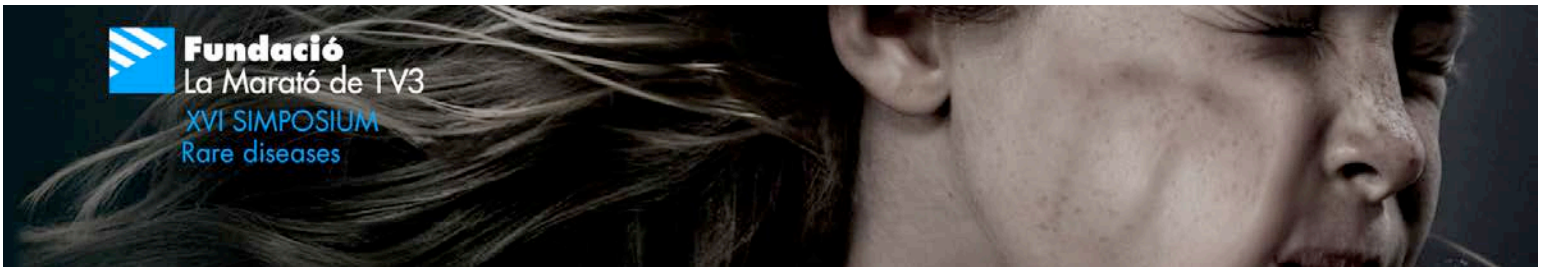

Does Ret signaling play an important role in Smith-Lemli-Opitz syndrome?

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1. Summary

Smith–Lemli–Opitz syndrome (SLOS; OMIM 270400, ORPHA818) is an autosomal recessive, multiple congenital malformation syndrome with mental retardation, with an incidence of approximately 1/50,000 live births. SLOS presents with a very heterogeneous phenotype, causing perinatal death in its most severe forms. In general, children affected by SLOS show growth retardation, developmental delay and intellectual disability. Craniofacial defects include microcephaly, micrognathia, and ptosis. Individuals affected by SLOS often display postaxial polydactyly of the hands or feet, and syndactyly of the 2nd and 3rd toes. Internal malformations include renal hypoplasia, ambiguous genitalia in male, pyloric stenosis, colonic aganglionosis (Hirschsprung's disease), holoprosencephaly and cardiac malformations. SLOS patients are hyperactive, display self-injurious behavior, and demonstrate autistic characteristics.

SLOS is caused by inherited mutations of the hydroxysterol-D7 reductase gene (DHCR7). DHCR7 gene product catalyzes the last step of cholesterol biosynthesis, namely conversion of 7-dehydrocholesterol (7DHC) into cholesterol. As a consequence, DHCR7 deficiency impairs cholesterol production, resulting in elevated 7DHC levels and typically decreased cholesterol levels. The molecular mechanisms underlying SLOS pathogenesis are uncertain. Cholesterol is a major lipid component of cellular membranes, and constitutes the precursor of steroid hormones and bile acids, among others. In addition, cholesterol is a critical component of plasma membrane microdomains known as lipid rafts.

Altered sonic hedgehog (SHH) signaling may explain some SLOS features. SHH is a morphogen involved in the patterning of many systems, including limbs, craniofacial structures, brain and spinal cord. Mutations of SHH in humans cause holoprosencephaly, or failure of forebrain to split into two hemispheres, a brain

malformation also found in some SLOS patients. On the other hand, it has been demonstrated that the altered sterol composition in SLOS affects the physiochemical properties and function of cellular membranes. More specifically, substitution of 7DHC for cholesterol alters both lipid raft stability and protein composition. SLOS membranes have altered membrane fluidity and synthetic membranes containing 7DHC studied have atypical membrane organization. These physical perturbations of membrane structure likely underlie functional defects in IgE receptor-mediated mast cell degranulation and cytokine production, NMDA receptor function and serotonin1A receptor ligand binding. In any case, given the multiplicity of functions of cholesterol, it is unlikely that a single pathological mechanism underlies the varied malformations and clinical problems found in these patients. Moreover, not only lack of cholesterol but toxic effects of 7DHC might account for the phenotype of SLOS patients.

