ELSEVIER

European Journal of Cancer 40 (2004) 1599–1603

European Journal of Cancer

www.ejconline.com

Truncated mouse adenomatous polyposis coli reduces connexin32 content and increases matrilysin secretion from Paneth cells

Trine Husøy [a,*], Hege B. Ølstørn [a], Helle K. Knutsen [a], Else Marit Løberg [b], Véronique Cruciani [c], Svein-Ole Mikalsen [c], Ingeborg L. Goverud [b], Jan Alexander [a]

[a] *Department of Food Toxicology, Norwegian Institute of Public Health, P.O. Box 4404 Nydalen, NO-0403 Oslo, Norway*
[b] *Department of Pathology, Ullevål University Hospital, NO-0407 Oslo, Norway*
[c] *Department of Environmental and Occupational Cancer, The Norwegian Radium Hospital, NO-0310 Oslo, Norway*

Received 14 January 2004; received in revised form 18 February 2004; accepted 26 February 2004
Available online 25 May 2004

Abstract

Heterozygous mutations in adenomatous polyposis coli (APC) is an early event in inheritable and sporadic colon cancer development. We recently found reduced connexin (Cx43) expression in intestinal cell lines with heterozygous *Apc* mutation. In this study we investigated Cx expression and the role of one mutated *Apc* allele in epithelia of multiple intestinal neoplasia (Min) mouse intestines by immunohistochemistry. Cx43 was not expressed in intestinal epithelia of Min and wild-type mice. Cx32 was specifically expressed in enterochromaffin cells in both mice types, and in Paneth cells of wild-type mice. In contrast, Min mice had nearly undetectable level of Cx32 in Paneth cells. Isolated small intestinal crypts from Min mice had markedly increased secretion of both lysozyme and matrilysin compared with wild-type mice. Absence of matrilysin in Min mice reduces adenoma development. Reduced Cx32 and increased matrilysin secretion from Paneth cells could be important to neoplastic development in the intestine.

Keywords: Adenomatous polyposis coli; Connexin; Paneth cells; Matrilysin; Colon cancer

1. Introduction

Mutations in the tumour suppressor gene *APC*, resulting in truncated APC protein, are important in the development of both sporadic and inherited (familial adenomatous polyposis; FAP) colon cancer. Somatic *APC* mutations have been observed in >80% of sporadic adenomas and carcinomas [1].

The functions of Apc in the intestine are only partly understood. It plays a key role in the Wnt-signalling pathway [2], where it participates in capturing the adhesion molecule and transcription factor β-catenin for degradation. Together with Tcf, β-catenin can turn on transcription of several genes [3]. However, it is believed that both *Apc* alleles must be inactivated to lose control of β-catenin-mediated transcription [4,5]. Apc also binds and stabilises microtubules [6].

Both *Apc* genes are inactivated upon development of intestinal adenomas. This occurs in two steps, where the mutation in the first *APC* allele might have a negative impact on the remaining intact allele. Truncated Apc proteins bind wild-type Apc and could function in a dominant negative manner [7]. In agreement with this, the normal intestinal tissue of multiple intestinal neoplasia (Min) mice, a FAP model with inherited heterozygous Apc^{Min} mutation, shows alterations in cellular functions such as adhesion, migration, differentiation and apoptosis [8].

Recently we found reduced gap junctional intercellular communication (GJIC) and connexin43 (Cx43) expression in intestinal cells heterozygous for Apc^{Min} mutation [4], suggesting that normal intestinal tissue in Min mice also could have reduced GJIC and Cx expression. Gap junction channels mediate the direct exchange of ions and small molecules between cells, and are built of proteins, the connexins [9]. Impairment of GJIC, caused by mutation, reduced Cx expression or

* Corresponding author. Fax: +47-22042243.
E-mail address: trine.husoy@fhi.no (T. Husøy).

