6. Discussion

Human MN/CA IX is a protein that was originally identified as a molecule involved in carcinogenesis and as a potential regulator of epithelial proliferation in the gastrointestinal tract (Pastorekova *et al.*, 1992; Zavada *et al.*, 1993; Pastorek *et al.*, 1994). It has been proposed that MN/CA IX protein may participate in acid-base balance, intercellular communication and cell proliferation (Pastorekova *et al.*, 1997; Saarnio *et al.*, 1998a; b). All the initial findings supported the notion that it might be a novel type of (proto)oncogene (Stanbridge et al, 1981; 1982; Pastorek *et al.*, 1994; Zavada *et al.*, 1997). Characteristically, MN/CA IX is abnormally expressed in various human carcinomas originating from MN/CA IX - negative tissues, but is absent or diminished in tumors derived from MN/CA IX - positive ones (Zavada *et al.*, 1993). Normally, its expression is restricted to only few tissues, particularly to mucosa of the alimentary tract (Pastorekova *et al.*, 1997). However, the role of MN/CA IX protein in physiological or pathological processes is largely unknown.

In the present work the construction of an animal model that would enable further studies of the role of MN/CA IX *in vivo* and *in vitro* is described. Gene targeting was chosen, as it is one of the most powerful approaches to fulfill this purpose (Capecchi, 1989).

First of all, in order to generate mice deficient in MN/CA IX protein, it was necessary to identify, clone and characterize the mouse *MN/CA9* cDNA. The human *MN/CA9* cDNA in the Southern blotting hybridized with mouse genomic DNA (Pastorek *et al.*, 1994; Opavsky *et al.,* 1996). Therefore a combination of RT-PCR with human degenerative primers, 5' and 3'RACE and primer extension methods was used to determine the complete mouse *MN/CA9* cDNA sequence.

The total size of the isolated mouse *MN/CA9* cDNA is 1982bp. The open reading frame of the mouse *MN/CA9* cDNA predicts a putative protein of 437 amino acid residues with a theoretical molecular mass of the mature protein of 47,3 kDa. An alignment of the predicted amino acid sequence of the murine and human *MN/CA9* cDNA showed 69,5% of sequence identity. Although the level of homology is not high, the murine sequence contained all extracellular and intracellular domains characteristic for the human MN/CA IX protein. It displays features typical of a carbonic anhydrases with all three histidine residues representing the CA core (His94, His96, His119, numerated as for mature CA I isozyme

(Christianson and Cox; 1999)). However, to maintain a catalytically competent structure of CAs, Glu106 and Thr199 also contribute. In the mouse MN/CA IX one substitution (Thr199→Ser) of these two residues was found, while in the human amino acid sequence of MN/CA IX both residues are conserved. Although the CA domain shares high homology between human and mouse, this one substitution in the mouse sequence might indicate an *MN/CA9* gene evolution, where CA activity would potentially be a secondary feature. On the other hand, mouse MN/CA IX protein still most likely retains the CA activity, as becomes evident from the purification experiment of MN/CA IX protein. This was based on the active binding of the potent CA inhibitor - sulfonamide (Zavada *et al.*, 2000). In the CA domain both sequences at the same position, (mouse aa 325 and human aa 346) have a consensus sequence for potential N-glycosylation site. Also a myristilation consensus pattern (mouse aa 420 and human aa 442) was conserved in both proteins. The comparison of conservation of the adhesion site in the mouse polypeptide could not be done, since for the human MN/CA IX protein its amino acid sequence still remains to be specified.

By Northern blot analysis and the primer extension experiment the presence of a single mRNA transcript coding for mouse *MN/CA9* was detected. This result was confirmed by finding a single band of molecular weight 54kDa after purification of the mouse MN/CA IX protein. Also, the human *MN/CA9* mRNA has a single splicing transcript as was shown by Northern blotting and the primer extension (Pastorek *et al.*, 1994).

