Transcription factor GATA6 in ductal metaplasia of hepatocytes

Emmi Liljeström

Bachelor of science
University of Oulu
Finland

Faculty of science

Abstract

GATA6 is evolutionary highly conserved transcription factor and a newish indicator and possible causer of ductal metaplasia in biliary atresia (BA). BA is a rare neonatal cholestatic liver disease caused by fibroinflammatory obstruction of bile ducts. BA causes proliferation of bile ducts which is also called ductal metaplasia. In ductal metaplasia hepatocytes phenotype starts resembling more cholangiocytes. When bile gathers inside the liver it (liver) starts to create new bile ducts. This reaction is called ductal reaction what is a consequence of ductal metaplasia.

In this thesis work we, study the role of GATA6 in ductal metaplasia. We have two different kinds of mice: control mouse (ctrl) and knock out mouse (cKO). Both mice were operated with bile duct ligation (BDL) to resemble the BA-like condition. Our control (Ctrl) mouse only went over BDL and cKO mouse's GATA6 gene was silenced in addition of the BDL.

Missing of GATA6 has shown histology changes in liver tissue – there is no clear bile duct structure like in normal BA-like tissue. Post-mortem tells also a bad condition of the liver of cKO mouse. Similarly, gene expression studies support hypothesis that ductal reaction hardly ever happens when GATA6 is missing.

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Abbreviations

BA = biliary atresia

DR =ductal reaction

CK =cytokeratin

HNF = hepatocyte nuclear factor

EpCAM = epithelial adhesion molecule

Review of the literature

1. The Liver

1.1 Development

Liver organogensis is a seven-stage series of development. Those seven stages are a priming phase, a phase of increasing specification, expansion of the liver bud, migration of hepatoblasts into the transverse septum mesenchyme, a phase of liver vascularisation, an organ growth stage, and terminal differentiation of cholangiocyte and hepatocyte lineages. (Zaret, 2001) Liver starts to differentiate from the foregut of the fetus. Endoderm and two other germ layers, ectoderm and mesoderm, are formed during gastrulation. (Jung, Zheng, Goldfarb, & Zaret, 1999) All parts of the liver arise from hepatic diverticulum, that derives from endoderm. (Färkkilä, Isoniemi, Heikkinen, & Puolakkainen, 2018) Normally, a part of the endoderm starts to express genes which are specific for liver tissue. Next, those newly-specified cells start multiply and create a new tissue bud and so liver morphogenesis begins. (Jung et al., 1999). Hepatoblasts are stem cells of the liver and they develop from the definitive endoderm. Hepatocytes and bile duct epithelial cells originate from hepatoblasts. (Zhao & Duncan, 2005). (Zhao et al., 2005)

1.2 Physiology and function

Liver is the largest, approximately 1500g weighing organ inside the human body and it has many important functions such as maintaining homeostasis. (Färkkilä, Isoniemi, Heikkinen, & Puolakkainen, 2018) Liver is located on the right side of the abdominal cavity and under the diaphragm and it is protected by the rib cage. (Monga & Cagle, 2011). The liver can be classified into endocrine and exocrine compartments. Endocrine functions comprise secretion of different hormones and growth factors like angiotensinogen, thrombopoietin and insulin-like growth factors. The most important function of exocrine compartment is secretion of bile. Furthermore, the liver has many other essential functions, e.g. liver is a storage of glycogen, it detoxificates drugs, regulates metabolism – also urea metabolism as well as controls cholesterol synthesis and transport. The liver also secretes

plasma proteins, including apolipoproteins and albumin (Si-Tayeb, Lemaigre, & Duncan, 2010). Moreover, synthesis of bile occurs in liver.

