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Expression of Reg IV and Hath1 in neuroendocrine neoplasms

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Summary. Reg IV (RELP), a Regenerating protein family member, is constitutively expressed in neuroendocrine cells of the intestinal mucosa. The helix-loop-helix transcription factor Hath1 is the human homologue of murine Math1, which regulates the embryonic differentiation of neural and intestinal secretory lineage cells. Hath1 is constitutively expressed in a subset of mature secretory gastrointestinal cells. We investigated by immunohistochemistry the expression of Reg IV and Hath1 in 63 neuroendocrine tumors. Intestinal neuroendocrine neoplasms showed co-expression of Reg IV and Hath1, as did parathyroidal and Merkel cell tumors. Lung small-cell carcinoma and gastric mucocellular carcinoma expressed only Reg IV. Pancreatic islet-derived tumors, pheochromocytomas, and paragangliomas expressed only Hath1. Lymph node and liver metastases retained the tissue-specific expression patterns. These distinct expression profiles may be useful for differential diagnostics of metastatic lesions of neuroendocrine tumors. The dissimilar expression patterns suggest that the proteins belong to different signaling pathways and are activated at different stages of neuroendocrine differentiation. Local Reg IV expression may be influenced by the growth factors bFGF and HGF and/or their receptors CD138 and c-met, which were found to co-localize with Reg IV in intestinal neuroendocrine tumors.

Key words: Reg IV, Hath1, Neuroendocrine tumor, Carcinoid, Immunohistochemistry

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Introduction

The regenerating (Reg) proteins, a group of secreted c-type lectins of approximately 20 kD, fall into four superfamilies called Reg I-IV based on their primary structure. Reg proteins appear to be growth factors and anti-apoptotic survival signal mediators for pancreatic islet cells and neurons, inflammation-modifying acute phase reactants, and mucosal repair stimulating factors in the gastrointestinal tract (Zhang et al., 2003b). The only Reg IV superfamily member in human, Reg IV or Reg-like protein (RELP), was independently cloned and characterized by Hartupee et al. (2001) and by us (Kämäräinen et al., 2003). The genes encoding for the human Reg I-III proteins are clustered on the chromosome 2p12, whereas the gene for Reg IV is located on 1p12-13.1. It encodes for a secreted protein of 158 amino acids with approximately 45% similarity to the Reg I-III proteins.

Reg IV mRNA expression in normal human body is restricted to the gastrointestinal tract (stomach, small intestine, large intestine, rectum), the pancreas, the prostate, and the testes (Kämäräinen et al., 2003). Histologically, the neuroendocrine cells of the crypt epithelium of intestinal mucosa express Reg IV, as shown by immunohistochemistry and *in situ* hybridization (Kämäräinen et al., 2003). Goblet cells of the normal intestinal mucosa show a weak Reg IV staining, whereas the mucosa in inflammatory bowel disease (IBD, Crohn's disease and ulcerative colitis) showed robust accumulation of Reg IV. In stomach, normal parietal cells stain for Reg IV, while normal antrum is Reg IV negative. An accumulation of Reg IV was also found in goblet cells of the ventricular mucosa in areas displaying intestinal metaplasia, and in Barrett's esophagus (Kämäräinen et al., 2003). The upregulation of *Reg IV* mRNA expression in ulcerative colitis was confirmed by Nanakin et al. (2007). As for neoplastic

lesions, Violette et al. (2003) and Zhang et al. (2003a) showed *Reg IV* mRNA in colorectal cancer and in colorectal adenomas, respectively. *Reg IV* is up-regulated in scirrhous-type cancer of the stomach (Oue et al. 2004), and in nearly 30% of gastric cancers showing mucocellular or neuroendocrine differentiation (Oue et al., 2005). The majority of colorectal carcinoid tumors were positive for Reg IV (Oue et al., 2005). Moreover, we have reported a strong accumulation of Reg IV in appendiceal mucinous adenomas and their disseminated lesions, called pseudomyxoma peritonei (Heiskala et al., 2006). Expression of Reg IV has been found in primary ductal cancer of the pancreas (Oue et al., 2005).

A role has been proposed for murine Reg IV in the differentiation of intestinal epithelium (Schröder et al., 2006). Reg IV gene is activated at distinct times during intestinal differentiation and maturation, and a highly specific, spatially distinct expression pattern is seen in adult intestinal epithelium. Based on these observations and the selective expression of human Reg IV in the gastrointestinal epithelium, Reg IV has a putative physiological role in the support of mucinous and/or neuroendocrine differentiation of the gastrointestinal tract. Its ectopic expression in inflammation, metaplasia, adenoma, and cancer is probably induced by dysregulation of growth/differentiation balance-related proteins upstream.

