Prelamin A Farnesylation and Progeroid Syndromes*

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Hutchinson-Gilford progeria syndrome (HGPS) is caused by a LMNA mutation that leads to the synthesis of a mutant prelamin A that is farnesylated but cannot be further processed to mature lamin A. A more severe progeroid disorder, restrictive dermopathy (RD), is caused by the loss of the prelamin A-processing enzyme, ZMPSTE24. The absence of ZMPSTE24 prevents the endoproteolytic processing of farnesyl-prelamin A to mature lamin A and leads to the accumulation of farnesyl-prelamin A. In both HGPS and RD, the farnesyl-prelamin A is targeted to the nuclear envelope, where it interferes with the integrity of the nuclear envelope and causes misshapen cell nuclei. Recent studies have shown that the frequency of misshapen nuclei can be reduced by treating cells with a farnesyltransferase inhibitor (FTI). Also, administering an FTI to mouse models of HGPS and RD ameliorates the phenotypes of progeria. These studies have prompted interest in testing the efficacy of FTIs in children with HGPS.

Lamin A and Lamin C, Key Structural Proteins of the Nuclear Lamina

Lamins A, C, B1, and B2 are intermediate filament proteins and the major structural proteins of the nuclear lamina, a filamentous meshwork beneath the inner nuclear membrane. Each of the lamins contains an N-terminal globular domain, a central helical rod domain, and a C-terminal globular domain (1). The lamin proteins dimerize to form parallel coiled-coil homodimers, which then associate head-to-tail to form protofilaments that interact laterally to form higher order filaments. Whether the A-type lamins (lamins A and C) form heterodimers with each other or with the B-type lamins (lamins B1 and B2) is not known.

Prelamin A (the precursor to mature lamin A) and lamin C are alternatively spliced products of the same gene, LMNA (2). Prelamin A and lamin C are identical through their first 566 amino acids diverging at the C terminus (2). Prelamin A contains 98 unique amino acids at its C terminus, including a CAAX motif that triggers protein farnesylation. Lamin C contains six unique amino acids at its C terminus; it does not have a CAAX motif and is not farnesylated (2).

Lmna-deficient mice survive development but die by 6–8 weeks of age from muscular dystrophy (3). The mice also exhibit a neuropathy, with reduced axon density and increased numbers of nonmyelinated axons in peripheral nerves (4). The nuclei of Lmna−/− embryonic fibroblasts are grossly misshapen (3). Further studies have revealed that Lmna−/− nuclei have impaired nuclear mechanics and mechanotransduction properties (5, 6). When subjected to prolonged cyclic biaxial strain, Lmna−/− fibroblasts exhibit more necrotic and apoptotic cells than wild-type cells (5, 6).

Lamin A and lamin C are found in roughly equivalent amounts in human and mouse cells. However, the “physiologic rationale” for the two protein isoforms has remained obscure. Several reports have suggested that lamin A plays unique roles in cell biology, such as in the targeting of lamin C and emerin to the nuclear envelope (7, 8). To evaluate the in vivo relevance of the two lamin isoforms, Fong et al. (9) examined lamin C-only mice (LmnaLCO/LCO), which synthesize exclusively lamin C. Remarkably, these mice were healthy and fertile with no discernible disease phenotypes (9). The frequency of misshapen nuclei in LmnaLCO/LCO cells was slightly greater than in wild-type cells (6, 9) but much lower than in Lmna−/− cells. Of note, the targeting of lamin C and emerin to the nuclear envelope in LmnaLCO/LCO cells appeared to be normal (9).

The studies with LmnaLCO/LCO mice indicated that prelamin A and lamin A are dispensable in laboratory mice. Whether lamin C production is equally dispensable remains to be determined. In any case, the apparent dispensability of one or both of the two isoforms does not mean that these conserved proteins lack unique or important functions. Unique roles of the two isoforms will likely emerge with additional study.

