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Gene expression changes in the stomach of *Carbonic anhydrase 9* knockout mice

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Summary

Carbonic anhydrases (CAs) are Zn^{2+}containing metalloenzymes that catalyze the reversible hydration of carbon dioxide to protons and bicarbonate ions. The reaction of $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$ is important in pH regulation as well as ion and electrolyte transport in microcellular environments. Different studies have shown that there are 13 enzymatically active members in the CA family, and altogether 16 different isoforms of CA have been found. CA participates in various other physiological and pathophysiological processes such as bone resorption, respiration, gluconeogenesis, lipogenesis, ureagenesis, fertilization, and tumorigenicity as well as in the formation of gastric acid, cerebrospinal fluid, pancreatic juice, and saliva. This suggests that the expression of CA is widely distributed in the mammalian body.

One of the most enzymatically active isoforms of CA is transmembrane glycoprotein CA IX. This enzyme is a cell surface protein consisting of an extracellular N-terminal proteoglycan-like domain (PG), a catalytic CA region, an α-helix transmembrane domain (TM) and a short C-terminal intracytoplasmic tail. CA IX is predominantly expressed in epithelial cell linings of the gastrointestinal tract, being involved in cell adhesion, proliferation and invasion in these microcellular environments. Interestingly, CA IX has been found to be related in tumorigenicity under highly acidic extracellular microenvironments. Moreover, it has been reported that CA IX is expressed in kidney, breast, lung and ovarian cancers. The knockout mice studies have shown that deletion of carbonic anhydrase 9, (*Car 9*) gene results in various morphological changes in the gastric mucosa of these mice. The loss of *Car 9* led to an increased number of the mucus producing pit cells, whereas the pepsinogen producing chief cells and H^+/K^+-ATP producing parietal cells were significantly decreased.

The main aim of this project was to characterize gene expression changes that occurred within the gastric mucosa of C57BL/6 mice due to the elimination of the *Car 9* gene. Stomach tissue samples of 12 C57BL/6 mice (6 wildtype and 6 knockout) were studied by quantitative real-time polymerase chain reaction (qRT-PCR). Eight different regulated genes identified by a cDNA microarray (*mEgf*, *mEla2a*, *mIl1rl1*, *mNono*, *mPkp4*, *mPnlip*, *mSftpd* and *mSlc9a3*) in *Car* $9^{-/-}$ knockout mice were further investigated by qRT-PCR. A statistical Mann-Whitney test showed significant changes in the expression levels of 6 of the 8 genes after disruption of *Car 9*. All of these genes are involved in different processes such as pH regulation, immunoresponse, overproduction of cells as well as cell proliferation and cell differentiation in microcellular environments. In future studies it would be interesting to know how these genes are related to different metabolic processes in gastric environments.

Introduction

Carbonic anhydrase enzymes

The zinc-containing carbonic anhydrases (CAs) are enzymes that catalyze the reversible reaction of $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$. The zinc ion ($Zn^{2+}$), which is present in the active site of CA, is very important for catalysis. The hydration of carbon dioxide into bicarbonate ions and protons is essential for the regulation of pH homeostasis, electrolyte and water balance, and ion transport in mammals. In addition, CA isozymes participate in various other pathological and physiological processes such as in the formation of body fluids (cerebrospinal fluid, pancreatic juice, gastric acid and saliva), respiration, bone resorption, gluconeogenesis, lipogenesis, ureagenesis, fertilization, and tumorigenicity (Hilvo 2008, Hilvo et al. 2008, Kivelä 2003, Parkkila et al. 2000).

The family of mammalian CA isozymes (α-CAs) includes 16 different isoforms. These enzymes are not only expressed in vertebrates but also in algae, bacteria and green plants (cytoplasm) (Supuran 2008). There are altogether 13 enzymatically active members (CA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, XIV, XV) in this family. CA VIII, X and XI are inactive isoforms. The 13 active isozymes have different tissue distributions, catalytic activity and subcellular localizations (Parkkila et al. 2000, Table 1.)

In addition to α-CAs there are also other active CA enzyme families. β-CAs are expressed mainly in algae, bacteria and chloroplast, γ-CAs are predominantly present in archaea and some bacteria and δ-CAs have been reported in some marine plankton (Supuran 2008).

Table 1. Distribution, catalytic activity and the subcellular localization of 13 active CA isozymes (Hilvo et al. 2008, Leppilampi 2006, Pan et al. 2007).

CA	Activity	Subcellular localication	Organ
I	low / moderate	cytosol	Gastrointestinal tract (GI), erythrocytes
II	high	“	GI, erythrocytes, kidney, brain, testis, lung
III	very low	“	adipocytes, skeletal muscle
IV	low / moderate	membrane bound	pancreas, lung, kidney, heart muscle, colon, brain capillaries
VA	”	mitochondria	liver
VB	”	“	heart, skeletal muscle, kidney, pancreas, GI, spinal cord
VI	”	secreted	mammary and salivary glands

VII	”	cytosol	CNS
IX	high	membrane bound	GI mucosa, different tumours
XII	low / moderate	“	eye, intestinal, renal and reproductive epithelia, tumours
XIII	“	cytosol	brain, gut, lung, kidney
XIV	low (humans) high (mice)	membrane bound	brain, liver, kidney
XV	low / moderate (mice)	“	kidney (mice) not expressed in humans

The CA IX enzyme

Carbonic anhydrase IX (CA IX) is a transmembrane glycoprotein. It is a cell surface protein that consists of four domains: an extracellular N-terminal proteoglycan-like domain (PG), a catalytic CA region, an α-helix transmembrane domain (TM) and a short C-terminal intracytoplasmic tail (Fig. 1, Hilvo et al. 2008).

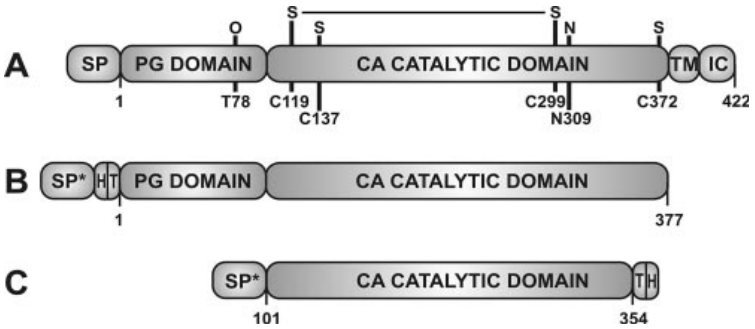


FIGURE1. **Human CA IX and the recombinant CA IX proteins.**
A, Human CA IX, B (PG +CA) domain and C (CA domain) shows the recombinant forms of the CA IX protein. The intramolecular cysteine bridge (C^{119} to C^{299}) that stabilizes the protein structure is shown in (A). Abbreviations PG = proteoglycan (may be involved in cell adhesion) SP = signal peptide (communications between other proteins), TM = transmembrane helix, IC = intracytoplasmic tail (Hilvo et al. 2008). Reproduced with permission from the copyright holder.

The other CA enzymes have quite similar structure as CA IX except for the PG-like region, which is present only in CA IX. It has been reported (Hilvo et al. 2008) that the PG-like domain somehow modulates the CO_2 hydration activity of the CA region, although the mechanism is still unknown. In addition, the PG-like domain is probably involved in the regulation of intercellular communication and contacts by cell adhesion (Ortova et al. 2002). The cDNA for human CA IX was first cloned and sequenced in 1994 (Pastorek et al. 1994). The genomic structure was later characterized by Opavsky et al. (1996). The *CA 9* gene is located on the chromosome 9 in humans (GenBank accession no. NM _001216) and the corresponding gene (*Car 9*) on chromosome 4 in mice (*Mus musculus*) (GenBank accession no NC_000070). Leppilampi (2006) reported that CA IX

can form homo-oligomers, most likely trimers, and migrates as a 58/54 kDa double band on reducing SDS-PAGE and as a 153 kDa band on non-reducing SDS-PAGE, suggesting that it could form disulfide-bonded trimers. Recently it has been shown (Hilvo et al. 2008) that CA IX may not be able to form trimers without the transmembrane helix and/or intracytoplasmic tail, and therefore further studies are needed to elucidate whether the C-terminal is involved in the regulation of oligomerization status.