Reg IV appears to enhance proliferation and inhibit apoptosis, but the detailed downstream mechanism of action remains unknown. A direct receptor of Reg IV has not been identified so far, but the apparent ability to transactivate epidermal growth factor receptor (Bishnupuri et al., 2006; Ohara et al., 2008) suggests that it might bind to a G-protein coupled receptor in the gastrointestinal tract epithelial cells. Reg IV stimulated cell proliferation, and an overexpression of the anti-apoptotic proteins Bcl-2 and Bcl-XL was also found in Reg IV treated cell cultures (Bishnupuri et al., 2006).

Taken together, Reg IV appears to be induced during physiologic differentiation in selected gastrointestinal tract epithelial cells, and inadvertently and/or ectopically in the course of inflammation, metaplasia and carcinogenesis, and to act as a mitogen and a pro-survival factor for regenerating and transformed cells.

The basic helix-loop-helix (bHLH) transcription factor Hath1 is a homologous human counterpart of the Drosophila Atonal and the mouse Math1. Math1 is a downstream protein in the Notch signaling pathway and it has been implicated in terminal differentiation of epithelial, neural and inner ear hair cells (Ben-Arie et al., 1997; Bermingham et al., 1999; Yang et al., 2001; Machold et al., 2007). Leonard et al. (2002) reported expression of Hath1 in Merkel cells and in Merkel cell carcinomas, suggesting a role in the establishment of neuroendocrine phenotype. In the intestine, a targeted deletion of the Math1 gene resulted in the absence of mature epithelial cells of the secretory lineage, including the intestinal goblet, enteroendocrine, and Paneth cells (Yang et al., 2001). Schroyer et al. (2007) reported that intestine-specific deletion of Math1 changed the genetic programming of enterocyte differentiation. Cells destined to a secretory phenotype became absorptive enterocytes.

In the gut, Hath1 is physiologically expressed in a subpopulation of small intestinal enteroendocrine cells, whereas in colon the expression is concentrated in the bottom of the crypts (Yang et al., 2001). Leow et al. (2005) reported a down-regulated expression of Hath1 in colorectal cancer, concomitantly with an absent goblet cell differentiation, thus suggesting a tumor suppressive function for Hath1. Overexpression of Hath1 in HT29 colon cancer cells led to slower proliferation and loss of ability to anchorage independent growth (Leow et al., 2005). Park et al. (2006) reported that Hath1 expression in intestinal neoplasms coincided with up-regulated expression of the goblet cell marker MUC2. They showed histological expression of Hath1 in the nuclei of normal goblet and colon enteroendocrine cells, but only in the cytoplasm of small intestinal enteroendocrine cells, despite the fact that Hath1 is a bHLH transcription factor. Moreover, Park et al. found upregulated expression of Hath1 in the nuclei of hyperplastic polyp cells, in adenomas and mucinous cancers, and in signet ring carcinomas of the intestine, but not in non-mucinous cancer of the gut. According to Aragaki et al. (2008), Hath1 protein is targeted by the Wnt-GSK3 axis, resulting in the proteosomal degradation and hence an apparently lower protein expression in human colon cancer. These observations suggest that Hath1 protein degradation may be required for maintaining the undifferentiated state of colon cancers.

Given the constitutive expression of Reg IV in gastrointestinal neuroendocrine cells, and the putative role of Hath1 in the development and maturation of the secretory lineage of gastrointestinal epithelial cells, and on the other hand their apparent involvement in the malignant transformation in these cells, we decided to investigate the possible interactions of these proteins by comparing their expression patterns in different neuroendocrine tumors, using immunohistochemistry.

Materials and methods

Tissue samples were formalin-fixed and paraffin-embedded specimens collected from the archives of the HUSLAB and the Department of Pathology, Haartman Institute, University of Helsinki, with the permission of the local ethical committee.

Antibodies

The monoclonal antibody to Reg IV was created as previously described (Heiskala et al., 2006). The rabbit polyclonal antibody to Reg IV was produced as described in (Kämäräinen et al., 2003).

Commercial monoclonal and polyclonal antibodies to Reg IV were obtained from R&D Systems (Minnesota, MN, USA). Rabbit polyclonal antibody to Hath1 was obtained from MBL International Corporation (Woburn, MA, USA). The CD138 and c-met antibodies and mouse monoclonal antibody to MIB were obtained from Dako (Glostrup, Denmark) and the antibodies to HGF from Biocare Medical (Concord, CA, USA) and to bFGF from Santa Cruz Biotechnology (Heidelberg, Germany).

Secondary antibodies, HRP-conjugated Rabbit anti-mouse and Goat anti-rabbit immunoglobulin (IgG), FITC-conjugated Goat anti-mouse, and TRITC-conjugated swine anti-rabbit were obtained from Dako (Glostrup, Denmark).

Immunohistochemistry

Four-µm sections from formalin-fixed, paraffin-embedded tissues were mounted on 3-aminopropyl-triethoxysilane-coated slides (Sigma), deparaffinized, and heated twice for 5 minutes in a microwave oven (650W) before exposure to the first antibody (monoclonal mouse anti-Reg IV antibody, dilution 1:500 or rabbit polyclonal antibody to Hath1, dilution 1:200). EliteKit (Vectastain; Vector Laboratories, Burlingame, CA) was used for immunoperoxidase staining, visualized with 3-amino-9-ethylcarbazole (Sigma).