0959-8049/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2004.02.024

loss of function, are involved in a number of diseases, including the development of cancer [10]. In support of this, Cx32-deficient mice had increased numbers of liver tumours [11], and Cx43 is mutated in advanced stages of human sporadic colon cancer [12]. Furthermore, Cx may function as tumour suppressors [13]. Interestingly, Wnt signalling affects GJIC and/or Cx expression [14,15], and Cx43 is a direct target for β-catenin/Tcf-mediated transcription [16].

The Cx have both specific tissue and cellular localisations. Small intestinal epithelium consists of four cell types. Little is known about the localisation and function of gap junctions in these cells. We studied the expression of Cx43 and Cx32 in the normal intestinal epithelium of wild-type and Min mice in order to determine whether Cx content and localisation is affected by $Apc^{Min/+}$ mutation also *in vivo*.

2. Materials and methods

2.1. Animals

C57/Bl6 $Apc^{Min/+}$ mice were bred at the Norwegian Institute of Public Health, Oslo, Norway, from inbred mice originally purchased from The Jackson Laboratory (Bar Harbor, ME) as described previously [17]. The $Apc^{Min/+}$ mice were identified by allele-specific polymerase chain reaction on DNA isolated from blood [18].

2.2. Immunohistochemistry

Paraffin-embedded formalin-fixed sections were prepared, deparaffinised and rehydrated. Epitope demasking was performed in microwave oven for 12 min in Target Retrieval Solution (DAKO, Glostrup, Denmark) pH 6.00–6.20 for anti-Cx32 (Cat. No. 71-0600; Zymed Laboratories) and anti-chromogranin A (CGA) (Cat. No. C21120; Transduction Laboratories), and in Tris/EDTA solution pH 9.1 for anti-lysozyme and anti-Cx43, as previously described [19]. Immunohistochemistry with Cx32 antibody was performed in a DAKO Autostainer system according to the manufacturer's instructions. Cx43 staining was done with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

Double immunofluorescence staining with anti-Cx32 and anti-CGA, and immunohistochemistry with anti-Cx43, were done manually. After blocking in normal goat serum for 20 min, the sections were incubated overnight at 4 °C with anti-Cx32 (1:200) and anti-CGA (1:50). Cx32 was visualised with anti-rabbit-Texas red (1:50) (Amersham, Buckinghamshire, UK) and CGA by anti-mouse–fluorescein isothiocyanate (1:25) (Bio-Rad, Hercules, CA), and the nuclei were stained with Hoechst dye.

Table 1
Survival of isolated crypts measured by exclusion of trypan blue

Time (h)	Survival (%)
1.5	>80
4.5	>70
7.5	~50
10.5	25
22.5	10

2.3. Crypt isolation

The isolation of epithelial crypts from mouse (9–13 weeks) intestine was mainly based on the method described by Ayabe and colleagues [20]. In brief, the intestine was rinsed with ice-cold Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) and the small intestine was cut into three equal segments denoted proximal, middle and distal intestine, in addition to the large intestine. The segments were turned inside out, the villi were removed by scraping with an object glass and were shaken in PBS containing 30 mM EDTA for 10 min. The segments were vigorously shaken for 15 s using a motorised pestle to remove more villi, then transferred to another tube with EDTA and shaken for 25 min. Crypts eluted during the last shaking were deposited by centrifugation and resuspended in iPIPES buffer (10 mM PIPES, pH 7.4, and 137 mM NaCl). The number of crypts was counted using a haemocytometer, and 2×10^4 crypts were used for experiments. Isolated crypts had a survival of more than 70% after 4.5 h measured by trypan blue exclusion (Table 1), as reported for pig crypts [21].

