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# Elevated autophagy and mitochondrial dysfunction in the Smith–Lemli–Opitz Syndrome

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ABSTRACT

Smith–Lemli–Opitz Syndrome (SLOS) is a congenital, autosomal recessive metabolic and developmental disorder caused by mutations in the enzyme which catalyzes the reduction of 7-dehydrocholesterol (7DHC) to cholesterol. Herein we show that dermal fibroblasts obtained from SLOS children display increased basal levels of LC3B-II, the hallmark protein signifying increased autophagy. The elevated LC3B-II is accompanied by increased beclin-1 and cellular autophagosome content. We also show that the LC3B-II concentration in SLOS cells is directly proportional to the cellular concentration of 7DHC, suggesting that the increased autophagy is caused by 7DHC accumulation secondary to defective DHCR7. Further, the increased basal LC3B-II levels were decreased significantly by pretreating the cells with antioxidants implicating a role for oxidative stress in elevating autophagy in SLOS cells. Considering the possible source of oxidative stress, we examined mitochondrial function in the SLOS cells using JC-1 assay and found significant mitochondrial dysfunction compared to mitochondria in control cells. In addition, the levels of PINK1 which targets dysfunctional mitochondria for removal by the autophagic pathway are elevated in SLOS cells, consistent with mitochondrial dysfunction as a stimulant of mitophagy in SLOS. This suggests that the increase in autophagic activity may be protective, i.e., to remove dysfunctional mitochondria. Taken together, these studies are consistent with a role for mitochondrial dysfunction leading to increased autophagy in SLOS pathophysiology.



## 1. Introduction

Smith–Lemli-Opitz Syndrome (SLOS) is an autosomal recessive disorder of cholesterol biosynthesis caused by mutations in the gene that encodes 3β-hydroxysterol-$\Delta^7$-reductase (DHCR7), the final enzyme in the cholesterol biosynthetic pathway. Affected individuals typically exhibit multiple anatomic malformations and intellectual disability, though the phenotypic expression of this condition is extremely variable. The clinical features of SLOS are thought to be primarily related to cholesterol deficiency and/or accumulation of cholesterol precursors and their metabolites. The primary metabolite that accumulates in SLOS is the immediate precursor to cholesterol in the Kandutsch–Russell cholesterol synthesis pathway, 7-dehydrocholesterol (7-DHC) [1,2]. 7-DHC contains a double bond at carbon seven, which is reduced by DHCR7 to form unesterified cholesterol, but is otherwise structurally identical to cholesterol (Fig. 1). Tint et al. [2] first described the biochemical defect in SLOS patients by virtue of the accumulation of 7-DHC in the plasma of affected individuals [2]. This finding has become diagnostic for SLOS and has led to the detailed description of a large variety of *DHCR7* mutations with over 154 mutations reported to date which include 130 missense, 8 nonsense, 8 deletions, 2 insertions, 1 indel, and 5 splice site mutations [3] and which may explain the large phenotypic variation observed for this disorder [4,5]. In contrast with the genetics of SLOS, relatively little work has been done to address the cell biology of this debilitating disease. The discovery that 7-DHC accumulation might participate in the pathogenesis of SLOS stems from the early work of Honda et al. [6] who demonstrated that 7-DHC accumulates in skin fibroblasts cultured from patients with SLOS. This observation was confirmed by

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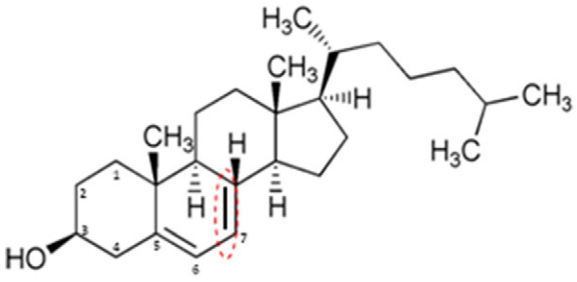
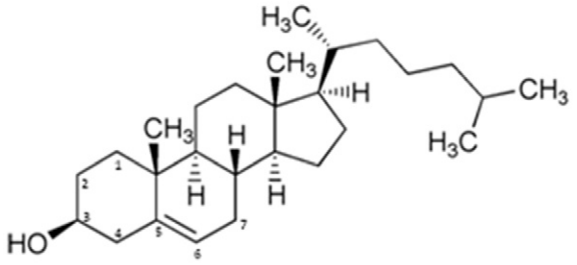



7-dehydrocholesterol




Cholesterol

**Fig. 1.** Chemical structures of cholesterol and 7-dehydrocholesterol (7DHC). Note that the only difference between the two molecules is the presence of a double bond between carbons 7 and 8 in 7-DHC.

Wassif et al. [7] and extended by us in studies demonstrating that cell membranes from SLOS fibroblasts contain 7-DHC which alters membrane structure/function [8,9].