Processing of Prelamin A

Prelamin A terminates with a CAAX motif (-CSIM) and undergoes four processing steps (Fig. 1) (10). First, a 15-carbon farnesyl lipid is added to the thiol group of the cysteine by a cytosolic enzyme, protein farnesyltransferase. Second, the last three amino acids of the protein (i.e., the -AAX) are clipped off by either ZMPSTE24 or RCE1, or both (11, 12). Which of these enzymes plays the predominant role for prelamin A has not been established. Mammalian ZMPSTE24 can function as a CAAX endoprotease in yeast (13–16) and in other eukaryotic cell types (17), and the ability of mammalian RCE1 to act as a CAAX endoprotease has been well documented (13, 14, 16, 18). In yeast as well as in mammals, the sequence of the CAAX motif influences substrate specificity for two different endoproteases (14, 16, 19). In yeast, the CSIM motif of prelamin A appears to be a better substrate for Rce1p (20). Third, the newly exposed farnesylcysteine is carboxyl-methylated by ICMT, a prenylprotein-specific methyltransferase of the endoplasmic reticulum (Fig. 1) (21, 22). Fourth, the last 15 amino acids of the protein, including the farnesylcysteine methyl ester, are clipped off by

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ZMPSTE24 and degraded, releasing mature lamin A (Fig. 1) (12, 23, 24).

The enzymatic machinery for converting prelamin A to mature lamin A is so efficient that prelamin A is virtually undetectable in wild-type cells. However, all of the processing can be blocked with a protein farnesyltransferase inhibitor (FTI)3 (24). In the absence of farnesylation, none of the subsequent processing steps occur, and nonfarnesylated prelamin A accumulates in cells (11, 24).

ZMPSTE24 is required for the fourth and final processing step, the release of mature lamin A from farnesyl-pretamin A. In the absence of ZMPSTE24, no mature lamin A is generated and farnesyl-pretamin A accumulates in cells (11, 25). Methylating the farnesylcysteine improves the efficiency of lamin A biogenesis but is not required (26). In the absence of ICMT, some farnesyl-pretamin A accumulates in cells, but the majority is converted to mature lamin A (26).

Why evolution designed and maintained such a complicated system for lamin A biogenesis is not fully understood. However, it is likely that the farnesylcysteine methyl ester helps to target prelamin A to the nuclear envelope (27–29). A large fraction of the farnesyl-pretamin A that accumulates in ZMPSTE24-deficient cells is located at the nuclear rim (25, 30), whereas the majority of nonfarnesylated prelamin A in FTI-treated cells is located in the nucleoplasm (28). Also, when cultured cells are transfected with a cDNA encoding mature lamin A, the lamin A reaches the nuclear rim but less efficiently than when lamin A is produced from prelamin A (27). Thus, it seems likely that the posttranslational processing of prelamin A has evolved to optimize the delivery of lamin A to the nuclear envelope. However, the importance of the posttranslational modifications at the “whole-animal” level remains to be tested. It would be interesting to create mice that express exclusively mature lamin A (rather than prelamin A) and then determine whether the absence of the posttranslational processing has adverse consequences.

Defects in Prelamin A Processing in Progeria

Studies during the past few years have established that several progeroid syndromes in humans are caused by genetic defects that interfere with the processing of farnesyl-pretamin A to mature lamin A. Hutchinson-Gilford progeria syndrome (HGPS) is caused by point mutations in LMNA that activate a cryptic splice donor site in exon 11 (31). The use of the exon 11 splice donor results in the in-frame deletion of the last 50 amino acids encoded by exon 11 (amino acids 607–656) but does not affect the CAAX motif (which is encoded by exon 12). Thus, the mutant prelamin A in HGPS (often called progerin) is farnesylated but lacks the site for the endoproteolytic cleavage reaction that would normally release mature lamin A (Fig. 1). The aberrant splicing in HGPS is incomplete; hence, some wild-type prelamin A is synthesized by the mutant allele (31). Lamin C production is unaffected.

Progerin is targeted to the nuclear rim, where it disrupts the integrity of the nuclear lamina, causing grossly misshapen nuclei (31–34). Children with HGPS appear normal for a few months after birth but then develop failure-to-thrive, loss of subcutaneous fat, prominent scalp veins, stiff joints, and sclerodermatous changes of the skin. Affected children ultimately develop multiple skeletal abnormalities, including micrognathia, osteoporosis, and osteolytic lesions of the clavicle, and death generally occurs from occlusive vascular disease (31).

