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**MOUSE MODELS FOR UNDERSTANDING THE
MOLECULAR MECHANISM OF BONE DISEASE IN
HUTCHINSON-GILFORD PROGERIA SYNDROME**

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Stockholm 2011

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ISBN 978-91-7457-454-8

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Für Papa

..... Das Leben ist schön

ABSTRACT

Aging is a complex process affecting all people. Intense research is applied to elucidate the biological basis of aging and disease that develop with aging. Studies of progeroid syndromes, where aging happens in an accelerated speed, might be useful to understand the molecular mechanisms in physiological aging. Hutchinson-Gilford progeria syndrome (HGPS or progeria) is a very rare, fatal genetic disease, with an incidence of 1 in 4-8 million live births, which causes segmental premature aging in children. Affected individuals are born looking healthy, but develop symptoms of disease within their first years of life. Signs of progeria include growth retardation, loss of body fat and hair, skin changes, stiffness of joints, hip dislocations and generalized atherosclerosis and cardiovascular disease. Children with HGPS die of heart disorders or stroke at an average age of 13 years. The aim of this thesis is to increase the understanding of the molecular mechanisms underlying progeria. For this purpose, we developed tissue-specific inducible mouse models for the most common HGPS mutation in the *LMNA* gene (c.1824C<T, p.G608G).

We demonstrate that the disease pathology in HGPS is reversible in a mouse model with keratin 5-targeted transgenic expression of the HGPS mutation. The mice had a progressive phenotype with similar abnormalities to those seen in the skin and teeth of HGPS patients. These abnormalities improved in mice following 6 and 13 weeks of suppressed expression of the HGPS mutation. Our results give hope for the future development of treatments for children with HGPS.

We report our negative experience with a previously functional transactivator mice that failed to target the expression of the lamin A minigenes to the bone. To share this result with the scientific community will help to raise caution and to point out the importance for controls.

LIST OF PUBLICATIONS

- I. Sagelius H, Rosengardten Y, Schmidt E, Sonnabend C, Rozell B, Eriksson M. **Reversible phenotype in a mouse model of Hutchinson-Gilford progeria syndrome.**
Journal of Medical Genetics. 2008; 45 (12): 794-801.
- II. Schmidt E, Eriksson M. **A previously functional tetracycline-regulated transactivator fails to target gene expression to the bone.**
BMC Research Notes. 2011; 4 (1): 282.
- III. Schmidt E, Nilsson O, Koskela A, Tuukkanen J, Ohlsson C, Rozell B, Eriksson M. **Expression of the Hutchinson-Gilford progeria mutation during osteoblast development leads to irregular bone mineralization and impaired skeletal integrity.**
Manuscript submitted
- IV. Schmidt E, Rodríguez S, Rozell B, Mugnaini E, Eriksson M. **Effects from brain-specific expression of the Hutchinson-Gilford progeria syndrome mutation.**
Manuscript

TABLE OF CONTENTS

1	Background	1
1.1	Lamins	1
1.2	The nuclear lamina	2
1.3	Laminopathies	4
1.4	Hutchinson-Gilford progeria syndrome	5
1.4.1	Skeletal phenotype in HGPS	6
1.5	Molecular basis in HGPS	7
1.5.1	Post-transcriptional processing of prelamin A in HGPS	7
1.6	Cellular phenotype in HGPS	8
1.7	Therapeutics for HGPS	9
1.8	Lamins and aging	10
1.9	Lamins in bone	11
1.10	Bone development and remodelling	12
1.11	An inducible and tissue specific mouse model system	14
1.11.1	Tetracycline-inducible transactivator system	14
1.11.2	Lamin minigenes	15
1.11.3	Transactivator mice	16
2	Aims of the thesis	18
3	Notes on methodology	19
3.1	Laboratory animals	19
3.2	Optimization of human lamin A/C immunohistochemistry on decalcified bone tissue	20
4	Results and discussion	22
4.1	Paper I	22
4.2	Paper II	23
4.3	Paper III	Error! Bookmark not defined.
4.4	Paper IV	Error! Bookmark not defined.
5	Conclusions and future perspectives	25
6	Acknowledgements	26
7	References	30

LIST OF ABBREVIATIONS

HGPS	Hutchinson-Gilford progeria syndrome
AR	Antigen retrieval
BMD	Bone mineral density
CMT2	Charcot-Marie-Tooth disease type 2B1
DCM	Dilated Cardiomyopathy
Dox	Doxycycline
DXA	Dual energy x-ray absorptiometry
EDMD	Emery-Dreifuss Muscular Dystrophy
ER	Endoplasmatic reticulum
FPLD	Familial Partial Lipodystrophy
FTIs	Farnesyl transferase inhibitors
IHC	Immunohistochemistry
INM	Inner nuclear membrane
IRES	Internal ribosome entry site
K5tTA	Keratin 5 tetracycline transactivator
LGMD	Limb-Girdle Muscular Dystrophy
LINC	Linker of nucleoskeleton and cytoskeleton complex
MADA	Mandibular Dysplasia type A
NPC	Nuclear pore complex
NSE-tTA	Neuron specific enolase tetracycline transactivator
ONM	Outer nuclear membrane
PHH3	Phospho-histone H3
pQCT	Peripheral quantitative computed tomography
pTRE	Tetracycline responsive promoter element
RD	Restricted Demopathy
rtTA	Reverse tetracycline responsive transcriptional transactivator
Sp7-tTA	Osterix tetracycline transactivator
TOR	Target of rapamycin
TRAP	Tartrate-resistant acid phosphatase
tTA	Tetracycline responsive transcriptional transactivator
TUNEL	Terminal transferase dUTP nick end labelling
α 1p-rtTA	Collagen type 1 α 1 reverse tetracycline transactivator

1 BACKGROUND

Exploring the molecular and cellular mechanisms of Hutchinson-Gilford Progeria Syndrome (HGPS) in animal models will help elucidate the pathophysiology of the disease in patients with this rare condition and thus lead to improved treatment options for affected individuals. HGPS affects only 1 in 4-8 million live born and the disease is present in all ethnic backgrounds throughout the world (Hennekam 2006). Signs of accelerated aging in several, but not all tissues and organ-systems, characterize HGPS and define it as a segmental premature aging disorder (DeBusk 1972, Brown 1992). Understanding the molecular and cellular mechanisms of HGPS may thus help to uncover general principles of the aging process and diseases that develop with aging. HGPS is caused by a *de novo* single point mutation in exon 11 of the *LMNA* gene (De Sandre-Giovannoli *et al.* 2003, Eriksson *et al.* 2003).

1.1 LAMINS

Lamins are intermediate filament proteins that polymerize and form the lamina network underlying the inner nuclear membrane (Fisher *et al.* 1986, Aebersold *et al.* 1986, Lin and Worman 1993). The *LMNA* gene codes for the two major isoforms of the A-type lamins, lamin A and lamin C, by alternative splicing. Lamin A is encoded by exon 1-12 and lamin C is encoded by exon 1-10 (Figure 1) (Fisher *et al.* 1986). A-type lamins are only expressed in terminal differentiated cells. In mice, lamin A and C first appear at embryonic day 12 in muscle cells of the trunk, head and the appendages. Three days later, they are also seen in the cells of epidermis. In the simple epithelial of lung, liver, kidney and intestine, as well as in the heart and the brain, lamins A and C do not appear until well after birth (Röber *et al.* 1989). Two minor additional isoforms, laminA Δ 10 and lamin C2, have also been identified. The LaminA Δ 10 protein lacks exon 10 compared with lamin A and has been found in the normal colon and in various carcinoma cell lines (Machiels *et al.* 1996). Lamin C2 differs in an alternative exon 1 compared to lamin C and is expressed in germ cells and fibroblasts (Furukawa and Hotta 1993, Rodríguez *et al.* 2009). In addition to the A-type lamins, there are also the B-type lamins. *LMNB1* encodes lamin B1 while *LMNB2* encodes lamin B2 and the minor isoform lamin B3 (Dechat *et al.* 2010). B-type lamins are expressed in all cells during development and in adult tissues. A- and B- type lamins share structural features of having a central α helical rod domain flanked by a small non-helical amino N-terminal sequence and a larger C-terminal globular domain (Figure 1) (Fisher *et al.* 1986). Lamin A, B1 and B2 are expressed as prelamins carrying a C-terminal CAAX motif and need to undergo post-translational modification including farnesylation and methylation to become mature lamins. Lamin C does not have a CAAX motif and does not require further modification (Figure 1) (Stuurman *et al.* 1998). Mature lamin B1 and B2 remain permanently farnesylated and carboxymethylated (Stuurman *et al.* 1998) while prelamins A and C get further

processed and mature lamin A lacks the C-terminal farnesylated and carboxymethylated modification (Corrigan *et al.* 2005).

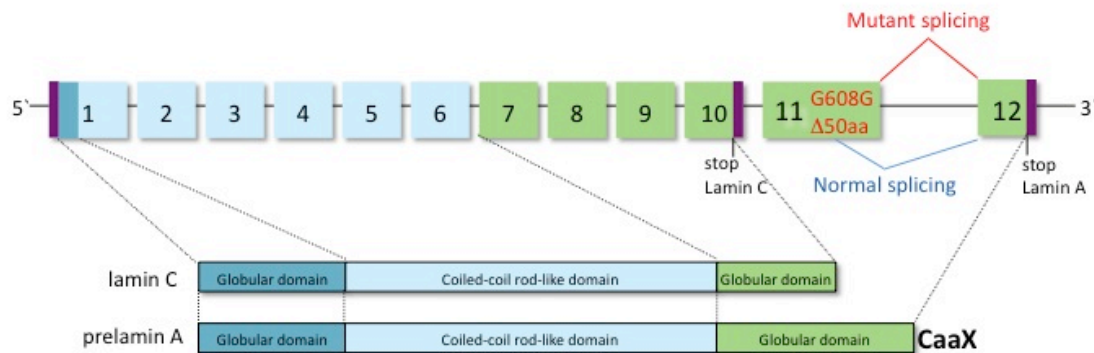


Figure 1. Schematic picture of the *LMNA* gene and the protein products lamin C and prelamin A. In the C-terminal globular tail domain prelamin A has a CAAX motif, which is absent in lamin C. The drawing was inspired by Burke and Stewart 2006 and Capell and Collins 2006.

