was progerin found in normally aged individuals,\(^{62}\) but skin biopsy tests showed levels increased with age.\(^{63}\) In vitro studies showed that as fibroblasts age in culture, the amount of mutated protein or mRNA increases with the number of passages.\(^{64,65}\) These results suggest that the presence and accumulation of progerin might contribute to normal physiological ageing.
Figure 7. HGPS patient skin fibroblasts. Healthy (left) and HGPS patient (middle) skin fibroblasts, with a detailed view of the diseased nuclei on the right. Immunofluorescent (IF) staining shows progerin in black, DNA in yellow, mitochondria in red and the endoplasmic reticulum in blue.

1.8 The Cellular Effects Of HGPS

HGPS patient cells exhibit irregularly shaped nuclei (Figure 7), which get more effected as the cells age in culture, demonstrating the accumulation of progerin goes hand-in-hand with increased toxic effects. Progerin disrupts the functions of lamin A and the lamina, and insoluble progerin aggregates in the INM causing the formation of blebs in the NE, thickened lamina and loss of peripheral heterochromatin. Normally lamin A/C mediates the peripheral location of chromatin during differentiation, while lamin B receptor (LBR) does so during early development.

The changes bought about by progerin are thought to cause abnormal transcription and a high level of misregulated genes. In vitro experiments with progerin-expressing human mesenchymal stem cells resulted in abnormal differentiation of the cells into bone- and adipose-related cell types. Cells expressing progerin also show a mislocalisation and clustering of NPCs, which is thought to impair the normal movement of proteins and mRNA into and out of the nucleus. Defects in genomic stability with an increased level of double-strand breaks have also been shown in HGPS studies.
1.9 The Skin

The skin is the first place where symptoms are visible in HGPS, and a tight and relatively inelastic skin is the cause of death for RD patients. Therefore skin is the organ chosen for study in Papers I and II. It is the largest organ in the body, and serves several important functions. As a tough and watertight barrier, it protects the body from pathogens, dehydration, as well as mechanical and chemical damage. It also contains a variety of nerve endings which allow for sensation of touch, pressure, pain and temperature. Skin also thermoregulates the body, with heat conservation aided by hair and subcutaneous adipose tissue, and heat loss aided by sweat glands and dilated blood vessels. The skin is composed primarily of three layers; the epidermis, dermis and hypodermis.

The epidermis is the outermost layer and interfaces with the environment. It consists of approximately 95% keratinocytes (Figure 8), with Merkel cells, Langerhans cells and melanocytes making up the remainder. The thicker dermal layer underlies and supports the epidermis, and is composed of fibroelastic tissue. It contains many sensory receptors, and is highly vascular. Finally beneath the dermis is the hypodermis, which contains mainly adipose tissue.

There are extensive prior studies on skin which provide many tools for the examination of the well characterised stem cell population in skin.

![Figure 8. The epidermis. IF is used to differentiate keratinocytes, which are labelled in blue. DNA is yellow and lamin B1 is red. Some non-keratinocytes can be seen in the epidermis labelled in red, these could be Merkel or Langerhans cells. The layers of the skin are listed on the right.](image)
The epidermis is made up of the following layers (listed from the lower to upper layers)\textsuperscript{72,73};

i. **Stratum basale (basal layer)**

The epidermis is constantly renewed by the division of a layer of stem cells in the basal layer. As the daughter cells detach from the basement membrane and migrate towards the skin surface, they change shape and lose their proliferative potential.

ii. **Stratum spinosum (spinous layer)**

As the cells migrate into the spinous layer, they begin to cornify, that is they begin to produce keratins and other proteins as well as begin to lose their nuclei and organelles. Keratins are very tough, fibrous proteins that make up the bulk of hair, nails and the outer layer of skin.

iii. **Stratum granulosum (granular layer)**

In this layer the cells become more cornified, and the cells also secrete lamellar bodies into the extracellular matrix (ECM), forming a hydrophobic barrier. Programmed cell death occurs.

iv. **Stratum corneum (horny layer)**

The outermost layer of skin consists of 15-20 layers of dead, flattened cells, which are continually shed, and continually replaced by the cells of the inner layers migrating outward.

