Altered expression of inflammasomes in Hirschsprung's Disease

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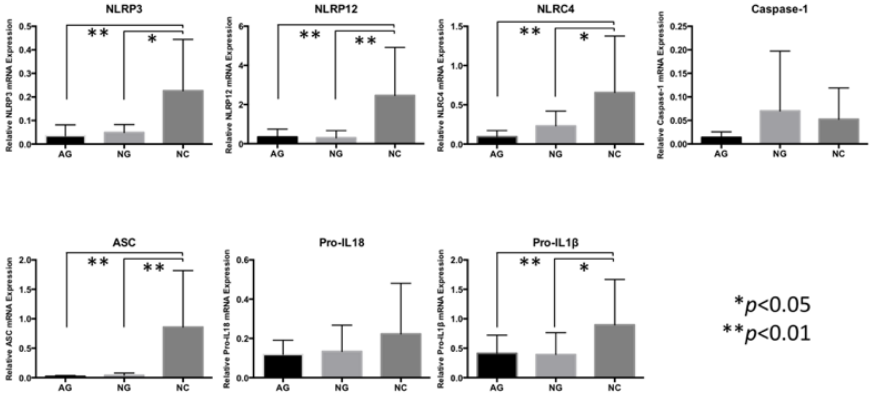
Abstract Body

Aim of the Study: The pathogenesis of Hirschsprung's disease associated enterocolitis (HAEC) is poorly understood. Inflammasomes are a large family of multiprotein complexes that are formed to mediate host immune responses to microbial infection and have a regulatory or conditioning influence on the composition of the microbiota. Inflammasomes and the apoptosis-associated speck-like protein (ASC) lead to caspase-1 activation. The activated caspase-1 promotes secretion of proinflammatory cytokines (IL-1β and IL-18) from their precursors (pro-IL-1β and pro-IL-18). Inflammasomes have been implicated in a host of inflammatory disorders. Among the inflammasomes, the NLRP3, NLRP12 and NLRC4 are the most widely investigated inflammasomes. Knock-out mice models of inflammasomes (NLRP3, NLRP12, NLRC4), caspase-1 and ASC are reported to have higher susceptibility to experimental colitis. The purpose of this study was to investigate the expression of NLRP3, NLRP12, NLRC4, caspase-1, ASC, pro-IL-1β and pro-IL-18 in the bowel specimens from patients with HSCR and controls.

Methods: Pulled-through colonic specimens were collected from HSCR patients (n=6) and healthy controls from the proximal colostomy of children with anorectal malformations (n=6). The gene expression of NLRP3, NLRP12, NLRC4, caspase-1, ASC, pro-IL-1β and pro-IL-18 was assessed using qPCR. The protein distribution was assessed using immunofluorescence and confocal microscopy.

Main Results: qRT-PCR analysis revealed that NLRP3, NLRP12, NLRC4, ASC and pro-IL-1β expression was significantly downregulated in the aganglionic and ganglionic colon of patients with HSCR compared to controls (Figure). Confocal microscopy revealed a markedly decreased expression of NLRP3, NLRP12, NLRC4, ASC and pro-IL-1β in colonic epithelium of aganglionic and ganglionic bowel of patients with HSCR compared to controls.

Conclusions: This is the first report of significantly decreased NLRP3, NLRP12, NLRC4, ASC and pro-IL-1β expression in patients with HSCR. Decreased expression of NLRP3, NLRP12, NLRC4, ASC and pro-IL-1β in the aganglionic and ganglionic bowel of HSCR may increase susceptibility to HAEC in HSCR patients.



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Expression of Dispatched RND Transporter Family Member 1 is Decreased in the Diaphragmatic and Pulmonary Mesenchyme of Nitrofen-induced Congenital Diaphragmatic Hernia

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Abstract Body

Aim of the Study: Congenital diaphragmatic hernia (CDH) and associated pulmonary hypoplasia (PH) are thought to be caused by a malformation of diaphragmatic and pulmonary mesenchyme. Dispatched RND transporter family member 1 (Disp-1) encodes a transmembrane protein that regulates release of cholesterol and palmitoyl, which is critical for normal diaphragmatic and airway development. Disp-1 is strongly expressed in mesenchymal compartments of fetal diaphragms and lungs. Recently, *Disp-1* mutations have been identified in CDH patients. We hypothesized that diaphragmatic and pulmonary Disp-1 expression is decreased in the nitrofen-induced CDH model.