CA IX has different functions in the human body. It is expressed mainly in the gastrointestinal tract (gastric mucosa) and is predominantly involved in cell adhesion, cell proliferation and cell invasion. In addition, it has been reported that CA IX could be related to tumorigenicity due to overexpression under highly acidic extracellular microenvironments (Parkkila et al. 2000, Leppilampi et al. 2003, Ortova et al. 2002). CA IX is highly expressed in several forms of cancer including renal, lung, ovarian and breast cancers (Ortova et al. 2002, Hilvo et al. 2008, Hilvo et al. 2004, Leppilampi et al. 2005). The studies on CA IX knock out mice have provided convincing evidence that CA IX is essential in gastric morphogenesis (Ortova et al. 2002, Hilvo et al. 2008, Hilvo et al. 2004). It is also important to notice that CA IX may play a role in Na^+-driven Cl^-/HCO_3^- exchange, which is coupled with proton secretion by gastric H^+ K^+ ATPase in gastric epithelial cells. In addition, CA IX seems to has a special role in conversion of CO_2 back into HCO_3^- and H_2CO_3 and is involved in H^+/HCO_3^- transport which is essential for pH regulation in the gastric epithelium (Chiche et al. 2009, Purkerson et al. 2007). Moreover, studies have shown that CA inhibitors such as sulfonamides can inhibit the relative invasion rate of cancer cell lines (Parkkila et al. 2000). One very recent study interestingly showed that protein tyrosin kinase (PTK) inhibitors - clinically widely used drugs in cancer therapy - effectively inhibited the activity of the 13 mammalian CA isoforms (CAI-CAXV) (Parkkila et al. 2009).

Recent studies have shown that the different domains of CA IX isozyme have special functions. To study biochemical properties of CA IX, two soluble forms of human CA IX were produced by baculovirus-insect cell expression system – one form containing the CA + PG domains (Fig. 1 B) and the other containing only the CA domain (Fig. 1 C). Both forms were expressed without the TM and intracytoplasmic regions at the C-terminus. Hilvo et al. (2008) reported that the catalytic activities of PG + CA and CA regions increased 23-fold and 10-fold, respectively, when $ZnCl_2$ was added to solutions of these two soluble recombinant CA IX forms. This is the highest catalytic activation ever measured among CAs. It is interesting that additional $ZnCl_2$, $CoCl_2$, $MgCl_2$ or $MnSO_4$ did not show any effect on the catalytic activity of the recombinant CA form but addition of $MnSO_4$ increased the catalytic activity of PG +CA regions as much as when $ZnCl_2$ was added. This could be due to metal ions which bind to PG-region while this region includes various negatively charged amino acid residues and therefore stabilizes the electrostatic repulsions. Hilvo et al. (2008) showed that both CA IX forms have an intramolecular Cys^{119} to Cys^{299} linkage (S-S) that can stabilize the protein structure (Fig. 1, A). Based on the amino acid sequence there could be only one additional cysteine residue (Cys^{137}) in the CA domain whereas two cysteine residues (Cys^{137} and Cys^{372}) in the PG +CA form (Fig. 1, B). Biochemical studies further showed that the CA and PG + CA regions were expressed as monomers as well as disulfide-linked dimers.

Wang et al. (2008) reported that CA IX can also function as a chaperone-like protein assisting the non-covalent folding or unfolding of other molecular structures. They showed that CA IX has an ability to activate an immune response by forming complexes with antigens. In addition, they reported that CA IX was able to bind and fold the denatured protein. In the same research Wang et al. (2008) reported also that the expression of *CA 9* in renal-cell carcinoma may respond to IL-2 cytokine therapy which could be involved in maintaining the antitumor immune response.

CA IX knockout mice

The CA IX isozyme is one of the enzymatically most active isoforms of the CA enzymes. It is expressed predominantly in the gastric mucosa and in the epithelial linings of the lower gastrointestinal tract (GI). Its physiological relevance has been shown in CA IX deficient (*Car 9*$^{-/-}$) mice. These mice had homologous mutation (*Car 9*$^{-/-}$), meaning that both of the loci of *Car 9* gene were inactivated. The *Car 9* gene was cloned and CA IX knockout mice with the null mutation were generated by targeted disruption of this gene (Ortova et al. 2002). After disruption of *Car 9* gene some significant morphological changes were observed in the gastrointestinal tract of these mice.

Interestingly, there was no expression of CA IX in embryos studied at days E10.5, E15.5 and E17.5. At embryonic day E18.5 there was a signal for CA IX in the mouse embryo (Ortova et al. 2002). The newborn *Car 9*$^{-/-}$ mice showed the first mild physiological changes in the stomach such as the thickening of the gastric mucosa predominantly in the corpus region of the stomach. The targeted disruption of *Car 9* led to gastric hyperplasia (overproduction of certain epithelial cells) and production of numerous cysts (Fig. 2 (C), Ortova et al. 2002). The proliferative zone of the gastric mucosa cells was strongly expanded in *Car 9*$^{-/-}$ deficient mice.

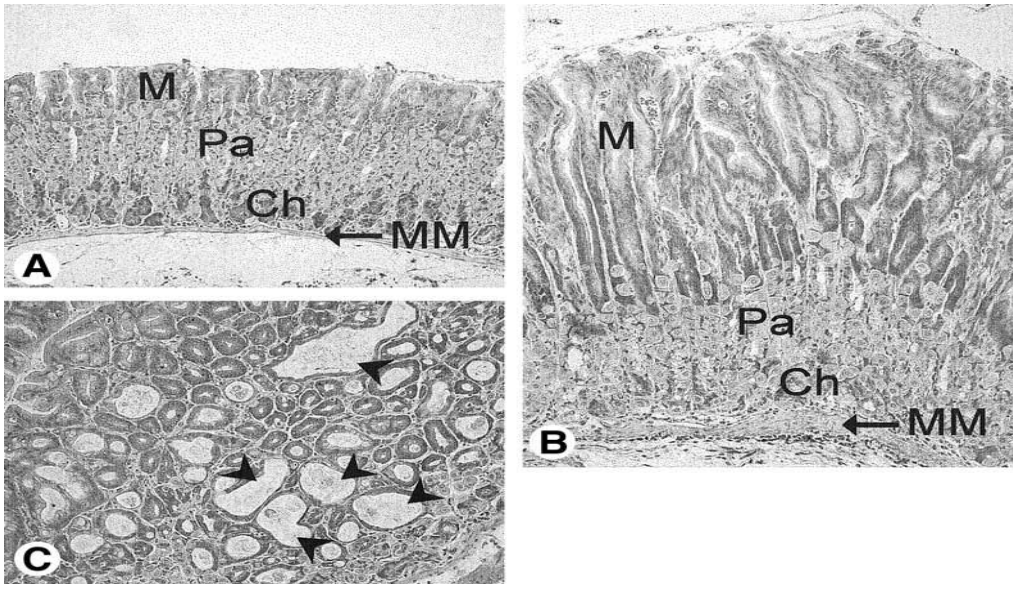


FIGURE 2. **Stomach mucosa in wild type (*Car 9*$^{+/+}$) mouse and knockout (*Car 9*$^{-/-}$) mice.**
Mucus producing cells (M) produce bicarbonate into the gastric juice. Parietal cells (Pa) are H^+/K^+ -ATP positive cells. They are involved in the regulation of hydrogen and potassium exchange within the gastric mucosa. Chief cells (Ch) produce pepsinogen in the gastrointestinal tract. Muscularis mucosa (MM) is a thin layer of smooth muscle. Among *Car 9*$^{-/-}$ mice (B and C) the disruption of the *Car 9* gene led to overproduction of mucus producing pit cells, a significant downregulation of parietal cells and the reduction of chief cells. The gastric epithelium in *Car 9* $^{-/-}$ mutant mice (C) consisted of numerous cysts that are shown with arrows (Ortova et al. 2002). Reproduced with permission from the copyright holder.