Notably, the epidermis in mice from postnatal day 5 onwards has a depth of only one cell layer. This is markedly different from mature human skin which has a thickened appearance. However when progerin is expressed in keratinocytes using the K5tTA promoter, the skin undergoes a striking hyperplasia which causes it to become thicker (Figure 8).

1.10 **Adult Stem Cells**

The segmental tissue specificity of HGPS seems to selectively affect tissues that undergo continuous growth and regeneration such as hair follicles, as well as tissues that undergo high levels of mechanical stress (such as joints, or cardiac blood vessels). These tissues have specific caches of continuously proliferating adult stem cells. Indeed, tissues which are more independent of the need for continuous regeneration, and those tissues that are protected from mechanical stress, seem to be less affected or even unaffected in HGPS (such as the brain, or liver).\textsuperscript{74} This hypothesis is supported by in vitro studies where progerin expression induced differentiation in human mesenchymal stem cells.\textsuperscript{75} In another study using induced pluripotent stem cells from HGPS patients suggested that the exhaustion of mesenchymal stem cells could be the cause of increased hypoxia sensitivity.\textsuperscript{76}
1.11 Animal Models

In 1974 the first genetically modified animal was created when a DNA virus was introduced into a mouse embryo, resulting in the inserted genes being present in all cells of the animal.\textsuperscript{77} In 1981 the first mouse model was developed that transmitted the exogenous genetic material to following generations.\textsuperscript{78} The transgenic mouse has since proven to be an invaluable tool for medical research, with models developed for the study of many diseases, such as heart disease, arthritis and ageing.

The first transgenic mouse model specific for HGPS was published in 2006 by random integration of a human bacterial artificial chromosome (BAC) with the c.1824C>T \textit{LMNA} mutation to a mouse-line. Both human lamin A/C and progerin were overexpressed in all tissues of these mice, however they lacked the gross pathologic features of HGPS. The mice did however lose vascular smooth muscle cells (VSMCs) in the medial layer of large arteries, as seen in HGPS patients, although this had no effect on their life expectancy.\textsuperscript{79}

A progeriod mouse model with mechanistic similarities to RD was developed by completely knocking out the \textit{zmpste24} gene,\textsuperscript{50} resulting in the accumulation of farnesylated prelamin A at the INM. These mice appear normal at birth, but thereafter develop skeletal abnormalities with spontaneous bone fractures, abnormal teething, progressive hair loss and muscle weakness. They die prematurely at the age of 6-7 months.\textsuperscript{80,81}

The recently created knock-in \textit{lmna}\textsuperscript{G609G} mouse model had the wild-type mouse \textit{lmna} gene replaced with a copy containing the murine equivalent of the human HGPS \textit{LMNA} c.1824C>T, p.G608G mutation, which is \textit{lmna} c.1827C>T, p.G609G. These mice had clinical features that were very similar to those found in HGPS. They accumulate progerin, displayed growth retardation, weight loss, bone abnormalities, cardiovascular problems and had a shortened lifespan.\textsuperscript{91}

During the last decade many other mouse models have been developed targeting prelamin A processing or with various mutations in the \textit{lmna} gene.\textsuperscript{82,83} In our lab we have focused on tissue specific inducible mouse models expressing the \textit{LMNA} c.1824C>T, p.G608G mutation. This is detailed in section 1.13.

