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EXAMINATION OF THE BOVINE IgG TRANSPORTER FcRn RECEPTOR EXPRESSION IN VIVO IN TRANSGENIC MOUSE MODEL

Thesis

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1. INTRODUCTION AND GOALS

1.1. History

Among immunoglobulins (Ig) the most important and also the most abundantly present in blood circulation is the antibody – IgG –which protects against viruses, bacteria, or parasitic infections. Following an encounter of IgG with an antigen, allergic and inflammatory reactions are triggered, which activates the immune cells and the extravasation process directs them to the desired tissue location. Only one type of Fc receptors is known, which is expressed in the epithelial cells. Namely, the receptor for MHC type I Fc receptor (FcRn), which transports through different epithelial barrier IgG, and in some species participates in the maternal IgG transport to newborns. The FcRn is a heterodimer, consists of an α-chain and a β2-microglobulin. The FcRn present in the epithelial cells transports IgG, while in the endothelial cells it plays a role in IgG homeostasis. Kacskovics and his colleagues cloned the bovine FcRn α-chain, and its expression was shown with Northern analysis in the small intestine and the mammary gland (Kacskovics et al. 2000). Mammals ensure through complex processes the transport of IgG pre-and postnatally (maternal immunity), and the permanently high blood levels of IgG. Based on our knowledge the FcRn receptor is in charge of both activities.

In order to analyze the *in vitro* identified regulatory elements, an *in vivo* transgenic animal is the theoretically most accurate way of modeling the tissue and developmental specific gene expression, as well as the level of protein expression. The traditional transgene constructs in transgenic animals are the optimal combinations of regulatory elements and the coding region of the protein of interest, however they show frequently integration site-dependent gene expression, which is often associated with ectopic gene expression (expression in a tissue where the endogenous gene was not expressed) and with variable amounts of gene product. A possible solution would be the so-called „knock in" transgenic animal in which the coding region of the FcRn gene would be replaced with an indicator gene and this transgenic animal would serve as a model to examine the role of regulatory factors (e.g. hormones) in the FcRn receptor tissue- and developmental stage-dependent expression. However transgenic cattle, to that aim has not been produced worldwide. Mouse FcRn receptor could be an appropriate model since cattle (ruminant) FcRn receptor has ruminant specific features. Therefore, to our best knowledge, the optimal solution to answer the questions above is the creation of a transgenic mouse model, which carries the whole

expression domain of the bovine FcRn receptor α-chain, and therefore the transgene will show copy number dependent, integration site independent expression.

Artificial chromosomes or artificial chromosome-type vectors (YAC, BAC, PAC) are suitable for cloning large genomic fragments. At the beginning of my experiments, it was a novel pioneering way of creating transgenic animals. Since FcRn is a heterodimer it should be pointed out, that in our experimental model, the bovine alpha chain and the mouse light-chain will compose the receptor. Question arose whether such a hybrid molecule preserve its function? Based on earlier data obtained in *in vitro* rat cell lines, we assumed that the bovine FcRn α-chain will form functional receptor with the mouse β2 microglobulin, since the rat and the mouse β2 microglobulin are higly similar. We also hoped that this model helps to reveal so far unexplored physiologically important characteristics of the bovine Fc receptor.

To underline the effectiveness and usefulness of BAC transgenesis I also present data on creating the human glycine transporter 1 (SLC6A9)-expressing transgenic mouse line.

1.2. Goals

- To create a transgenic mouse model to study the bovine FcRn receptor (bFcRn) function *in vivo*.
- To determine the integrated transgen copy numbers and characterize the transgenic animals at protein and nucleic acid level, revealing physiological differences among the lines and analyzing the integration site and copy number dependency of the gene expression.
- To establish a functional marker system to prove the biological activity of the heterodimer receptor.
- To breed a double transgenic mouse model, without the mouse endogenous Fc receptor, for direct analysis of the bovine Fc receptor.

