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ADDENDUM

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Pelger-Huët anomaly and Greenberg skeletal dysplasia: LBR-associated diseases of cholesterol metabolism

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ABSTRACT

Lamin B Receptor (LBR) is an inner nuclear membrane protein associated with the rare human diseases Pelger-Huët anomaly and Greenberg skeletal dysplasia. A new study has used CRISPR/Cas9-mediated genetic manipulations in a human cell system to determine that the molecular etiology of these previously poorly understood disorders is a defect in cholesterol synthesis due to loss of LBR-associated sterol C14 reductase activity. The study furthermore determined that disease-associated LBR point mutations reduce sterol C14 reductase activity by decreasing the affinity of LBR for the reducing agent NADPH. Moreover, two disease-associated LBR truncation mutants were found to be highly unstable at the protein level and are rapidly turned over by a novel nuclear membrane-based protein quality control pathway. Thus, truncated LBR variants can now be used as model substrates for further investigations of nuclear protein quality control to uncover possible implications for other disease-associated nuclear envelopathies.

Introduction

Lamin B Receptor (LBR) is a bi-functional polytopic membrane protein of the inner nuclear membrane (INM).[1,2] The N-terminus of LBR resides inside the nucleus and directly interacts with both chromatin and the nuclear lamina, the meshwork of intermediate filaments that underlies and supports the inner nuclear membrane (Fig. 1).[2-5] This is the function for which the protein is named, but LBR has a second, seemingly unrelated role in sterol metabolism as the C-terminal, multi-pass transmembrane domain of the protein is a functional C14 sterol reductase.[6,7] This second function of LBR has until now been somewhat puzzling as human cells contain another C14 sterol reductase called TM7SF2 or DHCR14 which, like most other enzymes in the cellular sterol biogenesis pathway, resides in the ER membranes and is responsive to the sterol regulatory element-binding protein (SREBP) pathway.[8-10] LBR, conversely, is constitutively expressed, unresponsive to the SREBP pathway, and resides instead at the INM.[8,11] Thus it has long been assumed that TM7SF2/DHCR14 is the primary cellular C14 sterol reductase while LBR provides at most redundant or cell type/developmental stage specific sterol biogenesis activity. However, a recent study by Tsai *et al.* has demonstrated not only that the sterol reductase activity of LBR is strictly required for viability under sterol-restrictive growth conditions in several human cell types, but also that two rare human diseases associated with mutations in LBR, Pelger-Huët anomaly and Greenberg skeletal dysplasia, are caused by the loss of LBR-based sterol reductase activity.[11]

Lamin B receptor and human disease

Mutations to proteins of the nuclear lamina are known to cause a wide spectrum of human genetic diseases with phenotypes including muscular dystrophy, lipodystrophy, cardiomyopathy and the premature aging disorder Hutchinson-Gilford progeria syndrome.[12-15] Collectively, these disorders are known as nuclear laminopathies, but in most cases the molecular pathways by which particular laminopathic mutations manifest as specific disease phenotypes
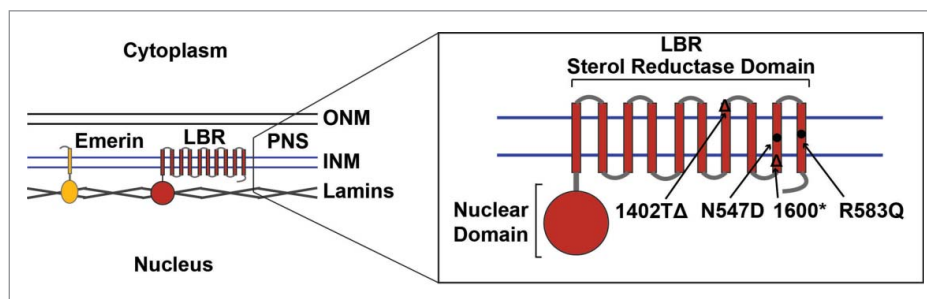
Figure 1. Localization and topology of LBR and Emerin at the inner nuclear membrane. Disease-associated LBR point mutations N547D and R583Q are indicated by filled circles and frameshift mutations by a Δ symbol at the point of truncation. (INM) inner nuclear membrane, (ONM) outer nuclear membrane, (PNS) perinuclear space.