1.12 The Control System

In order to study one of the most fascinating aspects of HGPS, the segmental phenotype, a mouse model with tetracycline controlled gene expression was used. These model systems can control transgenic expression spatially, by using a tissue specific promoter, and temporally, by adding or removing the tetracycline derivative, doxycycline (dox). This powerful binary control system allows for the deliberate and controlled expression of a protein in restricted tissues, at regulated time-points (Figure 9).\textsuperscript{84,85} With this control system studies can be performed on mature animals even when gene products would be lethally toxic if expressed in embryogenesis. The effects of transgene expression in specific tissues can also be examined, and even the possibility of disease reversal can be studied by ceasing the expression of the protein after the disease phenotype has been developed.
Two control elements define the system, the regulatory and the response elements. A tissue specific promoter controls the expression of the regulatory element, either a tTA or an rtTA protein (corresponding to the Tet-Off or Tet-On systems). The response part is a tetop element which is upstream from the target transgene. When a cell contains both elements, the gene of interest is expressed once the tTA/rtTA element binds to the tetop motif.

The difference between the Tet-on and Tet-off system is that in the former, the rtTA molecule binds when tetracycline is present, and in the latter the tTA molecule binds when tetracycline is absent. We have used the Tet-off system in Papers I and II, and the Tet-on system in Paper III. In order to study the effects of progerin expression on skin, the K5 promoter was used in Papers I and II, and to study the effects on the cardiovascular system the sm22α promoter was used in Paper III.

1.13 The Minigene

A human lamin A minigene using the Tet-system was generated to study the effects of progerin expression (Figure 9). It consists of exons 1-11, intron 11 and exon 12 of the human *LMNA* gene, followed by an internal ribosomal entry site (IRES), and the coding region of enhanced green fluorescent protein (eGFP). The IRES allows for independent translation of eGFP. The minigene terminates with an SV40/poly A tail to aid in nuclear export, mRNA stability and translation. On codon 608 the construct carries the most common HGPS c.1824C>T, p.G608G mutation (tetop-LAG608G). Mice with this minigene were crossed to mice with the K5 (Paper I and II) and the sm22α (Paper III) transactivators.

As skin is the first organ to display a typical disease phenotype in HGPS, and as it undergoes such extreme effects including scleroderma, loss of subcutaneous fat and alopecia, the K5 promoter was chosen to direct minigene expression to the basal cells of the interfollicular epidermis and the hair follicle. In the K5tTA model the tetop-LAG608G+; K5tTA+ mice successfully expressed human lamin A and progerin in basal cells of the interfollicular epidermis, (as well as in ameloblasts, and in the basal epithelia of salivary glands, oesophagus, stomach, tongue, nose cavity and trachea). These mice were used to study postnatal expression in Paper I, and had expression from both postnatal day 0 and 21. In Paper II expression was induced embryonically in order to assess the mouse model as a model for RD.

The organs of the cardiovascular system are severely affected in HGPS, they undergo a progressive depletion of vascular smooth muscle cells, and most often death from HGPS is caused by cardiovascular disease and complications due to atherosclerosis. The sm22α model was designed to express the minigene in vascular smooth muscle cells to allow for a detailed study of the effects of progerin on these cells, however no expression was detected. This is detailed in Paper III.

Figure 9. The Tet systems (Opposite page). In the Tet-systems gene expression is controlled by administration of doxycycline (dox). In the tTA (Tet-Off) system transcription is activated in the absence of dox. In the rTA (Tet-On) system transcription is activated in the presence of dox.
1.14 Treatment for HGPS

As an autosomal dominant disease, HGPS would require gene therapy with anti-sense oligo morpholinos to target the splice site, as the production or effect of progerin would need to be eliminated. Antisense oligonucleotides directed against the aberrant exon 11 and exon 12 junction found in mutated pre-mRNAs, or the lamin A splice donor site found in exon 10, have been tested in HGPS cell lines, with positive results. However these treatments are not yet ready for clinical trials.

A phase II clinical trial used a farnesyltransferase inhibitor (FTI) as treatment, which works by inhibiting the farnesylation of prelamin A. Previous in vitro research with FTIs showed a significant reduction in the numbers of misshapen nuclei. In vivo results were also impressive, with various HGPS and zmpste24-deficient mouse models showing an improvement in disease phenotype. However the disease was not completely cured by FTIs. Although FTI treatment inhibits the farnesylation of prelamin A by farnesyltransferase, another modification pathway (via geranylgeranyltransferase-I) allows prelamin A to be processed into progerin despite the FTI.