Liver circulation is devided in two distinct parts: approximately 60-70% of the liver blood flow goes through the portal vein and rest (30-40%) is the blood of hepatic artery. Portal vein and hepatic artery are situated in area called porta hepatis, which is the bottom of the liver. Inside the liver, veins branch according to liver segments and the blood is carried all over the liver. The liver has also three hepatic veins which unite to inferior vena cava. Liver's lymphatic ducts end up to lymph follicle of the gullet, porta hepatics, abdominal cavity and inferior vena cava. (Färkkilä et al., 2018) Inside the liver there are also small sinusoids, which are basically free space between hepatocytes. They consist of hepatic sinusoidal endothelial cells and stellate cells. (Si-Tayeb, Lemaigre, & Duncan, 2010)

Liver has many different cell types (figure1) which are classified into two groups: parenchymal cells and non-parenchymal cells. Parenchymal cells include hepatocytes. Hepatocytes are abundant in the liver and form the structural base of the organ. Non-parenchymal cells cover all the other cell types, like Kupffer cells, hepatic dendritic cells, periportal fibroblasts, sinusoidal endothelial cells and stellate cells. (Monga & Cagle, 2011) Recent reports have shown that stellate cells have many special functions which affect liver function and development. (Friedman, 2008).

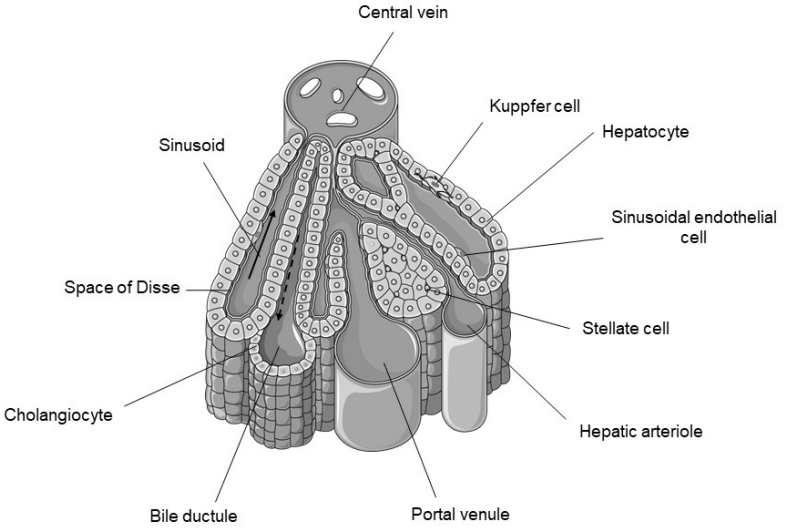


Figure1. Cell types and structure of the liver lobule

Arrows in picture are showing flowing direction of bile and blood. Bile flows in bile ductules and blood flows in sinusoids. Created using image vectors from Servier Medical Art (http://smart.servier.com/), licensed under the Creative Commons Attribution 3.0 Unported License.

1.3 Hepatocytes

The hepatocytes differentiate from liver stem cells, hepatoblasts. Hepatocytes maturate in the liver parenchyma. They are polarized epithelial cells and the most common parenchymal cell type in the liver. Because the big quantity of hepatocytes the liver is efficient in the purification of proteins with biochemical methods. Hepatocytes are located tightly next to each other and this structure of cells create a canaliculus. The main function of canaliculus is to collect and transport bile acids and bile salts. (Si-Tayeb et al., 2010)

1.4 Cholangiocytes

Cholangiocytes, in other words biliary epithelial cells, develop from same stem cells than hepatocytes: hepatobalst. Location of cholangiocytes inside the liver is bile duct

epithelium. Cholangiocytes cover approximately 3% of liver cell population. Main functions of cholangiocytes are to form bile ducts that transport bile from liver to gall bladder, control flow and pH of bile and secrete bicarbonate and water (Si-Tayeb et al., 2010) In this study we are interested in genes which express in cholangiocytes. Notch2 and JAG1 expressed during cholangiocytes synthesis (Grochowski, Loomes, & Spinner, 2016). HnF6 expressed while bile ducts develop. (Falix et al., 2014) Cytokeratin19 together with cytokeratin 7 expressed in the normal adult liver in cholangiocytes. (Jain, Fischer, Serra, & Chetty, 2010)