2.4. Western blotting

Proteins in the supernatant from 2×10^4 crypts were precipitated by adding an equal amount of cold acetone (1 ml) and centrifuged at 10,000*g* for 5 min. The pellet was resuspended in 12 μl sample buffer and 4 μl glycerol, loaded and separated on a discontinuous sodium dodecyl sulphate–polyacrylamide gel electrophoresis gel as described previously [4]. The blots were treated with anti-lysozyme (Cat. No. A0099; DAKO), and anti-matrilysin (Cat. No. AB8118; Chemicon, Temecula, CA). Quantification of band intensities was done in MatLab, and protein concentrations were calculated from the area under the curves. A two-tailed *t*-test was used for the statistical calculations. Secretions of crypts isolated from six and five animals were used to measure secretion of lysozyme and matrilysin, respectively.

3. Results and discussion

3.1. Cx localisation

Cx32 has, to our knowledge, not previously been studied in the intestine. Cx32 was highly expressed in the

cell membrane of the Paneth cells in the bottom of the small intestinal crypts from wild-type mice (Fig. 1A). Most interestingly, the Cx32 staining in Paneth cells from Min mice was much weaker and even below the detection limit in some preparations (Fig. 1B). While Cx43 was present in an intestinal epithelial cell line, it was not present in the epithelial cells *in vivo*, but only in the intestinal muscular layer as reported for other species (Fig. 1G) [22]. No difference in Cx43 intensity was observed between Min and wild-type mice (not shown). In the intestinal cell lines, heterozygous for the Apc^{Min} mutation, we found that the Cx43 content was much lower than in wild-type cells [4]. Cx43 is identified as target for Wnt signalling, in which Apc is an important participant [15,16]. Cx43 content in the muscular tissue of Min mice is apparently not affected by the heterozygous Apc^{Min} mutation, although Apc also is expressed in the muscular tissue [23]. It is therefore possible that the regulation of Cx in muscular cells and epithelial cells is different. For Cx32, however, no direct connection between Wnt and Cx32 is known. The observed reduction in Cx32 in normal intestinal cells of the Min mouse and in Cx43 of the $Apc^{Min/+}$ cells [4] is probably not a result of induced Wnt signalling, since β-catenin was unaffected [4,5]. Most probably, Wnt signalling in the intestinal epithelium is only induced when two *Apc* alleles are mutated. One mutated *Apc* allele, as found in normal intestinal epithelia of Min mice, probably affects Cx in the cells through other mechanisms. Furthermore, horizontal sections showed that Cx32 was highly expressed in the cytoplasm and the cell membrane of enterochromaffin cells as demonstrated by colocalisation with CGA (Fig. 1C–F). All enterochromaffin cells contained Cx32, and no difference in intensity between Min and wild-type mice was found (not shown). We found no difference in the distribution of enterochromaffin cells (not shown) and Paneth cells (Fig. 2) between normal intestine in wild-type mice and Min mice.