The combination of statins and bisphosphonates was used to inhibit the synthesis of farnesyl pyrophosphate, which is a co-substrate of farnesyltransferase and a substrate precursor for geranylgeranyltransferase-I. This combination resulted in increased lifespan, reduced oxidative stress, reduced cellular senescence and an improved phenotype in mouse models of laminopathies.

The positive outcomes from these studies lead to clinical trials in HGPS patients, which generated promising results, including improvement of the vascular stiffness. However there is still a need to assess complementary treatments.

HGPS cells treated with rapamycin, which has been used in transplant patients as an anti-rejection drug, showed enhanced progerin degradation, reduced levels of senescence, and decreased levels of nuclear blebbing. The enzyme ICMT has also been shown to be a potential target for progeria treatment, with beneficial effects in a progeria mouse model. Additionally, treatment of zmpste24 knock-out mice with resveratrol arrested their loss of adult stem cells, slowed down body-weight loss, improved bone abnormalities and mineral density, as well as significantly extending the life span.
2. Aims Of The Thesis

The aim of this thesis was to increase the understanding of the molecular mechanisms behind HGPS and RD, with a focus on skin and the cardiovascular system.

**Paper I.**

To test the hypothesis that premature exhaustion of adult stem cells contributes to the progressive phenotype.

**Paper II.**

To test the hypothesis that the more severe phenotype in RD compared to HGPS is due to higher levels of farnesylated prelamin A or progerin.

**Paper III.**

To develop a mouse model with tissue-specific expression of the progeria mutation in vascular smooth muscle cells, and to examine the effects of this expression.
3. Results and discussion

3.1 Paper I

We induced expression of the \textit{LMNA} c.1824C>T, p.G608G HGPS mutation in mice with the K5 transactivator. Expression was induced from the date of birth (D0), or from three weeks after birth (D21). The D0 mice exhibited a more severe and rapid progression of the phenotype than D21 animals.

The stem cell population in skin can be identified with a label-retention experiment, where animals are given Bromodeoxyuridine (BrdU) injections. BrdU is a thymidine analogue that is incorporated into newly synthesised DNA during cell division. After a series of injections almost all epidermal cells are labeled. As the cells divide, the BrdU labels grow weaker, and 70 days after the final injection, only cells that rarely divide still retain their BrdU labels. This label retention is an indicator of the level of cell division each cell has undergone. As stem cells are slow cycling by definition, cells that were still BrdU labelled after 70 days were considered to be stem cells.\textsuperscript{107} BrdU can also be used with a short chase period to identify actively proliferative cells. The animals are injected with a dose of BrdU one hour before the tissue is collected. The BrdU is incorporated into dividing cells and marks them as proliferating cells.

\textbf{Figure 10. Epithelial label-retaining cells.} IF skin sections with BrdU labelled cells shown in red and keratin 5 in blue. On the left a short chase period was used, and BrdU incorporation highlights proliferating cells along the edge of a four-day-old wound. On the right a long chase was used, and BrdU marks slow cycling cells in a hair follicle.

Using long-chase BrdU to identify stem cells, we showed that the progeria mice had a reduced population of adult stem cells in dorsal skin. This was confirmed with fluorescence-activated cell sorting using two markers (α6-integrin and CD34) that have been shown to mark cells with stem cell characteristics. Further tests assessed the ability of keratinocytes to form colonies \textit{in vitro}. Stem cells should form large self-renewing colonies, while non-stem cells divide only a small number of times before undergoing terminal differentiation.
Keratinocytes from progeria animals produced fewer colonies that were smaller and less dense compared to wild-type colonies. Additionally, stem cell markers Lrig1, CD34, and Krt15 were analysed with quantitative RT-PCR, and were seen to be downregulated in progeria mice.