A more severe progeroid disorder, restrictive dermopathy (RD), is caused by homozygous loss of ZMPSTE24 (35, 36). The absence of ZMPSTE24 abolishes the production of mature lamin A and leads to an accumulation of farnesyl-pretamin A, which accumulates at the nuclear rim and causes misshapen nuclei (35, 36). RD patients exhibit intraterine growth retardation, tight and rigid skin, bone mineralization defects, osteolytic lesions, loss of fat, and generally die within a few weeks after birth (35, 36). In one kindred, the phenotype elicited by homozygous loss of ZMPSTE24 was reduced by a LMNA mutation that truncates prelamin A and prevents protein farnesylation, underscoring the importance of farnesyl-pretamin A accumulation in disease pathogenesis (37). Partial loss of ZMPSTE24 activity has been associated with milder progeroid syndromes (38, 39).

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3 The abbreviations used are: FTI, protein farnesyltransferase inhibitor; HGPS, Hutchinson-Gilford progeria syndrome; RD, restrictive dermopathy.
Mouse Models of Defective Prelamin A Processing

Zmpste24<sup>−/−</sup> mice manifest phenotypes resembling those in humans with progeroid syndromes (11, 25). The nuclei of Zmpste24-deficient mouse fibroblasts are grossly misshapen (25, 30). Also, Zmpste24<sup>−/−</sup> mice appear normal at birth but then grow very slowly and develop micrognathia, osteolytic lesions, and osteoporosis. A hallmark of Zmpste24<sup>−/−</sup> mice is osteolytic lesions in the ribs, which lead to spontaneous rib fractures (11, 30). By ~24 weeks of age, nearly all of the ribs in Zmpste24<sup>−/−</sup> mice are fractured at the costovertebral junction. Zmpste24<sup>−/−</sup> mice also exhibit muscle weakness (11, 25, 30).

Yang and co-workers (34) created a “knock-in” mouse model of HGPS in which the mutant Lmna allele (Lmna<sup>HG</sup>) produced exclusively progerin. As in human HGPS fibroblasts, progerin was targeted to the nuclear rim and caused misshapen nuclei. Lmna<sup>HG/</sup> mice exhibited phenotypes similar to those observed in human HGPS patients and/or Zmpste24<sup>−/−</sup> mice, including retarded growth, reduced amounts of adipose tissue, micrognathia, osteoporosis, osteolytic lesions in bone, and very frequent rib fractures near the costovertebral joints (40). Unlike Zmpste24<sup>−/−</sup> mice, Lmna<sup>HG/</sup> mice do not develop muscle weakness.

Amelioration of Disease Phenotypes by Reducing Prelamin A Production

Fong et al. (30) hypothesized that farnesyl-prelamin A was intrinsically toxic to cells and that its accumulation was responsible for all of the phenotypes associated with Zmpste24 deficiency, including both the misshapen nuclei in cultured cells and the disease phenotypes in vivo. If this were true, they reasoned that these phenotypes might be ameliorated by reducing prelamin A synthesis. To test this idea, they bred Zmpste24<sup>−/−</sup> mice that produced half-normal amounts of prelamin A (i.e. Zmpste24<sup>−/−</sup>/Lmna<sup>+/+</sup> mice). As predicted, the Zmpste24<sup>−/−</sup>/Lmna<sup>+/+</sup> mice were protected both from the misshapen nuclei and all of the disease phenotypes normally associated with Zmpste24 deficiency. These findings have been confirmed by an independent group (41). The elimination of disease phenotypes was clearly because of reduced accumulation of farnesyl-prelamin A (rather than to reduced lamin C expression) because subsequent studies revealed that the disease phenotypes in Zmpste24<sup>−/−</sup> mice could also be eliminated with a single copy of a Lmna<sup>LCO</sup> allele (9).