The GDNF family ligands (GFLs) constitute a group of neurotrophic factors (GDNF, NRTN, PSPN, ARTN) that regulate several aspects of the developing and injured nervous system. Outside the nervous system, these factors play a crucial role in kidney morphogenesis and maturation of the spermatids. GFLs signal through a common tyrosine kinase receptor (Ret), and one of the four coreceptors known as GFRalpha, which bind directly to GFLs providing ligand specificity: thus, GDNF binds preferentially to GFRα1, NRTN to GFRα2, ARTN to GFRα3 and PSPN to GFRα4. GFRα coreceptors are attached to the outer leaflet of the plasma membrane via a glycosyl-phosphatidyl inositol anchor, which targets them to lipid rafts. Stimulation by GFLs prompts recruitment of Ret to lipid rafts, where the GFL-GFRα-Ret ternary complex interacts with Src family kinases to elicit maximal biological responses. Interestingly, stimulation of Ret by soluble GFRα also drives Ret to lipid rafts where it interacts with FRS2. Finally, partitioning of Ret to lipid rafts not only promotes association with key signaling intermediates, but prevents its proteasomal-mediated degradation. In conclusion, lipid rafts integrity is critical for optimal Ret signaling, and their disruption results in severely decreased downstream signaling and bioactivity.

In vivo, Ret signaling is necessary for the proper development of the autonomic nervous system and the genitourinary system. The enteric nervous system is formed when vagal neural crest cells migrate to colonize the gut in response to Ret signaling. Mice lacking Ret have no enteric neurons distal to the stomach, whereas some Ret point mutations cause different degrees of colonic aganglionosis, thus mimicking Hirschsprung's disease in humans. Sympathetic neuron precursors migrate to their final position and extend axons guided by a gradient of ARTN expressed by blood vessels. Mice lacking either ARTN or its GFRα3 develop ptosis owing to failure of sympathetic neurons from the cervical superior ganglion to innervate the tarsus muscle, thus causing dropping of the eyelid. Finally, Ret signaling is crucial to kidney development. Development of the metanephric kidney begins when an outgrowth of the Wolffian Duct, the ureteric bud (UB) invades the surrounding metanephric mesenchyme and branches repeatedly to ultimately form the collecting system of the kidney. The UB, which expresses Ret, evaginates and branches in response of GDNF secreted by the metanephric mesenchyme. Both GDNF and Ret knockout mice are born without kidneys owing to the failure of UB to grow out from the Wolffian Duct. In the present proposal we aimed to ascertain whether congenital abnormalities found both in SLOS and Ret mutant mice (ptosis, kidney malformations and Hirschsprung's disease) are due to suboptimal or aberrant Ret signaling owing to failure of 7-DHC to correctly form lipid rafts. Our data show that Ret signaling is largely normal in a mouse model of SLOS.

2. Results

Urinary and enteric nervous systems are largely normal in newborn DHCR7 knockout mice

Although no major defects in kidney and digestive system were described in DHCR7 knockout mice, we sought to investigate whether subtle abnormalities reflecting

compromised Ret signaling could be identified in these mice. Macroscopic examination of the genitourinary system of newborn mutant mice revealed no overt malformations consistent with aberrant Ret signaling such as kidney aplasia or hypoplasia, cystic or supernumerary kidneys, or ureter abnormalities. Hematoxylin-eosin staining showed that mutant kidneys had normal architecture, with well-organized cortex and medulla, a prominent nephrogenic zone, and no cystic dilations or signs of hydronephrosis. The number of glomeruli was also similar between DHCR7 knockout mice and their wild type littermates, consistent with proper branching of the ureteric bud. Immunostaining of colon from newborn mice with PGP9.5 showed no signs of colonic aganglionosis, the hallmark of Hirschsprung's disease. Enteric plexus density was assessed throughout the entire gut by means of acetylcholinesterase staining, revealing no differences between genotypes.