Epidermal adult stem cells are of paramount importance in the tissue formation stage of wound healing, when cellular proliferation and migration close the open wound. To study this aspect of wound healing, skin biopsies were taken and the wound site was analysed four and seven days post-wounding. Four-day-old wild-type wound edges had well organised proliferation and migration and after seven days were completely re-epithelialised. The wound edges in progeria animals were uneven and disorganised after four days and there were still wounds that were not re-epithelialised after seven days.

Short-chase BrdU was used to identify proliferating cells in the wound edges. Progeria animals showed a significantly higher number of BrdU-labeled cells compared to wild-type, seven days after wounding. However, progeria keratinocytes showed an impaired migration, delaying the wound healing relative to wild-type animals. The impaired keratinocyte migration could be the result of reduced integrin expression. Transcripts from progeria and wild-type keratinocytes were analysed to test this. The results showed reduced expression of β1-integrin and α6-integrin in cells from progeria mice.

DNA damage in keratinocytes was analysed with IF, using an antibody that can highlight double-stranded DNA breaks. Progeria mice had an increased frequency of cells with higher amounts of DNA double-strand breaks. This suggests that they had a higher number of cells with severe DNA damage or that the cells had an impaired DNA damage repair mechanism. Severe or irreparable DNA damage is one trigger of cellular senescence, which we examined with an assay for senescence-associated β-galactosidase. This test showed a tendency of increased senescence in the interfollicular epidermis of progeria skin.

It has been proposed that senescent cells secrete pro-inflammatory factors that cause surrounding cells to enter into a pro-inflammatory state. This set of characteristics is termed the “senescence-associated secretory phenotype”. We analysed selected inflammatory markers in keratinocytes from wild-type and progeria animals with quantitative RT-PCR. Progeria mice had a significant increase in all of these markers. Senescence-associated secretory phenotype proteins are known to promote proliferation, and this matches the increased proliferation that is seen in this mouse model. Chronic inflammation is also known to impair stem cell function, and is associated with normal ageing, further supporting the correlation of progerin expression and stem cell dysfunction and ageing.

We also examined the expression of p63, an epidermal stem cell maintenance protein, in the epidermis. It was downregulated on both the mRNA and protein level, which may account for the loss of adult stem cells. The expression of TAp63, an isoform of p63, was also analysed in HGPS patients and was found to be downregulated.
3.2 Paper II

In order to ascertain if the more severe symptoms in RD, as compared with HGPS, are due to the higher levels of farnesylated lamin A or progerin produced in RD, the LMNA c. 1824C>T, p.G608G was expressed embryonically. IF examination of skin from 17.5 day old embryos (E17.5) showed transgenic expression, using an antibody against human lamin A and progerin. The expression corresponded to keratin 5 positive skin cells. To quantify the levels of expression Western blots were used, showing an increase in transgenic overexpression of lamin A, prelamin A, and progerin from E17.5 to D3 and to D5. RT-PCR comparing D8 to D5 showed that the transgenic expression of progerin was unchanged, and the expression of lamin A was slightly reduced. This was reflected in an increased ratio of progerin to lamin A. These results suggest that the increased levels of progerin, lamin A, and prelamin A proteins were caused by their accumulation.

The transgenic expression led to a severe phenotype with dry scaly skin, and death within the first two weeks of life. Histological assessment of dorsal skin sections showed no abnormalities at D3, but at D4 the wild-type animals showed the beginnings of skin maturation, developing from a multilayered into single-layered epithelium, and this process was complete by D5. For animals expressing the mutation however, the skin maturation steps never took place, they still had the immature, multilayered epithelium. These animals showed a hyperplastic epidermis, disorganised sebaceous glands and an acute inflammatory dermal response at D5. By D9 the skin pathology was even more pronounced, with dense collagen fibres in the progeria animals that were not present in their wild-type littermates. These progeria animals had a diminished size, as well as hair thinning and dry scaly skin. There was no detected decrease in elastin, which is a histological feature seen in skin biopsies from RD patients. This is probably due to the mutation being expressed only in the epidermis, and not the dermis.