Critical Role of Protein Farnesylation in Pathogenesis of Disease

In the studies of Zmpste24<sup>−/−</sup>/Lmna<sup>+/+</sup> mice, Fong et al. (30) concluded that prelamin A was toxic and suggested that the farnesylated version of prelamin A might be the real culprit. To explore the idea that farnesylation of prelamin A might be crucial to toxicity, Yang and co-workers (34) created multiple early passage, genetically identical Lmna<sup>HG/</sup> fibroblast cell lines and tested whether blocking protein farnesylation would mislocalize progerin away from the nuclear rim and reduce the percentage of cells with misshapen nuclei. The results were unequivocal: an FTI caused progerin to be targeted to the nucleoplasm and reduced the percentage of cells with misshapen nuclei. Toth et al. (42) extended these studies by showing that an FTI improved nuclear shape in mouse Zmpste24<sup>−/−</sup> fibroblasts, human HGPS fibroblasts, and fibroblasts from a human patient with RD. The laboratories of Drs. Susan Michaelis, Francis Collins, and Thomas Glover reported similar observations (43–45).

In the cell culture studies testing FTIs in Zmpste24-deficient and Lmna<sup>HG/</sup> cells by Yang et al. (34) and Toth et al. (42), the blockade of farnesylation was assessed with Western blots for HDJ-2 and prelamin A. (HDJ-2 is a farnesylated CAAX protein, and the electrophoretic migration of HDJ-2 is retarded when the farnesylation of HDJ-2 is blocked (46, 47).) The Western blots revealed that the FTI had completely blocked the farnesylation of HDJ-2. Also, the FTI caused nonfarnesylated prelamin A to accumulate in wild-type cells; this nonfarnesylated prelamin A was easily detectable on Western blots with a prelamin A-specific antibody and with an antibody against lamin A/C. In the latter studies, nonfarnesylated prelamin A was more abundant than mature lamin A; thus, the FTI resulted in a substantial inhibition of prelamin A processing. Of note, the amount of nonfarnesylated prelamin A plus mature lamin A in the FTI-treated cells was low, relative to the amount of lamin A in untreated cells, suggesting the possibility that nonfarnesylated prelamin A may have reduced stability in the mouse (34, 42). In contrast, FTI treatment of human fibroblasts does not substantially reduce prelamin A/lamin A levels in cells (34, 42).

The favorable effects of the FTIs on nuclear shape in cultured cells raised the issue of whether an FTI might improve disease phenotypes in mouse models of progeria. Fong et al. (48) tested the ability of a potent FTI, ABT-100 (49), to ameliorate disease phenotypes in Zmpste24<sup>−/−</sup> mice. Peak plasma concentrations of the drug were 0.24–0.90 µg/ml, overlapping with levels achieved in experiments in which ABT-100 was shown to block the growth of human tumor xenografts (49). The FTI significantly improved the body weight curves of both male and female Zmpste24<sup>−/−</sup> mice (p < 0.0001). In contrast, treatment of wild-type mice with the FTI reduced body weight. The FTI treatment also improved the survival of Zmpste24<sup>−/−</sup> mice. Moreover, many of the FTI-treated Zmpste24<sup>−/−</sup> mice appeared robust at 5 months of age, whereas most of the vehicle-treated mice were inactive and nearly moribund. The median number of rib fractures was much lower in FTI-treated Zmpste24<sup>−/−</sup> mice than in vehicle-treated controls (p = 0.0002). The FTI treatment also significantly improved muscle strength in Zmpste24<sup>−/−</sup> mice.

In these studies, the FTI affected protein farnesylation in vivo, as judged by Western blots, but the levels of inhibition were not nearly as high as those observed in the cultured cells (34, 42). In the FTI-treated Zmpste24<sup>−/−</sup> mice, only ~20–50% of the HDJ-2 in tail extracts was nonfarnesylated. The FTI resulted in the appearance of prelamin A in the tail extracts from wild-type mice, as judged by Western blots with the prelamin A-specific antibody (48). However, when Western blots were performed with antibodies against lamin A/C, prelamin A was virtually undetectable, and the levels of mature lamin A were not appreciably diminished. Thus, in contrast to the cell

4 L. Fong, unpublished observations.
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culture studies, the amount of inhibition of prelamin A farnesylation in vivo was small, probably less than 5% of the total.

Treatment with an FTI also improved disease phenotypes in LmnaHG/+ mice (40). The FTI improved body weight curves in both female and male LmnaHG/+ mice (p < 0.0001). In addition, the FTI increased the weight of the major fat pads in LmnaHG/+ mice (p = 0.002) and significantly reduced the number of rib fractures (p < 0.0001). FTI treatment of LmnaHG/+ mice also led to an improvement in bone mineralization and cortical thickness (40).