2. MATERIALS AND METHODS

2.1. Gene construction production

Three bovine BAC clones, which harbour the whole FCGRT gene (the FcRn coding gene) were available. The 90α BAC clone was originated from a BAC library created from a 2-years old Jersey cattle lymphocytes (Resource Center / Primary Database of the German Human Genome Project www.rzpd.de). The 189H02 and 128E04 BAC clones were isolated from an independent BAC library (Eggen et al. Gen. Sel. Evol. 33543-548. 2001). Following *BamH*I digestion of the 90α BAC clone and *Not*I digestion of the 189H02 and 128E04 clones, pulse field gel-electrophoresis (BIO-RAD) and Expand Long Template (ELT) PCR (Roche) were adapted to determine the insert and the 5' and 3' bordering region's sizes. In case of the 128E04 BAC clone neither the 5' nor 3' size could be determined. With this method a maximum of about 20 kb long regions could be amplified, which ment that this clone in both directions from the FCGRT gene is longer than 20 kb. The use of 90α clone was rejected because the 5' boundary region was only 8.5 kb long. Based on the ELT-PCR data, the size of the BAC 189H02 clone is around 130 kb, and the 128E04 is about 100 kb. The exact size of the BAC clones could only be determined later when the cattle genome database became publicly available (www.ncbi.nlm.nih.gov). As some pieces of the 5' and 3' end sequence of the contigs of the 128E04 clone was already sequenced, I could BLAST these sequences against the bovine genome library, and could determine precisely the total size of the 128E04 BAC clone (102 kb). As the characterization of the three BAC clones showed that the 128E04 BAC clone harboured the FCGRT gene at the most optimal configuration, we chose this BAC clone to be injected. The 128E04 BAC clone was grown overnight in LB at 37 °C overnight and isolated with Plasmid Maxi Kit (Qiagen) according to manufacturer's instructions. Subsequently, it was submitted to digestion with *Not*I restriction enzyme (Promega) and the restriction fragments were separated in 1% PFCA gel (BIO-RAD), 0.5 x TBE buffer at 14 °C for 12-hour-lock PFGE (Bio-Rad). The insert was isolated from the gel and re-run in a 1% Low Melting Point gel and the desired fragment was again isolated and digested with Gelase enzyme (Epicentre), cleaned with Microcom YM50 column (Millipore) and eluted with 1x injection buffer (10 mM Tris, 0.1 mM EDTA, 100 mM NaCl, 1x polyamine mix, pH 7.5). Concentration of the isolated fragment was verified with gel electrophoresis.

2.2. Creation of BAC transgenic mouse lines and analysis of transgene integration

The creation of transgenic animals by genetic manipulation at early embryonic stage (one cell) was performed by microinjection with Nomarski differential interference contrast and 3 dimensional motion providing arms micromanipulators (Narishige, Japan) equipped with IMT-2 invert microscope (Olympus, Japan). The DNA concentration was adjusted to 0.4 ng/μl using microinjection buffer (10mM Tris-HCl, pH 7.5, 0,1 mM EDTA, 100 mM NaCl) and injected into fertilized FVB/N mouse oocytes. Recipients were 10 weeks old CD1 females. In order to detect the presence of the bFCGRT in the mice, genomic DNA was isolated from tail biopsies of the offsprings and screened by two PCR amplifications. The two primer pairs were designed based on the bFCGRT sequence of the 128E04 BAC clone. The first primer pair was composed of the fcrnprf: 5'-CGG CTA CCA CCT CAT TCA TT-3' as sense and fcrnprr: 5'-TGC ATT CAC ACT GAC TGG TT-3') as antisense; while the second primer pair was bFcRnex4f: 5'-CCA TTG CCC AGT TGA ACG-3' as sense and bFcRnex4r: 5'-GTG TGG GTA GCA GGA GAG GA-3'.

The integrity of the transgene in the three transgenic lines was evaluated by specific primer pairs designed for the 5' and 3' ends of the BAC 128E04 and for the five putative protein encoding genes that are localized on the injected BAC based on the bovine genomic map (GenBank MapViewer bovine chromosome 18; region between 48,368K-48,934K bp).