The progeria animals were found to have a higher rate of dehydration than their littermates, and a study of their transepidermal water loss showed a significantly higher rate. The epidermal barrier function was tested, to check if the skin could successfully prevent ingress of a dye. The function of tight junctions was also tested, these are a barrier found in the epidermis which prevent egress of water from the skin (Figure 11). Both of these systems were functional. The increased rate of dehydration could be an effect of the increased exposure area of nucleated cells in a hyperplastic epidermis, as these cells lie outside the protection of the tight junctions.

Another factor in the increased rate of dehydration may be from the decreased efficacy of dry, cracked skin. Keratinocytes in progeria skin aberrantly retain intact nuclei (parakeratosis) instead of undergoing terminal differentiation by transforming into corneocytes that lack nuclei (Figure 11). Corneocytes are filled with, covered with and release lipids. These lipids are necessary for healthy skin barrier function. The arrested development of progeria keratinocytes may lead to a failure to maintain correct hydration of the stratum corneum, as well as a failure to form a functional lipid matrix in the stratum corneum intracellular space.

RT-PCR was also used to analyse inflammation effects in skin from progeria mice. Although no inflammatory response was seen at E17.5, by D3 multiple inflammatory response genes were upregulated, and this response was even more marked by D5.
Cells from HGPS patients show a loss of peripheral heterochromatin. The peripheral localisation of heterochromatin during early development is thought to be mediated by LBR. As cells undergo terminal differentiation, this function is taken over by lamin A/C. Postnatal dorsal skin from progeria mice was analysed with IF using an antibody for LBR. D9 wild-type animals showed less LBR-positive cells compared with D3, in agreement with reduced expression of LBR as development proceeds. For progeria animals, LBR-positive cells were found both in the basal and suprabasal cell layers.

An analysis of the DNA distribution in suprabasal LBR-positive cells of D9 progeria animals showed a dense and depleted DNA distribution, with contiguous regions of little or no DNA, a phenomena referred to as DNA condensation. Most suprabasal LBR-negative cells showed normal DNA distribution, with DNA dispersed in the intranuclear space. Notably a recent paper has indicated that induced chromatin condensation can stimulate damage-independent upstream signalling of the DNA damage response (DDR), which in concert with these results, where an increased level of DNA condensation was seen, might mean that indicators for double-strand breaks seen in Paper I do not necessarily imply the presence of actual double-strand breaks.

This unusual suprabasal LBR expression may be the cause for the failure of keratinocytes to terminally differentiate, possibly by sequestering peripheral heterochromatin. Tightly regulated cytoplasmic and nuclear localisations, and chromatin association of IKKa (the master kinase for NF-κB), are required for the activation of the NF-κB pathway. So the disruption of the nuclear lamina, as well as the loss of peripheral heterochromatin in progeria cells may have lead to the inappropriate activation of the NF-κB pathway. This NF-κB pathway activation may then have caused the observed inflammatory response. The expression of the LMNA c.1824C>T mutation appears, in summary, to interfere with terminal cell differentiation, and to cause an arrest of skin development possibly by the aberrant sustained expression of LBR. Further studies are need to assess the contribution of LBR to the disease pathology.
3.3 Paper III

Death in HGPS is most often caused by complications from cardiovascular disease, including atherosclerosis, and a depletion of vascular smooth muscle cells was seen in the cardiovascular system of patients.\textsuperscript{14,15} In order to develop a mouse model that could further elucidate the mechanical mechanisms of HGPS, as well as act as a testing platform for possible HGPS treatments, the \textit{LMNA} c.1824C>T, p.G608G mutation was to be expressed in the vascular smooth muscle cells of the aortic arch and thoracic aorta. The reverse tetracycline-controlled transactivator, sm22α-rTA, was selected to control this expression.