remain to be determined. Two known human diseases are associated with mutations in LBR: Pelger-Huët anomaly and Greenberg skeletal dysplasia (Table 1).[16-19] Pelger-Huët anomaly is a relatively benign autosomal dominant disorder characterized by hypolobulation of the normally hyperlobulated nuclei of granulocytes, a type of white blood cell.[19] Although studies of cells derived from the partial LBR knockout *ic/ic* mouse model have demonstrated a significant defect in the growth and derivation of myeloid progenitors, the multipotent cells from which all bone marrow-based blood cells are derived,[20] individuals displaying Pelger-Huët anomaly in granulocytes are otherwise asymptomatic. Conversely, Greenberg skeletal dysplasia (also known as hydrops-ectopic calcification-moth-eaten or HEM skeletal dysplasia) is a perinatally lethal autosomal recessive condition characterized by highly abnormal bone development (with abnormal calcification and a "moth eaten"

appearance) and excessive fluid accumulation (fetal hydrops).[18,21-25] Interestingly, some LBR mutations found in the heterozygous state in individuals displaying Pelger-Huët anomaly have also been found in HEM/Greenberg dysplasia fetuses, and Pelger-Huët anomaly has been observed in Greenberg skeletal dysplasia parents, suggesting that at least in some cases, the two disorders represent different allelic states of the same genetic lesion, rather than distinct and unrelated genetic diseases.[18,21,26] As LBR is a bi-functional protein, with both nuclear lamina/chromatin binding and C14 sterol reductase activities, there has been some debate as to whether these disorders should be classified as nuclear laminopathies or as diseases of cholesterol metabolism.[22,27] Notably, elevated levels of the direct upstream cholesterol intermediate acted upon by the C14 sterol reductases LBR and TM7SF2 were detected in a Greenberg dysplasia fetus,[22] raising the question of why a deficiency in LBR is not

Table 1. Congenital diseases associated with Lamin B receptor.