These symptoms became prominent among 4-week-old mice when the gastric morphogenesis was over, remaining similar even in 1.5-year-old mice. The disruption of the *Car 9* gene led to reduction of chief cells (Ch), which are pepsinogen-producing cells in the GI (Fig. 2, (A and B) Ortova et al. 2002), upregulation of pit cells which are involved in the production of bicarbonate-rich mucus (M). Mucus cells also produce important proteins that protect the epithelial cells in the GI tract (Fig.2, Ortova et al. 2002). Loss of CA IX significantly decreased the relative proportion of H^+/K^+ -ATP parietal cells (Pa) but the absolute number of Pa did not decrease. Parietal cells are involved in the regulation of hydrogen and potassium exchange in the GI tract and therefore are pivotal to gastric

acidification (Ortova et al. 2002). There were no other significant changes in *Car 9*$^{-/-}$ deficient mice. Interestingly, these knockout mice had normal gastric acid secretion, plasma electrolyte values and gastric pH. In conclusion, these results indicated that CA IX has an important role in the regulation of the gastric morphogenesis and the differentiation and proliferation of certain cells in the gastric mucosa of the stomach.

Microarray

DNA microarrays are small, solid supports onto which the sequences from thousands of different genes are attached, at fixed locations. The supports are usually tiny glass microscope slides, but can also be silicon chips or nylon membranes. The whole microarray hybridization process is based on probing the array with fluorescently labeled (red, green, blue) nucleic acid molecules (cDNA, generated from isolated mRNA) to identify complementary molecules, sequences that are able to base-pair with one another. Hybridized fluorescently labelled cDNA is detected by using laser technology.

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) measures the amount of PCR product (the target cDNA of the genes) after each PCR cycle. To detect the amount of the target cDNA after each PCR cycle fluorescent dyes, such as SYBR Green that bind to double stranded DNA (dsDNA), can be used. A computer-based program monitors the fluorescence signal during the reaction and the amount of the formed product correlates with the intensity of these fluorescent signals (Bustin 2002, Kubista et al. 2006).

In the beginning the fluorescence signal is very weak and cannot be distinguished from the background. The cDNA sequence starts to double in concentration with each cycle and the fluorescence signal level increases exponentially when it has become detectable above background. The cycle threshold (Ct) (crossing point, Cp) value represents the number of cycles needed to reach a set threshold fluorescence signal level. The background value is based on the relatively stable fluorescence level on the first cycles. To detect the fluorescence above background the threshold value is determined (Kubista et al. 2006)

The Ct values are obtained when the fluorescence from a sample crosses the threshold. By plotting fluorescence against cycle number on a logarithmic scale the relative concentrations of cDNA present during the exponential phase of the reaction can be determined. A standardcurve with a linear relationship between the Ct values and the starting amounts of template is generated by known concentrations of serially diluted PCR products. The concentrations of unknown samples based on their Ct values are determinated from the standard curve. In qRT-PCR procedure it is important to use housekeeping genes (control genes) to correct the variability in gene expression. Thus, appropriate use of housekeeping genes is an important step in normalization of the gene expression values (Bustin 2002, Kubista et al. 2006).

Housekeeping genes

The following control genes are always expressed in cells because they encode essential proteins that are constantly required by the cell. They are known as housekeeping genes and are widely used as housekeeping controls in qRT-PCR.

Beta-actin
This gene encodes actin which is the major component in cytoskeleton. Actin is a highly conserved protein that has been shown to exist in the nucleus. Nuclear actins have several functions such as transcription initiation, elongation and chromatin remodelling. Thus, these proteins are involved in the regulation of cell integrity, structure and motility (Tang et al. 2009).

Hypoxanthine guanine phosphoribosyl transferase 1
This gene is involved in the purine metabolism, in the formation of nucleotide monophosphates from purines and pyrimidines. It helps to recover the nucleosides and bases during the DNA and RNA degradation (Duan et al. 2004).

Succinate dehydrogenase complex, subunit A
This gene has functions in the respiratory chain and in the citric acid cycle. Indeed, this gene encodes a subunit of succinate dehydrogenase (SDH) that converts succinate to fumarate in the citric acid cycle. The SDH protein complex functions in the inner membrane of the mitochondria. Oxidation of succinate to fumarate is important for H+ transport in the respiratory chain. Thus, succinate dehydrogenase complex, unit A is an important regulator in essential metabolic pathways (Yankovskaya et al. 2003).

Aims

One of the aims of my research was to verify cDNA microarray results with qRT-PCR in gastric specimens obtained from CA IX deficient (*Car 9*$^{-/-}$) and control mice. The gene expression analysis varies between different molecular biological approaches. The sensitivity and accuracy of QRT-PCR method for analysing gene expression changes is very high therefore the microarray results were controlled with qRT-PCR. The major aim was to characterize the gene expression changes occurred within the gastric mucosa of 6 C57BL/6 mice due to the elimination of the *Car 9* gene. The first CA deficient mouse model (Ortova et al. 2002) created by targeted disruption of *Car 9* gene in C57BL/6 was published seven years ago. These *Car 9*$^{-/-}$ mice showed very interesting and restricted phenotype and the major phenotypic change involved stomach which is the main site of CA IX expression in normal mice. The *Car 9*$^{-/-}$ mice exhibited gastric hyperplasia and cystic changes within the gastric mucosa. According to previous studies *Car 9* deletion increased cell growth in the stomach of these mice and decreased significantly the production of parietal cells. Overall, according to previous studies, it could be expected in my research that the different genes will show either up or down regulated expression levels after the deletion of *Car 9*.

Results

One of the aims of this study was to characterize the gene expression changes that occurred within the gastric mucosa of six C57BL/6 knockout mice due to the disruption of *Car 9* gene. cDNA microarray analysis identified total of 69 target genes with changed expression levels in the corpus region of the stomach in *Car 9*$^{-/-}$ mice (Table 2.).

Microarray

The isolated RNA, from the stomach (corpus region) of 12 C57BL/6 mice (6 wild type mice (WT, *Car 9*$^{+/+}$), 3 male and 3 female and 6 knockout mice (KO, *Car 9*$^{-/-}$, 3 male and 3 female) was reverse transcribed into cDNA and sent to Finnish DNA Microarray Center in Turku for gene expression analysis. The mRNA expression data obtained from the Finnish DNA Microarray Center was analyzed by Chipster program to see if these genes were either up or downregulated in the stomach of the KO mice.

After deletion of *Car 9* a total of 25 genes showed upregulated expression levels while the expression of 44 target genes were decreased (Table 2). Eight important gene targets were selected for detailed quantitative assays and 3 housekeeping genes was used for normalization. genes was selected depending on their expression levels after deletion of *Car 9* $^{-/-}$ and their The different important functions in the stomach. These eight target genes are involved in essential immunological processes (*mIl1rl1*, *mSftpd*), regulation of intracellular pH (*mSlc9a3*), fatty acid absorption (*mPnlip*), cell-cell adhesion (*mPkp4*), cell proliferation (*mEgf*), proteolysis of proteins (*mEla2a*) and different nucleic acid processes (*mNonO*).