2.3. Transgene copy number determination with real-time quantitative polymerase chain reaction

The 128E04 BAC transgene copy numbers were determined with TaqMan method, using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Primer and probe oligonucleotide sequences were designed with the Primer Express v2.0 program (Applied Biosystems). Conventional phenol/chloroform method was used for DNA extraction from tail samples of hemyzigous animals with an additional chloroform extraction step. Mouse β-actin and bFCGRT genes were quantified in each sample by absolute quantification using calibration curves. Standard curves using five points, diluted over a 32-fold range led to a high linearity with the primer sets. Linearity and efficiency of PCR quantification were validated before quantification. Samples were run in duplicate. The endogenous β-actin gene which is represented by two copies in each cell was used as internal standard to determine the DNA concentration. Mouse genomic DNA was used to set up the

calibration curve for the β-actin gene. Absolute quantification of the bFCGRT gene were based on a standard curve generated from serial dilutions of the 128E04 BAC supplemented with mouse genomic DNA. The standard curves enabled us to determine the copy numbers of bFCGRT gene based on the following calculations: the exact amount of DNA determined the number of diploid genomes in the samples, while the bFCGRT gene calibration curve determined its copy number in DNA samples of hemizygous animals from lines TG14 and TG19.

2.4. Establishment and maintenance of transgenic lines

Establishment of the founder lines of transgenic specimens were paired with wild-type mice. The offspring born was analyzed with the transgene specific PCR to select the transgenic individuals These heterozygous individuals were crossed with one another and in the G2 generation the transgene was segregated according to the Mendelian laws indicating that the integration occurred in one locus. The transgenic G2 mice were crossed with wild type animals and those transgenics, whose progenies were all positive for the transgene were accepted as homozygous.

2.5. Production of double transgenic mice lines

To exclude the interference caused by the endogenous mouse Fcrn α-chain, we made a double transgenic mouse strain. The bFcRn transgenic mice were crossed with the mouse FcRn knock-out mice ($mFcRn^{-/-}$). This transgenic strain was obtained from the Jackson Laboratory (http://jaxmice.jax.org). A multiplex PCR was planned for the genotypization of the G3 mice, by which all possible genotypes were detected in one reaction (mFcRn, bFcRn, NEO). With this multiplex PCR we could select those mice that had only bovine FcRn without mouse FcRn ($mFcRn^{-/-}$).

2.6. Examination of RNA expression levels

Total RNA was extracted by using RNAzol™ B (TEL-TEST INC) from liver, lung and mammary gland of six weeks old females and from the intestine of newborns. Two micrograms of RNA was reverse transcribed by using reverse transcriptase enzyme and the (dT)17-adapter primer as recommended by the manufacturer (Acces RT-PCR System; Promega). PCR was performed to obtain a 367 bp long bFCGRT specific amplicon (914-1280

bp) by the primer pairs: B7 5'-GGCGACGAGCACCACTAC-3'and B8 5'-GATTCCCGGAGGTCWCACA-3'. The amplified segment was separated by electrophoresis on 1% agarose gel and stained with ethidium-bromide.

2.7. Northern analysis examination of mRNA expression

Total RNA was isolated from liver of young adult female mice and 5 µg of total RNA was size fractionated on 1% agarose/2.2 M formaldehyde gel, transferred to Hybond N^+ membrane (Amersham) and hybridized with the ^{32}P-labeled cDNA probe synthesized by PCR with the B7-B8 primers described above. The signals obtained were evaluated using a PhosphorImager™ and quantified with STORM™ imaging system (Molecular Dynamics). Comparison between the bFcRn mRNA specific signal densities of the two, four, five and ten copies transgenic mice was done by using the Student's t-test.

2.8. Examination of copy number dependent expression with Northern blot

Five µg total RNA isolated from bovine liver, and livers of wild type mouse and transgenic mice carrying 2, 4, 5 and 10 copies of bFcRn were used to prepare Northern blot. The density of the fragments in the autoradiography was quantified with a Soft Imaging System AnalySYS Pro 3000 program and the results were processed in MS Excel program.

2.9. Detection of the recombinant protein with Western blot

Protein extracts were resolved on polyacrylamide denaturing Tris-glycine gel; blots were probed with affinity purified rabbit antiserum (raised against the peptide CLEWKEPPSMRLKAR representing the highly conserved 173-186 aminoacid residues of bFcRn β-chain plus an N terminal Cys for conjugation to KLH (Mayer et al. 2002). Bound bFcRn β-chain antibody was detected with horseradish peroxidase-conjugated goat anti-rabbit antibody and enhanced chemiluminescence, using luminol-based solution as substrate. The bovine mammary epithelial cell line (B4) stable transfected with bFcRn β-chain was used as positive control (Kacskovics et al. 2006).