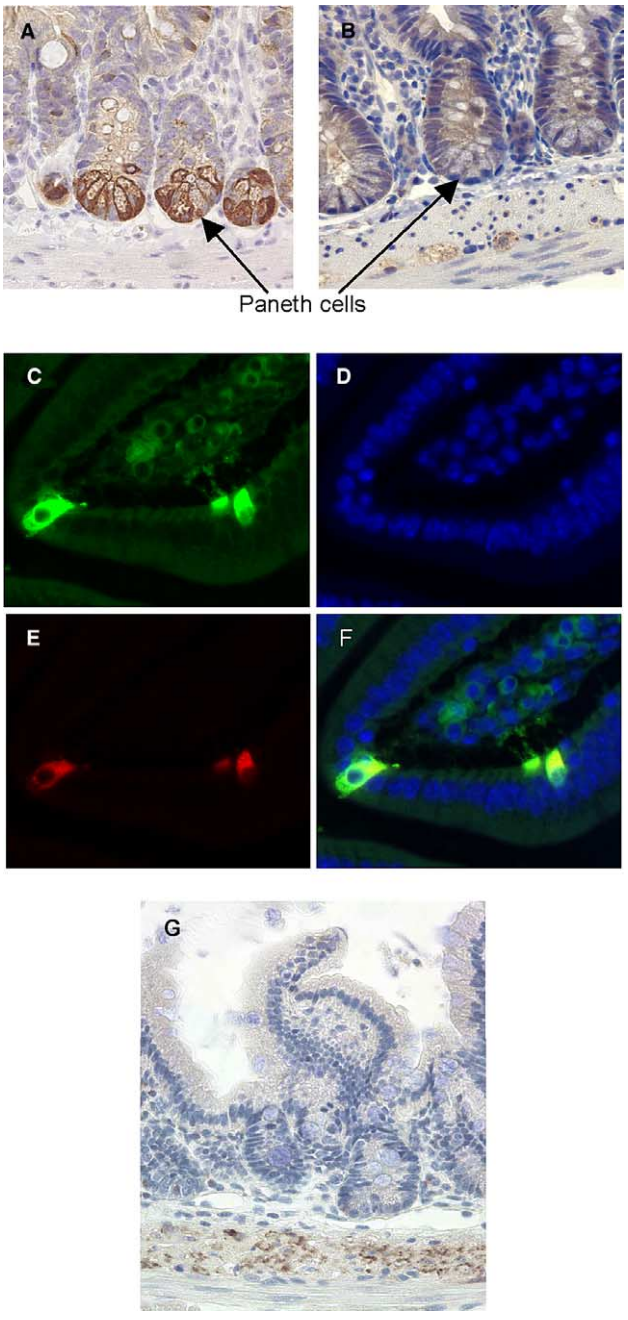

Fig. 1. Cellular localisation of connexin (Cx) 32 and 43 in the intestine. Vertical sections from small intestine of wild-type mouse (A) and multiple intestinal neoplasia (Min) mouse (B) treated with anti-Cx32. Controls without primary antibody gave no signal. Note the low expression of Cx32 in Paneth cells of Min mouse. Horizontal sections from wild-type mouse small intestine treated with antibodies against chromogranin A (CGA) (C) and Hoechst dye (D), and Cx32 (E). Merging (C), (D) and (E) showed colocalisation of CGA and Cx32 (F). Vertical sections from Min mouse small intestine treated with antibody against Cx43 (G).

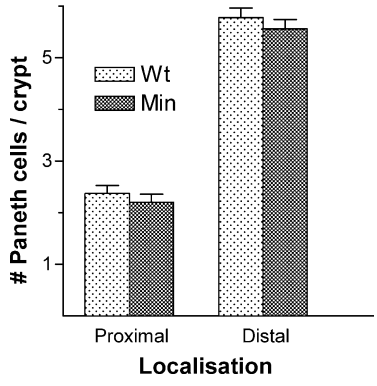

Fig. 2. The number of Paneth cells in each crypt of the small intestine of Min and wild-type mice. Three microscopic fields (20× objective) in the proximal and distal part of the intestine was counted in sections from two animals of each genotype. A significant increase in Paneth cells was found towards the distal part of the small intestine in both mice ($P < 0.001$). The error bars show SEM for each measurement with $n = 41$–60.