Table 2. Expression changes for 69 genes in the stomach of *Car 9* $^{-/-}$ mice.

Gene	GenBank	Fold Change [a] (FC)	P-value
NeoR		76,82	0
Slc9a3	XM_127434	8,07	0
LOC634449	XM_913205.2	10,46	0
Prss1	NM_053243	-10,57	1,00E-04
Try4	NM_011646	-12,14	1,00E-04
Amy2	NM_009669	-9,85	1,00E-04
Il1rl1	NM_010743	5,38	4,00E-04
Blm	NM_007550	-6,27	6,00E-04
9130204L05Rik	XM_130997	4,19	8,00E-04
Dmbt1	NM_007769	5,68	9,00E-04
Tm4sf5	NM_029360	4,26	0,001
Gdf9	NM_008110	-7,04	0,0011
OTTMUSG00000008911	XM_135581	-7,12	0,0024
U46068	NM_153418	5,95	0,0029
Sostdc1	NM_025312	-4,17	0,0033
Abpg	NM_194338	-3,67	0,0037
Sftpd	NM_009160	4,11	0,004
Slc38a5	NM_172479	-3,64	0,0046
Egf	NM_010113	-3,68	0,0046
Slc27a2	NM_011978	-3,80	0,0048
Tmed6	NM_025458	-4,07	0,0049

LOC100043836	XM_284375	**-3,41**	0,0049
Mug1	NM_008645	**-4,30**	0,0084
Pkp4	AK021168	**3,54**	0,0103
OTTMUSG00000016644	AK090072	**3,45**	0,012
Nono	NM_023144	**-3,37**	0,0137
1810007D17Rik		**-3,86**	0,0142
Ela2a	NM_007919	**-7,59**	0,0153
Nrn1	NM_153529	**-3,36**	0,0165
Gper	XM_355659	**-2,96**	0,018
LOC100045250	NM_177912	**2,88**	0,0188
Pnlip	NM_026925	**-6,23**	0,0192
Sprr1a	NM_009264	**3,30**	0,0238
Bhlhb8	NM_010800	**-2,77**	0,027
Ly6d	NM_010742	**2,69**	0,0319
1190020J12Rik	XM_133360	**3,81**	0,0328
Sycn	NM_026716	**-2,91**	0,0448
Cfd	NM_013459	**-5,35**	0,0451
Scd1	NM_009127	**-3,27**	0,0452
Chia	NM_023186	**-3,52**	0,0468
Ivl	NM_008412.2	**3,08**	0,0482
Slc5a8	NM_145423	**-2,68**	0,0501
Adipoq	NM_009605	**-3,74**	0,0515
Car3	NM_007606	**-3,72**	0,0519
Car3	NM_007606	**-2,94**	0,0636
Spink3	NM_009258	**-3,32**	0,0707
Isg15	NM_015783	**2,44**	0,0739
Oas1g	NM_011852	**2,15**	0,1051
Ela3	NM_026419	**-3,48**	0,1086
Cpb1	NM_029706	**-2,73**	0,1115
Cyp2e1	NM_021282	**-2,22**	0,1116
BAI3	XM_284174	**-3,95**	0,1154
Prss1	NM_053243	**-2,42**	0,1175
Il33	NM_133775	**2,15**	0,1197
Sprr2i	NM_011475	**3,31**	0,1199
Gcg	NM_008100	**-2,27**	0,1201
Dbp	NM_016974	**-2,08**	0,1208
Ctrb1	NM_025583	**-5,00**	0,1227
Ctrl	NM_023182	**-3,19**	0,1251
Trex2	NM_011907	**2,11**	0,1306
Cpa2	XM_133021	**-2,32**	0,1337
5430420C16Rik	NM_175165	**2,35**	0,1425
Igh-6	XM_354710	**-3,13**	0,1556
LOC640340	XM_001006032.1	**-2,31**	0,1784
Lep	NM_008493	**-1,93**	0,1899
LOC330581	XM_144778	**-1,90**	0,1912
Sprr2d	NM_011470	**3,46**	0,1915
Mup1	NM_031188	**2,25**	0,1958
Igh-VJ558	XM_354700	**1,97**	0,1978

a: Red and blue colours indicate up- and downregulation, respectively.

Verification of over- and underexpression

Polymerase chain reaction and Chipster

Conventional reverse transcription polymerase chain reaction (RT-PCR) was performed to test the quality of the selected primer pairs. These primer pairs had a good quality according to RT-PCR. and After that the microarray data analyzed by Chipster was verified by highly sensitive and efficient quantitative real-time PCR (qRT-PCR). 3 control genes (*mActb*, *mHPRT-1* and *mSDHA*) were studied as housekeeping genes to normalize the variability of the gene expression data. After verifying the Chipster results by qRT-PCR there were totally 4 genes with downregulated values (*mEgf, mEla2a, mNono and mPnlip*) and 4 genes with upregulated values (*mIl1rl1, mPkp4, mSlc9a3 and mSftpd*) (Table 3.). The expression levels of *mPkp4* and *mPnlip* between WT and KO mice showed no significant changes and therefore H_0 was accepted (data not shown).

Table 3.The gene expression changes after the deletion of *Car 9* gene.

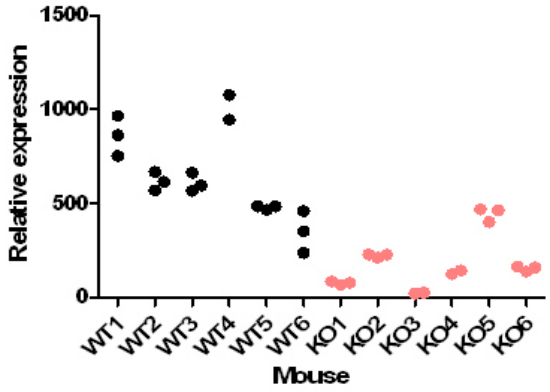
Gene	GenBank (Accession.)	Chipster (FC) [b]	qRT-PCR (FC)	P-value [c]
mEgf	NM_010113	-3,68	-3,71	0,004
mEla2a	NM_007919	-7,59	-16,23	0,004
mIl1rl1	NM_010743	5,38	8,95	0,002
mNonO	NM_023144	-3,37	-1,24	0,015
mPkp4	NM_026361	3,54	1,09	-
mPnlip	NM_026925	-6,23	-23,88	-
mSftpd	NM_009160	4,11	3,69	0,009
mSlc9a3	NM_001081060	8,07	10,72	0,002

b FC=Fold change

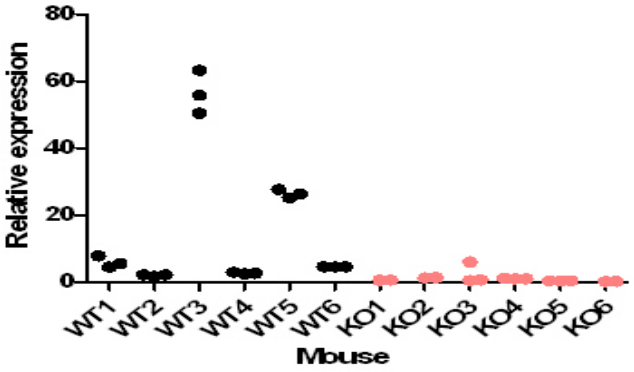
c The Mann-Whitney test was used to analyze the statistical significance. Statistically significant gene expression changes were accepted at the $P < 0.05$.