2.10. Identification of genes in the BAC clone

As BAC clones are formed of large regions of genomic DNA, it is possible that other genes also integrated with the transgene. So we checked if the entire BAC clone integrated. For this, primers based in the already known bovine genome were designed for the genes positioned close to the FCGRT gene and that might have integrated together. According to the PCR results we can determine the size of the integrated BAC.

2.11. Fluorescent In Situ Hibridization (FISH)

To visualize the genomic integration of the 128E04 transgene, fluorescence in situ hybridization (FISH) was performed. The 128E04 BAC DNA was labeled by nick-translation with biotin-14-dATP (BioNick labeling kit, Invitrogen, USA). Mitotic chromosomes were obtained from vinblastine treated fibroblasts, which were isolated from 13.5 day old homozygote TG14 and TG19 embryos respectively, following standard protocols involving hypotonic treatment and methanol:acetic acid (3:1) fixation. FISH was performed essentially as published. The biotinylated probe was denatured and allowed to hybridize with denatured chromosome spreads, overnight at 37 °C. Hybridization sites on chromosomes were amplified with an anti-biotin antibody raised in goat (Vector Laboratories Inc, Burlingame, CA), and visualized by further incubation with fluorescein conjugated rabbit anti-goat IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands). Chromosome preparations were counterstained with diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and observed with a Nikon Eclipse E600 epifluorescence microscope (Nikon Instruments; Kawasaki, Japan). Fluorescence images were captured using a Cohu 4912 CCD camera (Cohu, Inc.; San Diego, CA, USA) and digitized with MacProbe 4.3 FISH software (Applied Imaging; Newcastle upon Tyne, UK) running on an Apple Macintosh G4 computer.

2.12. The functional marker system

Following a prebleed, five animals from the homozygote 14 line and from control mice were microinjected intravenously with 10 mg/kg bodyweight (BWkg) of anti-OVA mouse IgG1 (Sigma) in 50 mg/ml saline solution and during the next 216 hours, periodic blood samples (50 μl/occasion) were collected from retroorbital plexus. A quantitative ELISA employing OVA (Sigma) as capture reagent and an HRP-conjugated affinity-purified

polyclonal goat anti-mouse IgG (α-chain specific) (Southern Biotech Associates Inc., Birmingham, AL, USA) as detecting reagent was used to evaluate plasma concentrations of anti-OVA mouse IgG1 during the course of the experiment. The samples were assayed in triplicates. Concentrations of Ig are reported based on a reference standard. Analysis of the mean IgG concentrations of the mice in the first ten days was done by fitting the data to the two-compartmental model using WinNonLin professional, version 5.1 (Pharsight, Mountain View, CA).

2.13. Effect of *in vivo* LPS over the expression of bRcRn

When the appropriate ligand (LPS, TNFα) binds to cell surface receptors a signal cascade is trigerred, the lκB protein breaks down and because of this NF-κB is released to the nucleus inducing transcription factors which will bind to their appropriate promoter inducing the gene of interest. To prove this, we created three separate groups (A, B, C) each of them included eight double-transgenic mice with four bFcRn copies. One of the animals was used as a control and the other 7 received 250 ug/100g LPS (SIGMA L-3129) injected ip.. One animal of each group was sacrificed 1, 2, 4, 6, 8, 12, 24 hours after LPS injection followed by collection of liver and spleen. Rectal body temperature (Multi Precision Thermometer, Roth) of each animal was measured. Sepsis was confirmed as symptoms like decreased temperature, ruffled fur, loss of appetite, depression were detected continuously. Sepsis was strengthened by post mortem autopsy. Transgene expression was analyzed with Northern blot and Real-Time PCR. Signal density was evaluated with the GeneTools program from Syngene and values are represented using Excel.