2. Biliary atresia

Biliary atresia (BA) is a rare and serious neonatal disease. It is an inflammatory condition which causes progressive fibrosis in extrahepatic bile ducts. That leads to obstruction of bile ducts and consequent liver cirrhosis. (Sanchez-Valle et al., 2017) Primary treatment of BA is Kasai portoenterostomy. In this operation extrahepatic bile ducts are replaced with part of small intestine fastened into the porta hepatis. (Färkkilä et al., 2018). (Gouw, Clouston, & Theise, 2011) BA is the most common pediatric indication for liver transplantation. (Feldman & Mack, 2015) Without treatment progressive liver cirrhosis leads to death by two years of age. Origin of BA is still unknown, but it is suggested that an inflammatory process damages the bile ducts. (Yoon et al., 1997) The incidence of BA in Western countries is around 0.5 to 0.8 per 10,000 live births. Prevalence in Asia and French Polynesia is roughly double compared to Western countries. Differences between continents depend on surveillance methods, diagnostic practice or ethnic or genetic factors, environment, or infectious pathogens. (Sanchez-Valle et al., 2017) There might be a little predominance with females when compared affected infants, especially in the embryonic form of the biliary atresia. Most of the BA cases are sporadic and no genetic mutations have been reported. (Monga & Cagle, 2011)

BA is classified into three different types (type I-III). Location of extrahepatic bile duct obstruction determines the type of BA. In type I the obstruction is in common bile duct. The incidence of this type is 5%. Type II is even rarer: only 2% of cases represent this type. In type II obstruction is located in the common hepatic ducts. In types I and II the intrahepatic ducts are normally preserved even if there might be morphologically abnormalities. The most common type is type III which covers over 90% of BA cases. In this type, obstruction is at the level of the porta hepatis. In this case most of the proximal part of the extrahepatic biliary tract inside the porta hepatis is fibrotic. (Sanchez-Valle et al., 2017)

BA can be also be classified into non-syndromic and syndromic forms. Roughly 80% of cases are non-syndromic. It is thought that these cases develop for genetically susceptible individuals as a consequence of prenatal virus infection. Also, environmental factors can

cause auto-immune mediated inflammation and scarring of bile ducts. Rest of the cases (20%) are syndromic where the disease relates to disturbed embryonic development. Syndromic patients also have other inborn abnormalites in lateral development, like heart disease, torsion disorder of intense, abnormal structure of pancreas, a divergent location of viscera, missing or abnormal spleen, abnormally ambulatory portal vein or missing inferior vena cava. (Färkkilä et al., 2018)

2.1 Ductal reaction

Distinctive pathological process in BA is expansion of intrahepatic bile ductules. The process is also known as ductular reaction (DR). Epithelial cell adhesion molecule (EpCam) express during ductal reaction. (Dollé et al., 2015)

The cellular origin of newly formed bile ductules in BA livers is not fully understood. Some of the new ductal structures derive from proliferating cholangiocytes, but hepatocytes similarly partake this process via ductal metaplasia. (Gouw, Clouston, & Theise, 2011) DR is an active process where different cell types interactive with each other. In DR, bile duct like-cells are expanded, accompanied by inflammatory and mesenchymal cells. This leads to liver fibrogenesis. Fibrogenesis is a key major cause of portal hypertension, in both chronic hepatocellular damage and cholangiopathies. Excessive and sustained activation of tissue repair mechanisms can cause fibrogenesis driven by ductal reaction (DR). (Fabris & Strazzabosco, 2011) The origin of epithelial cell type of ductal reaction is controversial. Many of the cells in DR area derive from proliferating cholangiocytes, but also hepatocytes participate to this process via ductal metaplasia meaning that hepatocytes around portal area transdifferentiate towards cholangiocyte-like phenotype. This can be thought as an escape mechanism where the liver tries to adapt to the injury caused by cholestasis. (Nishikawa et al., 2005) In liver there are also hepatic progenitor cells (HPC). These cells are located in Hering's canals and can differentiate into cholangiocytes or hepatocytes. HPCs also participate in DR by producing transitional ductal- or hepatocyte-like cells. (Roskams, 2003)