1.2 THE NUCLEAR LAMINA

The nuclear envelope separates the cytoplasmic cell content from the cell nucleus (Figure 2). The interaction of the different cell compartments is highly regulated by nuclear membrane domains. The inner and outer nuclear membranes build together with the nuclear pore complexes (NPC), a complex regulatory protein network for the nucleo/cytoplasmic transport of proteins and RNA. The outer nuclear membrane (ONM) is physically connected with the endoplasmatic reticulum and shares many of its proteins. The inner nuclear membrane (INM) embeds numerous integral membrane proteins and is also connected to the underlining nuclear lamina. This lamina consists of polymers formed by the A- and B-type lamins (Strelkov *et al.* 2004). Polymerization of A- and B-type lamin homodimers takes place separately, but the forming filamentous networks interact with each other (Sullivan *et al.* 1999, Muchir *et al.* 2000, Vergnes *et al.* 2004, Shimi *et al.* 2008). The nuclear lamina is disassembled and reassembled by phosphorylation and dephosphorylation during mitosis, along with the rest of the nuclear envelope (Hutchinson *et al.* 2001). The lamina meshwork plays an important role in the nuclear architecture by determination of the shape and mechanical properties (Furukawa and Hotta 1993, Sullivan *et al.* 1999, Liu *et al.* 2000, Goldman *et al.* 2008). The anchoring of chromatin and the correct localization of the nuclear pore complex in the nuclear envelope are additional important functions of the lamina. The minor fraction of the nucleoplasmic lamins is believed to influence DNA replication and transcription (Dechat *et al.* 2008). Lamins have been shown to play a role in the translocation of transcriptional regulators and to have an impact on gene expression (Hutchison 2002, Burke *et al.* 2002, Mounkes and Stewart 2004, Maraldi *et al.* 2011). Several studies have identified the interaction and binding of various proteins, including lamina-associated proteins (LAP 1 and Lap2 α) (Foisner and Gerace 1993, Dechat *et al.* 2000), Emerin (Clements *et al.* 2000), the SUN proteins (SUN1 and SUN2) (Crisp *et al.* 2006), the nuclear envelope

spectrin repeat proteins (nesprin 1 and 2) (Mislow *et al.* 2002, Zhang *et al.* 2002), actin (Sasseville and Langelier 1998), pRb (retinoblastoma gene product) (Ozaki *et al.* 1994) and SREBP (sterol regulatory element-binding protein 1) (Lloyd *et al.* 2002). Furthermore lamins bind with one or more components of RNA polymerase II dependent transcription complexes and DNA replication complexes (Spann *et al.* 1997). The linker of nucleoskeleton and cytoskeleton complex (LINC) provides a physical connection between the nucleoskeleton and the cytoskeleton and its localization to the nuclear envelop depends on the lamins (Méjat and Misteli 2010). At the inner nuclear membrane the LINC complex depends on the SUN proteins. All SUN proteins contain a C-terminal transmembrane domain and a N-terminal domain that interacts directly with A-type lamins (Crisp *et al.* 2006). Nesprins are components of the LINC complex at the outer nuclear membrane where nesprin 1 and 2 bind to the actin cytoskeleton. The nesprins are also in connection with the SUN proteins by their transmembrane KASH (Klarsicht/ANC/Syne homologue) domain (Haque *et al.* 2006). Emerin, another lamin binding protein, also binds to nesprin, thus providing an additional connection between the lamins and the cytoskeleton (Zhang *et al.* 2002, Warren *et al.* 2005). The physical bridging of the nuclear lamina with the cytoskeleton has a major impact on cellular functions including cytoskeleton organisation, organelle positioning and cellular division (Méjat and Misteli 2010).

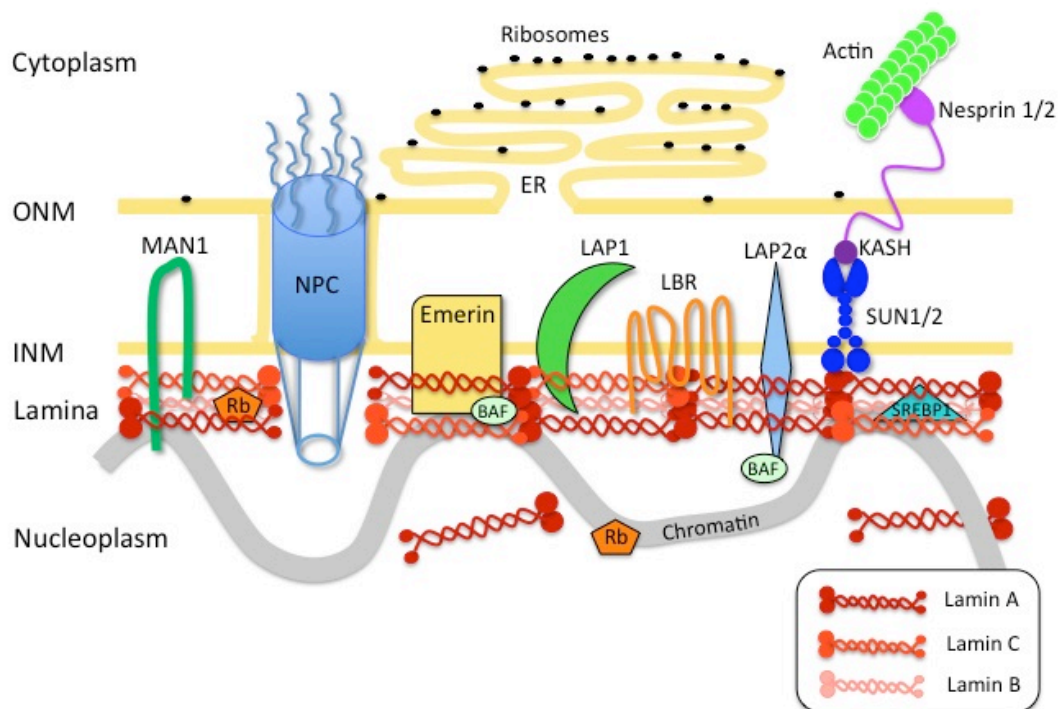


Figure 2. Schematic picture of the nuclear envelope and the nuclear lamina.

The lamina filaments (lamin A, C and B) located underneath the inner nuclear membrane (INM) interact with several trans-membrane proteins. Lamins play a role in anchoring of chromatin and the correct localization of the nuclear pore complexes (NPC). Through the binding of SUN domain proteins the lamina is also active in the linker of nucleoskeleton and cytoskeleton complex (LINC) involving KASH domains as well as Nesprin 1 and 2 and thereby establish a physical connection to the cytoskeleton (Actin). The drawing was inspired by Goldman *et al.* 2002 and Burke and Stewart 2006.

1.3 LAMINOPATHIES

There are more than 10 different heritable conditions known to be due to mutations in the *LMNA* gene. These disorders are collectively named laminopathies and are often subdivided based on their shared phenotypes into four distinct categories: muscular dystrophies, lipodystrophies, neuropathies and segmental progeroid syndromes (Figure 3). There are more the 200 mutations associated with these conditions and the disease mechanisms are still far from clear (Capell and Collins 2006, Worman *et al.* 2010).

Muscular dystrophies include Emery-Dreifuss Muscular Dystrophy (EDMD), Limb-Girdle Muscular Dystrophy (LGMD) and Dilated Cardiomyopathy (DCM). The muscular dystrophies are characterized by progressive skeletal muscle weakness, also affecting the heart. Defects in muscle proteins, death of muscle cells and gradual wasting of the muscles with replacement by scar tissue and fat are typical clinical features. Patients with EDMD show slowly progressive contractures in childhood and teenage years. Muscle weakness and wasting starts in the distal limb muscles. Most patients also suffer from cardiac conduction defects and arrhythmias (Bonne *et al.* 1999, Morris 2001). Slowly progressive shoulder and pelvic muscle weakness and wasting followed by development of contractures and cardiac disturbances are typical for LGMD (Muchir *et al.* 2000). In DCM affected individuals display impaired contractility, arrhythmias and conduction defects. The muscle weakness in DCM is a cardiac-specific dystrophy without any affects on the skeletal muscle (Fatkin *et al.* 1999).

The generalized or localized loss of body fat represents the typical clinical features of the **lipodystrophies**, like Familial Partial Lipodystrophys (FPLD), generalized Lipodystrophy type 2 and Mandibular Dysplasia typ A (MAD). In FPLD loss of subcutaneous white adipose tissue is seen in the extremities, the trunk and the gluteal region while the face, neck and abdominal region accumulates fat cells. FPLD (Peters *et al.* 1998, Hegele *et al.* 2005) and also generalized Lipodystrophy type 2 (Csoka *et al.* 2004) are associated with an increased risk of early endpoint atherosclerosis and the development of metabolic diseases like high blood levels of triglycerides (hyperglyceridemia) and insulin resistance, leading to diabetes. Clinical characteristics for MAD include general lipodystrophy, delayed closure of cranial sutures, dental crowding, joint contractures, mandibular and clavicular hypoplasia, acroosteolysis and insulin resistance. MAD patients also show short stature and alopecia (Novelli *et al.* 2002, Simha *et al.* 2003).

Chartoc-Marie-Tooth disease type 2B1 (CMT2) is a **neuropathy** that affects the nerves that control the muscles. CMT patients slowly lose normal use of their extremities as the nerves degenerate and the muscles in the extremities become weakened because of the loss of stimulation by the affected nerves (De Sandre-Giovannoli *et al.* 2002).

Under the category of the **segmental progeroid syndromes** are atypical Werner syndrome, Restrictive Demopathy (RD), classical and atypical HGPS. Werner syndrome is also known as progeria of the adult with death first occurring in the fifth or sixth decade in life due to atherosclerosis or neoplasia. Growth retardation manifesting from the second decade, cataracts, type 2

diabetes, osteoporosis, alopecia, sclerodermatous skin, loss of adipose tissue and increased tendency for cancer are typical clinical features for Werner syndrome (Werner 1985). Classical Werner syndrome is caused by mutations in the *WNR* gene, which encodes a RecQ helicase (Yu *et al.* 1996). Atypical Werner syndrome has similar features as classical Werner syndrome but is caused by mutations in the *LMNA* gene (Chen *et al.* 2003). Restrictive Dermopathy (RD) is a lethal tight skin contracture syndrome. Intrauterine growth retardation, joint contractures, tight and rigid skin and prominent vessels are typical for this neonatal disease. Affected individuals die due to pulmonary hypoplasia and subsequent respiratory insufficiencies during gestation or early after birth (Navarro *et al.* 2004, Navarro *et al.* 2005, Moulson *et al.* 2005, Burke and Stewart 2006).