2.14. Production of human GLYT1 construct

From the RPCI human BAC library (Children's Hospital Oakland Research Institute, USA), the RP11-107-F21 and RP11-445-G20 BAC clones containing the gene of GLYT1 transporter were acquired. The transgenic mice were produced with the same methods used for the production of the bFcRn transgenic mice. Founders were detected with the following primers: GLY1_L: 5'-CTCTGGAGGCTGTGGTTGAG - 3'; GLY1_R: 5'-GTCCACCTACARCGGCATCT - 3'. The copy number was defined with dot-blot method using a probe produced with the same primers mentioned above.

3. RESULTS

3.1. Creation of transgenic mice with pronucleus microinjection and transgene integration analysis

Microinjection of FVB/N oocytes was performed based on a protocol previously suggested with minor changes in the composition of microinjection buffer (supplied or not with spermine/spermidine). A total of 41 pups were born and genotyped from tail DNA for the presence of the bFCGRT. Three independent transgenic mouse lines were established. Two of these lines TG14, TG19 showed Mendelian pattern of the transgene inheritance in the first generation (17 and 12 from a total 30 and 34 litters carried the transgene), however the third line TG9 indicated a degree of mosaicism in the founder animal. Transgenic mice were indistinguishable from their wild type littermates based on their weight and overall health. The integrity of the transgene was tested by multiple PCR on tail DNA with seven pairs of primers covering both ends of the insert as well as all five putative protein coding gene sequences. All primer pairs gave the same PCR products as the 128E04 BAC and the bovine genomic DNA indicating integration of the intact BAC except line TG9 DNA in which the LOC 511234, LOC 522235 and the BAC128E04 3'-end specific PCR did not result PCR products. Therefore, we concluded that in this transgenic line an estimated 30 kb long fragment from the 3' end of the integrated BAC transgene was missing. The loss of genomic fragments both from 5' and 3' ends of large transgenes is a common phenomenon. Nevertheless to avoid the possibility of altered bFCGRT expression due to the absence of not characterized regulatory elements which might lie in the missing part of the BAC DNA, line TG9 was not included in further studies.

3.2. Expression analysis with RT-PCR

All three founder lines (TG9, TG14, TG19) express the transgene in the expected size. As the α-chain of FcRn is also expressed in the endothelial cells of blood vessels, ectopic expression was therefore not examined, FcRn α-chain would be detectable in all tissues.

3.3. Examination of copy number dependent expression with Northern blot

In order to evaluate the copy number dependence of transgene expression and to compare it with the quantity of the endogenous bFcRn α-chain mRNA, liver RNA samples from bovine and individuals of lines TG14 and TG19 were analyzed using Northern blot. The 18S RNA signal was used as an internal standard to estimate RNA loading on the gels. The level of mRNA expression in the liver of the line TG14 heterozygous transgenic mice, carrying two copies of the BAC transgene reached 90% of that observed in the bovine liver. Quantitative analysis and statistical evaluation of signal intensity from two or three hetero- and homozygote animals from both transgenic lines revealed that the amount of bFcRn α-chain mRNA in the liver of transgenic mice strictly correlates with their transgene copy numbers and the differences were significant at a $p<0.01$ probability level. This result along with the fact that the level of FcRn α-chain mRNA expression in the liver of transgenic mice carrying two transgene copies was similar to the level of mRNA in the bovine liver indicates that the 128E04 BAC carries all the necessary regulatory elements which ensure copy number dependent, position independent expression for the bFCGRT.

3.4. Detection of the transgenic protein with Western blot

Expression of the bFcRn α-chain at protein level was examined by Western analysis. In heterozygous lung samples from both transgenic lines - consistently with the known molecular weight of bFcRn α-chain a 40 kD protein was detected, which has not been found in the wt mouse sample used as negative control. The molecular weight of the transgenic FcRn α-chain was compliant with the recombinant protein produced by the B4 bovine mammary epithelial cell line which had been stable transfected with bFcRn. Moreover, this data confirmed our Northern blot analysis indicating that the sample from TG19 mice expressing 5 copies of transgene bFcRn shows much more bFcRn protein that was detected in line TG14 mice expressing 2 transgene copies.