3. GATA Transcripion factors

GATA is a common name for all of the already found six (GATA1- to 6) GATA transcription factors. (Viger, Guittot, Anttonen, Wilson, & Heikinheimo, 2008) (Bouhlel, Lambert, & David-Cordonnier, 2015). These factors are important regulators of genes important for cell differentiation and proliferation. (Lentjes et al., 2016) GATA factors are evolutionary concerved and they all contain zinc finger DNA-binding domains. Those domains follow a characteristic amino acid sequence. (Tang et al., 2014) GATA factors can be classified into subgroups based on spatial and temporal expression patterns and all of them have significant roles in differentiation and development of all eukaryotic organism. GATA1- to 3 play roles in differentiation of hematopoietic cell lineages. Those GATAs are necessary for differentiation of erythroid and megakaryocyte cells, proliferation of hematopoietic stem cells, and development of T lymphocytes. They are also expressed in the brain, spinal cord, and inner ear. GATA4- to 6 are expressed mainly in mesodermal and endodermal tissues for example gut, heart, and gonads. (Viger et al., 2008) Studies have shown that GATA factors have essential roles in controlling early development of the mammalian liver. (Watt, Zhao, Li, & Duncan, 2007) Transcription factor GATA6 together with GATA4 are also necessary for the liver normal development. Especially for the early hepatic development and the expansion of the liver bud

3.1 GATA6

GATA6 is the most recently found member of GATA family. It is expressed highly levels in adult's heart, lung, ovary and pancreas, and lower levels in liver and spleen. To be precise, GATA6 does not express in adult hepatocytes but it expresses in cholangiocytes. (Soini et al., 2018) In embryonic tissue, GATA6 is expressed partly similarly: expression is higher in heart and lungs and lower in brain, liver and kidney. (Suzuki et al., 1996)In hepatocytes, GATA6 expressed only during early gestation. (Soini et al., 2018) In developing liver GATA6 is an important factor because it regulates expression of hepatocyte nuclear factor 4 (HNF4) that is critical for liver development. (Zhao et al., 2005).Studies have shown that GATA6 expression is elevated in BA. The expression is

significantly higher in BA than in other cholestatic diseases. In BA, expression of *GATA6* messenger-RNA (mRNA) is found both in hepatocytes and bile duct epithelium. The expression is especially high in periportal hepatocytes that udergo ductal metaplasia. (Soini et al., 2018).

In this thesis is studied the role of GATA6 in biliary atresia by using GATA6-silenced mice-model.