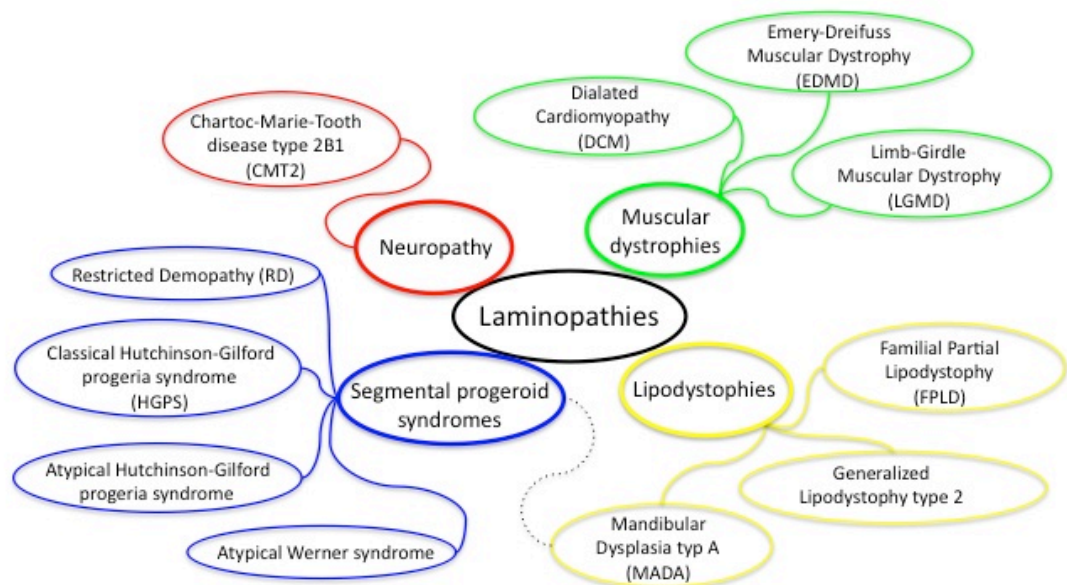


Figure 3. Schematic presentation of the laminopathies in four different categories: neuropathy, muscle disorder, lipodystrophy and progeroid syndrome. The clinical characteristics of MADA include lipodystrophic features as well as features implicated in segmental progeroid syndromes.

1.4 HUTCHINSON-GILFORD PROGERIA SYNDROME

Hutchinson-Gilford Progeria Syndrome (HGPS, progeria, OMIM#176670) is an extremely rare genetic disorder affecting one in 4-8 million live births (DeBusk 1972) and it is classified as a segmental progeroid syndrome (Brown 1979, Brown *et al.* 1985, Sarkar and Shinton 2001). Affected individuals appear normal at birth and develop the first symptoms of disease usually within the first two years of life. Growth retardation together with dermatological changes like scleroderma and loss of subcutaneous fat are the early indications. The skin tightens over the abdomen and can restrict motion. Regional changes with wrinkled, thin, dry and atrophic skin, with signs of hyperkeratosis and hyperpigmentation, are found. Children usually gradually develop complete alopecia by the age of 3 years (DeBusk 1972, Gillar *et al.* 1991, Erdem *et al.* 1994, Jansen and Romiti 2000, Sarkar and Shinton 2001, Ackerman

and Gilbert-Barness 2002, Sevenants *et al.* 2005, Hennekam 2006, Merideth *et al.* 2008). Short stature, low weight, prominent scalp veins and eyes, a pyriform chest, a high-pitched voice, a small and beaked nose, dental crowding and oral soft tissue alteration, thin lips and dystrophic fingernails are additional clinical features. Affected children do not enter puberty and lack sexual maturation (Hennekam 2006). The intellect of HGPS patients is unaffected and affected children do not develop signs of senility, cataract, cancer or metabolic diseases (DeBusk 1972, Stables and Morley 1994, Sarkar and Shinton 2001, Hennekam 2006, Mazereeuw-Hautier *et al.* 2007, Merideth *et al.* 2008, Domingo *et al.* 2009). With increasing age, affected children develop shortness of breath with exertion, increased pulse rate and high blood pressure. The most common causes of death are heart failure and stroke at a median age of 13 years, ranging from about 8-21 years (Baker *et al.* 1981, Shozawa *et al.* 1984). Autopsy studies have shown abnormalities in the blood vessel layers with small diameters of the intima and media and increased tunica thickness. Histological observations include extensive loss of smooth muscle cells and their replacement with fibrous tissue (Markous *et al.* 1962, DeBusk 1972, Stehbins *et al.* 1999, Stehbins *et al.* 2001, Ackerman and Gilbert-Barness 2002, Nair *et al.* 2004, Gordon *et al.* 2005, Hennekam 2006, Merideth *et al.* 2008).

1.4.1 Skeletal phenotype in HGPS

The disease progression in HGPS includes several developmental abnormalities of the skeleton. Growth retardation with short stature and low weight is a clinical feature that is always present (Badame 1989). A horse-riding stance is described, as the patients typically stand with flexed knees and increased distance between the thighs. In motion affected children display a wide based, shuffling gait. Decreased mobility is associated with joint stiffness usually starting in knees, followed by elbows, phalanges and hips. The limbs appear very thin with prominent joints (Moen 1982, Hennekam 2006). One of the earliest abnormal findings is acro-osteolysis, the distal resorption of fingers and toes, which gets more severe with disease progression (de Paula Rodrigues *et al.* 2002, Gordon *et al.* 2007). Roentgenographic analysis of the skull and facial bones reveals an overall decreased size and thinning of facial bones together with reduced head circumferences. The size of the cranial vault is normal compared to brain size, but appears relatively large compared to the decreased size of the facial bones. A glyphic beaked nose or convex nasal profile is a common characteristic of the facial features (Badame 1989, de Paula Rodrigues *et al.* 2002). The chin becomes smaller with an increased angle of the mandibular leading to dental crowding and double rows of teeth (Hennekam 2006). Among the oral abnormalities, delayed tooth eruption, dysmorphic teeth and hypodontia, have been reported (Merideth *et al.* 2008, Domingo *et al.* 2009). The upper part of the thorax often gradually narrows with progressive osteolysis of the clavicles, as well as thinning and tapering of the ribs ending in a pyramidal thorax configuration and pear shaped appearance (Hennekam 2006, Gordon *et al.* 2007, Merideth *et al.* 2008). Spinal malformations include dislocated shoulders, decreased spinal flexion, kyphosis and scoliosis. The vertebral bodies display an ovoid and fish-mouth appearance. Hip dislocations

are characteristic for coxa valga, a deformity caused by an increased angle between the femoral neck and shaft and coxa plana, the disintegration of the capital femoral head leading to diminished blood supply of the bone and subsequent osteonecrosis (de Paula Rodrigues *et al.* 2002, Hennekam 2006, Gordon *et al.* 2007). The long bones are described to be more slender and bowed (Hennekam 2006). Earlier reports of clinical investigators interpreted the abnormal mineralization observed on radiographs from HGPS patients as focal osteoporosis restricted to the distal ends of the long bones (Gordon *et al.* 2007). Others regarded the decreased bone mineral density (BMD) as histopathological features that score in the osteoporotic range (Merideth *et al.* 2008). Recently a study utilizing dual energy x-ray absorptiometry (DXA) and peripheral quantitative computed tomography (pQCT) concluded that the pathological conditions represent a unique skeletal dysplasia, which differs from defective bone turnover, the normal cause for senile osteoporosis (Gordon *et al.* 2011). In addition, this study analyzed the daily caloric and nutrient intake and showed that the skeletal pathology could not be ascribed to malnutrition-induced bone loss. Along with normal bone age, neither proliferative changes, like osteoarthritis or rheumatism, nor abnormalities in growth plate development and fracture risks were found as orthopedic aspects of progeria (Gordon *et al.* 2007, Gordon *et al.* 2011).

1.5 MOLECULAR BASIS IN HGPS

The majority of the classical cases of HGPS are caused by a *de novo* single point mutation within exon 11 of the *LMNA* gene (c.1824C<T, p.G608G). The mutation activates a cryptic splice site, removing 150 nucleotides of exon 11, and results in a prelamin A protein, named progerin, with an internal deletion of 50 amino acids (De Sandre-Giovannoli *et al.* 2003, Eriksson *et al.* 2003). Since the mutation is located in exon 11, it will just affect the lamin A protein. Atypical HGPS describes diseases with similar progeroid features of classical HGPS, which are caused by missense mutation in the *LMNA* gene, but do not result in abnormal splicing within exon 11 (Eriksson *et al.* 2003, Csoka *et al.* 2004, Verstraeten *et al.* 2006, Liang *et al.* 2009).

1.5.1 Post-transcriptional processing of prelamin A in HGPS

Lamin A is synthesized as a precursor molecule, prelamin A. Prelamin A is modified in different processes before it becomes mature lamin A (Figure 4) (Lutz *et al.* 1992, Sinensky *et al.* 1994, Hennekes *et al.* 1994). Farnesylation of the cysteine residue of the CAAX box initiates a sequence of processing steps starting with the -AAX motif cleavage by the endopeptidases Rce1 (Ras-converting enzyme 1) or Zmpste24 (FACE1 in humans) (Beck *et al.* 1990, Zhang and Casey 1996). The cleavage step is followed by carboxymethylation of the C-terminal cysteine by isoprenylcysteine carboxy methyltransferase (Icmt) (Winter-Vann and Casey 2005). The endoprotease responsible for the final cleavage of the prelamin A molecule is FACE1 or Zmpste24 (Pendas *et al.* 2002, Bergo *et al.* 2002). In HGPS cells, where the mutation by cryptic splice site activation of the pre-mRNA induces a deletion in the carboxy terminal end of

lamin A, the deletion would include removal of the recognition motif necessary for the final endoproteolytic step. This incompletely processed prelamina A molecule is predicted to go through initial post-translational modification steps but the final step is omitted and leave a prelamina A protein with a carboxymethylated and farnesylated cysteine in the carboxyterminal end. Previous *in vitro* studies of the post-translational processing of the lamin A protein predict that cells from HGPS patients have partially processed and truncated prelamina A protein attached to the inner nuclear membrane (Hennekes *et al.* 1994, Stuurmann *et al.* 1998, Capell *et al.* 2009).