Removal of one Ret allele does not cause renal or enteric abnormalities in DHCR7 knockout mice

The above results were not surprising in light of the relatively mild phenotype of DHCR7 null mutants when compared to SLOS patients. Besides cleft palate and immature lungs, DHCR7 knockout mice show no overt developmental abnormalities reminiscent of the human disease. It is usually assumed that such discrepancies are due to differences in transplacental cholesterol transport between species. Thus, maternal cholesterol transfer to embryos in rodents is quantitatively much more important than in humans, and therefore maternal cholesterol would compensate cholesterol deficiency in murine but not human embryos. To determine whether reduction in Ret gene dosage could unveil suboptimal Ret signaling in these mice, we crossed DHCR7 mutant mice to heterozygous Ret knockin mice expressing one copy of EGFP from the Ret locus, thus allowing easy tracking of Ret-expressing cells. Removal of one copy of Ret did not affect ureteric bud branching or enteric plexus density, thus reinforcing the idea that no major abnormalities related to abnormal Ret signaling are found in DHCR7 knockout mice. Taken together, the above observations

indicate that Ret signaling is not disturbed in the developing urinary and nervous systems of DHCR7 knockout mice.

Simvastatin treatment affects kidney and enteric nervous system development

We next sought to lower maternal cholesterol levels in an effort to reduce cholesterol transfer rate to pups. We treated pregnant dams with simvastatin in drinking water from post coitum day 0.5 and analyzed kidney and enteric nervous system development at birth. Of note, plasma cholesterol levels of treated dams was dramatically reduced after simvastatin treatment. At the doses used no gross external abnormalities were found in either wild type or mutant pups. However, closer examination of mutant kidneys revealed an altered morphology, with thinner cortex and expanded medulla, whereas kidney from wild type littermates appeared normal. Moreover, the number of glomeruli was reduced in knockout mice. On the other hand, simvastatin treatment caused a reduction in colonic enteric plexus density in mutant but not wild type pups, as judged by GFP fluorescence. Thus, simvastatin treatment caused kidney and enteric nervous system abnormalities consistent with reduced Ret signaling.

Ureteric bud branching of DHCR7 mutant metanephroi is reduced in vitro

To examine Ret signaling avoiding the confounding effect of maternal cholesterol transfer, we decided to place embryonic metanephroi from DHCR7 mutant embryos in culture, in the absence of any exogenous source of cholesterol. As stated in the introduction, ureteric bud branching is critically dependent on GDNF-Ret signaling, both in vivo and in explant culture. We explanted metanephroi from E12.5 wild type and null embryos in defined medium containing retinoic acid, and counted ureteric bud tips after two and five days in vitro. We pooled data from wild type and DHCR7 knockout mice irrespectively of the Ret allele expressed as no differences on ureteric bud branching were found between $Ret^{+/EGFP}$ and $Ret^{+/+}$ embryos within a given DHCR7 genotype. We then stained $Ret^{+/+}$ metanephric explants with a calbindin antibody or

directly photographed $Ret^{+/EGFP}$ metanephroi under a fluorescence microscope. Small but significant differences between wild type and DHCR7 knockout metanephroi were found after two days in culture, which increased with time in culture. Thus, ureteric bud branching is reduced in DHCR7 null embryonic kidneys when cultured in defined medium and are therefore devoid of exogenous cholesterol.