3.2. Secretion from Paneth cells

Paneth cells are secretory cells, which secrete antibacterial peptides such as lysozyme and matrilysin. Since Cx32 has been found to regulate secretion from cells in pancreas and liver [24,25], we studied whether the reduced expression of Cx32 in Paneth cells in Min mice was associated with changes in secretion. Immunohistochemical staining of small intestine from wild-type and Min mice showed that the Paneth cells contained similar amounts of lysozyme (not shown). Paneth cell secretion was studied in isolated small intestinal crypts. Following stimulation with lipopolysaccharide for 1 h at 37 °C, supernatants from isolated crypts were collected, and lysozyme and matrilysin contents were determined by Western blots (Fig. 3A). Crypts from Min mice secreted much more lysozyme and matrilysin than crypts from wild-type mice as verified by quantification of lysozyme and matrilysin from Western blots (Fig. 3B). As expected, no lysozyme or matrilysin was secreted by crypts isolated from colon epithelia (Fig. 3A), since normal colon tissue does not contain Paneth cells. Total matrilysin, both inactive promatrilysin and active matrilysin, were revealed in Paneth cell secretions (Fig. 3A), although the distribution varied between experiments. In line with this observation, Cx32-deficient mice had an increased basal release of amylase from pancreatic acinar cells [24]. In transgenic mice, where Cx32 was specifically expressed in pancreatic β-cells, a marked reduction of insulin secretion was observed after stimulation with glucose [26]. Secretion of lysozyme and matrilysin from Paneth cells of Min mice was increased, possibly as a result of reduced Cx32 content. However, we cannot completely exclude the possibility that the reduction in Cx32 and the increased secretion of lysozyme and matrilysin from Paneth cells are separate events caused by mutation in one *Apc* allele. The amount of lysozyme and matrilysin secreted by small intestinal crypts isolated from various segments increased from the proximal to the distal part (Fig. 3A). This increased secretion correlated well with an increasing number of Paneth cells towards the distal intestine (Fig. 2). Production of matrilysin has a large impact on cancer development in Min mice, where a 60% reduction in the development of small intestinal adenoma was observed in the absence of matrilysin [27]. Additionally, increased expression of matrilysin is shown in human colorectal cancer [28]. Interestingly, adenomas in Min mice appear in the most distal part of the small intestine [18,29], the same part which contains most Paneth cells and secretes the highest amount of matrilysin. We therefor hypothesise that increased matrilysin secretion from Paneth cells also may affect early stages of adenoma development in the intestine of Min mice. Two recent publications show that matrilysin mediates extracellular cleavage of E-cadherin [30,31], implying an important function for matrilysin in modulating adhesion. Reduced adhesion has indeed been found in the normal tissue of the Min mouse intestine [32].

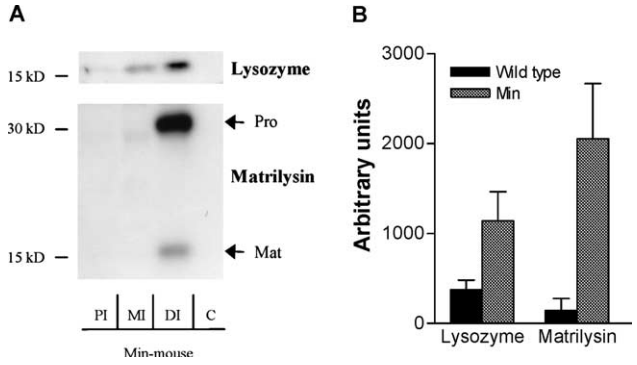

Fig. 3. Secretion from isolated crypts analysed by Western blots. (A) Secretion from isolated crypts from the small intestine divided in three equal segments; proximal (PI), middle (MI), and distal (DI), and colon (C) were loaded in different lanes. Lysozyme was detected as one band at about 17 kDa. Matrilysin was detected as inactive (Pro) and active (Mat) matrilysin at about 30 and 18 kDa, respectively. The figure shows a blot from one representative experiment. (B) Quantification of lysozyme and matrilysin content on Western blots. Since we did not achieve successful crypt isolation from each segment of the intestine in each experiment, the small intestinal segments with the highest number of successful crypt isolations were chosen for quantification. Lysozyme secretion from the middle part of the small intestine was analysed from six independent Western blots, while total matrilysin (promatrilysin and active matrilysin) from the distal small intestine was analysed from five independent blots. Secretion in wild-type and Min mice was always analysed from the same blot, while quantification of lysozyme and matrilysin content was done on different blots and could not be directly compared. The error bars show SEM. Both lysozyme and matrilysin secretion are significantly higher in Min mice compared with wild-type, with $P = 0.030$ and $P = 0.034$, respectively.