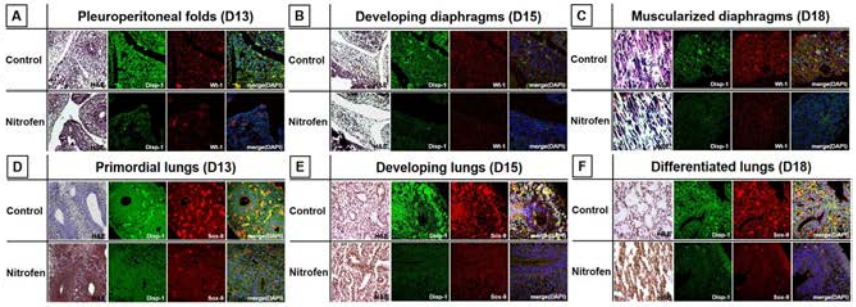
Methods: Time-mated rats received nitrofen or vehicle on gestational day 9 (D9)(Ethics: REC668b). Fetal diaphragms and lungs were microdissected on selected endpoints D13, D15 and D18, and divided into control and nitrofen-exposed specimens (*n*=12 per sample, time-point and experimental group). Diaphragmatic and pulmonary *Disp-1* expression was evaluated by qRT-PCR. Immunofluorescence-double-staining for Disp-1 was combined with diaphragmatic and pulmonary mesenchymal markers Wt-1 and Sox-9 to localize protein expression in fetal diaphragms and lungs.

Main Results: Relative mRNA levels of *Disp-1* were significantly decreased in pleuroperitoneal folds /primordial lungs on D13, developing diaphragms/lungs on D15 and fully muscularized diaphragms/differentiated lungs on D18 of nitrofen-exposed fetuses compared to controls (**Table**). Confocal-laser-scanning-microscopy demonstrated markedly diminished Disp-1 immunofluorescence predominately in diaphragmatic and pulmonary mesenchyme of nitrofen-exposed fetuses on D13, D15 and D18, associated with a reduction of proliferating mesenchymal cells (**Figure A-F**).

Disp1

	Endpoints	Control	Nitrofen
Diaphragms	D13	0.46±0.41	0.18±0.08*
	D15	0.44±0.23	0.18±0.06*
	D18	0.32±0.23	0.22±0.18*
Lungs	D13	1.34±0.79	1.06±0.27*
	D15	1.16±0.27	0.73±0.36*
	D18	0.77±0.14	0.56±0.16*

*$p < 0.05$ *vs.* Control.



Conclusions: Decreased Disp-1 expression during diaphragmatic development and lung branching morphogenesis may interrupt mesenchymal cell proliferation, thus leading to diaphragmatic defects and PH in the nitrofen-induced CDH model.

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Effective spheroids transplantation of SHED-derived hepatocytes-like cells in liver fibrosis mice

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Abstract Body

Aim of the Study:

SHED (Stem Cells from Human Exfoliated Deciduous Teeth) has been shown to share MSC characteristics and one of ideal cell sources of regenerative medicine. Intrasplenic cell transplantation has long been used as a route of cell delivery for liver diseases. This methods might have some risk including the cell may reside in the spleen or cause portal vein embolism. To improve the number and survival of engrafted cell and preserve their function, we transplanted SHED-derived hepatocytes-like cells as spheroid system.

Methods:

SHED was differentiated to Hepatocyte-like cells using several cytokines in vitro. They were seeded on low-attachable wells/tubes and cultured until 28 days, and then spheroids, approximately 0.5 mm in diameter were naturally created in wells/tubes. The characters of spheroid were examined, and 10 spheroids (approximately 1x10^5cells/spheroid) were transplanted on the surface of left lobe of liver of mice (C57BL/6 mice, 6-8-week-old) treated with CCl4 for 4 weeks (n = 5). After 8 weeks, mice were sacrificed and examined histologically, biochemically, and genetically.

Main Results:

The histological examination of spheroid showed PAS positive and cord-like structure, resembling normal liver tissue. Gene assay of spheroid showed human albumin and human factor VIII expressions. The size of lobe of transplanted mouse was bigger than that of non-transplanted mouse. Liver function test (AST, ALT) showed normal level in transplanted mice. Hepatocyte-like cells derived SHED was successfully settled in transplanted site as well as non-transplanted site of the liver. Transplanted hepatocyte-like cells settled and showed antifibrotic effects, and secrete human albumin.

Conclusions:

Spheroid transplantation of SHED-derived hepatocytes-like cell showed antifibrotic effect for CCl4 induced liver fibrosis in mice. SHED-derived hepatocytes-like cells settled and migrated into non-transplanted lobe. SHED transplantation using spheroid may become clinical therapeutic tool for pediatric liver disease.