Tissue specific expression of the mouse *MN/CA9* mRNA was evaluated by an RNase protection assay. High levels of the *MN/CA9* transcript were detected in the stomach. Considerable levels of expression were seen in the proximal intestine and also in the distal colon. A similar expression pattern was already described for the rat MN/CA IX protein (Pastorekova *et al.*, 1997). Important for the construction of gene targeted animals is to determine whether the targeted protein is expressed in the embryonal stem cells. Inactivating of a protein with an important function in an early stage of development might render success hard or even impossible to reach (Joyner, 1993). In the embryonal stem cells and in the embryo E10,5 the level of *MN/CA9* mRNA was below the detection threshold of this sensitive technique. These results suggest that MN/CA IX protein probably does not play a role in the early stages of development. On the contrary, *MN/CA9* mRNA was detectable in embryo from E18,5. Interestingly the development of the gastric epithelium begins at this time point (Gordon and Hermiston, 1994; Rubin *et al.*, 1994). This result supports the idea that MN/CA IX might play an important role in the development of the gastrointestinal tract.

The association of the human MN/CA IX with oncogenesis (Stanbridge *et al.*, 1981;

1982), its expression in the neoplastic clinical specimens and cell lines (Zavada *et al.*, 1993; Liao *et al.*, 1994; Costa *et al.*, 1995; Brewer *et al.*, 1996; Liao and Stanbridge, 2000; Turner *et al.*, 1997; Vermylen *et al.*, 1997; Liao *et al.*, 1997; McKiernan *et al.*, 1999; Saarnio *et al.*, 1998b; Pastorekova *et al.*, 1997; Osterwijk *et al.*, 1986; 1993) is certainly the most respected feature of MN/CA IX protein. However, to date the expression of MN/CA IX in mouse tumor cell lines and tumor samples has not been reported. Using RT-PCR it was possible to confirm the presence of *MN/CA9* cDNA in several cell cultures originating from a mouse mammary gland carcinoma (TS/A, GR/3, and MM5). In a cell line representing normal mammary gland (NMG) no expression of *MN/CA9* cDNA was detected. Also in the cell lines RENCA (renal carcinoma) and J558 (B lymphoma) *MN/CA9* cDNA was absent. One sample of spontaneous mouse mammary gland tumor was isolated, investigated and demonstrated to express the *MN/CA9* cDNA. These results together strongly support the idea that in mouse the MN/CA IX is also implicated in tumorigenesis and that the mouse model of null mutation in MN/CA IX will be very useful for studying of the role of MN/CA IX in cancer.

In order to identify and characterize the murine MN/CA IX, the protein was purified and isolated. The method of affinity chromatography on gel containing p-aminomethylbenzensulfonamide coupled to agarose beads was previously described by Falkbring *et al.* (1972) and modified by Zavada *et al.* (2000). This method is applicable only for enzymatically active carbonic anhydrases that bind sulfonamides (Falkbring *et al.*, 1972). Purified human MN/CA IX appeared as a twin band of size 54 and 58kDa (Zavada *et al.*, 2000). The murine MN/CA IX purified on sulfonamide-coupled agarose was detected by Coomassie blue staining on acrylamide gel as a single band of size 54kDa. The identity of this band was confirmed by MALDI-MS. Similarly, rat MN/CA IX protein showed also a single band of 53.5kDa (Zavada *et al.*, 1993). It is still not known why the human protein after purification and staining by Coomassie blue and Western blotting is detected as a twin band (Pastorekova *et al.*, 1992; Zavada et al; 2000). The reason for this interesting discrepancy between the human and the mouse or rat homologues in appearance remains unclear.

However, this result confirms the conservation of certain CA activity of the murine MN/CA IX, at least its sensitivity to sulfonamide, an inhibitor of the CAs. To estimate the CA catalytic activity of the mouse MN/CA IX, additional experiments would be necessary.