Autophagy is an ancient cellular degradation pathway for long-lived and excess proteins, lipids, nucleotides, etc., along with unneeded or damaged cellular organelles including mitochondria, peroxisomes and endoplasmic reticulum. The autophagosomes are formed from a double membrane precursor phagophore and delivers their cargo to lysosomes by fusion where they are degraded to biologically active monomers, e.g., amino acids for cellular recycling [10]. In this way, autophagy continually "refreshes" the cytoplasm and thus plays a homeostatic role which is particularly important in terminally differentiated cells like neurons. A well defined set of autophagy-related genes (ATG 1–35) are required for autophagy and its related processes which are highly conserved among eukaryotes, and numerous studies have revealed a variety of physiologic roles of autophagy [11]. Autophagic activity has both selective and nonselective features which vary by how substrate cargo is delivered to the lysosome. For example, the chaperone-mediated class of autophagy is highly selective targeting proteins containing a KFERQ motif while the microautophagy class is largely nonselective and involves continuous degradation of cytosolic materials close to lysosomes by inward budding of the lysosomal membrane. Lastly, macroautophagy, the most widely studied autophagy class, can be largely selective specifically targeting defective proteins and organelles for engulfment into the phagophore which fuses with lysosomes for cargo degradation. However, microautophagy can also be nonselective, for example during nutrient starvation whereby autophagosomes envelop random cytosolic proteins and organelles for lysosomal degradation to re-supply the cell with essential amino acids and carbohydrates for protein, energy and neosynthesis. An important function of microautophagy is cargo-specific and responsible for the clearance of defective organelles and its specificity has been delineated along functional lines. Hence, "mitophagy" clears dysfunctional mitochondria, "pexophagy" clears peroxisomes, "xenophagy" clears invading bacteria, etc. In this way, selective autophagy serves as an important and essential cellular quality control preserving the steady-state content of functional organelles thereby maintaining a healthy cytosolic milieu. Importantly, while autophagy was initially thought to be a pro-survival mechanism, it is now appreciated that defective autophagy contributes to cellular pathology, notably in cancer, neurodegenerative diseases and defective immunity [10].

A recent report from our lab showed impairment of IP3 synthesis in SLOS cells [9]. Because impaired IP3 synthesis activates autophagy [12,13], we set out to determine whether SLOS cells might demonstrate enhanced autophagy. Using skin fibroblasts obtained from children with confirmed diagnosis of SLOS, we found that numerous markers of autophagy are elevated under basal conditions including the hallmark protein of autophagy LC3B-II as well beclin-1 and increased cytoplasmic autophagosome inclusions. Taken together, we present for the first time data supporting the notion that autophagy is enhanced in SLOS cells, and that dysfunctional mitochondria likely stimulate mitophagy in SLOS cells. However, a defect in autophagic flux may also exist in SLOS cells thereby preventing the efficient clearance of defective mitochondria contributing further to cytopathology in children with SLOS.

## 2. Methods

Beclin-1 and LC3B rabbit polyclonal antibodies were from Cell Signaling (Danvers, MA). Actin mouse monoclonal antibody was from Chemicon (Billerica, MA). AY9944 (trans-1,4 bis-(2-dichlorobenzylaminomethyl)cyclohexane dihydrochloride) (1 μM as a working solution), N-acetylcysteine (NAC) and monodansylcadaverine (MDC) were from Sigma (St. Louis, MO). Lipoprotein-deficient serum was from Cocalico Biologicals, Inc. (Reamstown, PA). LC3-GFP was from Addgene and transfast transfection kits were from Promega. Lipofectamine 2000 and the antioxidant cocktail supplement B27 (50× stock solution) were from Invitrogen (Grand Island, NY. JC-1 Mitochondrial Membrane Potential Assay Kit was from Cayman Chemical Company (Ann Arbor, MI).

### 2.1. Cell lines and culture

All fibroblast cell lines were obtained from our pediatric collection at OHSU (RDS PI). SLOS fibroblast cell lines were established from skin biopsy specimens obtained from SLOS patients with confirmed biochemical and genetic diagnosis. Table 1 lists the specific genotypes in fibroblasts isolated from children with SLOS. Control fibroblast cell lines were established from skin specimens obtained from healthy age-matched individuals during reconstructive surgery. The OHSU Institutional Review Board approved this study and a written consent to use

**Table 1**
Patient genotypes. For each patient a clinical severity score was calculated using the published anatomical measure of severity [50,51].

| Patient | Clinical severity score | Nucleotide | Amino acid | Nucleotide |
|---|---|---|---|---|
| 1 | 17 | c.529T>C | p.Trp177Arg | c.724C>T |
| 2 | 11 | c.906C>G | p.Phe302Leu | c.1409T>A |
| 3 | 44 | c.278C>T | p.Thr93Met | c.964-1G>C |
| 4 | 17 | c.461C>T | p.Thr154Met | c.292C>T |
| 5 | 30 | c.1384T>C | p.Tyr462His | c.964-1G>C |
| 6 | 20 | c.278C>T | p.Thr93Met | c.976G>T |
| 7 | 10 | c.1349G>T | p.Arg450Leu | c.964-1G>C |
| 8 | 11 | c.470C>T | p.Ala247Val | c.964-1G>C |
| 9 | 33 | c.1228G>A | P.Gly410Ser | Same mutation |
| 10 | 5 | c.1139G>A | p.Cys380Tyr | c.964-1G>C |