Immunofluorescence

Four-µm sections from formalin-fixed, paraffin-embedded tissues were deparaffinized and heated twice for 5 minutes in a microwave oven (650W) before exposure to the first antibody (monoclonal mouse anti-Reg IV antibody, dilution 25 mg/ml or rabbit polyclonal antibody to Hath1, dilution 1:30). The secondary antibodies FITC-conjugated Goat anti-mouse, and TRITC-conjugated swine anti-rabbit antibodies (1:30) were used to visualize the protein.

In situ hybridisation

We used the TA cloning kit (Invitrogen, San Diego, CA) to prepare probes. A PCR-amplified 0.4-kilobase of Hath1 cDNA was ligated into the pCR-II vector (Invitrogen). The templates for Hath1 antisense or sense RNA probes were generated by linearizing the appropriate vector construct (3' to 5' or 5' to 3', respectively). The RNA Labeling Kit of Boehringer-Mannheim (Mannheim, Germany) was used according to the manufacturer's instructions to generate digoxigenin-labeled RNA probes by in vitro transcription.

Tissue sections were deparaffinized and specially prepared for *in situ* hybridization. Prior to hybridization, the sections were treated with 0.3% triton X-100 and then with 10mg/ml proteinase K for 15 min at 37°C. The sections were then post-fixed with 4% paraformaldehyde for 5 min. After acetylation of the sections (2x5 min with TEA buffer containing 0.25% acetic anhydride (v/v)), they were incubated with a pre-hybridization buffer (4x SSC containing 50% deionized formamide) for 15 min at 37°C.

The pre-hybridization buffer was drained and the sections were overlaid with hybridization buffer (Sigma, St. Louis, MI) containing 10 ng of digoxigenin-labeled RNA probe. The sections were covered with plastic coverslips and incubated overnight in a humid chamber at 42°C.

After hybridization, the coverslips were removed and the sections were washed twice at 37°C in 2x SSC for 15 min each, then twice with 1x SSC for another 15 min each. Unbound RNA probe was digested with NTE buffer containing 20 mg/ml of RNAse A at 37°C, after which the sections were washed twice with 0.1x SSC for 30 min each. The labeled probe was detected using the DIG nucleic acid detection kit (Roche, Germany) according to the manufacturer's instructions.

Results

The immunohistochemical staining results of the different tumors, as well as the patient data, and the WHO classification of the tumors are summarized in Table 1. Occasionally, tumors showed heterogeneity, only a subset of the malignant cells expressing the target protein. If less than 5% of the tumor signaled the presence of the target molecule, the result of the staining was considered negative.

Expression of Reg IV and Hath1 in normal intestinal mucosa

Reg IV is found in the cytoplasm of scattered cells in normal intestinal epithelium, in particular in the proximal parts of the villi and in crypts (Fig. 1a). Reg IV is located in cytoplasmic granules where it is co-expressed with the neuroendocrine marker chromogranin A (Kämäräinen et al., 2003). Selected intestinal epithelial cells displayed strong cytoplasmic staining for Hath1 as in the work of Park et al. (2006), with an anatomical distribution similar to that of the Reg IV positive cells (Fig. 1b). Co-staining experiments nevertheless revealed that Reg IV and Hath1 are expressed in different cells, with only occasional cellular co-expression (Fig. 1c-e). The specificity of the antibody to Hath1 in the normal intestinal epithelium was confirmed with *in situ* hybridization, showing that cells in which the Hath1 protein was detected by IHC also expressed the Hath1 specific mRNA (Fig. 1f-g).

Expression of Reg IV and Hath1 in neuroendocrine tumors (carcinoids) of ileum and appendix

Reg IV and Hath1 had a similar expression pattern

Table 1. Summary of the stained neuroendocrine tumor samples.