The mouse monoclonal antibody (M75) raised against human MN/CA IX protein

recognizes a twin-band protein of 54/58kDa in Western blot analysis (Pastorekova *et al.*, 1992). To identify and investigate the protein product of the mouse *MN/CA9* gene, a polyclonal rabbit antiserum (anti-PG-MN) against a synthetic oligopeptide was prepared. The oligopeptide was deduced from the *MN/CA9* cDNA sequence of the proteoglycan-like domain. Western blot analysis with the anti-PG-MN serum was performed using the whole cell extract of the mouse fibroblast cell line NIH 3T3 transfected with the murine *MN/CA9* cDNA. In contrast to non-transfected NIH 3T3 cell extract, analysis of the cell extract from the transfected cells revealed a single band of 54kDa, that roughly corresponded to the predicted size estimated to 47,3kDa. This assumption was confirmed with purified murine MN/CA IX protein.

Because the targeting frequency can be affected by sequence polymorphism (Deng and Capecchi, 1992) the mouse genomic library from the identical strain as the later used ES cell line strain for electroporation was chosen. Three overlapping subclones (EcoRI 6,5kb; KpnI 1,4kb and KpnI 10kb), encoding together the *MN/CA9* gene, were isolated from a mouse embryonic stem (ES) cell 129/Ola genomic library (Genome Systems) in pBAC108L (Bacterial Artificial Chromosome108L). Structural characterization of the three genomic subclones in pBluescript was conducted by DNA sequencing, Southern blot analysis and restriction mapping. The sequencing revealed the genomic organization of the murine *MN/CA9* gene into 11 exons and 10 introns. Identical genomic organization was reported for the human *MN/CA9* gene (Opavsky *et al.*, 1996). In both genomic sequences the signal peptide and the proteoglycan-like domain are situated in the first exon, the CA domain in exons 2-8, the transmembrane domain in exon 10 and the intracytoplasmic tail in exon 11. The exon-intron boundaries within the coding sequence are very similar in the two homologues. The high evolutionary conservation of the gene structure and the identical hybridization pattern of the human and murine cDNA with the mouse genomic DNA, allows to conclude that the isolated mouse gene is the human counterpart of *MN/CA9*.

The isolation, cloning and characterization of the mouse genomic DNA sequence and its comparison with the human one, was carried out as a next step in the development of a transgenic knockout model for MN/CA IX. In terms of criteria for design of a targeting vector for homologous recombination, murine *MN/CA9*, like human (Opavsky *et al.*, 1996), is present as a single-copy gene per haploid genome. The genomic map facilitated the selection of a specific genomic DNA fragment for construction of the targeting vector (Torres and Kuhn, 1997).

To inactivate the *MN/CA9* gene the strategy of a replacement vector was chosen (Rossant and Nagy, 1995). To assure the resulting null mutation of the gene, it was necessary to concentrate on several points: a) The first exon of the *MN/CA9* which encompasses also the signal peptide was selected because MN/CA IX is a transmembrane protein and it is led by the signal peptide across the membrane of endoplasmic reticulum. The deletion of 14bp from the first exon and insertion of a PGK-neo cassette shifted the open reading frame of the sequence coding for the mature protein and separated it from the sequence coding for the signal peptide, b) At the same time, the insertion of PGK-neo cassette introduced multiple stop-codons in all reading frames, c) Since the PGK-neo cassette contains a strong promoter, the cassette was inserted in the reverse orientation. This would support destabilization of any secondary transcript (Joyner, 1993).

After the electroporation of the target vector (p*MN/CA9*-neo), the clones resistant to G418 were screened for homologous recombination by Southern blot analysis. The proportion of homologous recombination incidence to not homologous integration of the vector into genome is usually in the ratio between 1:6 to 1:2000, as referred by Zhang *et al.* (1994). It depends on the method of targeting that was used, length of the region of homology and the level of polymorphism at the locus of homology. For the p*MN/CA9*-neo vector, with 5,3kb of total homology to the endogenous gene locus, the incidence of homologous integration was 1 : 175. This indicates a good frequency of homologous recombination for this system.