In the studies on LmnaHG/+ mice, more than half of the HDJ-2 in the livers of FTI-treated mice was nonfarnesylated (40). Also, the FTI resulted in the appearance of nonfarnesylated prelamin A (40). Once again, however, the amount of nonfarnesylated prelamin A in cells was small, as judged by lamin A/C Western blots (40).

The FTI treatment did not completely cure the Zmpste24–/– mice or the LmnaHG/+ mice. The FTI-treated mice still had an abnormal growth curve; some still developed rib fractures; and some still manifested abnormal muscle strength (in the case of the Zmpste24–/– mice). It is unclear whether higher doses of an FTI (with more complete inhibition of protein farnesylation) would provide further benefit.

Important Issues Surrounding Use of FTIs for HGPS

In the Zmpste24–/– and LmnaHG/+ mice, the FTI ameliorated disease phenotypes with only a small effect on lamin A biogenesis, at least as judged by lamin A/C Western blots (i.e. no apparent reduction in mature lamin A and only a very small amount of prelamin A) (40). One potential explanation is that the small reduction in prelamin A farnesylation was entirely sufficient to reduce disease phenotypes. Another interpretation is that FTIs simply strengthen the bones in the progeria mice by some unrelated mechanism (i.e. involving another farnesylated protein), leading to a global improvement in the health of the mice. Finding out whether FTIs actually improve disease directly by interfering with prelamin A farnesylation is an important issue. It would be interesting to determine whether an FTI would improve disease phenotypes in mice expressing a geranylgeranylated form of progerin. If they had no effect in those mice, one could conclude that the favorable effects of the FTIs in the recent studies were because of the inhibition of prelamin A farnesylation.

Another issue is that FTI treatment leaves HGPS cells with a pair of structurally abnormal lamin proteins, nonfarnesylated progerin and nonfarnesylated prelamin A. At this point, there is every reason to believe that nonfarnesylated progerin is less toxic than farnesylated progerin. However, it is sobering to remember that missense mutations in the C-terminal domain of prelamin A cause disease (51,52), so it is conceivable that the nonfarnesylated proteins could have their own toxicities over the long term. One way to determine whether nonfarnesylated progerin has long term toxic effects is to generate and then analyze mice expressing a mutant form of progerin that cannot undergo farnesylation.

Another issue is whether progerin might undergo alternate prenylation in the setting of an FTI. Other CAAX proteins that terminate in methionine are alternately prenylated by geranylgeranyltransferase type I in the setting of an FTI (53). It is conceivable that the same thing could happen with prelamin A or progerin (54), although there is little evidence so far that this is the case (42,44).

A significant issue in the progeria field is the molecular mechanisms underlying disease and figuring out whether these mechanisms are altered by an FTI. There have been several efforts to define gene expression perturbations associated with progerin expression (33,55), but few of these studies have been replicated in multiple cell lines, in multiple laboratories, or with mouse models where the genetic background issues can be controlled. No one has identified a molecular marker of disease that is normalized by FTI treatment.

Over the past few years, there have been reports that progerin and/or prelamin A leads to defective DNA repair and genome instability (56), overexpression of p53 target genes (41), and changes in histone methylation that affect heterochromatin organization (57). Also, progerin has been shown to affect the mechanical stability of the nucleus (58), and a recent study has indicated that progerin interferes with lamin A and lamin B1 homopolymer formation and results in the production of structurally abnormal progerin-containing heteropolymers (50). In the future, it will be important to define the extent to which these abnormalities are affected by an FTI.

Finally, it will be important to determine whether FTIs will be a useful therapy in children with progeria. A trial of FTIs in human patients with HGPS should begin soon (www.progeriaresearch.org). The outcome of the mouse studies (40,48) provides a reason for optimism, although it is worth pointing out that the mouse studies simply showed that an FTI could prevent the emergence of disease, not that an FTI could reverse or ameliorate advanced disease. Patients afflicted with HGPS will be hoping for reversal of existing disease phenotypes.

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