Diagnosis	age	sex	Reg IV	Hath1	WHO class
Tumors of the stomach					
Well-differentiated neuroendocrine tumor, stomach	64	m	+	-	1
Well-differentiated neuroendocrine tumor, stomach	43	f	-	-	1
Well-differentiated neuroendocrine carcinoma, stomach	63	f	+	-	2
Poorly differentiated neuroendocrine carcinoma, duodenum (gastrinoma)	54	f	+	NS	3
Tumors of the small intestine					
Well-differentiated neuroendocrine carcinoma, ileum	48	f	++	++	2
Well-differentiated neuroendocrine carcinoma, ileum, liver metastasis	48	f	+	-	2
Well-differentiated neuroendocrine carcinoma, ileum, lymph node metastasis	48	f	++	++	2
Well-differentiated neuroendocrine carcinoma, ileum	64	f	++	+/-	2
Well-differentiated neuroendocrine carcinoma, ileum, lymph node metastasis	64	f	+	-	2
Well-differentiated neuroendocrine carcinoma, ileum	73	m	+++	+/-	2
Well-differentiated neuroendocrine carcinoma, ileum	77	f	+	+	2
Well-differentiated neuroendocrine carcinoma, ileum, lymph node metastasis	77	f	++	+	2
Well-differentiated neuroendocrine carcinoma, ileum	57	m	+++	+/-	2
Well-differentiated neuroendocrine carcinoma, ileum	57	m	+++	+/-	2
Well-differentiated neuroendocrine carcinoma, papilla vater	41	m	-	-	2
Tumors of the appendix					
Well-differentiated neuroendocrine tumor, appendix	25	f	++	++	1
Well-differentiated neuroendocrine tumor, appendix	49	f	++/-	++	1
Well-differentiated neuroendocrine tumor, appendix	49	m	++	+++	1
Well-differentiated neuroendocrine tumor, appendix	73	m	+	+	1
Mixed exocrine-neuroendocrine carcinoma, appendix, Goblet cell carcinoid	42	m	+++	+	4
Mixed exocrine-neuroendocrine carcinoma, appendix, Goblet cell carcinoid	69	m	++	++	4
Mixed exocrine-neuroendocrine carcinoma, appendix, Goblet cell carcinoid	45	m	++	++	4
Tumors of the rectum					
Well-differentiated neuroendocrine tumor, rectum	49	f	-	++	1
Poorly differentiated neuroendocrine carcinoma, rectum	78	m	++	+	3
Poorly differentiated neuroendocrine carcinoma, rectum	69	m	+	-	3
Tumors of the pancreas					
Well-differentiated neuroendocrine tumor, pancreas (insulinoma)	58	m	-	+++	1
Well-differentiated neuroendocrine tumor, pancreas (non-functional)	27	f	-	+	1
Well-differentiated neuroendocrine tumor, pancreas (insulinoma)	26	f	-	+	1
Well-differentiated neuroendocrine carcinoma, pancreas (MEN1)	54	f	-	+	2
Well-differentiated neuroendocrine carcinoma, pancreas, lymph node metastasis	54	f	-	+	2
Well-differentiated neuroendocrine carcinoma, pancreas (vHL)	38	f	+	+	2
Well-differentiated neuroendocrine carcinoma, pancreas (glucaconoma)	36	f	-	++	2
Well-differentiated neuroendocrine carcinoma, pancreas	43	m	-	NS	2
Well-differentiated neuroendocrine carcinoma, pancreas, liver metastasis	53	f	-	+	2
Well-differentiated neuroendocrine carcinoma, pancreas (insulinoma), liver metastasis	42	m	-	+	
Tumors of the thymus					
Well-differentiated neuroendocrine carcinoma, thymus	42	m	-	-	2
Poorly differentiated neuroendocrine carcinoma, thymus	37	f	-	-	3
Tumors of the lung					
Typical carcinoid, lung	65	f	-	+/-	
Typical carcinoid, lung	61	m	-	-	
Typical carcinoid, lung	61	f	-	+/-	
Typical carcinoid, lung	19	f	-	-	
Typical carcinoid, bronchus	39	f	-	+/-	
Small-cell lung carcinoma	54	f	++	-	
Small-cell lung carcinoma	64	f	++	-	
Small-cell lung carcinoma	64	f	-	-	
Small-cell lung carcinoma	64	m	-	-	
Small-cell lung carcinoma	54	f	-	+/-	
Small-cell lung carcinoma	63	m	-	-	
Small-cell lung carcinoma	64	m	-	-	
Tumors of the skin					
Carcinoma Merkel	67	m	+	+/-	
Carcinoma Merkel	75	m	++	-	
Tumors of the parathyroid					
Parathyroid adenoma	52	f	+	NS	
Parathyroid adenoma	66	m	+	NS	
Parathyroid adenoma	19	m	+	+++	
Parathyroid carcinoma	58	f	-	++	
Parathyroid carcinoma	61	f	+	NS	
Tumor of the thyroid					
Medullar carcinoma, thyroid	64	f	-	+	
Tumors of the adrenal gland					
Pheochromocytoma, adrenal gland	57	f	-	-	
Pheochromocytoma, adrenal gland	62	m	-	+	
Pheochromocytoma, adrenal gland	62	m	-	+	
Paraganglioma	82	m	-	++	
Paraganglioma	40	m	-	+	
Paraganglioma	34	m	-	+	

Staining intensity of Reg IV and Hath1 immunohistochemistry of the tumor samples. +++, very intensive staining; ++, clear staining; +, weak staining or only a part of the tumor stained; +/-, alternating areas of negative and intensive staining, the positive area representing a small part of the tumor; -, negative, less than 5% of the tumor stained, and/or staining very weak; NS, the sample was not stained.

in well differentiated intestinal and appendiceal neuroendocrine tumors. The peripheral cell layer directly interacting with the surrounding tissue typically showed strong staining for both Reg IV and Hath1, whereas the cells inside the tumor islets were mostly negative for them (Fig. 1h-k). The similar staining pattern was maintained in ileal neuroendocrine tumor-derived lymph node and liver metastases (Fig. 1l-m). The proliferation marker MIB-1 was expressed in a subset of the tumor cells, but its expression did not correlate to that of Reg IV or Hath1 (data not shown). A specific form of appendiceal neuroendocrine tumor, goblet cell carcinoid, also stained strongly for both Reg IV and Hath1 (Table 1.; data not shown).