3.5. Results of FISH

In order to exclude the possibility that the bovine BAC 128E04 clone which was used to create the bFcRn transgenic mice was accidentally integrated at identical segments on the mouse chromosome in both transgenic lines, and thereby possibly making the phenotypes of

the transgenic mouse lines TG14 and TG19 resulted from insertional mutagenesis of unidentified gene(s) at the transgene integration sites, we performed FISH analysis and found that the fluorescently labeled BAC 128E04 hybridized to entirely different chromosome segments in the TG14 and TG19 mice strains, respectively. Moreover, the single spots in the chromosomes indicate that the transgene integration most probably occurred in the form of tandem repeats. Based on this analysis, we concluded that the phenotypes TG14 and TG19 mouse lines were determined by the transgene expression independently from the transgene integration sites.

3.6. *In vivo* effects of LPS injection in bFcRn receptor expression in the double transgenic mouse model

The FcRn receptor expression changes in the LPS-treated mice spleen as revealed by Northern hybridization. These results were confirmed by Real Time PCR. According to my data, in the LPS treated transgenic mice, induction of the bovine FcRn mRNA expression occurs very rapidly, whereas 24h after treatment a decreasing amplitude close to the baseline was observed. This result indicates that the transcription factor NFκB plays an important role in the regulation of the bovine FcRn expression.

3.7. The results of the functional marker system

The first test case in order to analyze the expression of the bFcRn α-chain in sites that are considered to be involved in IgG catabolism as well as to test if the bFcRn α-chain and the mβ2 microglobuline are able to form a functional receptor, we have analyzed the pharmacokinetic behavior of the mIgG in these animals. Ten micrograms per gram OVA-specific mouse monoclonal IgG1 were injected in the wild-type and transgenic animals carrying 4 copies of bFcRn (line TG14, homozygous) and its levels in serum were measured with a sandwich ELISA. The clearance curves were biphasic, with phase 1 (alpha phase) representing equilibration between the intravascular and extravascular compartments, phase 2 (beta-phase) representing a slow elimination. Mathematical modeling of phases 1 and 2 until hours 216 have shown good correlation to the general scheme of FcRn mediated IgG pharmacokinetics hence we calculated the alpha and beta phase half-lives of mIgG in this time frame. The estimated alpha phase half-lives were similarly around 5 hours in the wt and transgenic mice, respectively. In contrast, there was a significant difference ($p<0.05$) in the

beta phase half-lives, as it was 125.4 ± 3.2 hours (mean ± SEM) in the wild-type and 165.1 ± 7.8 hours in the transgenic animals, based on the two-compartmental modeling analyses.

In the second study by the bFcRn associated with high affinity human IgG 4 copies injected transgenic (TG14 homozygous) and wild-type mice vena caudalis two different doses. The studies have shown that the serum half-life of hIgG in bFcRn transgenic mice had an average 168 hours, the wild-type mice, although this value is only 105 hours. Results show that in the blood of transgenic mice over time higher concentration of human IgG was detected as in the wild-type mice. This underlines, that the transgene product is present in the endothelial cells and demonstrates that the recombinant bovine FcRn α-chain forms a functional receptor with mouse β2 microglobulin.

3.8. Production of the human GLYT-1 transporter carrying mouse lines

Three series of microinjection were performed. The first series of 87 mice born from microinjected and recipient females implanted embryos. PCR tests could not show a single founder bearing the transgene. In the second series 105 animals were born and one mouse was showed by PCR to be transgenic (TG71). To confirm, the PCR fragment was sequenced and BLASTed which showed 99% homology with the human SLC6A9 gene which is the GLYT1 gene in the sequence data base. In the last microinjection, 24 animals were born and according to PCR, three more founders were identified (TG11, TG13, TG18). As TG71 line produced a progeny with larger number of positive animals, it was the chosen strain we used to create a transgenic line by pairing. G1 and G2 generations originated from TG71 line obeyed Mendelian inheritance law.

3.8.1. The determination of transgene copy number

The copy number of integrated transgene was determined with dot-blot. Compared to a dilution row of P-11-445-G20 BAC clone, transgenic animals from line TG71 showed to have the transgene integrated as 2 copies meanwhile the TG8, TG11 and TG18 heterozygous animals have 1 copy. This result does not explain why this transgene is inherited in a lower proportion than the expected in the TG8, TG11 and TG18 lines.