Materials and methods

1. Generation of GATA6 conditional knockout mouce

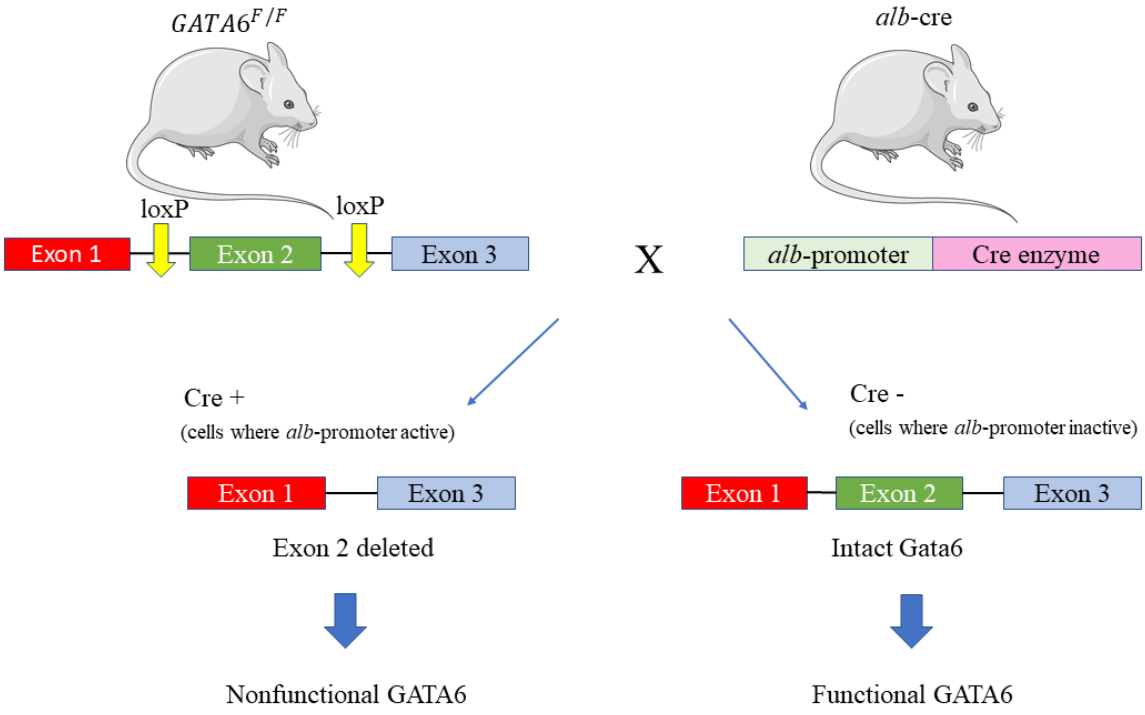


Figure2. Generation of GATA6 cKO mouse

A formal presentation of the Cre-loxP recombination system. Created using image vectors from Servier Medical Art (http://smart.servier.com/), licensed under the Creative Commons Attribution 3.0 Unported License.

The GATA6 knockout mouse was generated by cre-lox recombination (figure2). The main idea was crossed mouse with loxP sites and mouse with albumin promoter and cre-enzyme. In cells where alb is active cre enzyme is produced and it catalyzes the recombination of loxP sites leading to a non-functional GATA6 protein. GATA6 cKO mice were born by following Mendelian ratio (1:4) and expected sex ratio (1:1).

1.1 Mice with BDL

In this study was used two different kind of mice: control (Ctrl) mice (XX pieces) and knock out (cKO) mice (XX pieces) – in all XX mice. All mice were operated with bile duct ligation (BDL). On account of BDL, mice resemble BA-like condition. In BDL bile ducts was bind and bile were started gathered inside the liver. Ctrl-mouse was only going over BDL but GATA6 of cKO mouse was silenced in addition of the BDL. In this study was used liver samples of the above-mentioned mice.

2.0 Laboratory works

The goal of this experiment was to figure out the expression of genes in mice' liver. Gene which was studied were: cytokeratin7, cytokeratin19, Hnf6, Notch2, Jag1, Hes1, and Epcam. Methods used was: RNA extraction, RT-reaction, and qPCR.

2.1 RNA extraction

In laboratory works research was started with an extraction of RNA from a liver. Before the experiment was cut a tiny piece from a liver sample. The sample was homogenized by lysis before extraction. RNA was extracted using NuceloSpin ® RNA/protein kit according to manufacturer's instructions.

2.2 RT-reaction

RT-reaction was doing after RNA extraction. Main function of RT-reaction is changing RNA into the complementary DNA (cDNA). RT-reaction was performed using Reverse

Transcriptase Core kit ® according to manufacturer's instructions. Samples were needed to change into the cDNA for qPCR reaction.

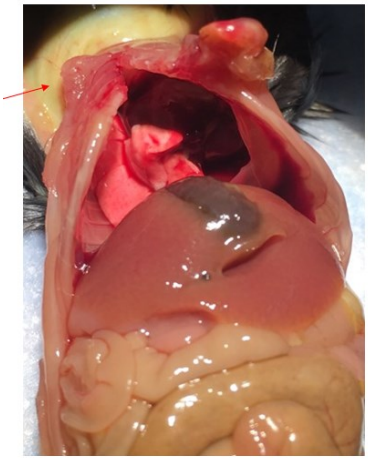
2.3 Real-Time PCR (qPCR)

The main function of qPCR is the same than in normal PCR: characterize, detect and quantify nucleic acids for numerous applications. It consists of three repeating steps which are denaturation, annealing, and elongation. The difference compared to normal PCR is a fluorescent label which makes collection of data possible during the run. ("What are the differences between PCR, RT-PCR, qPCR, and RT-qPCR? - Enzo Life Sciences," n.d.)