Altogether, the observed reduced content of Cx32 in Paneth cells in Min mice and our recently published results on the reduction in Cx43 in a cell line heterozygous for the Apc^{Min} [4] mutation strongly suggest that Cx are downstream targets following mutation in one *Apc* allele. Further studies are needed to identify the mechanisms involved and to verify the importance of Paneth cell function in the development of cancer in the intestine.

Acknowledgements

We thank Hege H. Haugen for excellent technical assistance. We thank Dr. E. Rivedal for making the anti-Cx43 antiserum available to us. Véronique Cruciani is supported by the Research Council of Norway. This study was supported with grants from the Research Council of Norway and the Norwegian Cancer Society.

References

1. Smith KJ, Johnson KA, Bryan TM, *et al*. The APC gene product in normal and tumor cells. *Proc Natl Acad Sci USA* 1993, **90**, 2846–2850.
2. Munemitsu S, Albert I, Souza B, Rubinfeld B, Polakis P. Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. *Proc Natl Acad Sci USA* 1995, **92**, 3046–3050.
3. Huelsken J, Behrens J. The Wnt signalling pathway. *J Cell Sci* 2002, **115**, 3977–3978.
4. Husøy T, Cruciani V, Knutsen HK, Mikalsen SO, Ølstørn HB, Alexander J. Cells heterozygous for the Apc^{Min} mutation have decreased gap junctional intercellular communication and connexin43 level, and reduced microtubule polymerisation. *Carcinogenesis* 2003, **24**, 643–650.
5. Maretto S, Cordenonsi M, Dupont S, *et al*. Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. *Proc Natl Acad Sci USA* 2003, **100**, 3299–3304.
6. Zumbrunn J, Kinoshita K, Hyman AA, Nathke IS. Binding of the adenomatous polyposis coli protein to microtubules increases microtubule stability and is regulated by GSK3 beta phosphorylation. *Curr Biol* 2001, **11**, 44–49.
7. Su LK, Johnson KA, Smith KJ, Hill DE, Vogelstein B, Kinzler KW. Association between wild type and mutant APC gene products. *Cancer Res* 1993, **53**, 2728–2731.
8. Mahmoud NN, Bilinski RT, Churchill MR, Edelmann W, Kucherlapati R, Bertagnolli MM. Genotype-phenotype correlation in murine Apc mutation: differences in enterocyte migration and response to sulindac. *Cancer Res* 1999, **59**, 353–359.
9. Evans WH, Martin PE. Gap junctions: structure and function (Review). *Mol Membr Biol* 2002, **19**, 121–136.
10. Trosko JE, Ruch RJ. Cell–cell communication in carcinogenesis. *Front Biosci* 1998, **3**, D208–D236.
11. Temme A, Buchmann A, Gabriel HD, Nelles E, Schwarz M, Willecke K. High incidence of spontaneous and chemically induced liver tumors in mice deficient for connexin32. *Curr Biol* 1997, **7**, 713–716.
12. Dubina MV, Iatckii NA, Popov DE, Vasil'ev SV, Krutovskikh VA. Connexin 43, but not connexin 32, is mutated at advanced stages of human sporadic colon cancer. *Oncogene* 2002, **21**, 4992–4996.
13. Eghbali B, Kessler JA, Reid LM, Roy C, Spray DC. Involvement of gap junctions in tumorigenesis: transfection of tumor cells with connexin 32 cDNA retards growth in vivo. *Proc Natl Acad Sci USA* 1991, **88**, 10701–10705.
14. Olson DJ, Christian JL, Moon RT. Effect of wnt-1 and related proteins on gap junctional communication in Xenopus embryos. *Science* 1991, **252**, 1173–1176.
15. Ai Z, Fischer A, Spray DC, Brown AM, Fishman GI. Wnt-1 regulation of connexin43 in cardiac myocytes. *J Clin Invest* 2000, **105**, 161–171.