3.8.2. Analysis of expression of the integrated transgene

The transgene integration and its inheritance were tested with parallel RT-PCR to verify if the human SLC6A9 (GLYT-1) gene was transcribed into mRNA in the G1 generation of the TG71 mouse line. All examined tissues of these mice showed the expected cDNA fragment in the identical place as the human control showing that the human SLC6A9 (GLYT-1) gene is expressed in the transgenic mice.

4. NEW SCIENTIFIC RESULTS

1. Bacterial artificial chromosome (BAC) type transgenic mice were produced first time in Hungary in the frame of my PhD thesis work and our publication pioneered in creation of a transgenic mouse model with bovine BAC clone, worldwide. This result underlines the feasibility of creating BAC transgenic mouse models of economically important bovine genes.
2. Based on my results to achive the expression of the bovine transgene, which fully reflects the expression pattern of the endogenous gene, the 44 kb 5' and 70 kb 3' boundary regions of the FCGRT gene were sufficient.
3. The bovine BAC transgene shows copy number dependent, integration site independent and tissue-specific expression both at mRNA and on protein level.
4. Based on the physiological marker system, which was developed to evaluate the *in vivo* function, the bovine α-chain and mouse β2 microglobulin form functional heterodimer.
5. Overexpression of the α-chain from the transgene extends the half-life of circulating IgG in the blood.
6. For the direct *in vivo* examination of LPS effect on the FCGRT gene expression I developed a double-transgenic mouse line in which only the bovine FcRn receptor was present and clarified that the bovine FcRn mRNA induction occurs very rapidly, suggesting important role of the transcription factor NFκB in the transcriptional regulation.
7. A human glicintransporter (GLYT-1) receptor-expressing transgenic mouse line was established with BAC transgenesis.

5. CONCLUSIONS AND SUGGESTIONS

The transgenic technologies created a novel platform to analyse traits, which could not be accurately assessed without model animals. The quick development of embryology in combination with the recombinant DNA technology made possible the alteration of mammalian genomes. The most widely used method for the production of transgenic animals is pronuclear microinjection, but in this case the transgene integration site and copy number is random. The random incorporation results in variable transgene expression levels and sometimes leads to mutation of important genes. Contrary to this the artificial chromosome-based vectors could ensure integration site independent, copy number depend transgene expression. Therefore, in most cases, the BAC transgenic animals reveal the same tissue and developmental stage specific gene expression as the endogenous gene independently from the choice of species, from which the transgene originate. Beyond the regulatory elements located at large distances from the coding region could be also examined in BAC transgenic models the transgenic mouse lines I established at first harboured a 102 kb BAC clone isolated from a bovine genomic BAC library. This BAC clone carries the FCGRT gene and its boundary regions 44 kb, 50 kb at 5' and 3' directions respectively.

The transgene product expressed in the same organs and tissues as of the bovine endogenous receptor. Therefore, these animals are suitable to evaluate the consequences of increased amounts of FcRn in the whole animal level. This mouse model is also suitable to examine the *in vivo* efficacy and tissue specificity of hormones and other bioactive compounds. The double transgenic mouse model, which we also created, could be used especially to evaluate the *in vivo* role of the regulating factors in the expression of the bovine FcRn. Based on the earlier *in vitro* data of our collaboration partners (Dr. Imre Kacskovics Department of Immunology, ELTE) the bovine FCGRT gene's promoter has three inducible NFκB transcription factor binding sites whose activity could be abolished by mutations.

In ruminants, which have epitheliochorialis (syndesmochorialis) placenta, the transfer of maternal immunity, i.e. the transport of maternal IgG, does not happen through the placenta during pregnancy, it takes place in the colostrum, during the first day following parturition. The first step of this process, the secretion of IgG to the colostrums in the udder has long been studied. The double transgenic mouse model can help to clarify the precise role of FcRn in the transport of maternal IgG. Earlier and recent data show that the bovine neonatal Fc receptor (FcRn) expressed not only in the udder, but also in the mucous membranes of other organs, which play a role in the immunodefense. Further analysis confirmed that the bovine receptor

is also expressed in the endothelial cells of blood vessels, to prevent the rapid degradation of circulating IgG as in other species. We still need to understand the exact mode of transcitosis in the epithelial cells and the role of the factors which affect the intracellular localization of the receptor. It is also needed to be clarified what is the consequence of the rearrangements in the localization of the FcRn in the lactating udder. Another important question is what are the factors, which affect the receptor expression in the mammary gland and how could it be altered experimentally.