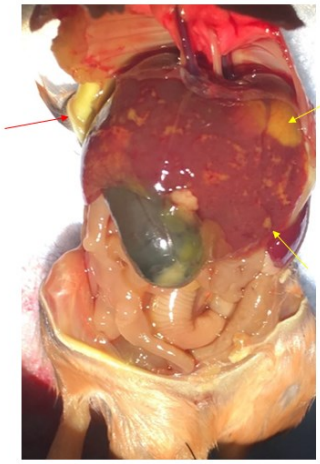
Real-Time PCR was performed using SYBR GREEN (qPCR MasterMix Plus, Eurogentec). Expression of above-listed genes normalized to housekeeping genes XXX. Before qPCR was combined samples and primers of genes.

Results and discussion

1. Cross phenotype



Ctrl mouse



cKO mouse

Figure3. The phenotype of bile duct ligated Gata6 cKO and control mice

Post mortem after 14 days of bile duct ligation. Red arrows show the yellow colour of the skin which is consiquense from cholestasis. Yellow spots within cKO mouse's liver are bile lakes. Bile lakes have developed because of cholestasis.

Phenotype of murines were GATA6flox/flox (crtl) and alb-cre; GATA6flox/flox (cKO). Colour of the skin of mice were yellowish because of BDL which have caused cholestatic. Normally liver processes bilirubin and secretes it into the gallbladder. In BA and BDL bile gathers to liver and causes cholestasis because bilirubin can not get off from a body. Bilirubin starts accumulates into hepatocytes and from there to circulation. ("Terveyskirjasto - Duodecim," n.d.)

Liver condition of cKO mouse looks macroscopically more indisposed than liver of ctrl mouse. Figure 3 was shown sign of more cholestatic condition of cKO mouse. Missing of GATA6 probably is an originator of bile lakes. Bile lakes are necrotic part of a tissue.