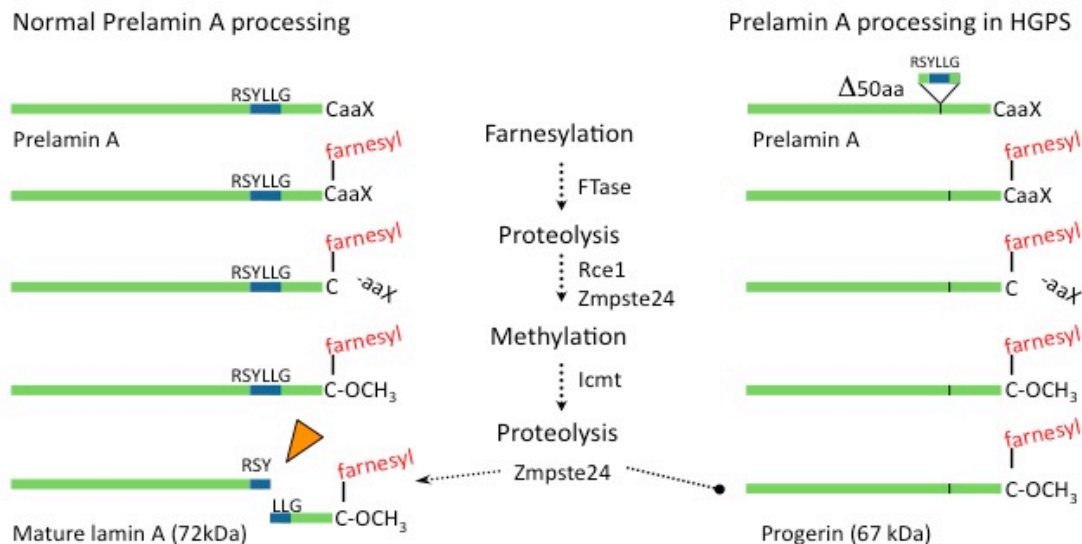


Figure 4. Multi step modification of the CaaX motif at the C-terminal end involving farnesylation, proteolysis and methylation processed by different enzymes. The recognition site for the last proteolytic step, to yield mature lamin A, is marked in blue. In HGPS this cleavage site is absent due to the aberrant splicing of the *LMNA* gene and the internal deletion of 50 amino acids in exon 11. The subsequent protein product in HGPS is a farnesylated and carboxymethylated truncated prelamina A named progerin. The drawing was inspired by Young *et al.* 2006.

1.6 CELLULAR PHENOTYPE IN HGPS

Immunofluorescence studies of cells from HGPS patients with an antibody directed to lamin A/C show structural defects with irregularly shaped nuclear membranes that get more severe as the cells age in culture and confirms the hypothesis of a toxic effect from mutated prelamina A accumulation (Eriksson *et al.* 2003, Goldman *et al.* 2004). Progerin forms insoluble aggregates at the nuclear rim during mitosis, causes chromosome segregation and alters the cell cycle progression (Dechat *et al.* 2007, Cao *et al.* 2007). Multiple structural abnormalities have been reported from studies of HGPS cells and include nuclear blebbing and lobulation, altered distribution of nuclear pore complexes, loss of peripheral heterochromatin, thickened lamina and mixed heteropolymer formation of A- and B-type lamins (Goldman *et al.* 2004, Delbarre *et al.* 2006). Transcriptional misregulation and altered expression of various genes have been detected in cells from HGPS patients (Ly *et al.* 2000,

Csoka *et al.* 2004). All of these changes could result in downstream effects on gene regulation and DNA replication. Reduced telomere length in HGPS fibroblasts has also been demonstrated to mediate the onset of premature cell senescence through progerin-induced DNA damage pathways (Allsopp *et al.* 1992, Decker *et al.* 2009, Benson *et al.* 2010). The segmental phenotype of HGPS affects tissues with continuous growth (hair follicles, nails) or tissues subjected to high mechanical stress (joints, blood vessels). These tissues require permanently proliferative stem cells for repair and remodelling (skin, bone). Accelerated cell turnover with increased proliferation triggered by mutated prelamin A expression is likely to exhaust the stem cell pools. Age related conditions absent in HGPS like brain aging (dementia, Alzheimer disease, senility) and metabolic diseases (diabetes, hyperlipidemia) affect tissues that are well protected from mechanical forces or independent from stem cell regeneration (Halaschek-Wiener and Brooks-Wilson 2007). *In vitro* experiments with human mesenchymal stem cells expressing progerin demonstrated aberrant differentiation towards an osteogenic and adipogenic cell character (Scaffidi and Misteli 2006). Studies from a mouse model of HGPS that were recently conducted indicate that the cellular senescence, associated with HGPS, results in a pro-inflammatory phenotype stimulating inappropriate stem cell proliferation (Rosengardten *et al.* 2011). Downregulation of Notch and Wnt signalling pathways together with impaired function and depletion of stem cells have also been shown in mouse models with lamin mutations that developed typical progeroid features (Hernandez *et al.* 2010, Rosengardten *et al.* 2011).

1.7 THERAPEUTICS FOR HGPS

With the discovery of the HGPS mutation in the *LMNA* gene (De Sandre-Giovannoli *et al.* 2003, Eriksson *et al.* 2003) the post-transcriptional processing of prelamin A was soon identified as a target for possible treatment of the disease. The processing of proteins with farnesylation motifs, like the CAAX box in prelamin A, can be modified by farnesyltransferase inhibitors (FTIs). Previously FTIs compounds without major side effects have been identified and applied in different cancer treatments (Reuter *et al.* 2000, Caponigro *et al.* 2003). In HGPS the suppressed farnesylation results in a non-farnesylated progerin protein product. In the absence of *ZMPSTE24*, like in RD, the suppressed farnesylation results in the accumulation of non-farnesylated prelamin A (Young *et al.* 2006). Several *in vitro* studies have shown that FTI treatment reduces the proportion of misshapen nuclei and rescues nuclear blebbing and abnormal prelamin A and lamin A localization and aggregation in human HGPS and RD cells, transfected cell lines (Capell *et al.* 2005, Glynn and Glover 2005, Mallampalli *et al.* 2005, Toth *et al.* 2005, Pan *et al.* 2007) and cells isolated from lamin A mutant mouse models (Yang *et al.* 2005). Administration of FTIs to mouse models for mutant *Lmna* and *Zmpste24* deficient mice improved the progeroid phenotype (Fong *et al.* 2006, Yang *et al.* 2006, Capell *et al.* 2008, Yang *et al.* 2008). In May 2007 a first clinical trial was enrolled including twenty-eight patients with classical HGPS from sixteen countries. After an initial two-month tolerance test the oral application of the

FTI Lonafarnib (SCH66336) was enrolled for a two-year period. This first clinical trial ended in December 2009 (<http://www.progeriaresearch.org/clinical-trial.html>).

While the first FTI trial was initiated additional studies have shown that prelamin A and progerin undergo alternative prenylation by geranylgeranyltransferase in the presence of FTIs (Varela *et al.* 2008). The investigators concluded that the limited *in vivo* benefits from FTIs application in *Lmna*^{HG/+} and *Zmpste24*^{-/-} mice could be ascribed to this alternative pathway. The combined treatment of FTIs, statins and bisphosphonates has been shown to prevent the cross-prenylation of prelamin A and progerin and markedly improved the cell morphology of HGPS cells, as well as the aging like phenotype in *Zmpste24*^{-/-} mice. The combined treatment also resulted in significantly prolonged animal life spans (Varela *et al.* 2008). After dosage tolerance testing with five individuals, a triple drug trial for the combined administration of Lonafarnib (FTI), Zoledronic Acid (bisphosphonate) and Pravastatin (statin) was initiated in August 2009 including forty-five children from twenty-four countries. The duration of the triple drug trial was planned over a two-year period (<http://www.progeriaresearch.org/clinical-trial.html>).

More recently a study concentrating on an alternative age associated pathway has shown promising results for the potential of the antibiotic rapamycin in HGPS treatment (Cao *et al.* 2011b). The antibiotic targets a well-conserved aging pathway (TOR, target of rapamycin). Activated TOR has been shown to stimulate protein synthesis and cell growth, and regulates diverse cellular processes (Tsang *et al.* 2007). Reduced TOR signalling upon rapamycin treatment promotes cell maintenance processes like autophagy (Rubinshtein *et al.* 2011) and has been shown to enhance the maximum lifespan of mice, when administration was initiated in old animals at the age of 600 days (Harrison *et al.* 2009). In HGPS fibroblast rapamycin treatment rescued the abnormal nuclear morphology, slowed the progression of cellular senescence and reduced progerin concentrations. Rapamycin had previously been shown to promote the degradation of toxic compounds inside the cell (Berger *et al.* 2006). In cells from progeria patients treatment with rapamycin decreased the formation of insoluble progerin aggregates. Increased autophagy was demonstrated to support the clearance of progerin from HGPS fibroblasts (Cao *et al.* 2011b). Rapamycin is already approved for the use as an immunosuppressive drug and in cancer treatments (Wullschleger *et al.* 2006). Therefore rapamycin should be further tested in mouse models for HGPS and considered as a possible treatment option for HGPS patients.

1.8 LAMINS AND AGING

The complex processes driving physiological aging are still not understood and it is most likely that several molecular mechanisms are involved. Evidence for the involvement of lamins in aging has first been shown in studies on fibroblast cell cultures. The presence of low levels of progerin in cells from healthy individuals indicate a role of the aberrant splicing of the prelamin A RNA in general human aging (Scaffidi and Misteli 2006). Cultured

cells from individuals that have aged normally share several of the reported nuclear defects. The nuclear abnormalities were present in cell lines from young and old donors and increased with prolonged passages. However the nuclear changes observed in cells from older individuals appear more rapidly and show changes in lamin A/C localization with accumulation at the nuclear rim similar to HGPS patient cells (Goldman *et al.* 2004, Scaffidi and Misteli 2006). *In vitro* studies of fibroblast from healthy donors further demonstrated that the amount of both, mRNA and mutated protein increases with the number of passage (Cao *et al.* 2007, Rodriguez *et al.* 2009). Progerin expression has also been found in the skin of older individuals, predominately in cells that have reached terminal differentiation or senescence (McClintock *et al.* 2007). Indeed, a more recent study demonstrated that telomere shortening and dysfunction induces the activation of progerin splicing in normal human fibroblasts and triggers cellular senescence (Cao *et al.* 2011a).