Expression of Reg IV and Hath1 in pancreatic islets and islet-derived neoplasms

Although other members of the Reg protein family are expressed in pancreas, Reg IV was not found in that location (Fig. 2a). The negative finding was confirmed by using both our own rabbit polyclonal antibody, and the commercially available monoclonal and polyclonal antibodies to human Reg IV (data not shown). In contrast, Hath1 was expressed in virtually all the endocrine pancreas cells (Fig. 2b). Neither Reg IV nor Hath1 was expressed in exocrine pancreas (Fig. 2a-b). In islet-derived neuroendocrine tumors, a distinct positive staining for Hath1 was found, particularly in peripheral cells, whereas the tumors remained negative for Reg IV (Fig. 2c-d). The same pattern was also seen in liver and lymph node metastases from insulomas (not shown).

Expression of Reg IV and Hath1 in gastric cancer

Expression of Reg IV in gastric cancer has been previously reported (Oue et al., 2004, 2005). In the current study epithelial cells of diffusely growing gastric

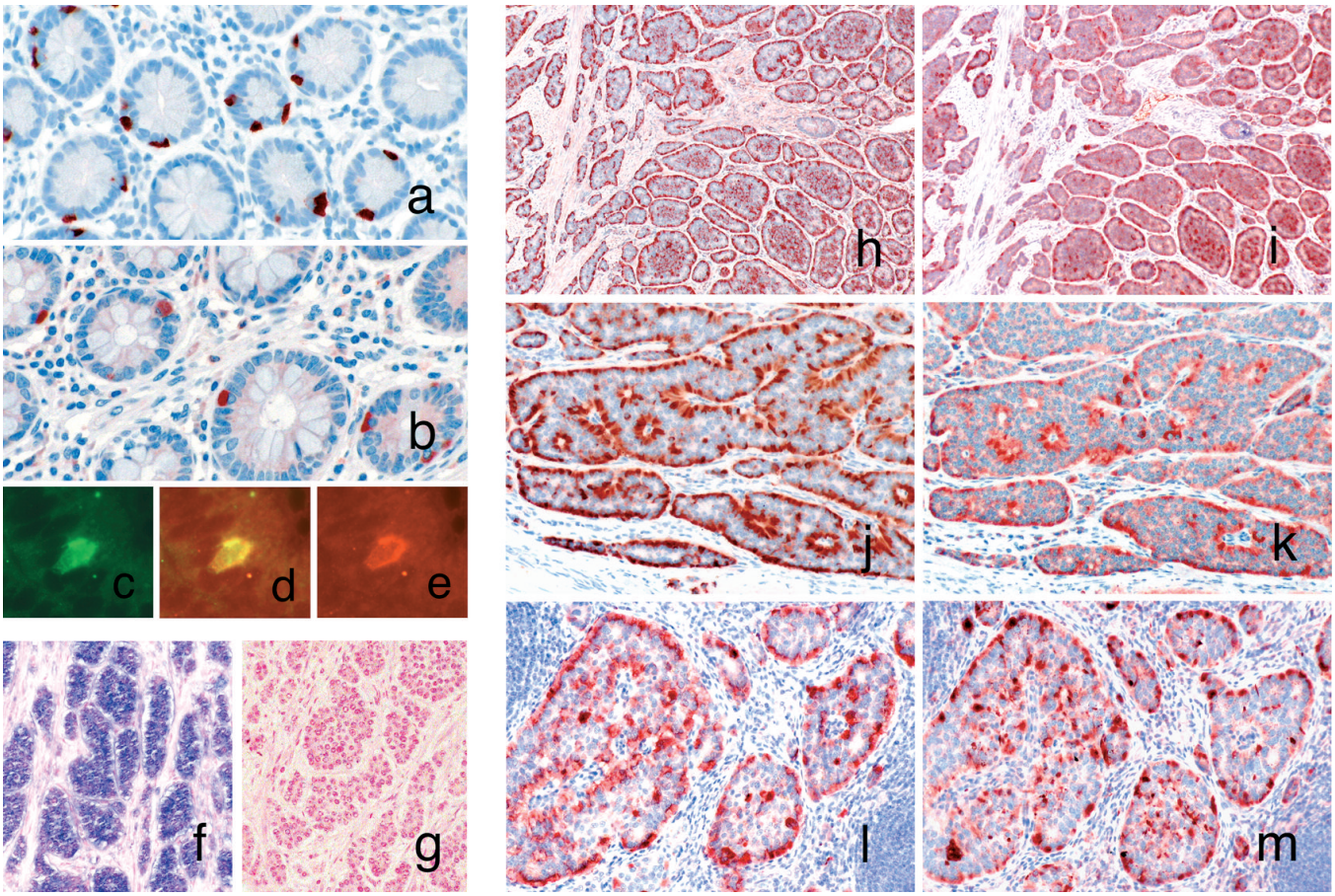

Fig. 1. Expression of Reg IV and Hath1 in normal mucosa and in neuroendocrine tumors of the ileum. Reg IV **(a)** and Hath1 **(b)** are mostly expressed in different subpopulations of enteroendocrine cells with only occasional cells displaying co-expression **(c-e)**: Reg IV immunofluorescence **(c),** Hath1 immunofluorescence **(e)** and merged **(d)**. *In situ* hybridisation of an ileal neuroendocrine tumor with *Hath1* antisense **(f)** and sense **(g)** probes. Expression of Reg IV **(h, j)** and of Hath1 **(i, k)** in the peripheral cell layers of ileal neuroendocrine tumors. Original magnifications 20x and 40x. **f-m.** Consecutive sections of a lymph node metastasis of an ileal neuroendocrine tumor stained with antibodies to Reg IV (l) and to Hath1 (m). a, l, m, x 200; b, x 400; c-e, x 630; f, g, j, k, x 100; h, i, x 50

cancer stained moderately for Reg IV, and mucocellular (signet ring) cancer showed a strong accumulation of Reg IV, whereas staining for Hath1 remained negative (Fig. 2e-h).

Expression of Reg IV and Hath1 in neuroendocrine tumors of the lung and skin