The Mann-Whitney test showed that 6 of 8 target genes had statistical significant changes in their expression levels after disruption of *Car 9*, among which 3 of them *mEgf*, *mEla2a* and *mNono*, were downregulated and 3 of them *mIl1rl1, mSlc9a3* and *mSftpd* were upregulated.

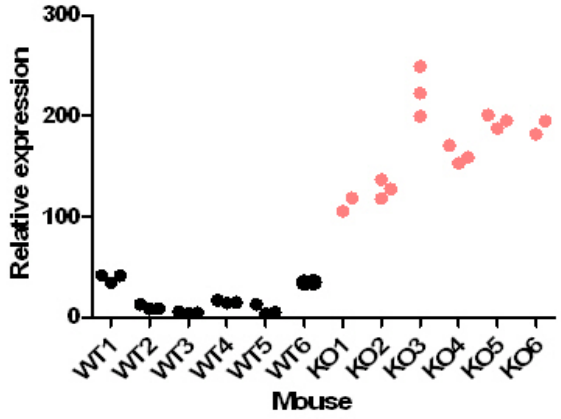
The actual values for the relative gene expressions in the six mice in each group (wild type and knockout) are shown in Fig.3.



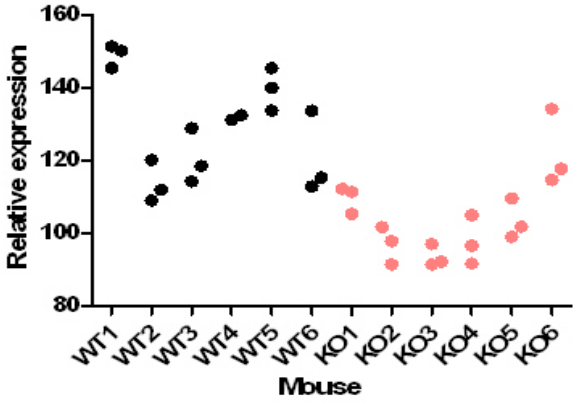
(A) ***mEgf***



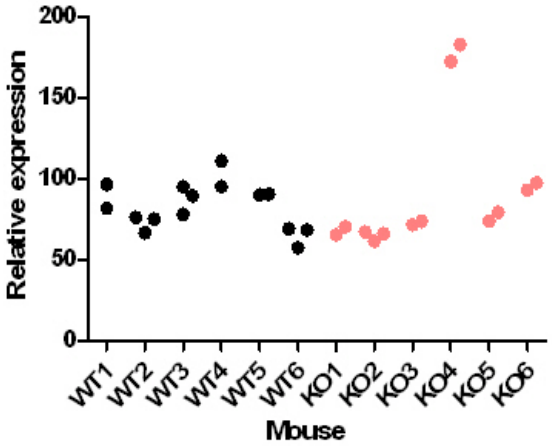
(B) ***mEla2a***



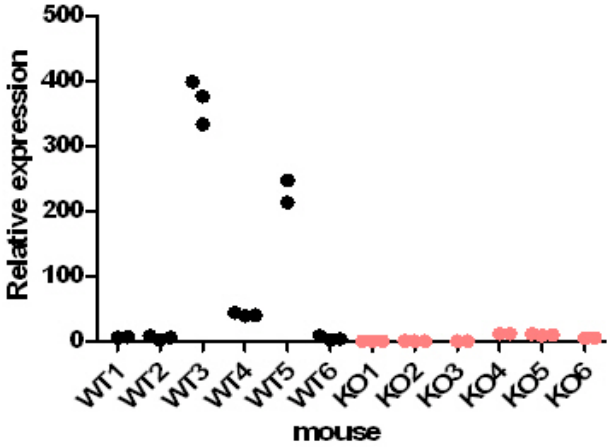
(C) ***mIl1rl1***



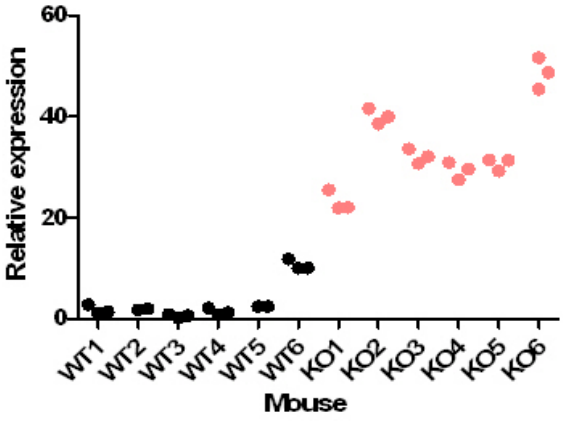
(D) ***mNonO***



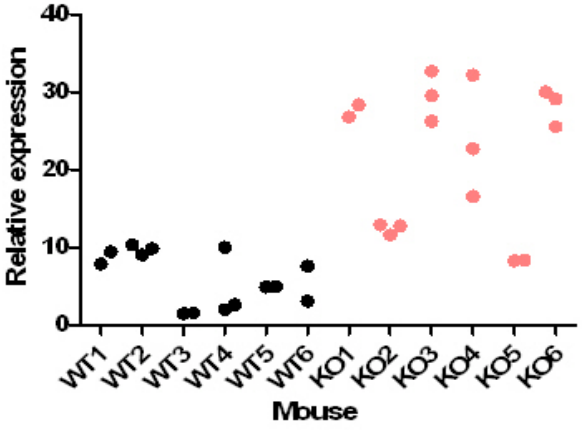
(E) ***mPkp4***



(F) ***mPnlip***



(G) ***mSlc9a3***



(H) ***mSftpd***

Figure 3. A-H, shows the relative expression of the studied genes in *Car 9* $^{-/-}$ as compared to in *Car* $9^{+/+}$ mice gastric mucosa. Expression levels determined in triplicate by qRT-PCR were normalized with respect to expression of 3 housekeeping genes encoding beta–actin (*mActb*), succinate dehydrogenase complex, subunit A (*mSDHA*) and hypoxanthine guanine phosphoribosyl transferase (*HPRT-1*). These relative expressions of certain genes are shown with black and pink colour among 6 WT; wild type and 6 KO; knockout mice, respectively. (A) *mEgf;* p=0.004, (B) *mEla2a*; p=0.004, (C) *mIl1rl1*; p=0.002, (D) *mNono*; p=0.015, (E) *mPkp4*; not statistically significant, (F) *mPnlip*; not statistically significant, (G) *mSlc9a3*; p=0.002 and (H) *mSftpd*; p=0.009.

Overall, the results show stronger individual variations in the gene expression of *mEgf*, *mIl1rl1*, *mNonO*, *mSlc9a3 and mSftpd* while the gene expression levels of *mEla2a*, *mPkp4 and mPnlip* were more constant.

Discussion

In this study, changes in gene expression due to the deletion of *Car 9* were studied in the corpus region of the stomach in 12 C57BL/6 mice. The apparent mRNA expression changes of 8 genes, were validated by qRT-PCR. Interleukin 1 receptor-like 1 (*mIl1rl1*), Solute carrier family 9, member 3 (*mSlc9a3*) and Surfactant associated protein D (*mSftpd*) were up-regulated, while the expression level of Plakophilin 4 (*mPkp4*) and Pancreatic lipase (*mPnlip*) was unaffected by deletion of the *Car 9* gene (data not shown). The Epidermal growth factor (*mEgf*), Elastase 2a (*mEla2a*) and Non-POU-domain-containing, octamer binding protein (*mNono*) genes were downregulated in *Car 9*$^{-/-}$ mice.