As well as *in vitro* studies with human progerin expressing cells, studies with *Zmpste24*^{-/-} mice have shown genomic instability associated with increased DNA damage (Liu *et al.* 2005, Varela *et al.* 2005, Liu *et al.* 2006). Defects in DNA repair mechanisms are a characteristic for progeroid syndromes and a hallmark of normal aging. Impaired DNA damage signalling activates the tumor suppressor gene p53, which triggers cellular senescence and accelerates an aging phenotype (Lans and Hoeijmakers 2006, Manju *et al.* 2006).

1.9 LAMINS IN BONE

In a study focusing on the osteoarticular system in C57BL6 wild-type mice lamin A/C was found in the trabecular and cortical bone. In chondrocytes lamin A/C was expressed at higher levels in endochondral compared to perichondral chondrocytes (Duque *et al.* 2006). Reduced expression of lamin A/C in aging bone was found in old mice (24 month old) compared to younger adult mice (4 month old). The numbers of lamin A/C expressing osteoblasts decreases with age from 76% in adult mice to 42% in old mice (Duque *et al.* 2006).

Knockdown of lamin A/C in *Lmna*^{-/-} mice (Sullivan *et al.* 1999) and mouse models with HGPS modifications of the *Lmna* gene (*Lmna*^{HG/+}) (Yang *et al.* 2005) developed skeletal abnormalities. Common features of bone disease in these mouse models included growth retardation, kyphosis, bone fractures and poor bone mineralization (Sullivan *et al.* 1999, Yang *et al.* 2005, Yang *et al.* 2006). Recent studies of bone turnover and bone structure in *Lmna*^{-/-} mice (Li *et al.* 2011) showed alteration in bone mass and skeletal microarchitecture and reduced osteoblast and osteoclast numbers and activity. Inhibited function of osteogenic transcription factor Runx2 to interact in the nuclear binding complex affected osteoblast differentiation. Studies with knockdown of lamin A/C in human osteoblasts and mice deficient in *Zmpste24* show inhibited osteoblastogenesis and simultaneously increased adipocyte differentiation (Akter *et al.* 2009, Rivas *et al.* 2009). The cellular mechanisms leading to these abnormalities in the bone remain unknown.

1.10 BONE DEVELOPMENT AND REMODELLING

The skeleton is a complex metabolically active organ that is formed by cartilage and bone. Important functions include the maintenance of mineral homeostasis, mechanical support, protection of vital organs, and assistance in locomotion and movement through its physical connection with muscles. The adult human skeleton consists of 213 bones, which undergo continuous remodelling throughout life. Bone remodelling involves the removal of mineralized bone followed by the formation of new bone matrix. Three different cell types, osteoblasts, osteoclasts and osteocytes, are involved in bone maintenance (Dempster 2006). Osteoblasts are responsible for the formation and organization of the extracellular matrix of bone and its subsequent mineralization. They are derived from mesenchymal precursor cells in the bone marrow and activated by Wnt and BMP signalling and induced by osteoblastic transcription factors like Runx2 and osterix (Sp7). Further on, the bone microenvironment supports a continuous supply of differentiation factors, which directs the cells towards proliferation, maturation and terminal differentiation. The principal product of the mature osteoblast is type I collagen (90% of the protein in bone) (Karsenty et al. 2009). As a terminal differentiation step, some osteoblasts become trapped in lacunae within the matrix of bone as osteocytes (Figure 5). Osteocytes are connected by an intercellular system of canaliculi. These canals are responsible for intercellular communication and there is evidence that osteocytes regulate the response of bone to the mechanical environment (Bonewald and Johnson 2008).

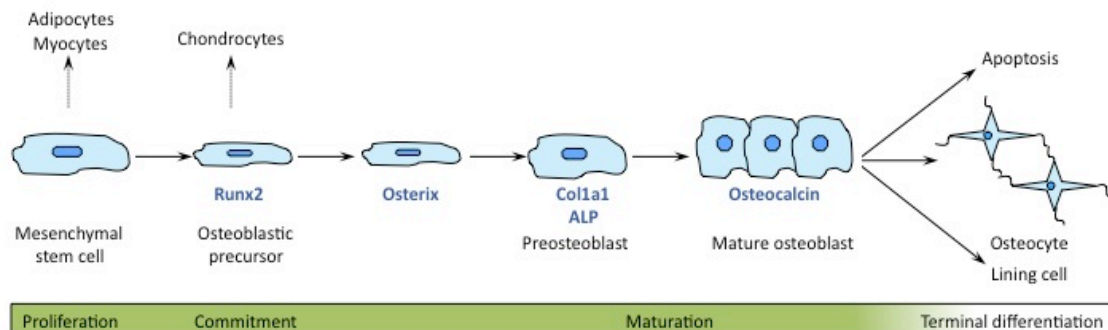


Figure 5. Schematic drawing of osteoblastogenesis

Osteoblastic precursors derive from the mesenchymal stem cell lineage. Runx2 is a transcription factor playing a key role in cell commitment towards osteoblastogenesis. Further cell maturation, matrix mineralization and differentiation is controlled by frequently used biomarkers. Selected examples are shown. The drawing was inspired by Walkley *et al.* 2008.

The cell types responsible for bone resorption are the osteoclasts, large and motile multinucleated cells located on bone surfaces. Osteoclasts are formed by the fusion of mononuclear cells derived from haematopoietic stem cells in the bone marrow. Osteoclasts are polarized cells, having a ruffled border region of the cell membrane that is surrounded by an organelle-free region. They adhere to the bone surface via integrins, which are specialized cell surface receptors. A pH gradient across the ruffled membrane is created by active transport

mechanisms, ATP-dependent proton pumps, and the enzyme carbonic anhydrase. Osteoclasts actively synthesize lysosomal enzymes (Teitelbaum 2007). Bone remodelling serves to adjust bone architecture to meet changes in mechanical needs and helps to repair microdamages in bone matrix, preventing the accumulation of old bone. It also plays an important role in maintaining plasma calcium homeostasis. The regulation of bone remodelling is both systemic and local. The major systemic regulators include parathyroid hormone (PTH), calcitonine, 1-25 hydroxyvitamin D (calcitriol), and other hormones such as growth hormones, glucocorticoids, thyroid hormones, and sex hormones. Furthermore, through the crosstalk between osteoclastic and osteoblastic cells through the RANK/RANKL/OPG system, the processes of bone resorption and formation are tightly coupled allowing bone formation to follow each cycle of bone resorption, thus maintaining skeletal integrity.

The remodelling cycle consists of four phases:

1. Activation phase

Activation of osteoclast precursors leads to cell maturation into multinuclear osteoclasts. The maturation step requires the essential cytokine M-CSF (macrophage colony stimulating factor) produced by marrow stromal cells and RANKL (receptor activator of NF kappa B ligand). The ratio between RANKL and the blocking agent OPG (osteoprotegerin), both produced by osteoblast cells, regulates osteoclastogenesis.

2. Resorption phase

Activated osteoclasts digest old bone and form a resorption cavity. Mature multinuclear osteoclast cells attach to the bone surface and secrete various enzymes and acids (TRAP, cathepsin K, Mmp9) that cause degradation and dissolution of the bone mineral matrix. The resorption phase ends with osteoclast apoptosis and is followed by the reversal phase.

3. Reversal phase

A variety of coupling factors (growth factors, cytokines and hormones) lead to osteoclasts apoptosis and activate osteoblast proliferation and differentiation. Thus mononuclear preosteoblast cells appear at the resorption surface and induce osteoblast maturation and their attachment to the resorption cavity.

4. Formation phase

Bone formation is a two-step process, where osteoblasts synthesize new bone matrix, the osteoid, and then regulate the mineralization by releasing small matrix vesicles that concentrate calcium and phosphate.

Under normal physiological conditions bone remodelling and the balanced interplay of all involved cell types is coupled, so that the relative amount of resorbed bone is replaced by newly formed bone matrix (Figure 6). However this balance is very sensitive to changes in hormone levels, mechanical stress and aging (Dempster 2006, Hadjidakis and Androulakis 2006, Manolagas and Parfitt 2010, Crockett *et al.* 2011).

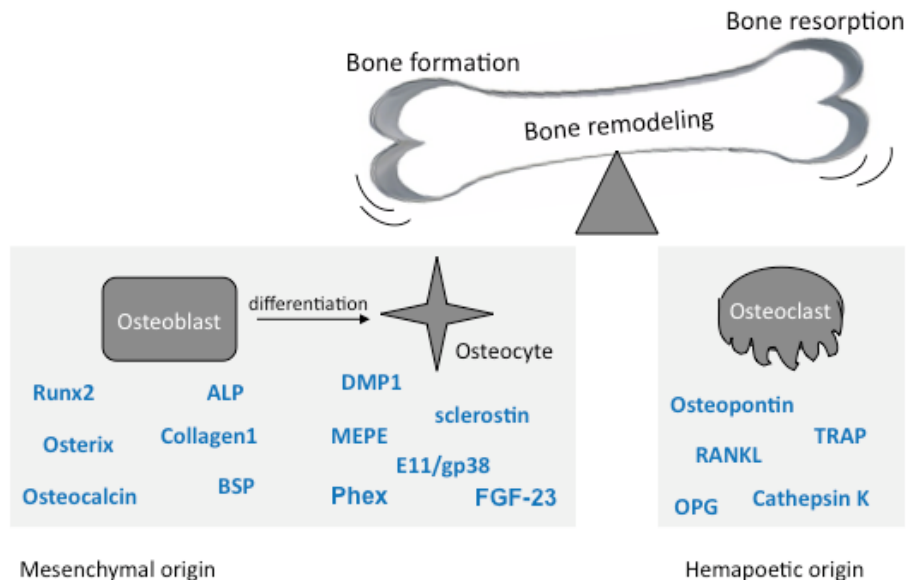


Figure 6. Bone remodelling is a cellular coupled and balanced process

Initiation of bone resorption by osteoclast is followed by osteoblast-mediated bone matrix formation and finally bone matrix mineralization. Multiple factors are involved in the activation and intracellular signalling between the involved cell types. A selection of important factors are indicated in blue.