Despite the fact that neuroendocrine tumors of the small intestine showed a clear expression of both Reg IV and Hath1, bronchial carcinoids were devoid of these intestinal epithelium growth and maturation-related markers (data not shown). Likewise small cell (oat cell) lung cancer did not express Hath1, but in two of the seven samples studied, areas of strong aberrant expression of Reg IV was seen, giving the tumors a reticular staining pattern (Fig. 3a,b).

Neoplastic cells from cutaneous mechanoreceptor tumors (Merkel cell carcinomas) showed punctuated cytoplasmic staining for Hath1 and strong positivity for Reg IV (Fig. 3c,d).

Expression of Reg IV and Hath1 in pheochromocytomas

Both Hath1 and Reg IV were weakly expressed in normal adrenal medulla (data not shown). Pheochromocytomas and extra-adrenal paragangliomas growing in a "zellballen" pattern showed strong, evenly distributed staining for Hath1 in the tumor cells (Fig. 3f), whereas ganglion cell pattern type pheochromocytomas strongly expressed the protein in a subset of cells only (Fig. 3h). Although both adrenal and extra-adrenal pheochromocytomas expressed Hath1, Reg IV was not found in these tumors (Fig. 3e,g).

Expression of Reg IV and Hath1 in normal and neoplastic parathyroid and thyroid

Both Reg IV and Hath1 were expressed in normal parathyroid tissue. Reg IV appeared as a granular cast in the cytoplasm of all cells, whereas Hath1 was found only in distinct areas of oxyphilic cells (Fig. 3i,j). In adenomas, Reg IV occurred frequently as a single intracytoplasmic aggregate (Fig. 3k), while Hath1 displayed a wider "map-like" distribution (Fig. 3l).

Normal thyroid gland did not express Reg IV, whereas occasional, possibly interstitial thyroid gland cells expressed Hath1 (data not shown). Tumors derived from the follicular epithelium were consistently negative for both Reg IV and Hath1. Scattered tumor cells in medullary carcinomas showed strong cytoplasmic staining for Hath1, resembling that of the ganglion cell type pheochromocytomas (Fig. 3n). Medullary thyroid tumors did not express Reg IV (Fig. 3m).

CD138, c-met, bFGF and HGF in ileal neuroendocrine tumors

Staining of ileal neuroendocrine tumors for bFGF showed an expression concentrated in the outer cell layer (Fig. 4a). Staining for CD138, the common receptor for bFGF, revealed distinct membrane positivity (Fig. 4b).