Accuracy of the quantitative real-time polymerase chain reaction

qRT-PCR values of the different gene expression changes were quite similar compared to values observed from Chipster analysis even though these methods are technically clearly different. The results show (Table 3.) that there were few genes with markedly higher FC values in qRT-PCR compared to those in Chipster analysis. These genes were *mPnlip*, *mPkp4* and *mEla2a*. In most cases, the FC values obtained by Chipster analysis were lower than those determined by qRT-PCR (Table 3.). Thus, the gene expression analysis varies between different molecular biological approaches. The sensitivity and accuracy of qRT-PCR for analysing gene expression changes is very high, and therefore this method could be considered more reliable than the Chipster analysis.

Interleukin 1 receptor-like 1

This gene is also known as ST2 and has been reported to be involved in the function of helper T-cells (Th), and therefore contributes to different immunological processes (Hayakawa et al. 2007, Trajkovic et al. 2004). The ST2 protein consists of a longer membrane bound form (ST2L) and a shorter secreted form (sST2). In addition to several other cells, the mast cells and fibroblasts have shown ST2 gene expression (Hayakawa et al. 2007). The expression of ST2L has been found in T helper type 2 cells, primary in macrophages, while the signals of the sST2 expression have been detected in fibroblasts, embryonic tissues and mammary tumours (Hayakawa et al. 2007, Trajkovic et al. 2004).

Both of these forms have special functions in immunological processes (Trajkovic et al. 2004). It is interesting that the ST2 protein has negative as well as positive feedback in the regulation of immunological and inflammatory responses. ST2L is strongly related to Th2 responses such as the production of inflammatory cytokines IL-4 and IL-5. These cytokines activate Immunoglobulin E (IgE) which dominates in allergy and parasitic infections (Trajkovic et al. 2004). IL-4 is required for Th2 development from helper T cells. Th2 cells express ST2L form and upon binding its putative ligand (ST2 binding protein) located on B lymphocytes, dendritic cells or other cells ST2L starts to send signals which help the proliferation of Th2 cells. This signal cascade induces also IL-4 and IL-5 release and helps to suppress the Th1 response. Thus, the ST2L form is responsible for maintaining this positive feedback in immunological processes (Trajkovic et al. 2004).

The soluble form of ST2 (sST2) has a special anti-inflammatory role in immunological responses. Macrophages express TLR4 (Toll-like receptor 4). These receptors attach to lipopolysaccharides (LPS) and activate macrophages which in turn produce pro-inflammatory cytokines such as IL-1, IL-6 and TNF (Trajkovic at al. 2004). These cytokines prevent inflammation in cell tissues. The activation of sST2 is maintained by fibroblasts or other cell types. When sST2 proteins bind to macrophages they inhibit the pro-inflammatory response and may down-regulate the TLR4

receptors. Thus, the sST2 form is involved in the maintainance of the negative feedback in immunological processes (Trajkovic et al. 2004). The relatively high fold change level (8,95) of this gene raised the question of whether *mIl1rl1* could activate the immunological system negatively or positively in C57BL/6 *Car9*$^{-/-}$ mice. Overall, the increased levels of *mIl1rl1* RNA in *Car 9*$^{-/-}$ mice show that the disruption of *Car 9* is associated with the functions in immunological responses. Thus, it could probably be considered that the increased expression of *mIl1rl1* is related positively or negatively to the immunological responses in C57BL/6 mice.

Solute carrier family 9, member 3

Solute carrier family 9, member 3, *Slc9a3* is an isoform (NHE3) of an Na^+/H^+ antiporter in the intestine and kidney. The regulation of intracellular pH is one of the important functions maintaining the cell homeostasis. It has been reported (Igarashi et al. 2002, Schultheis et al. 1998) that *Slc9a3* has a special role in intracellular pH regulation through the transepithelial transport and reabsorption of Na^+ (into the cell) and HCO_3^- (to blood) in intestinal epithelial cells and the renal proximal tubule. It has also been shown that NHE3 mediates electroneutral NaCl absorption in the gastric mucosa (Bachmann et al. 2003, Igarashi et al. 2002, Schultheis et al. 1998).

The highest upregulation level was detected for *mSlc9a3* expression. Schultheis et al. (1998) reported that *Slc9a3*$^{-/-}$ mutant mice were mildly acidotic, their blood pressure was reduced, they had absorptive defects in the intestine, fluid and HCO_3^- absorption were reduced in the proximal convoluted tubule. Increased epithelial Na^+ channel activity in the colon was detected and mRNA of H^+ K^+ ATPase was induced in the colonic area. In addition, the secretion of aldosterone was increased which means that the reabsorption of Na^+ and K^+ were increased in the kidney. The increased Na^+ and K^+ ion concentration caused strong reabsorption of H_2O in the kidney, resulting in diarrhoea among these mice (Schulteis et al. 1998). In conclusion, these arguments showed the fact that the acid-base balance and the homeostasis of Na^+ fluid volume is impaired when *Slc9a3* gene is disrupted (Schultheis et al. 1998). It could be speculated that the upregulation of this gene in intestine and kidney probably would cause a major changes in metabolic acidosis by lowering the luminal pH. This could be related to the acidification of microcellular environments. Moreover Bachmann et al. (2004) reported that NHE3 is activated by low intracellular pH.

Surfactant associated protein D

Surfactant associated protein D (*mSftpd*, SP-D) is a collagenous glycoprotein containing a C-type lectin domain. This protein forms homotrimers. It binds to ligands that consist of lipids and carbohydrates by its carbohydrate recognition domain (CRD) (Leth-Larsen et al. 2005, Mason et al. 1998). According to Murray et al. (2002) and Leth-Larsen et al. (2005) SP-D protein can bind to Gram-positive and Gram-negative bacteria such as *Helicobacter felis* and *Helicobacter pylori* via CRD attaching to lipopolysaccharides (LPS) that are present on the surface of these bacteria

Also the surfactant associated protein expression was increased in *Car 9*$^{-/-}$ mice. After infection of *Helicobacter pylori* (*H. pylori*) related *H. felis* to insulin/gastrin (INS-GAS) transgenic mice Kobayashi et al. (2007) showed that SP-D was found in different parts of the GI tract like in a deeper part of the gastric epithelial linings and on the gel layer of the surface mucosa and these parts were colonized by *H. felis*. This finding supported the previous studies that SP-D binds to Gram-negative bacteria. Interestingly, SP-D could be a possible protector against this kind of bacterial infection by binding to bacteria which could enhance the activity of macrophages and neutrophils during the killing and phagosytosis of the cells. This means that SP-D could be involved in the inhibition of the progressive helicobacter infections. Moreover, SP-D has shown to be

upregulated in *H. pylori*-induced gastritis (Namavar et al. 1998, Kobayashi et al. 2002). This could probably indicate that the increased *Sftpd* expression due to the deletion of *Car 9* gene may be related to infections by Gram-negative and Gram positive bacteria in gastric environments. Still, further studies are needed to verify these speculations.

SP-D is predominantly synthesized in the alveoles of the lungs as well as in the epithelial cells of the mucosa in the gastrointestinal tract. According to Ikegami et al. (2007) there is a linkage between lipopolysaccharide (LPS)-induced pneumonia and SP-D protein. Addition of exogenous human SP-D to *Sftpd*$^{-/-}$ deficient mice decreased the LPS-induced pneumonia among these mice. They reported also that the overall levels of the proinflammatory cytokines were lower in *Sftpd*$^{+/+}$ mice compared to *Sftpd*$^{-/-}$ mice. It has been reported (Kobayashi et al. 2007) that this innate immune protein plays a special role during hyperplasia by regulating surfactant metabolism. Expression of SP-D protein in adenocarcinoma and hyperplasia has been demonstrated immunohistochemically (Kobayashi et al. 2007). In addition Leth-Larsen et al. (2005) reported that SP-D is related to immune response to the different pathogenic molecular patterns and therefore could be a useful biomarker for some diseases. Since these studies have shown that *Sftpd* gene is related to bacterial infections it could be speculated that the increased levels of *Sftpd* in *Car 9*$^{-/-}$ deficient mice could decrease the levels of proinflammatory cytokines in these mice.