1.11 AN INDUCIBLE AND TISSUE SPECIFIC MOUSE MODEL SYSTEM

The development of mouse models are a critical step in further understanding the pathogenesis of human diseases and in testing new forms of treatment. To investigate the segmental nature of HGPS with clinical features only present in restricted tissues will help to further understand the disease mechanism. In particular, since lamin A is expressed in all differentiated cells (Dechat *et al.* 2008), it still remains unclear how to explain the partial impact the mutation has on patients. The tissue-restricted fashion is also one of the challenges in the generation of representative mouse models of the human disease. The tetracycline-inducible systems (tet-system) are binary transgenic mouse model systems for spatial and temporal regulated expression of target genes (Gossen and Bujard 1992, Zhu *et al.* 2002). By choosing the tet-system in combination with tissue-specific promoters, the investigation of distinct disease mechanism in restricted HGPS affected tissues (skin and bone) is possible. In contrast to general knock-out or knock-in animal models, the tissue restricted fashion of the tet-system also enables the study of effects from sustained expression of the HGPS mutation in isolated organ systems. This is particularly interesting when investigating the long-term effects of progerin expression in unaffected tissues (including the brain).

1.11.1 Tetracycline-inducible transactivator system

Tetracycline-responsive transcriptional transactivator (tTA, tet-OFF) and reverse tetracycline-responsive transcriptional transactivator (rtTA, tet-ON) transgenic mice temporally regulate expression of a gene of interest. The transcriptional transactivators (tTA and rtTA) bind to a tetracycline responsive promoter element (pTRE element, tetop) that controls the transcription of a

target gene construct (Figure 7). The treatment of mice with tetracycline, or its derivate doxycycline (Dox), allows in the tet-ON system the binding of the rtTA to the pTRE element and subsequently allows the temporal regulation of gene expression. Whereas the administration of doxycycline in the tet-OFF system represses the binding of the tTA to the pTRE element and blocks the gene expression (Figure 7) (Gossen and Bujard 1992, Gossen *et al.* 1995). This is particularly interesting if one might want to study the expression of genes with detrimental effects during development or expression effects at later time points (Kistner *et al.* 1996). Temporal control also enables reversal studies after phenotype development depending on the target gene expression. Furthermore the fusion of tissue-specific promoter elements upstream of the transactivators directs their expression in a tissue-specific manner. This makes it possible to control the distinct spatial target gene expression inside the mammalian organism in *in vivo* model systems or in *in vitro* studies in single cells (Furth *et al.* 1994).

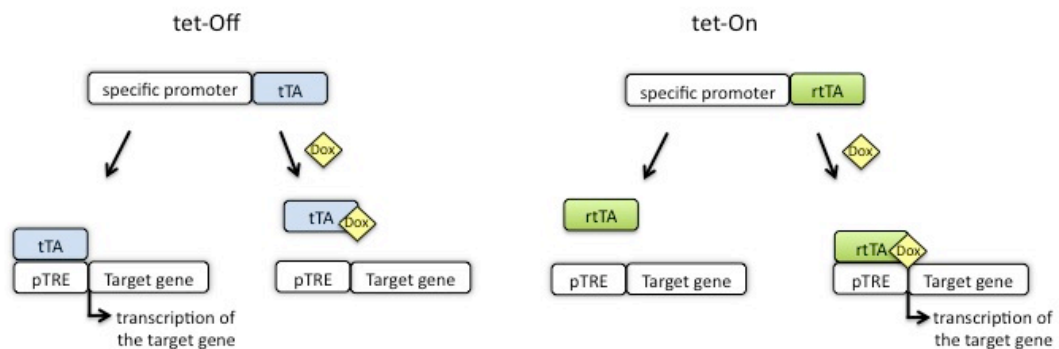


Figure 7. The tet-Off and tet-On expression systems

Schematic pictures of the gene regulation in the tet-system. Induction of gene expression can be controlled by administration of Doxycycline (Dox). In the tet-Off system tTA binds the pTRE element and activates transcription in the absence of Dox. In the tet-On system rtTA binds the pTRE element and activates transcription in the presence of Dox. The drawing was inspired by Gossen *et al.* 1995.

1.11.2 Lamin minigenes

Based on the tet-system, our laboratory generated transgenic mice with different minigenes of lamin A under the control of a pTRE element (tetop) (Figure 8) (Sagelius *et al.* 2008). Three lines of target mice show good expression levels with no leaky expression. The first two lines contain a minigene of human lamin A consisting of exon 1-11, intron 11 and exon 12, followed by an IRES, and the coding region of the green fluorescent protein (*eGFP*). The two lines only differ in codon 608, one construct carries the wild-type sequence of *LMNA* (tetop-LA^{wt}) and the second construct carries the most common HGPS c.1824C>T, p.G608G mutation (tetop-LA^{G608G}). The third line of mice carries the HGPS abnormal spliced cDNA lacking 150 nucleotides (tetop-LA^{progerin}) followed by an IRES, and *eGFP*. These transgenes enable tetop-LA^{wt} to express wild-type human lamin A, tetop-LA^{G608G} to express wild-type human lamin A and progerin, and tetop-LA^{progerin} to express exclusively progerin.

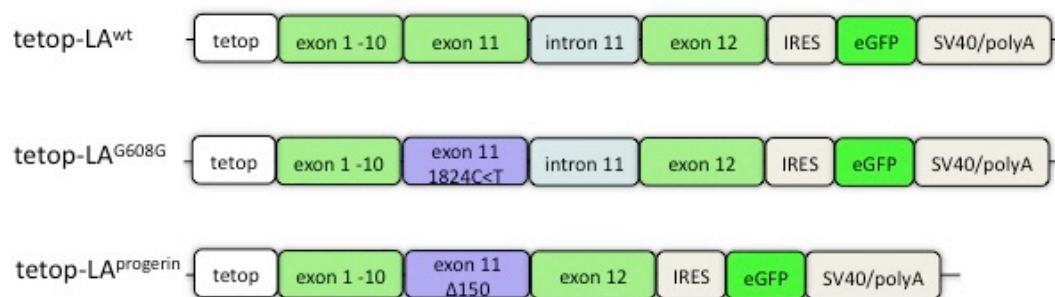


Figure 8. Schematic drawings of the lamin constructs

All three constructs contain a responsive pTRE element (tetop), which regulates the expression of the downstream gene in the presence of a transcriptional transactivator. Construct tetop-LA^{wt} over-expresses wild-type human lamin A. Construct tetop-LA^{G608G} over-expresses wild-type human lamin A and progerin. Both constructs utilize the mouse splicing machinery for mRNA transcription and protein translation. Construct tetop-LA^{progerin} over-expresses progerin. An internal ribosomal entry site (IRES) allows separate translation of enhanced green fluorescent protein (eGFP). Nuclear export, translation and mRNA stability is supported by the SV40/poly A tail. The drawing was modified from Sagelius *et al.* 2008.

1.11.3 Transactivator mice

The work included in this thesis is based on mouse models using the lamin A transgenes tetopLA^{G608G} and tetop-LA^{wt} in combination with four different transactivator mice to achieve expression of the transgene in different organ systems.

Keratin 5 promoter (paper I)

To obtain human lamin A and progerin expression in the skin Keratin 5 promoter mice (K5tTA, Diamond *et al.* 2000) were intercrossed with lamin minigene mice. Cytokeratins are intermediate filament proteins that play a central role in epithelial tissues. Keratin 5 is expressed from embryonic day E9.5 and onwards (Byrne *et al.* 1994). Bitransgenic offspring expressed the minigene in the skin (in the basal layer of the epidermis, sebaceous glands and throughout the outer root sheet of hair follicles), ameloblasts layer of the teeth, salivary glands, esophagus, stomach, tongue, nose cavity and trachea (Sagelius *et al.* 2008). Tetop-LA^{G608G}; K5tTA⁺ mice developed a phenotype in the skin with abnormal hair follicles and sebaceous gland development, fibrosis and loss of hypodermal adipocytes. Oral abnormalities included tooth fractures and inflammatory reactions of the surrounding tissues. No histopathological changes were detected in other organs (Sagelius *et al.* 2008).

Collagen type 1 promoter (paper II)

Transgene expression driven by the 2.3kb collagen type 1 α 1 promoter (α 1p-rtTA, Liu *et al.* 2004) has been shown at high levels in bone, at low levels in skin fibroblasts, but not to be present in any other tissues. Type 1 α 1 collagen is expressed from embryonic day 13-14 and onwards in wild-type mice (Rossert *et al.* 1996).

Osterix promoter (paper III)

Osterix (*Osx1*, *Sp7*) is an osteoblast specific transcription factor, active during early osteoblastogenesis and essential for bone development. Osterix is expressed from embryonic day E14.5. *Osx* null mutant mice totally lack bone formation (Nakashima *et al.* 2002). Sp7-tTA transgenic mice enable expression of target genes during osteoblast differentiation (Rodda *et al.* 2006).

Neuron specific enolase promoter (paper IV)

The 1.8kb NSE promoter (*NSE*, *ENO2*) is expressed from embryonic day E14.0 and throughout adulthood in the nervous system of mice (Forss-Petter *et al.* 1990). It was initially generated to study specific brain regions (Chen *et al.* 1998), but has been shown be active in additional tissues. Transgenic expression was detected at high levels in brain, bone and adipose tissue. Lower level expression was observed in skin and spleen (Sabatakos *et al.* 2000). NSE-tTA transgenic mice allow study of the effect of mutant lamin expression simultaneously in a variety of affected (bone, skin, fat) and unaffected (brain) tissues in HGPS.

2 AIMS OF THE THESIS

The overall aim of this thesis was to develop mouse model systems to elucidate the molecular mechanisms of HGPS in dental and bone tissue and to study effects that come with sustained progerin expression in the brain.

- Paper I The specific aim of this study was to test if the HGPS mutation causes an irreversible phenotype in skin and teeth or if the elimination of progerin production would reverse developed disease conditions. In addition the aim was to further characterize the dental abnormalities observed in the mouse model.
- Paper II The initial aim of this study was to develop an inducible mouse model system for bone disease in HGPS. However the analysis of the transactivator mice revealed insufficient expression. Therefore the amended aim of this study was to share our negative experience with the tet-system.
- Paper III The specific aim of this study was the development of a mouse model for the spatial expression of the *LMNA* c.1824C>T, p.G608G mutation in bone tissue. The purpose was to gain a better understanding of the molecular mechanisms underlying the skeletal phenotype in HGPS.
- Paper IV The specific aim of this study was to investigate effects of the expression of the HGPS mutation simultaneously in affected and unaffected tissues and to study long-term effects from sustained expression of the HGPS mutation in the brain.