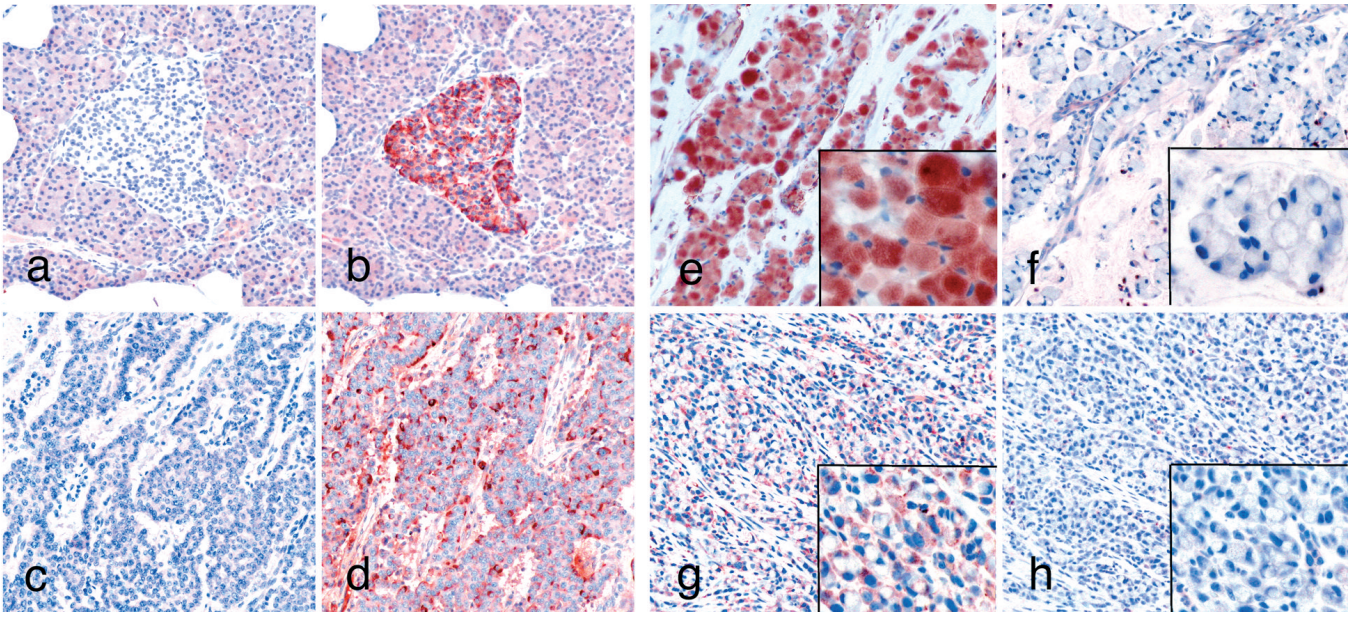

Fig. 2. The Langerhans islets do not express Reg IV **(a)** but stain positively for Hath1 **(b).** Insulomas are also Reg IV negative **(c)** but Hath1 positive **(d)**. Mucocellular and diffusely growing gastric cancers express Reg IV **(e, g)** but are Hath1 negative **(f, h)**. a, g, h, x 50; c-f, x 100; g, h, x 200; inserts in e, f, x 400

Positive staining for HGF was seen throughout the tumor, with scattered cells of higher expression (Fig. 4c). Immunohistochemistry also revealed an expression of c-met in the outer cell layer in a configuration similar to that of Reg IV (Fig. 4d).

Discussion

We investigated a panel of neuroendocrine neoplasms by immunohistochemistry for the expression of Reg IV and Hath1, two markers associated with neural or neuroendocrine differentiation. A systematic comparison of the expression of these proteins in neuroendocrine tumors has not previously been reported. The expression patterns were shown to be dissimilar in the majority of the tumors, with the exception of a consistent co-expression in tumors originating from ileum and appendix. The tumor origin-based differential expression of Reg IV and Hath1 may provide an additional tool for differential diagnosis and follow-up of neuroendocrine neoplasms.

Previous reports describing the expression of Reg IV and Hath1 in normal adult tissues and tumors support the current findings, although some discrepancies are also noted. In the current study, neuroendocrine tumors of the pancreas were Reg IV negative. In contrast to our findings, Oue et al. (2005) reported expression of Reg IV in the insulin-producing neuroendocrine cells of the pancreas. Others have reported Reg IV expression in tumors of the exocrine pancreas (Oue et al., 2005; Takehara et al., 2006). Our antibodies to Reg IV gave only a weak hue, interpreted as background staining in the exocrine pancreas, while islet cells remained fully negative. We did not, however, include any tumors of the exocrine pancreas, in which the staining pattern could be different from the normal tissue. Apparently the difference of findings with regard to Reg IV expression is not due to a weak target binding of our antibodies, since the negative result in both the normal and malignant endocrine pancreas was confirmed using