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Exosome protein content plays a crucial role in the paracrine signaling of amniotic fluid stem cells

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Abstract Body

Aim of the Study: Exosomes are extracellular vesicles (EV) that contain cargo in the form of RNA and proteins. We have previously shown that amniotic fluid stem cell exosomes (AFSC-Exo) promote lung maturation and growth in experimental congenital diaphragmatic hernia (CDH), mainly through a microRNA-based mechanism. The aim of the present study was to investigate whether proteins contained in AFSC-Exo also contribute to their beneficial paracrine effect.

Methods: Exosomes were isolated from conditioned medium of c-Kit^{+} rat AFSCs, and assessed for size (nanoparticle tracking analysis), and morphology (transmission electron microscopy).
AFSC-EV proteomics: EV-proteins were extracted and digested for nanoscale liquid chromatography coupled to tandem mass spectrometry. Normalized spectral abundance factors from pooled samples were assessed. Protein set enrichment analysis was conducted using PSEA-Quant. Reported p-values represent probability that cargo proteins are independent of each other for that specific Gene Ontology dataset.

Main Results: Proteomics analysis showed that AFSC-Exo contain: 1) Hspa and CD63, EV canonical markers that confirm their EV nature (**Table1A**); 2) Annexins and Hnrnps, microRNA-stabilizing proteins that are crucial for microRNA function (**Table1B**); 3) proteins belonging to pathways important for EV structure and function (**Table1C**); and 4) no specific proteins related to fetal lung development.

Conclusions: In this study, we demonstrated that AFSC-Exo contain proteins that are crucial for formation of vesicles and stabilization of microRNA. The absence of proteins linked to lung development pathways confirms that AFSC-EVs exert their beneficial effects likely through an RNA-mediated mechanism. Exosome based therapy could be a promising option for fetal lung regeneration in babies with CDH.

Table 1A

EV-related proteins	# Mass spectrometry spectral counts in AFSC-EV
Hspa1a	14
Hspa2	8.3
Hspa4	2
CD63	2

Table 1B

RNA-stabilizing proteins	# Mass spectrometry spectral counts in AFSC-EV	RNA-stabilizing proteins	# Mass spectrometry spectral counts in AFSC-EV
Annexin 4	9	Hnrnpk	4.6
Annexin 7	6.3	Hnrnpf	4
Annexin 11	3.3	Hnrnpa1	2.6
Annexin 3	2	Hnrnpa2b1	2.3
Hnrnph1	8	Hnrnpul2	2
Hnrnpm	6.3	Hnrnpl	4.6

Table 1C

Gene Ontology Term	Gene Ontology Annotation	p-value	# AFSC-EV proteins	Total # proteins in annotations
extracellular region part	GO:0044421	0.000001	350	3479
extracellular vesicular exosome	GO:0070062	0.000001	330	2632
extracellular membrane-bounded organelle	GO:0065010	0.000001	330	2632

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CircularRNAs Are Highly Dysregulated in Lungs from Patients with Congenital Diaphragmatic Hernia.

Author(s)

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Abstract Body

Aim of the Study: In Congenital Diaphragmatic Hernia (CDH), epigenetic changes are involved in the underlying pathogenesis. MicroRNAs have been shown to be crucially involved in the cause of CDH. Circular RNAs are powerful upstream regulators of microRNAs, that can influence gene expression, without changing the DNA sequence. Their involvement in normal and abnormal lung development has not been shown yet. Our objective was to compare circular RNA profiles between human hypoplastic CDH lungs and age-matched controls.

Methods: Lung tissues for CDH (n=6) and healthy Controls (n=6) were obtained for mid-pregnancy cases and end-pregnancy cases from deceased subjects from our pathology department. After total RNA isolation we profiled the circular RNA expression via circular RNA microarray (Arraystar Inc., Rockville, MD, USA). In depth statistical data analysis was performed with R Studio. Pathway analysis was performed with KEGG and Ingenuity Pathway Analysis (Qiagen).

Main results: CDH lungs showed an altered circular RNA profile compared to lungs from healthy controls. PLS-DA analysis revealed significant clustering that differentiated between CDH and Control. VIP score analysis demonstrated the most important circular RNAs that drove the change. In total, 16 circular RNAs were significantly (Fold change > 1.5; p-value < 0.05) altered at mid-pregnancy and 35 circular RNAs at end-pregnancy.

Conclusions: We analysed the circular RNA profile of human hypoplastic CDH lungs and healthy controls at two different developmental time points and identified significant differences between the two groups. We are now validating these findings with RT-qPCR. Our results may uncover potential biomarkers for CDH.

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