Ret signaling is not affected by deletion of DHCR7 in sympathetic neurons

Even though signaling by the GDNF-GFRα1-Ret axis is the major player in ureteric bud branching, the above observations do not directly prove suboptimal Ret signaling in this system. To directly assess biological responses downstream of Ret, we chose sympathetic neurons from the superior cervical ganglion as a model system. We first used immunoblots to phosphorylated ERK as a readout of Ret signaling in sympathetic neurons cultured for five days in the presence of lipid-depleted serum, and stimulated with GDNF in the presence soluble GFRα1. No major differences on GDNF-mediated ERK phosphorylation were found between wild type and mutant neurons. Survival of sympathetic neurons in the presence of increasing concentrations of GDNF was assessed and no differences between genotypes were observed at any of the concentrations assayed. Neurite outgrowth of SCG explants in response to GDNF was also similar between wild type and DHCR7 null mice. Thus, at least for sympathetic neurons, mutation of DHCR7 does not appear to have any consequence on Ret signaling.

Ret signaling components partition to lipid rafts in brains from DHCR7 mice

We next investigated whether lack of effect of DHCR7 mutation on Ret bioactivity was due to normal partition of critical components of Ret signaling to lipid rafts. To this end, we generated flotation gradients from neonatal brain extracts of both control and DHCR7 null mice. Importantly, cholesterol does not cross the blood-brain barrier and thus the contribution of maternal cholesterol to brain tissues is negligible. Localization of GFRα1, and c-Src, two critical components of Ret signaling known to

partition to lipid rafts, was indistinguishable between wild type and DHCR7 knockout mice. GFRα1 was mainly found in the high-buoyant, lipid raft fractions, whereas c-Src showed a more uniform distribution among different fractions. Distribution of lipid raft (GM1 ganglioside, caveolin-1 and flotillin-1) on non-raft (transferrin receptor) markers indicated correct fractionation of the samples. Thus, loss of DHCR7 does not affect distribution of critical components of Ret signaling to lipid rafts in brain

7DHC can efficiently support Ret signaling.

To directly test the hypothesis that 7-DHC can efficiently substitute cholesterol in terms of Ret signaling, we conducted cholesterol depletion / 7-DHC replenishment experiments. We first confirmed that depletion of membrane cholesterol by means of methyl-β-cyclodextrin affected signaling downstream of Ret in sympathetic neurons. GDNF-mediated phosphorylation of both ERKs and Akt were affected by increasing concentrations of methyl-β-cyclodextrin to different extents, the PI3-K/Akt pathway being more sensitive to cholesterol depletion. Since methyl-β-cyclodextrin was toxic at high concentrations even for short exposure times, we focused on p-Akt phosphorylation for replenishment experiments. Replenishment of cellular membranes with 7-DHC efficiently supported GDNF-mediated Akt phosphorylation.

3. Relevance and implications for therapy

This is essentially a basic science proposal aimed to test one putative molecular cause of some of the developmental defects found in SLOS patients. The rationale of the proposal is based on two observations: first, lipid rafts have been shown to be important for Ret signaling in vitro and, second, SLOS patients show developmental defects consistent with impaired Ret signaling. I believe this is of great relevance to the field of signal transduction because although the composition and function of lipid rafts has been analyzed in great detail with regard to signal transduction, controversy

continues as to whether lipid rafts are membrane structures that exist under normal physiologic conditions. Ret dysfunction in mice deficient for DHCR7 would strongly suggest that lipid rafts are important for signal transduction in vivo.

As for the translational implications of this project, one potential therapy aimed to prevent developmental defects found in SLOS patients would have been in utero treatment with Ret agonists such as XIB4035, which has shown efficacy in treatment of small fiber neuropathy in two mouse models and unlike GFLs has the potential to cross the blood-brain barrier. However, lack of evidence supporting a role of Ret signaling in SLOS pathogenesis does not warrant such intervention.

Finally, one important finding derived from our studies relevant to SLOS therapy is that in utero administration of simvastatin is detrimental rather than beneficial to mutant embryo development. These findings provide a note of caution as simvastatin treatment has been proposed as a therapeutic intervention for children affected with SLOS.

4. Publications

A manuscript is under preparation describing these findings.