| LBR Mutation (gene)	LBR Mutation (protein)	Type	Phenotype (heterozygous)	Phenotype (homozygous)	Reference
c.1639A>G	p.N547D	point mutation	Unknown	Greenberg Dysplasia	Clayton et al [26] Konstantinidou et al [18]Tsai et al[11]
c.1748G>A	p.R583Q	point mutation	No Phenotype	Greenberg Dysplasia	Clayton et al[26] Tsai et al[11]
c.1402delT	p.Y468TfsX475	frameshift	Unknown	Greenberg Dysplasia	Clayton et al [26]Tsai et al[11]
c.1599-1605 TCTTC TA®CTAGAAG	p.X534	nonsense	Pelger-Huët Anomaly	Greenberg Dysplasia	Waterham et al[22]Tsai et al[11]
c.32delTGGT	p.V11EfsX24	frameshift	Pelger-Huët Anomaly	Greenberg Dysplasia	Clayton et al[26]
c.355C>T	p.P119L	point mutation	Pelger-Huët Anomaly	Unknown	Best et al[19]
c.1706C>G	p.P569R	point mutation	Pelger-Huët Anomaly	Unknown	Best et al[19]
c.1308G>A	p.X436	nonsense	Pelger-Huët Anomaly	Unknown	Hoffman et al[16]
c.1173del	p.G382DfsX393	frameshift	Pelger-Huët Anomaly	Unknown	Hoffman et al[16]
c.1129C>T	p.X377	nonsense	Pelger-Huët Anomaly	Unknown	Hoffman et al[16]
c.500G>C; 501-504 delCCTT	p.S167TfsX176	frameshift	Pelger-Huët Anomaly	Unknown	Hoffman et al[16]
IVS11-9A>G		splice acceptor	Pelger-Huët Anomaly	Unknown	Best et al[19]
IVS2-2A>G		splice acceptor	Pelger-Huët Anomaly	Unknown	Hoffman et al[19]
IVS12-5-10del		splice acceptor	Pelger-Huët Anomaly	Unknown	Hoffman et al[19]
IVS11+1G>A		splice donor	Pelger-Huët Anomaly	Unknown	Hoffman et al[19]
IVS13-2A>G		splice acceptor	Pelger-Huët Anomaly	Unknown	Hoffman et al[19]
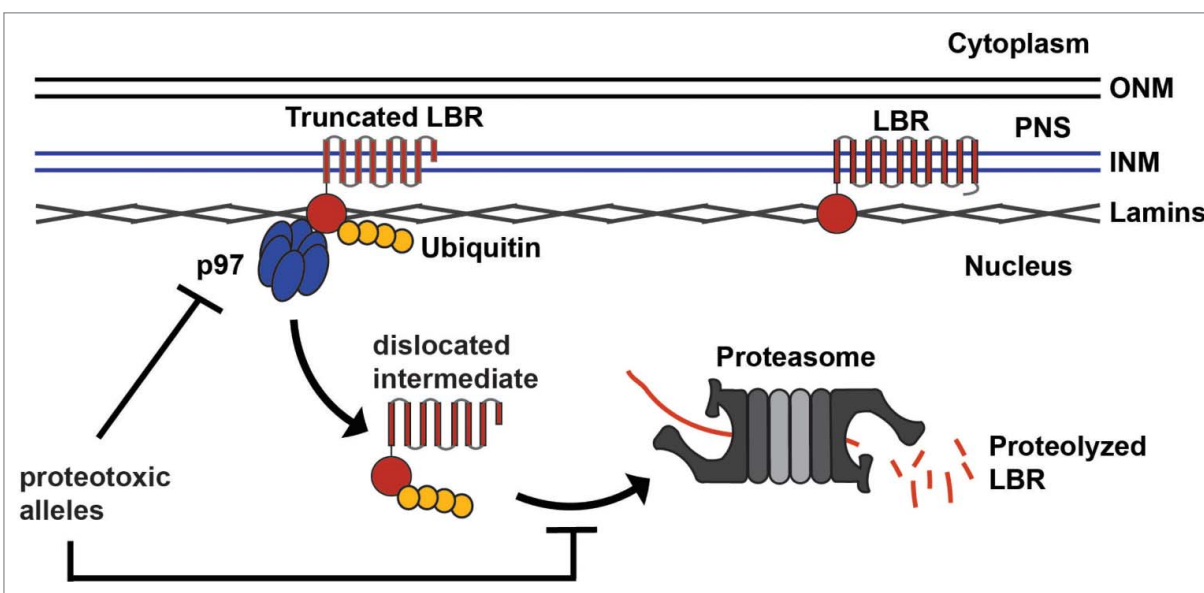
Figure 2. Emerging model of nuclear membrane-associated protein quality control. Truncated LBR is trafficked to the inner nuclear membrane, ubiquitylated, and dislocated into the nucleoplasm via a p97-dependent mechanism (note that p97 cofactors were omitted for clarity). Dislocated LBR is then degraded by nuclear proteasomes. Laminopathy-associated alleles may exert proteotoxic effects on nuclear protein quality control by competing for limited components of the degradation machinery or by inhibiting the pathway at the stage of ubiquitylation, INM dislocation, or proteasomal degradation. (INM) inner nuclear membrane, (ONM) outer nuclear membrane, (PNS) perinuclear space.

compensated for by the putative primary C14 sterol reductase TM7SF2, in which no defect was detected. Thus the molecular etiology of these related disorders has until now remained unclear.

Pelger-Huët anomaly and Greenberg skeletal dysplasia are loss-of-function diseases of cholesterol metabolism

Using a combination of CRISPR/Cas9 mediated knockout and Flp/Frt recombination-based gene integration, we have established a system for conducting classical genetics in a human cell system and revealed the molecular basis of the rare human diseases Pelger-Huët anomaly and Greenberg skeletal dysplasia.[11] We determined that LBR is required for cell viability under sterol-restrictive growth conditions in 3 different human cell lines and directly detected a decrease in cellular cholesterol content in LBR knockout cells, challenging the assumption that the C14 sterol reductase TM7SF2/DHCR14 is a redundant enzymatic activity. Interestingly, we also found no defect or change in the structure of the nuclear lamina in an LBR knockout cell line, arguing against a primarily structural role of LBR at the INM, albeit only in a tissue culture cell system.