3 NOTES ON METHODOLOGY

Material and methods used in this thesis are described in the specific section of each paper and included:

- PCR genotyping
- Bone tissue homogenization for RNA and protein extraction
- Reverse-transcriptase PCR on cDNA
- Western Blotting
- Immunohistochemistry
- Immunofluorescence
- Real time RT-PCR
- Isolation of primary osteoblast explant cultures
- 3-point bending test
- Peripheral quantitative computed tomography (pQCT)
- Bone mineralization assay
- Perfusion fixation

3.1 LABORATORY ANIMALS

Animals were used in accordance with the guidelines for care and use of experimental animals at the Karolinska Institutet. Stockholms Södra Djurförsöksetiska Nämnd approved all applied experimental procedures. The animals were housed in a 12-hour light/dark cycle at 20-22°C and 55-65% humidity in pathogen-free animal facilities at the Karolinska University Hospital. Doxycycline treatment and a soft food diet were applied as described in the respective study (paper I - paper IV). The genetic background of the mice and transgene integration have been discussed as possible factors for variation in expression levels of transactivators and target gene transcription (Dobie *et al.* 1997, Opsahl *et al.* 2002, Robertson *et al.* 2002). To circumvent possible problems with differences in genetic background we generated congenic mouse strains by backcrossing the original mouse strain for ten generations to the new genetic background strain. For the lamin minigene tetop-LA^{wt} and tetop-LA^{G608G}, both generated on FVB/N, mice were maintained on FVB/N and backcrossed to form B6.Cg-Tg(tetop-LA^{wt},-EGFP)SF1-04 and B6.Cg-Tg(tetop-LA^{G608G},-EGFP)VF1-07, respectively. The transactivator mice α 1p-rtTA, generated on C57BL/6XSJL (Liu *et al.* 2004) was maintained on C57BL6 and backcrossed to form FVB/N.Cg-Tg(α 1p-rtTA). Transactivator line NSE-tTA

(Chen *et al.* 1998), provided on CD1, was maintained on CD1 and backcrossed to form FVB/N.Cg-Tg(NSE-tTA). Transactivator strain B6.Cg-Tg(Sp7-tTA,tetO-EGFP/cre)1Amc/J, here referred to as Sp7-tTA (Rodda *et al.* 2006), was maintained on C57BL6. The transactivator mice K5tTA (Diamond *et al.* 2000) was generated and maintained on FVB/N mice.

3.2 OPTIMIZATION OF HUMAN LAMIN A/C IMMUNOHISTOCHEMISTRY ON DECALCIFIED BONE TISSUE

Standard protocols for immunohistochemistry (IHC) on formalin fixed paraffin-embedded sections include application of an antigen retrieval method (AR) to avoid the partial or complete loss of immunoreactivity of the antigen. The principle of AR is based on heat incubation in an aqueous solution. This procedure has been shown to be the ultimate technique for standardization of IHC protocols. It is essential to recover the antigen from cross-linking of unrelated proteins caused by formalin fixation. The cellular location of the target antigen can be an additional challenge in AR method optimization to reach sufficient antigen unmasking (Shi *et al.* 2001).

IHC using a mouse anti-human Lamin A/C antibody (mab3211, JOL2, Millipore) has been used to investigate the cellular location and expression pattern of the human transgene in our tissue specific mouse models. The protocol for lamin A/C staining of paraffin embedded sections of mouse tissues captured and baked on coated SuperfrostPlus Gold slides (Menzel-Gläser) needs the use of 1mM EDTA, pH 8.0 in a pressure cooker for optimal antigen unmasking (Sagelius *et al.* 2008).

However this AR protocol was not applicable to decalcified bone, since the tissue sections detached from the glass slides during the harsh boiling procedure. To obtain optimal antigen unmasking in skeletal tissue, different retrieval solutions (1mM EDTA, pH8.0 vs. 10mM Citrate buffer, pH6.0), either containing no or different supplemented detergents (TWEEN vs. TritonX-100) and multiple heating techniques for AR (water bath vs. microwave oven vs. heating plate), were tested. To enhance the unmasking effect of a milder AR method we also tested for different procedures for bone decalcification (EDTA vs. Formic Acid). Skeletal tissue sections tested for the protocol optimization included long bones (femur and tibia), rib cage, spine, lower jaw and skull.

The best results were obtained heating the bone sections in a glass beaker in the presence of boiling stones and 10mM Citrate buffer pH6.0 without any detergent supplement, as the AR solution. After deparaffinization and rehydration, the slides were placed in a plastic rack standing on a metal ring in a glass beaker. The solution was slowly brought to 95°C using a heating plate and kept at this temperature for 15 minutes under observation of correct temperature and double aluminium foil to cover the glass beaker. The beaker was then taken from the heat source and the solution was allowed to cool down to room temperature. The following staining procedure included blocking for endogenous peroxidase activity with methanol containing 1% H₂O₂, blocking of non-specific binding using 20% normal goat serum followed by mouse-to-mouse blocking reagent (Scytex Laboratories). Primary antibody

incubation was performed at a 1:30 dilution in PBS at 4°C over night using a MIST tray. The slides were washed and then incubated with a secondary biotin-goat-anti-mouse antibody prior to visualization and development of the peroxidase by ABC staining kit (Vectashield) and DAB Chromogen system (DakoCytomation), according to the manufactures recommendations, followed by brief incubation with DAB enhancer solution (Zymed). Counterstaining was performed with Mayer's hemalum solution prior to dehydration and mounting. All washing steps were performed by carefully dipping the slides in PBS. From all skeletal parts tested, the staining on lower jaw sections displayed the most intact bone tissue with specific nuclear signals for lamin A/C located in osteoblasts and osteocytes. However it is recommended to include several slides per samples to obtain usable stained bone sections for analysis, since tissue damage to various degrees invariably occurs.

4 RESULTS AND DISCUSSION

4.1 PAPER I

To find a cure for HGPS requires that the toxic effects that come with progerin expression are not irrevocable for the affected tissues. The aim of this study was to test if progerin causes irreversible disease or if it is possible to reverse an existing phenotype in HGPS mice by suppressing progerin expression. We developed a tissue specific mouse model, based on the tet-system, that allows the temporal regulation of the HGPS mutation. In this model, the administration of doxycycline suppressed the transgenic expression of the HGPS mutation (tetop-LA^{G608G}). By utilizing Keratin 5 transactivator transgenic mice (K5tTA, Diamond *et al.* 2000) we directed the progerin production to the skin and dental tissue from the date of birth until 7 weeks of age. Keratin 5 is expressed in the basal layer of the skins epidermis, the outer root sheath of hair follicles and the ameloblast layer in the teeth (Ramirez *et al.* 1994, Diamond *et al.* 2000, Ravindranath *et al.* 2003). At postnatal week 7 bitransgenic animals, both genotype positive for K5tTA and tetop-LA^{G608G}, displayed severe abnormalities including growth retardation, lower weight, hair thinning, skin crusting and dental changes in the incisors. The skin pathology included varying degrees of hyperplasia, hyperkeratosis, hypergranulosis and enlarged and displaced sebaceous glands. Regions with inflammatory cells and fibrosis were present in the dermis. Analysis of the distribution of keratinocytes in dorsal skin sections showed mislocalization of keratin 5 and 6, and the thickening of the spinous and granular layer with normal keratin 10 expression. Increased proliferation was detected using an antibody directed against phospho-histone H3 (PHH3).

In the jaws pathological analysis revealed embedded hairs and regional acute inflammatory reactions in the incisors. The enamel matrix appeared non-mineralized. Enamel is formed in a two-phase process. First ameloblast cells are required for secretion of enamel matrix followed by matrix mineralization (Long and Leininger 1999). The expression of the mutated HGPS protein in the ameloblast cell layer most likely leads to inhibition of enamel matrix secretion and improper enamel mineralization. The abnormal mineralized and softened enamel enables hairs to enter the tooth and cause massive inflammatory reactions in the surrounding tissues.

To suppress the expression of the G608G mutation, we fed the mice doxycycline in drinking water from postnatal week 7 until week 20. Animals were terminated and tissues were collected at the age of 7, 13 and 20 weeks and the tissues from the different time points were analyzed and examined to check for phenotype improvement. That the strong transgenic expression seen in 7 weeks old bitransgenic mice was efficiently turned off after 6 and 13 weeks of doxycycline treatment was demonstrated by RT-PCR and Western blot on skin extractions for RNA and protein. Immunofluorescence staining using a human lamin A/C specific antibody revealed only single positive cells remaining in a few hair follicles, while the expression in the ameloblast layer in the incisors was fully suppressed.

Regrowing hair and healing skin crusting was already evident after one week of doxycycline treatment and the external skin phenotype was completely recovered after 6 weeks of transgenic suppression. The skin pathology was almost completely normal with only a few regions of epidermal hypoplasia, hyperkeratosis, fibrosis and inflammatory reactions. Progressive fibrosis generated permanent fibrotic scars, which were hard to reverse (Wynn 2008) and ongoing and previous wound healing most likely explained the remaining inflammation. In bitransgenic mice the aberrant localization of keratin 5 and 6 normalized with doxycycline treatment. The thickening of the epidermal layers and detectable increased proliferation in bitransgenic mice reversed and was indistinguishable from wild-type littermates.

After 6 weeks of doxycycline treatment the overall teeth phenotype was milder with some remaining embedded hairs and inflammatory regions. After 13 weeks of transgenic suppression the incisors had regenerated with only low-grade inflammation in single areas. The transgenic suppression of progerin led to almost complete functional tooth regeneration in bitransgenic mice.

Upon doxycycline treatment the animals only slightly improved their reduction in body weight, which remained lower compared to wild-type littermates. We reasoned that the suppression of transgenic expression did not have any effect on body weight, since the major growth phase of mice was already past at the time of doxycycline introduction.

In summary this study showed that regenerative tissues including skin and incisors have the potential to reverse an existing phenotype when the expression of progerin was inhibited. The results raise the possibility for the development of future therapies that cure the conditions of the HGPS disease. Additional studies are necessary to investigate if the phenotype reversal is also possible in other tissues affected in HGPS.

4.2 PAPER II

The tet-systems are helpful tools to generate tissue specific mouse models that enable the temporal regulation of the expression of target genes. In this study we wanted to take advantage of a previously published reverse tetracycline regulated transactivator (rtTA) under the control of the collagen type 1 promoter ($\alpha 1p$ -rtTA, Liu *et al.* 2004) to develop a bone specific expression model for the HGPS mutation.