2. Histology

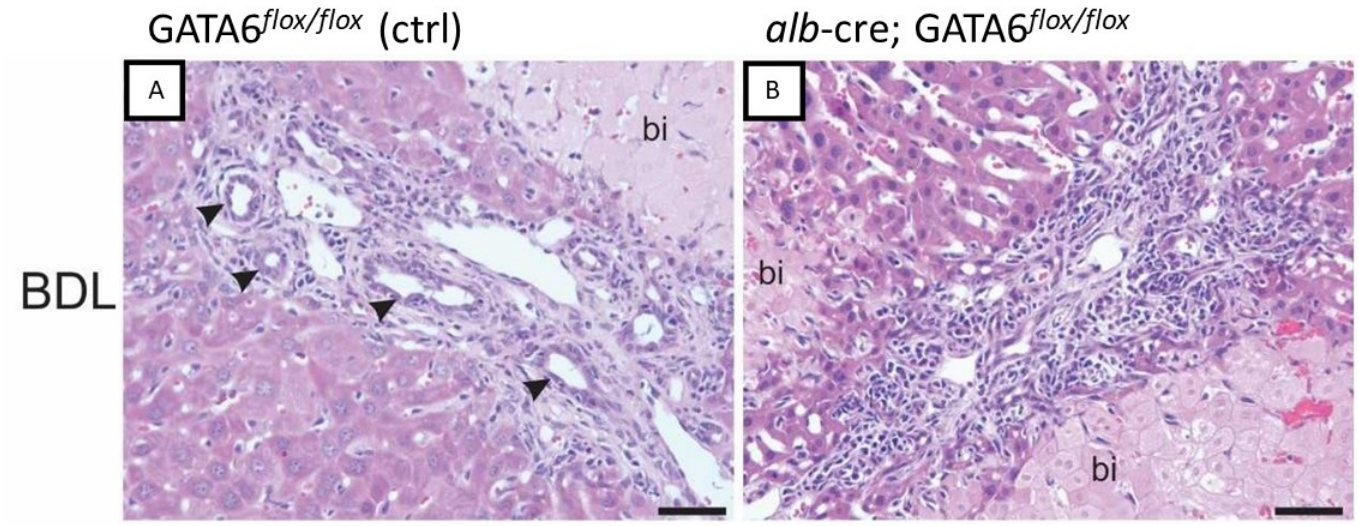


Figure4. Histology of ctrl and cKO mouse

Picture A (ctrl) shows abundant occurrence of bile ducts. Bile ducts were caused in consequence of ductal reaction. In picture B bile ducts are not clear and tissue is inflammatory.

Histology of mice' liver display difference between ctrl and cKO mice. BDL was caused BA-like condition for both mice. Missing GATA6 of cKO mouse caused more inflammatory tissue and lows ductal reaction in liver -there is not any clear bile duct structure. Surrounded by inflammation, cells of the tissue are unidentified. Missing of GATA6 probably prevent ductal reaction. Ctrl mouse's histology exhibits abundant occurrence of bile duct. Finding looks typical cholestatic liver which has appeared bile duct proliferation.

3. Gene expression

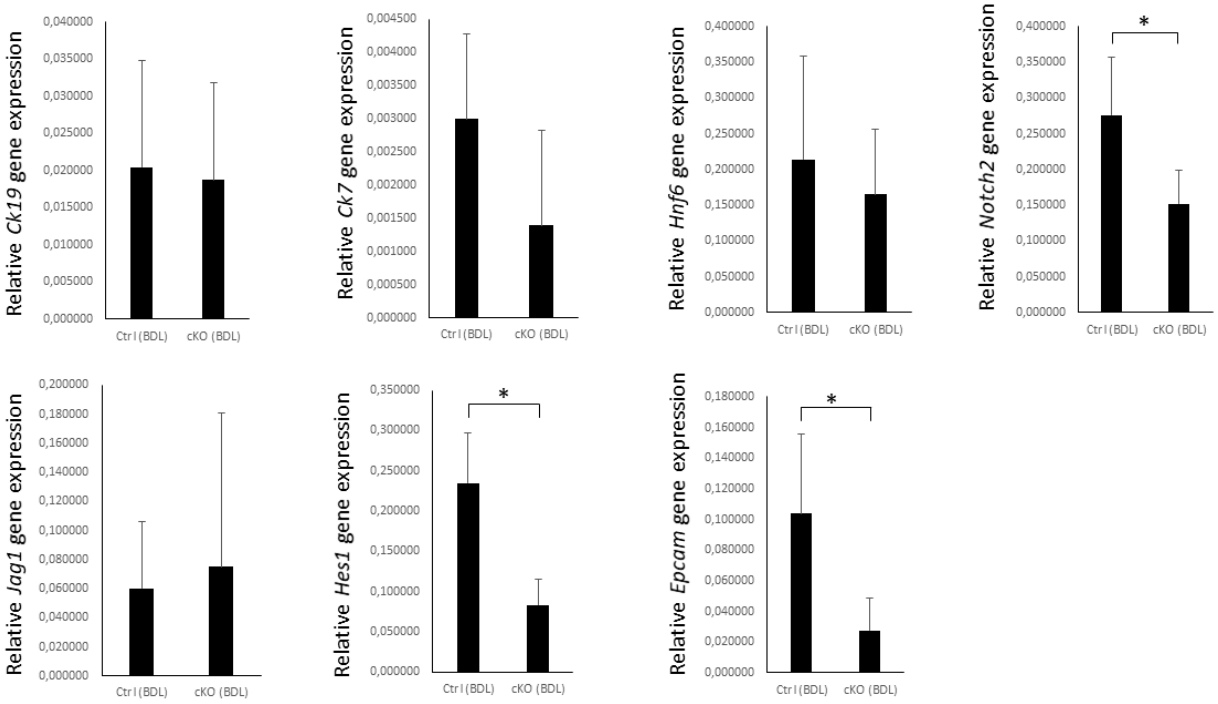


Figure5. Diagrammatic tables of gene expression calculated from geomean

*Tables of relatives of genexpression. Black column shows average of expression and thin line top of clomun shows standard deviation. If difference between standard deviation is markable it has marked with *.*