16. van der Heyden MA, Rook MB, Hermans MM, *et al*. Identification of connexin43 as a functional target for Wnt signalling. *J Cell Sci* 1998, **111**(Pt 12), 1741–1749.
17. Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 1990, **247**, 322–324.
18. Steffensen IL, Paulsen JE, Eide TJ, Alexander J. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine increases the numbers of tumors, cystic crypts and aberrant crypt foci in multiple intestinal neoplasia mice. *Carcinogenesis* 1997, **18**, 1049–1054.
19. Husøy T, Mikalsen SO, Sanner T. Effects of five phorbol esters on gap junctional intercellular communication, morphological transformation and epidermal growth factor binding in Syrian hamster embryo cells. *Carcinogenesis* 1993, **14**, 73–77.
20. Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ. Secretion of microbicidal α-defensins by intestinal Paneth cells in response to bacteria. *Nat Immunol* 2000, **1**, 113–118.
21. Leschelle X, Robert V, Delpal S, *et al*. Isolation of pig colonic crypts for cytotoxic assay of luminal compounds: effects of hydrogen sulfide, ammonia, and deoxycholic acid. *Cell Biol Toxicol* 2002, **18**, 193–203.
22. Mikkelsen HB, Huizinga JD, Thuneberg L, Rumessen JJ. Immunohistochemical localization of a gap junction protein (connexin43) in the muscularis externa of murine, canine, and human intestine. *Cell Tissue Res* 1993, **274**, 249–256.
23. Reinacher-Schick A, Gumbiner BM. Apical membrane localization of the adenomatous polyposis coli tumor suppressor protein and subcellular distribution of the β-catenin destruction complex in polarized epithelial cells. *J Cell Biol* 2001, **152**, 491–502.
24. Chanson M, Fanjul M, Bosco D, *et al*. Enhanced secretion of amylase from exocrine pancreas of connexin32- deficient mice. *J Cell Biol* 1998, **141**, 1267–1275.
25. Temme A, Stumpel F, Sohl G, *et al*. Dilated bile canaliculi and attenuated decrease of nerve-dependent bile secretion in connexin32-deficient mouse liver. *Pflugers Arch* 2001, **442**, 961–966.
26. Charollais A, Gjinovci A, Huarte J, *et al*. Junctional communication of pancreatic β cells contributes to the control of insulin secretion and glucose tolerance. *J Clin Invest* 2000, **106**, 235–243.
27. Wilson CL, Heppner KJ, Labosky PA, Hogan BL, Matrisian LM. Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. *Proc Natl Acad Sci USA* 1997, **94**, 1402–1407.
28. Brabletz T, Jung A, Dag S, Hlubek F, Kirchner T. β-Catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. *Am J Pathol* 1999, **155**, 1033–1038.
29. Paulsen JE, Elvsaas IK, Steffensen IL, Alexander J. A fish oil derived concentrate enriched in eicosapentaenoic and docosahexaenoic acid as ethyl ester suppresses the formation and growth of intestinal polyps in the Min mouse. *Carcinogenesis* 1997, **18**, 1905–1910.
30. Noe V, Fingleton B, Jacobs K, *et al*. Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. *J Cell Sci* 2001, **114**, 111–118.
31. Davies G, Jiang WG, Mason MD. Matrilysin mediates extracellular cleavage of E-cadherin from prostate cancer cells: a key mechanism in hepatocyte growth factor/scatter factor-induced cell–cell dissociation and in vitro invasion. *Clin Cancer Res* 2001, **7**, 3289–3297.
32. Carothers AM, Melstrom Jr KA, Mueller JD, Weyant MJ, Bertagnolli MM. Progressive changes in adherens junction structure during intestinal adenoma formation in Apc mutant mice. *J Biol Chem* 2001, **276**, 39094–39102.