Expression analysis by RT-PCR showed that the expression of $\alpha 1p$ -rtTA transgenic mice was too low to drive the expression of the human lamin minigene, since only scant amplification products could be detected after 35 cycles of PCR. RNA and DNA samples were included to test for genomic DNA contamination and demonstrated that there was no significant DNA contamination. Adequate cDNA samples were included as positive controls for the PCR assay for rtTA, human lamin A and progerin.

On protein level we performed Western Blot on bone protein extracts and immunofluorescence staining on primary osteoblast explant cultures with a human lamin A/C specific antibody. No transgenic expression driven by $\alpha 1p$ -rtTA could be detected on Western films or osteoblast cells for any of the tested

backgrounds. Positive control samples were included from tetop-LA^{G608G+}; Sp7tTA⁺ mice (paper III) and demonstrated the functionality of the experiments.

To prevent possible expression variation or limitations based on genetic mouse background (Dobie *et al.* 1997) we generated congenic mouse lines for the involved transgenic mice. α 1p-rtTA was generated on C57BL6 and backcrossed to FVB/N.Cg-Tg(α 1p-rtTA). Tetop-LA^{G608G} (Sagelius *et al.* 2008) was generated on FVB/N and backcrossed to B6.Cg-Tg(tetop-LA^{G608G}). Tetop-LA^{wt} was generated on FVB/N and backcrossed to B6.Cg-Tg(tetop-LA^{wt}). All breedings were preceded for ten generations and enabled the analysis of transgenic expression on mixed (FVB/N; C57BL6) and two pure genetic backgrounds, FVB/N and C57BL6 respectively. When we intercrossed α 1p-rtTA transgenic mice with tetop-LA^{G608G} transgenic mice, expressing human lamin A and progerin, no phenotype differences were detectable despite the genotype and genetic background of the offspring.

Reasons for the inadequate expression of α 1p-rtTA transgenic mice were not further investigated. Long-term instability dependent on epigenetic modification, unexpected expression pattern, positional- and dosage dependent effects and problems due the usage of small construct sizes have been reported and discussed before by others as explanations for variable efficiencies reported for the tet-system (Furth *et al.* 1994, Böger and Gruss 1999, Fedorov *et al.* 2001, Ryding *et al.* 2001, Robertson *et al.* 2002, Lee *et al.* 2006, Sun *et al.* 2007, Bao-Cutrona and Moral 2009).

In summary, in this study, we reported our negative experience with a previously functional transactivator mice. To share unfortunate research approaches with the scientific community is of importance to raise cautions in the use of established model systems and to reinforce the obligation for controls. To monitor the long-term stability of generated transgenic mouse lines will help to eliminate dysfunctional expression systems and contribute to save valuable resources.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The work included in this thesis demonstrated that the tissue restricted inducible model systems are useful tools to test for the possibility of phenotype reversal after disease manifestation. The expression of progerin in tissues with a high turnover like the skin and the regenerative teeth did not cause incurable damage. Investigation of the recovery potential of tissues and organ systems affected in HGPS gives hope for the development of future treatments of children suffering from progeria.

For the initial approach in the second study we concluded that the previously functional collagen type 1 specific reverse tetracycline-controlled transactivator failed to target the HGPS mutation to the bone. Possible mechanisms leading to the inactivity of the promoter were not further investigated. However we believe that the report of our negative experience is an important contribution to the scientific community. Especially in the context of established model systems that are widely used and commonly shared it is of importance to raise caution and to reinforce the need for controls.

6 ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to the people that made this thesis possible!

Maria Eriksson, my principal supervisor. Thank you for guidance, helpful advices and financial support throughout the years and sharing your broad scientific knowledge with me. You gave me the opportunity to work with these challenging projects, and that is where it all starts!!!

Ola Nilsson, my co-supervisor. Thank you for your support and especially your never-ending enthusiasm for my projects. I am grateful for the introduction to bone biology and your help with the experimental challenges that come with mineralized tissues. I will never forget our exciting x-ray session and the day you showed me how to microdissect bones, while you had a broken arm.

Mikael Holst, my co-supervisor. Thank you for all the discussions at our project meetings. It was of great help to get your scientific advice and you had an excellent timing for positive support. Special thanks also for your flexibility to meet me in various KI locations and parking places.

Karin Dahlman-Wright, chairman, and **Jan-Åke Gustaffson**, former chairman of the Department of Biosciences and Nutrition, for providing excellent research facilities.

Ylva Rosengardten. You shared the entire time of my PhD studies with me and you have been really supportive when things were not so easy. Om ett år eller två...Säkert! Thank you for your positive attitude and your friendly nature that really lifts the atmosphere in the research group.

Sofia Rodríguez. It was so refreshing to have you around in the group with your mouse free projects. Thank you for help and advice with cell culture issues. Beside all the interesting scientific things you made me learn how to use earplugs in the proper way.

Tomás McKenna. Thank you for bringing a little bit more chaos to my life. You are the most impossible colleague to work with and at the same time one of the most fun persons to hang out with when it comes to climbing and hiking.

Hanna Sagelius. I learned so many of the laboratory methods from you, the first PhD student in the ME research group. With your work you set the basis for my projects. For my private life, you are one (if not the) decisive reason, that encouraged me to move my life from Germany to Sweden. You are a wonderful friend and you make others becoming better persons. Stockholm i mitt hjärta och Hanna vid min sida!

Diana Grochová. Thank you for your guidance into the world of qRT-PCR, primer efficiency testing and C_t-values. For me, your scientific passion represents the ultimate researcher. Your stress about your ASHG presentation distracted me from my own nervousness, thanks for that.

I would also like to thank the past group members **Fabio Coppedè** and **Mubashir Hanif** as well as all the students that have been part of the ME research group: **Antje Petzold**, **Caroline Sonnabend**, **Charlotte Westerberg**, **Gaëlle Wambiecke Kiyeko**, **Hasina Nasser**, **Johanna Olsson**, **Léa Maitre**, **Malin Arenås** and **Sarah Hanna**. Thank you for helping with my projects, giving me the chance to test teaching and for contributing to the nice work environment.

Björn Rozell. Thank you for amazing help with the work on the pathological analysis, taking nice microscope pictures, for experimental suggestions, for extensive comments on the manuscripts and your refreshing enthusiasm and interest in the dental aspects of the mice phenotype.

Thanks to all scientific collaborators. **Antti Koskela**, **Claes Ohlsson** and **Juha Tuukkanen** for collaboration in paper III. **Enrico Mugnaini** for collaboration in paper IV. **Adam Glick**, **Andrew McMahon**, **Francis S. Collins**, **Roland Baron** and **Xu Cao** for distributing mice. **Mikael Zmarzlak**, **Moustapha Hassan** and all the staff at the animal facilities for their work and help with the mice. **José Inzunza** for providing reagents for the mouse sperm freezing. **Xiuzeh Wang** for help with mouse perfusion. **Emma Eriksson** for showing me how to use the x-ray machine. **Carin Lundmark** for technical advice and assistance around sectioning and staining procedures. **Annica Rönnbäck** for showing me how to microdissect different brain regions. **Åsa Bergström** for technical consultation and for sharing various reagents.

Thanks to all other people at the Department of Biosciences and Nutrition that helped with equipments and reagents over the past years.

I also like to thank the staff at the administration unit and IT support at the Department of Biosciences and Nutrition. Special thanks to **Monica Ahlberg**, **Marie Franzén**, **Lena Magnell**, **Gunnel Almberger**, **Christina Thulin Andersson**, **Inger Moge** and **Lars Nybom** for always helping with a smile on their faces. Ni är underbara!

Thanks to all members of the Book Club and Journal Club for all the project presentations and scientific discussions.

I like to thank **Jesper Brohede** for being my external mentor and **Joachim Kruppa** and **Oliver Ullrich** for supporting my KID application with their nice reference letters.

I would like to thank the **Progeria Research Foundation** for organizing inspiring meetings. The opportunity to meet children with progeria and their families and listen to their stories was really special and motivating.

Special thanks to **Sarah Earnshaw** for checking the spelling and grammar of the thesis.

Beside my working life at the Karolinska Institutet the last five years have been a wonderful contribution to my private life starting with moving to a new country and culture, learning a new language and meeting a lot of interesting, wonderful persons.

My ladies **Andrea Kögel, Anne-Laure Brault, Daniela Aul, Mona Rödiger** and all members of the **Damklubb**. All of you contributed to making me feel **Stockhome** – thank you!!!!

Patrik Nordström, min allra bästa svenskalärare. Tack kompis för alla timmar som du har lagt ner på min svenska språkutbildning. Jag tycker att du har gjort ett kanonjobb.

Ich danke **Hannelore Tenbergen** und **Sonja Schmidt** für langjährige, reisefreudige, interessierte, liebevolle und treue Freundschaft. Vielen Dank für Gastfreundschaft in der Heimat und ständige Verpflegung mit deutschen Köstlichkeiten und schmerzlich vermissten Produkten.

Meiner Familie gehört mein größter Dank. Auch in der Ferne seid ihr mir nah! Jeder auf seine ganz individuelle Art und Weise. Ich liebe euch!

Meiner Schwester **Claudi** danke ich für ein einmaliges, spezielles, wichtiges, wunderschönes, gemeinsames Weihnachtsfest.

Bei meinem Bruder **Robert** bedanke ich mich für unvergessene Tanzeinlagen und legendäres Pistenvergnügen.

Meiner **Mutter** danke ich für treue Telefonseelsorge, das Wünsche-von-den-Lippen-ablesen bei Heimatbesuchen, hilfreiche Ratschläge und die Freiheit meine eigenen Entscheidungen treffen zu dürfen.

Keiner hat so schön mitgefiebert, mitgelitten, mitfinanziert, sich mit gefreut, sich gesorgt, sich gekümmert, unterstützt, Mut zugesprochen, an mich geglaubt und mich so häufig besucht wie mein **Vater**. Ich werde unsere Urlaube und Erlebnisse, ob zu Fuss, mit dem Fahrrad oder auf Skiern, ob an der Theke, auf dem Tisch, in der Kirche, in den Bergen oder am Strand nie vergessen – **DANKE**.

This work was supported by the Karolinska Institutet KID founding, the Loo and Hans Osterman Foundation, the Torsten and Ragnar Söderberg Foundation, the Swedish Research Council, the Swedish Foundation for Strategic Research, the Tore Nilsson Foundation, the Åke Wiberg Foundation, the Hagelen Foundation, the OE and Edla Johansson Foundation, the Lars Hiertas Minne Foundation and the Karolinska Institutet.

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