Tet-control systems are invaluable tools for disease studies. The ability to generate tissue specific mouse models while controlling the temporal expression of target genes allows research on mature animals with gene products that would be lethal if expressed during embryogenesis, expression in specific tissues can be examined, and stopping the protein expression after the disease phenotype has been developed allows for disease reversal to be studied. The sm22α-rTA transactivator should have allowed us to direct the expression to the cardiovascular system with tissue specificity.

However, the expression of this transactivator was almost undetectable in the arteries, and was insufficient to induce the expression of the minigene. RT-PCR using mRNA from the aortic arch showed only very weak amplification products for human lamin A, lamin AΔ150 and the reverse transactivator after 35 cycles of PCR. The same weak signal also came from samples that did not have the sm22α-rTA transactivator. This would suggest that the weak amplification product occurred due to the leakiness of the Tet-On and Tet-Off models, which has been reported in previous studies.\textsuperscript{109-111} Protein extracts from pooled aortic regions were examined with an antibody for human lamin A/progerin on a Western blot, but did not show transgenic expression.

The same antibody was used with IF to examine the aortic arches of progeria animals, and only a very few transgene positive cells were found. IF analysis of aortic arch tissue with an antibody against sm22α-actin failed to show any differences between the progeria animals and their wild-type littermates. Studies with haematoxylin and eosin staining, and immunohistochemical staining with an antibody against cleaved caspase 3, a marker for apoptosis, also showed no differences between the transgene positive animals and their littermates.

As the sm22α-rTA transactivator has been previously shown to induce target transgene expression in the aortic arch, abdominal aorta and thoracic aorta,\textsuperscript{89} we can only hypothesise that perhaps epigenetic modifications of the promoter sequence of the sm22α-rTA transgene may have occurred, resulting in suppression of the rTA expression.\textsuperscript{111-113}
3.4 Future Perspectives

Although the clinical trials in patients with HGPS have generated promising results, including improvement of vascular stiffness, there is still a need to assess complementary treatments. The availability of various animal models will be of importance in the future assessment of options for treating HGPS, both treatments being discussed now but also new candidates that will emerge from ongoing research.

Unpublished results from our laboratory with suppressed transgenic expression of the HGPS mutation after a phenotype had been developed (in bone and skin) indicated an improved phenotype, suggesting that treatments targeting aberrant progerin splicing can help HGPS patients. The treatment of \( \text{zmpste24}^-/- \) mice with resveratrol resulted in a significantly improved phenotype in addition to an increased life span compared to untreated mice.\(^{106}\) This suggests that treatment with resveratrol in our tissue-specific HGPS bone model might be associated with a positive outcome.

The results in papers I and II point towards an increased rate of inflammation in the early stages of progeroid skin pathology (evident already on postnatal day 3). Results from treatment with NF-κB inhibitors in progeroid mice have shown promising results,\(^{114}\) and our results support the notion that anti-inflammatory drugs might be good candidates for suppression of certain aspects of the progeroid disease phenotype.

Our animal models could also be of importance in the identification of novel potential targets for the treatment of progeria and ageing. Interestingly, when analysing the expression of LBR in the interfollicular epidermis of our progeria mice, we identified a significant fraction of suprabasal cells at postnatal day 9 with sustained LBR expression. These cells also showed an aberrant DNA distribution (Paper II). Future studies could use RNA sequencing to analyse early changes in gene expression that are induced by the expression of the progeria mutation in LBR positive suprabasal keratinocytes. Primary keratinocytes would be extracted from progeria mice (and littermate controls) and sorted using FACS into populations of suprabasal and basal keratinocytes. Then RNA would be extracted from these populations and processed for gene expression analysis. By taking this approach one might be able to identify early changes in gene expression. These would likely be primary effects preceding the pathological changes, which are not evident until postnatal day 4. This approach could potentially identify therapeutic targets for HGPS, and perhaps also physiological skin ageing.
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