Furthermore, we employed Flp/Frt based recombination[28] to re-introduce both wild-type and disease-associated LBR alleles into an LBR knockout cell background, achieving moderate protein expression under doxycycline control and allowing for a careful analysis of both genotype and phenotype. Using this system, we determined that re-integration of wild-type LBR fully rescues the observed cholesterol auxotrophy phenotype, but that 4 separate LBR alleles associated with Pelger-Huët anomaly and Greenberg skeletal dysplasia (Table 1)(Fig. 1) do not.

Further investigation of these four disease-associated LBR alleles revealed two separate and distinct mechanisms by which LBR mutation leads to a defect in cholesterol production. By comparison to a published structure of a related bacterial sterol C14 reductase,[29] we found that the LBR point mutations LBR N547D and LBR R583Q map to the NADPH binding pocket. As NADPH is the essential cofactor used by C14 sterol reductases to convert a carbon-carbon double bond in the cholesterol intermediate 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol to a single bond, any mutation which decreases the binding affinity of the enzyme for NADPH is expected to significantly decrease its reductive capacity. Indeed, we found that LBR R583Q has an approximately 7-fold decrease in

affinity for NADPH compared to wild-type LBR, while LBR N547D showed an almost 12-fold decrease in NADPH binding capacity, resulting in a near-complete loss of *de novo* cholesterol synthesis.[11]

Conversely, the two LBR truncation mutants, both of which result in the omission of several transmembrane helices from the C-terminus of the protein (Fig. 1), are highly unstable and are rapidly turned over by cellular protein quality control machinery, resulting in an extremely low steady-state protein expression level. Remarkably, it appears that truncated LBR is degraded by a mechanism that resembles the traditional ER-associated protein degradation (ERAD) pathway in that LBR ubiquitylation, a functional proteasome, and a functional p97 motor are required[30,31] (Fig. 2). However, unlike in the canonical ERAD pathway, which has until now only been found in the ER membranes, truncated LBR is first trafficked to the nuclear membranes before being dislocated directly into the nucleoplasm, where it is presumably degraded by nuclear proteasomes. This is, to our knowledge, the first instance in which an INM-based ERAD-like pathway has been observed in higher eukaryotes, and represents a second molecular mechanism by which LBR-associated disease mutations result in a loss of sterol C14 reductase functionality.

Nuclear protein quality control: Implications for other laminopathies

Having established a model system for the investigation of protein turnover at the INM, other human diseases resulting from mutations in proteins of the nuclear envelope or lamina[32-34] can now be examined from the perspective of protein quality control. We have previously proposed that some of these mutations may act as dominant-negative disease alleles, for example by exerting a proteotoxic effect causing aberrant protein accumulation.[35] Since suitable readouts for monitoring the turnover of INM proteins are now firmly established,[11] future studies could now determine if the molecular pathology of other laminopathy-associated alleles is related to a dominant negative overburdening or "poisoning" of the nuclear protein quality system, which would impede the otherwise timely removal of unrelated defective or otherwise undesirable proteins from the nucleoplasm or nuclear membranes (Fig. 2). This putative proteotoxic effect may be analogous to established proteotoxic mechanisms found in neurodegenerative diseases.[36,37] It is also tempting to speculate that INM-associated protein turnover machinery plays an important role beyond protein quality control, for example in regulatory proteolysis, as exemplified by INM-resident ubiquitin ligases in yeast which are involved in the regulation of sterol synthesis.[38,39] Additionally, it will be interesting to test whether human disease mutations which result in reduced levels of INM proteins can be explained by their degradation in the nuclear compartment, as was observed for truncated LBR.[11] In conclusion, a better understanding of the nuclear quality control network and its relationship to nuclear envelopathies and laminopathies is urgently required to facilitate the development of novel therapeutic avenues for the treatment of this collection of human genetic diseases.

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