Pancreatic lipase

Expression of Pancreatic lipase, *mPnlip* was not affected by disruption of the *Car 9*. Pancreatic lipase is produced by pancreas and related to food digestion. *Pnlip* can hydrolyze triglycerides to fatty acids and therefore is an essential factor in intestinal fatty acids absorption. According to Muramatsu et al. (2005) there could be a linkage between *Pnlip* expression and obesity in the Otsuka Long-Evans Tokushima Fatty rats (OLETF). Muramatsu et al. (2005) showed that the mRNA levels of the *Pnlip* increased in the pancreas of OLETF rats.

Plakophilin 4

The deletion of *Car 9* did not change the expression of *Pkp4* in *Car 9* $^{-/-}$ mice. Desmosomes, especially their extracellular cadherin regions, are essential for epidermal cell-cell adhesion by forming a linkage between epithelial plasma membranes. The cytoplasmic cadherin regions are associated with these extracellular regions with intermediate filament system through different interactions with desmoplakin, plakoglobin and plakophilin which are bound to keratin filaments in the cytoskeleton (Setzer et al. 2004). Desmosomes are mainly expressed in tissues that are exposed to mechanical stress, such as skin and heart. *Pkp4* is an important member of armadillo proteins and is related to the desmosomal proteins (plakophilins) as well as adherence junction protein (Setzer et al. 2004).

Plakophilin 4, *mPkp4* has reported to be involved in cell-cell adhesion by binding to adherence junctions and desmoplakins in desmosomes (Setzer et al. 2004). This suggests that *mPkp4* could be strongly related to the regulation of the molecular cross talk that may occur between different intercellular junctions thus, influencing the cellular events regulated by intermediate filament networks. Setzer et al. (2004) reported also that an addition of extracellular Pkp4 to epithelial cells caused a loss of plakoglobin and retraction of keratin filament. Overall, *Pkp4* seems to promote adherens junction assembly and dissasembly of desmosomes and inhibition of epithelial cell migration (Setzer et al. 2004).

Epidermal growth factor

The expression of epidermal growth factor, *mEgf* was significantly decreased after deletion of *Car 9* gene in C57BL/6 mice. Egf is produced by salivary glands and absorbed mainly through the oral cavity and gastrointestinal tract (Clarke et al. 2001). It has been reported (Clarke et al. 2001) that Egf is related to wound healing of the soft tissues and skin by the production and secretion from cells at wounded areas. According to Clarke et al. (2001) the disruption of *Egf* affect negatively to the cell proliferation of the liver. Moreover, it has been shown that *Egf* plays a major role in cell differentiation, gene expression and cell migration (Clarke et al. 2001). According to these studies the decreased levels of *Egf* could affect negatively the epithelial cell proliferation in different tissues especially in the GI tract were it is absorbed. Indeed, Ortova et al. (2002) reported that *Car 9* disruption led to depletion of pepsinogen producing chief cells in the GI tract. This could possibly support the fact that decreased *Egf* expression may be associated with the depletion of pepsinogen producing chief cells due to the disruption of *Car 9* gene.

Elastase 2a

Elastase 2a (*mEla2a*) gene is also known as neutrophil elastase and can hydrolyze different proteins. Ela2a is a member of serine proteases, which may have an important role in inflammatory and other degenerative diseases by the proteolysis of proteins such as elastin and collagen of the extracellular matrix (Belaaouaj et al. 2000). Elastin and collagen are important for regulating mechanical properties in connective tissues. It has been reported that neutrophil elastase has an antimicrobial role in inflammatory responses (Belaaouaj et al. 2000). During bacterial infections, neutrophil elastase attacks gram-negative bacteria such as *Escherichia coli* and kills them by degrading their outer membrane protein (OmpA) (Belaaouaj et al. 2000). Belaaouaj et al. (2000) also reported that lack of netrophil elastase could cause pulmonary (lung) disorders.

mEla2a was strongly downregulated in *Car 9*$^{-/-}$ mice. According to Belaaouaj et al. 2000 *Ela2a* is important during the formation of essential amino acids by hydrolyzing different proteins. It can be speculated that the decreased *Ela2a* in gastric microenvironment among *Car 9*$^{-/-}$ knockout mice may cause a depletion of valuable amino acids needed for cell proliferation. Ortova et al. (2002) reported that the deletion of *Car 9* gene in C57BL/6J mice led to the reduction of certain cells in the gastrointestinal tract. Thus, it would be interesting to know if the decreased *Ela2a* expression could be associated with depletion of pepsinogen producing chief cells or other mucus associated cells in *Car 9*$^{-/-}$ mice.

Non-POU-domain-containing, octamer binding protein

NonO genes have been identified in the nuclear matrix, nucleolus and nuclear membrane. Their products, p54^{nrb} (in humans) and NonO (in mouse) proteins are found as monomers as well as heterodimers in nuclear complexes during various nucleic acid processes such as during the regulation of pre-mRNA splicing, DNA pairing and transcriptional repression or activation (Shav-Tal et al. 2002).

The expression of *mNonO* was moderately reduced in *Car 9*$^{-/-}$ mice. This gene encodes p54^{nrb} among humans and NonO among mice. Both of these proteins have the same functions (Shav-Tal et al. 2002). In addition, it has been reported that p54^{nrb}/ NonO has similar enzymatic activity as carbonic anhydrase (CA) enzymes and immunological similarity with CA II (Karhumaa et al. 2000). Nucleic acid processes are essential for cells. During the cell proliferation these processes are

needed for DNA duplication and amino acid production. The decreased expression of *mNonO* could indicate that some of these processes may be altered in the GI tract due to the disruption of *Car 9*.

Compared to CA enzymes, $p54^{nrb}$/ NonO do not have histidin residues which are important for Zn^{2+} binding in enzymes. Karhumaa et al. (2000) observed that the expression of $p54^{nrb}$/ NonO was strongest in the colon, spleen, liver and ovary of mice. In most cases nuclear proteins bind to either DNA or RNA binding domain whereas $p54^{nrb}$/ NonO proteins have an exceptional ability to bind both of these regions. Indeed, they could be regarded as linking proteins during multiple nuclear processes. There have been observations about the modified expression of the multi-functional $p54^{nrb}$/ NonO protein in differentiating cells and in cancer cells such as papillary renal cell carcinoma cells (Karhumaa et al. 2000, Shav-Tal et al. 2002). Moreover, Ortova et al. (2002) reported that the absence of functional CA IX enzyme was linked to abnormal morphogenesis of the gastric mucosa. Overall, the decreased levels of this important $p54^{nrb}$/ NonO protein in *Car 9*$^{-/-}$ mice could possibly be one reason for the changes in gastric morphogenesis.

Thus, it is very surprising to notice that the expression of so many various genes changed after the deletion of *Car 9* gene in C57BL/6 knockout mice. All of these genes are physiologically important, and therefore, altered gene expression levels in these knockout mice may cause several problems in pH regulation, immunoresponses, as well as in tissue morphogenesis involving both the cell proliferation and the cell differentiation. In further studies it would be interesting to understand how these important genes are related to the function of CA IX enzyme.

Materials and methods

Mice
All of the 6 wild type, WT (3 male, m and 3 female, f) and 6 *Car 9*$^{-/-}$, KO (3m, 3f) mice were of a C57BL/6 strain. The WT mice were 8-12 months old and the KO mice were 10-12 months old. The mice were housed in the animal care center of the university of Oulu (Finland).