The half-life of IgG in the bFcRn transgenic mice is longer than in the wild-type. It is widely known that to combat certain bacterial toxins and severe viral infections the patients treated with blood plasma preparations which contain antibodies at high concentrations for the specific pathogen (such as Hepatitis B, rabies viruses and toxins Botulinus anti-human immunoglobulin preparations). As the foreign molecules from other species may induce adverse immunological reactions, recognized as foreign, and trigger neutralizing antibody production -ideally those treatments should be performed with antibodies from the same species, in case of humans with human serum samples. However, this is not a restricting factor in case of the intestine, since under physiological conditions molecules are not transported from here to the bloodstream, and therefore can not activate the immune system. However this provide almost unlimited possibilities for therapeutic interventions since the therapeutic molecules are equally efficient in the intestinal cells. This is especially true if the antibody-producing individuals were encountered or immunized with the antigen. Since human beings could not be immunized for subsequent antibody production, this solution is restricted to animals. Further advantage of the animal derived immunoglobulin preparations is that they cannot carry the risk of human pathogen contaminations (HIV, hepatitis C, etc.). The bovine colostrums contains huge amounts of maternal IgG, with which a number of positive experiences were already published. However to utilize the bovine or other ruminant species immunoglobulins in human therapy, it is important to clearly understand the species specific characters of those immunoglobulins and their effects in human beings. To that aim a special working group (CIgW) was founded by the International Federation of Immunological Societies (IUIS). Since the ruminants produce huge amount of IgG (about 500 grams of pure IgG) in the colostrum within a short period of the whole lactation, this phenomenon raised considerable interest in the scientific community. Theoretically, the period while the valuable IgG molecules are produced at high amount could be extended for the whole lactation, through understanding the regulatory mechanisms. Therefore, if we would immunize a cow against an intestinal pathogen, we could obtain neutralizing antibodies from its milk.

The immune system produces antibodies to combat pathogens and this process could be induced artificially through vaccinations. In a number of situations, it is not possible to be prepared for the attack of a life-threatening pathogen or toxin. In this case, the affected person receives antibody therapy produced in animals or isolated from human blood. Some people are unable to produce sufficient amounts of antibodies, and therefore receive a weekly dose of immunoglobulin (IVIG), to protect them from the development of side effects of viral or bacterial infections.

The therapeutic application of antibodies (especially monoclonal antibodies) has started in the eighties and now has become a priority on the antibody market, which is estimated to be 23-25 billion dollars. The worldwide human antibody market is estimated to be around 100 tons per year and its steadily increasing (this tendency parallely followed with the increasing demand for antibodies in therapeutic, diagnostic and *in vitro* research). However, antibodies from human serum preparations are available in limited amount, and there is a permanent risk of the contamination of patients with unrecognized infections. Therefore different companies were founded recently aiming the productions of humanized antibodies in transgenic animals (such as the transchromosomic cattle by Hematech, the transgenic rabbits by THP-Roche and the currently developed transgenic pigs (Revivicor). The use of animal antibodies is not limited to therapy, significant amount are used in research and clinical diagnostics (e.g. detection of pathogens). The MBK and the ELTE's joint international patent application was filed for producing transgenic animals with extended IgG half-life (I. Kacskovics, Z. Bősze, B. Bender, J. Cervenak, and L. Hiripi. 2007 Transgenic animal with enhanced immune response and method for the preparation thereof PCT/IB2007/054770). The ImmunoGenes Ltd. utilize the transgenic mouse models, created in the frame of this PhD thesis work for polyclonal antibody production and for creating transgenic rabbits and sheep. This innovation is encompassing a large potential, because of the use of antibodies in the life science research and clinical diagnostic is widespread in all areas, and has particular relevance in the human therapy. The so-called polyclonal antibody market has $ 2.5 billion annual turnover, while the total antibody market globally is around 23-25 billion dollars.

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