Cytokeratin 19 (CK19) is a member of keratin family. CK19 expressed in the bile duct epithelium. (Jain et al., 2010). CK19 expressed in both mice's liver. The expression is almost the same in both liver samples. An inflammatory area in cKO mouse might include cholangiocyte-like cells which could explain the expression of CK19 in cKO mouse's liver or primer might have been spoiled. Expression in ctrl mouse's liver is an explicable abundant volume of cholangiocytes – which are a consequence of ductal reaction.

Cytokeratin 7 (CK7) also a beloved family of keratins. CK7 normally expressed when happening proliferation of intraductal epithelial. (Reisenbichler, Ross, & Hameed, 2014) CK7 also expressed in bile duct epithelium. (Jain et al., 2010) CK7 expressed more in ctrl mouse's liver. Because ductal reaction only happened for ctrl mouse the expression of CK7 is higher. Still, difference compared cKO mouse is not markable.

HnF6 is a biliary marker and it expresses in cholangiocytes and during bile-duct development. (Falix et al., 2014). Expression of HnF6 is higher in ctrl mouse than in cKO mouse but no markable. Obscure tissue in cKO mouse's liver might rise value of Hnf6 expression - like in case of CK19. Expression of ctrl mouse liver is clearer because histology and postmortem prove proliferation of bile duct.

Notch2 and JAG1 express during cholangiocytes synthesis (NOTCH signaling) (Grochowski et al., 2016). Average of Jag1 shows that it expresses a little bit more in cKO mouse's liver. However, the standard deviation of diaphgrams points that difference is insignificant. Results of qPCR were slightly aberrant. Primers might have been inferior. Expression of Notch2 is markable between mice. Even though Jag1 does not work as expected, Notch2 worked well. Like already said, cholangiocytes and bile ducts expressed more in ctrl mouse's liver. That would explain the high level of Notch2.

Hes1 is a transcription factor and cholangiocytes marker. (Lu et al., 2016) It expressed markable highly in liver of ctrl mouse. Hes1 expressed in cholangiocytes like GATA6 - which is also a transcription factor. Difference between mice might be accountable missing of GATA6. When GATA6 is silenced ductal reaction can not start, and cholangiocyte-like cells does not arise. Hes1 express in liver only cholangiocytes, and without GATA6 value of cholangiocytes is low.

EpCam in other words epithelial cell adhesion molecule is a transmembrane glycoprotein. EpCam expresses during ductal reaction. (Dollé et al., 2015) Difference between cKO and ctrl mice is markable. BDL remind condition of BA and in BA it has proved that ductal reaction happens when bile gathers inside the liver. That could explain abundant expression in liver of ctrl mouse. Expression in liver of cKo mouse is markable lower which is probably a consequence of missing of GATA6. This also proved hypothesis:

GATA6 prevent a proliferation of bile duct. Histology of cKO mouse also supports the hypothesis.

Gene	Ctrl/BDL	P-value
Ck 19	1,092617	0,851737
Ck 7	0,184362	0,103017
HnF6	1,293569	0,572284
Notch2	1,815930	0,025661
Jag1	0,806072	0,769162
Hes1	2,813217	0,002263
EpCam	3,738941	0,025176

Table 1. Numerous values of genes' relation and P-value.
Ctrl/BDL tells average between geomean of genes'. Value of P tells standard deviation. When P-valuea is under 0.05 result is notable.

Expression of genes has listed to table 1. Relation between cltr and cKO mice does not tell is result markable. P-value in other words standard devitation tells difference of average values. When P-value is high enough – over 0.05 – it means that result is not markable. Low P-value tells minor difference of original values.

Diaphgrams of figure 5 was created by using values of table 1. Both tells same data in different way. Result we got was partly expected. Expression of some genes was unclear which might be consequence of bad primer or pipetting mistake.

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