RNA extraction and first strand cDNA synthesis
Total RNA from the stomach tissue specimens of these mice had been extracted in 2008 by MSc Heini Kallio, in prof. Seppo Parkkila's group at the University of Tampere (Finland) using the RNeasy RNA isolation kit (Qiagen, Valencia, USA) following the manufacturer's instructions. Residual DNA was removed from the samples using RNase-free DNase (Qiagen) (10 µg DNase + 70 µg RDD buffer). The RNA concentration and purity was determined by measurement of the optical density at 260 and 280 nm. 3 µg of RNA was converted into first strand cDNA using a First Strand cDNA synthesis kit (Fermentas, Burlington, Canada) using random hexamer primers according to the protocol recommended by the manufacturer.

cDNA microarray
Microarray studies were performed in the Finnish DNA Microarray Centre at Turku Centre for Biotechnology. The samples were analyzed individually. 400 ng of total RNA from each sample was amplified using the Illuminas RNA TotalPrep Amplification kit (Ambion) following the manufacturer's instructions. The in vitro transcription reaction, which was conducted for 14.5 h, included labelling of the cRNA by biotinylation, a fluorophore, which emit light at blue wavelength. Both RNA and cRNA concentrations were checked with Nanodrop ND-1000 and the quality was controlled by BioRad´s Experion electrophoresis station. 1.5 µg each cRNA sample was hybridized to Illumina´s Sentrix Mouse-6 Epression Bead Chips (Illumina, Inc., San Diego, CA) at 58 °C over night (18 h) according to manufacturer's instructions. Hybridization was detected with 1 µg/ml Cyanine 3-streptavidine. Chips were scanned with Illumina Bead Array´s Reader and the numerical results were extracted with Bead Studio. Microarray analysis was performed using Chipster software. (http://Chipster.sourceforge.net/index.shtml visited 09-07-28, Microarray: http://www.illumina.com/downloads/mouse68.pdf visited 09-08-11)

Polymerase chain reaction

Conventional reverse transcription polymerase chain reaction
After the RNA extraction, first strand cDNA synthesis and cDNA microarray the genes *mEgf, mEla2a, mIl1rl1, mNono, mPkp4, mPnlip, mSftpd* and *mSlc9a3* were amplified by PCR. All of the primer sequences for these genes are shown in Table 4. Every PCR reaction consisted of 45 µl Reddy Mix 1,5 mM $MgCl_2$ (Thermoprime Plus DNA polymerase, Tris-HCl, $(NH_4)_2SO_4$, $MgCl_2$, Tween® 20, dNTP mix), 1 µl forward primer, 1 µl reverse primer, 1 µl ddH_2O and 3 µl cDNA template (sample gene). The PCR program was: 96°C 5 min followed by 35 cycles of: 96°C 30s, xx°C 60s and 72°C 60s. Table 2 shows annealing temperatures (xx°C) for each primer pair. After PCR the DNA products were analyzed on 1.5% agarose gel ((BIO-RAD), 1xTAE buffer; 40mM Tris acetate and 1mM EDTA)) containing 0,01 µM of ethidiumbromide (SIGMA) for staining the gel. The gel electrophoresed at 90 V, 400 A, 40 min. Afterwards the gel was photographed and the DNA was cut and purified from the gel using recommendations (GE Healthcare illustra GFX™ PCR DNA and Gel Band Purification kit, Protocol 5.4) according to manufacturers. DNA concentrations were measured by NanoDrop.

Quantitative real-time polymerase chain reaction

Real-time PCR primers were designed based on the complete cDNA sequences deposited in GenBank. The house-keeping genes *mActb, mSDHA, mHPRT-1* were used as internal controls to normalize the cDNA samples for possible differences in quality and quantity. In order to avoid amplification of genomic DNA, the primers from each primer pair were located in different exons whenever possible. The primer sequences are shown in Table 4.

Table 4. Primer sequences for quantitative real-time PCR used in the study.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	PCR size (bp)	T_a [f] (°C)	Accession no. (8 genes) Identification no. (3 housekeeping genes)
mEgf	GCTCGGTGTT TGTGTCGTG	CTGTCCCATC ATCGTCTGG	163	55	NM_010113
mEla2a	TGATGTGAGC AGGGTAGTTGG	CACTCGGTAG GTCTGATAGTTG	178	60	NM_007919
mIl1rl1	ATTCTCTCCAG CCCTTCATC	AAGCCCAAA GTCCCATTCTC	180	55	NM_010743
mNono	GGCTCCTTCTT GCTGACTAC	CTGGGTGGCT GTTCTCTCTC	137	58	NM_023144
mPkp4	GAACATAACCA AAGGCAGAGG	GGTGGACAGA GAAGGGTGTG	196	58	NM_026361
mPnlip	CCCGCTTTCTCC TCTACACC	TCACACTCTC CACTCGGAAC	192	58	NM_026925
mSftpd	CCAACAAGGAA GCAATCTGAC	TCTCCCATCC CGTCCATCAC	186	58	NM_009160
mSlc9a3	TGACTGGCGTG GATTGTGTG	ACCAAGGACA GCAGGAAGG	100	56	NM_001081060
mActb [d]	AGAGGGAAATC GTGCGTGAC	CAATAGTGATG ACCTGGCCGT	138	57	634
mHPRT-1 [d]	AGCTACTGTAAA TGATCAGTCAACG	AGAGGTCCTTT TCACCAAGCA	198	55	10050
mSDHA [e]	GCTTGCGAGCT GCATTTGG	CATCTCCAGTT GTCCTCTTCCA	145	53	15030102a2

d obtained from Quantitative PCR Primer Database QPPD http://web.ncifcrf.gov/rtp/gel/primerdb/
e obtained from Primer bank http://pga.mgh.harvard.edu/primerbank/
f T_a: annealing temperature

Each qRT-PCR reaction was performed in a total volume of 20 µl containing 0.5 µl of first strand cDNA, 1x of QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) and 0.5 µM of each primer. The amplification and detection were carried out by Lightcycler detection system (Roche, Rotkreuz) as follows: initial 15 min. activation step at 95 ^{o}C, 45 cycles of: 95 ^{o}C 15 s, annealing at a temperature determined according to the T_m for each primer pair for 20 s (Table 4.), 72 ^{o}C 15 s (the ramp rate was 20 ^{o}C/s for all the steps), and a final cooling step at 40 °C 30 s. Melting curve analysis was always performed after the amplification to check the specificity of the qRT-PCR reaction. A standard curve was established for each gene using five-fold serial dilutions of known concentrations of purified PCR products generated with the same primer pairs. Every cDNA sample was tested in triplicate, and the obtained crossing point (Cp) value permitted the levels of the starting mRNA to be determined using the specific standard curve. The mean and standard deviation (SD) of the triplicate values was calculated. The SD cutoff 0.2 was used so that when the SD was above 0.2 the outlaying value was removed. This ensures the reliability of the results (Larionov et al. 2005). The geometric mean of the three internal control genes *mActb*, *mHPRT-1* and *mSDHA* was used to normalize gene expression levels. The final relative mRNA expression was indicated as the copy number of the target mRNA divided by the corresponding normalization factor and multiplied by 10^3.

Statistical analyses

The Mann-Whitney test was used for statistical analyses to analyze differences between the gene expression levels in WT and KO mice. The null hypothesis (H_0) was that the gene expression values do not change between WT and KO mice. H_1 was that the gene expression levels change between WT and KO mice. The H_0 was accepted if the statistical probability (P) that the data did not show differences between WT and KO mice was $P \geq 0.05$.

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