The transgenic experiment is successful if the ES cells enter the germline of the chimeric animals. Therefore attention had to be paid to the maintenance of ES cells pluripotency (Evans and Kaufmann, 1981; Longo *et al.*, 1997). Two positive clones (10B and 9F) of the homologous recombination were used and in both cases the disrupted *MN/CA9* gene was indeed transmitted to the offspring. Four fertile animals with relatively high chimerism resulted from the clone 10B. Two mice were not able to transmit the mutation at all (despite their high level of chimerism) while two other mice were capable of germline transmission by 80-100%. This discrepancy might have been due to a contamination of the clone 10B with a wild type clone during the isolation of resistant clones. In the clone F9 only one mouse was fertile and transmitted the mutation in 80%. The results confirm the optimal handling of the ES cell culture (Toreros and Kahn, 1997).

The ES cell lines are derived from the male (XY) and therefore, germline transmission is usually through male chimeras. However, the E14 ES cells have been reported to produce female chimeras with capacity of germline transmission (Schorle *et al.*, 1991). From both

clones together (10B and 9F) 3 female chimeras were obtained. An attempt to breed them was unsuccessful since all of them were found to be sterile.

To prove that the targeting of the *MN/CA9* gene resulted in a null mutation, the expression of the *MN/CA9* transcript was analyzed. As shown by the Northern blotting with the murine cDNA as a probe, a single band of practically the same size was detected from *MN/CA9*$^{+/+}$ and *MN/CA9*$^{-/-}$ mice. 5'RACE revealed a shortage of the *MN/CA9*$^{-/-}$ mRNA probably caused by post-transcriptional modifications. In the mutated mRNA the sequence coding for the 5' untranslated region, signal peptide, most of the sequence of the PGK-neo cassette and of the proteoglycan-like domain was missing. This result was confirmed by RT-PCR using gene specific primers. The RT-PCR also showed that the remaining coding region for carbonic anhydrase domain, transmembrane anchor, intracytoplasmic tail and that the 3' untranslated region was not affected. Despite the presence of a part of the coding region it is not likely that this would contribute as a functional protein to the phenotype of the *MN/CA9*$^{-/-}$ mice. First, in the PGK-neo sequence, that remained after the splicing, the first ATG initiation codon was found followed by multiple stop codons in all reading frames. However, in the remaining fragment of the coding sequence the next ATG codon is located in the open reading frame followed by the sequence coding for the CA domain. Even if the first ATG is ignored and the translational apparatus would recognize the next ATG, the resulting protein is missing the signal sequence that should conduct it across the membrane of the endoplasmic reticulum. In this case, proper post-translational modifications as well as transport to a correct location at the cell surface could not occur and the protein in the cytoplasm would become an easy target for ubiquitous proteinases.

Histological examination of the phenotypic consequences of the null mutation in most tissue samples did not reveal any pathological changes (lung, spleen, kidney, jejunum, ileum and colon). Remarkable abnormalities were found in the stomach of most (in 14 from 17) of the analyzed *MN/CA9*$^{-/-}$ animals. The differences between individuals of the same genotype are frequently observed in other gene-targeting experiments and have been attributed to variations in the genetic background. All of the analyzed mice were the F2 generation with a mixed 129/Ola x C57 BL/6J background. In order to generate a pure genetic background from a mixed strain 10 generations of backcrosses to the inbred mouse strain have to be done. Then the phenotypical differences of the homozygous mutant animals among the genetic backgrounds can be determined.

Additional pathological changes were encountered in pancreas and duodenum. Examination of heterozygous animals did not show any phenotypic difference compared to the normal mice.

Even though the mice were housed in a specific pathogen-free facility, we noted that 50% of analyzed adult *MN/CA9*$^{-/-}$ animals showed a presence of inflammatory cells in the pancreas. The histological picture was indicative of chronic pancreatitis. It is not clear whether the *MN/CA9*$^{-/-}$ animals are more sensitive to opportune infections or if this is a direct consequence of the genetic alternation.

In humans, MN/CA IX protein has been proposed as a marker for proliferative enterocytes locating in the Lieberkuhn crypts of the small intestine (Saarnio *et al.*, 1998a; b). Therefore special attention was paid to the intestinal phenotype in the *MN/CA9*$^{-/-}$ mice. The immunological staining for proliferation marker PCNA did not reveal any difference between the homozygous mutant and normal animals. Also incidences of tumorigenic changes were carefully followed in the intestinal samples. Out of 14 examined animals three cases of a mild dysplasia in the duodenal region of the small intestine were detected. Since this phenotypic trait was not registered in the wild type mice it might be due to the genetic alternation and might therefore be eminently significant.

Changes in the stomach of *MN/CA9*$^{-/-}$ animals were restricted to the epithelium. The first changes were detected at postnatal day P0,5 as a very mild thickening of the mucosa. By the time the gastric epithelium development is fully terminated (1 month of the postnatal age) (Gordon and Herminston, 1994; Rubin *et al.*, 1994), the gastric mucosa of *MN/CA9*$^{-/-}$ mice was affected by severe hyperplasia. This phenotype became even more pronounced with age. The architecture of the gastric units of the epithelium was distorted and concerned all the major epithelial cell types without any obvious defect in cell differentiation. This phenotype of *MN/CA9*$^{-/-}$ mice was accompanied by a number of large pathological cysts in the mucosal region.

The total number of epithelial cells was greatly increased in the *MN/CA9*$^{-/-}$ mice along the whole body region of the stomach, while the forestomach had a normal structure. Immunohistochemical staining of wild type mouse stomach samples revealed strong expression of MN/CA IX in all cells of the corpus. However, the forestomach turned out to be negative for MN/CA IX. This explains the absence of any phenotypic changes in the forestomach of *MN/CA9*$^{-/-}$ mice.

The immunohistochemical staining of the stomach for PCNA showed that the

proliferative zone was disorganized and enlarged in the *MN/CA9*$^{-/-}$ mice, spanning across the entire breadth of the mucosa. In contrast, the wild type mice showed a relatively narrow band of proliferative cells in the isthmus/neck regions of the gastric mucosa. Despite the distortion of the proliferative zone, the ratio between the number of proliferative cells and total number of cells in the proliferative area, was approximately the same in both *MN/CA9*$^{-/-}$ mice and wild type controls. By extension of nicks in the DNA mediated by dUTP (TUNEL) it was attempted to estimate whether the pathological changes could be attributed to decreased cellular death. No differences were identified in cells undergoing apoptosis between the wild type and the *MN/CA9*$^{-/-}$ mice. Therefore the architectural and cell number abnormalities in the gastric epithelium of *MN/CA9*$^{-/-}$ mice seem most probably to be caused by a defect in the regulation of cell proliferation.

Abnormal regulation of cell differentiation, proliferation or cellular death leads to development of gastric abnormalities also in humans. The gastric pathology of the *MN/CA9*$^{-/-}$ mice most closely resemble to the histological changes observed in Menetrier's disease (Menetrier, 1888), which is characterized by diffuse expansion of the superficial mucosa compartment of the stomach with a marked increase in the depth of the gastric pits caused by hyperplasia of mucin-secreting cells. The deep gastric glands are variable in height (Sleisenger and Fordtran, 1989; Ming and Goldman, 1992). The etiology of Menetrier's disease is so far unknown. It is possible that *MN/CA9*$^{-/-}$ animals could be useful for understanding of the molecular origin of this rare human disorder.

In developed countries stomach carcinomas are among the most common cancers and cancer-related mortalities (Kinzler and Vogelstein, 1996). As in other cases of carcinomas, the gastric carcinoma represents the result of a multistep process, which most likely begins with disturbing of cellular proliferation and commitment program of the epithelial cells. In human, the gastric carcinomas have been connected to down regulation or even loss of MN/CA IX protein (Pastorekova *et al.*, 1997). Also low expression of another adhesion molecule, E-cadherin, was shown to be a prognostic marker of poor outcome in many tumor models, notably gastric cancer (Guilford *et al.*, 1998). E-cadherin has a disorganized pattern of expression in the *MN/CA9*$^{-/-}$ mouse stomach, which is in line with the disrupted architecture of the mucosa itself. The E-cadherin immunostaining in the gastric mucosa of *MN/CA9*$^{-/-}$ mice seems to be less intense, although the expression was proven not to be different by Western blot analysis. However, it has to be stressed, that no dysplasia or neoplasia was observed in the stomach of MN/CA IX deficient mice. Also, a null mutation in IQGAP1, a negative regulator of cadherin mediated cell-adhesion, caused late onset

gastric hyperplasia (Li *et al.*, 2000). IQGAP1 is a widely expressed Ras-GAP related protein. IQGAP1 is among several targets for Cdc42 and Rac1, the small GTPases (Kuroda *et al.*, 1998; Fukata *et al.*, 1997). They negatively regulate IQGAP1 function by inhibiting the interaction of IQGAP1 with β-catenin, leading to stabilization of the E-cadherin-catenin complex. The slow process of late onset hyperplasia of the stomach in IQGAP1$^{-/-}$ mice might be a result of special sensitivity of gastric cells to defective cadherin-catenin adhesion.

Human MN/CA IX protein was found to have a highly enzymatically active CA domain. Both human and mouse MN/CA IX is mainly expressed in the stomach and thus it seemed possible that this enzyme might play a role in the maintenance of acid-base homeostasis. However, analysis of blood from adult homozygous mutant mice revealed no perturbation of acid-base balance. It was not surprising to find that the physiological values of pH were not disturbed in the MN/CA IX null mice. Alteration in the plasma pH and electrolytes would usually be expected in the case of severe defects of the stomach pH regulation, but minor deviations of these variables are corrected in lung and kidney. Also, in the stomach itself at least four other carbonic anhydrases are present that could compensate this defect. In parietal cells and surface cells, cytoplasmic CA II is expressed (Parkkila *et al.*, 1994). CA IV is expressed in the endothelium of capillaries (Fleming *et al.*, 1995) while mitochondrial CA V is cell-specifically expressed in parietal cells and in G-cells (Saarnio *et al.*, 1999). CA VI is not expressed in the gastric mucosa itself, but it is secreted by salivary glands. However, it is present in the gastric mucosa and it is thought to protect gastroesophageal mucosa from acid injury (Parkkila *et al.*, 1997).

Together, an influence of gastric pH on the mucosal architecture in the *MN/CA9*$^{-/-}$ mice is not probable. Nevertheless, additional experiments including measurement of gastric pH, could bring more insight.

The null mutation in the MN/CA IX protein in a mouse revealed its important physiological function in maintenance of the precise architecture and cell number during the development of the gastric epithelium. *MN/CA9*$^{-/-}$ mice are the first animal model for carbonic anhydrase isozyme deficiency constructed by gene targeting. It is also the first targeted adhesion molecule expressed in the gastrointestinal epithelium that exhibited involvement in the development of the stomach mucosa. It is still not clear whether disabling of the MN/CA IX protein carbonic anhydrase or the adhesion function (or their functional synchronization) is responsible for the phenotypic changes in the gastric

epithelium. Since any severe alteration in the acid-base balance regulation was not observed, it is likely that the gastric abnormalities in the *MN/CA9*$^{-/-}$ mice are due to perturbation of the MN/CA IX adhesion function. Our findings support the idea that the null mutation of MN/CA IX protein abrogates the proper processing of the intercellular signaling that normally define the level of proliferation and the architecture of gastric epithelial cells. This idea is consistent with the general view that the processes of proliferation, differentiation and cellular death are regulated in large by non-autonomous mechanisms. We also do not know whether the defect is caused by perturbation of reception or processing of signals imported from the mesenchyme underlying the stomach epithelium, or from the adjacent epithelial cells. We believe that identifying the molecular mechanism involved in the upstream and downstream signaling pathway(s) of MN/CA IX-mediated regulation of gastric epithelium proliferation would be very revealing for understanding the interactive complexity of the epithelial systems.