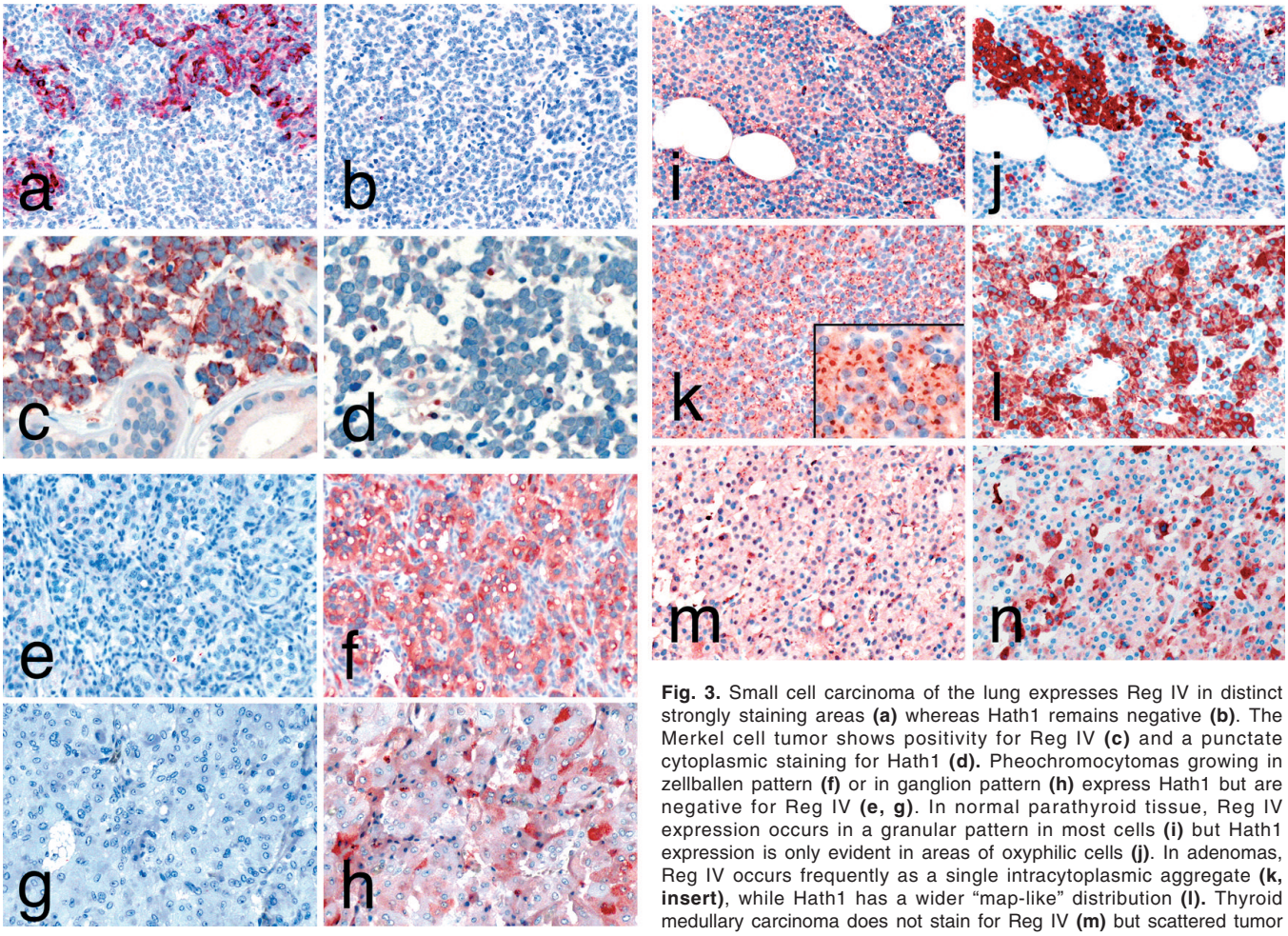

Fig. 3. Small cell carcinoma of the lung expresses Reg IV in distinct strongly staining areas **(a)** whereas Hath1 remains negative **(b)**. The Merkel cell tumor shows positivity for Reg IV **(c)** and a punctate cytoplasmic staining for Hath1 **(d).** Pheochromocytomas growing in zellballen pattern **(f)** or in ganglion pattern **(h)** express Hath1 but are negative for Reg IV **(e, g)**. In normal parathyroid tissue, Reg IV expression occurs in a granular pattern in most cells **(i)** but Hath1 expression is only evident in areas of oxyphilic cells **(j)**. In adenomas, Reg IV occurs frequently as a single intracytoplasmic aggregate **(k, insert)**, while Hath1 has a wider "map-like" distribution **(l).** Thyroid medullary carcinoma does not stain for Reg IV **(m)** but scattered tumor cells show strong cytoplasmic staining for Hath1 **(n)**. a, b, x 50; c, d, x 200; e-m, x 100; insert in k, x 200

commercially available monoclonal and polyclonal antibodies to Reg IV. As for Hath1, we showed that the neuroendocrine cells in normal islets of Langerhans consistently and strongly stained for Hath1, as did tumors derived from the endocrine pancreas (insulomas). This is inconsistent with the findings of Tsuchiya et al. (2007) who screened normal human tissues using Northern blot and found that the expression of *Hath1* was restricted to the lower gastrointestinal tract, lung and pancreas remaining negative. Differences in the longevity of different species of mRNA in different cell types may explain the discrepancies between the protein- and mRNA-based findings. Reports on Hath1 in neuroendocrine tumors of pancreas have not been published previously.

We found Reg IV expression in two of the seven small-cell lung carcinoma (SCLC) samples studied, while all of the samples were Hath1 negative. Oue et al. (2005) did not detect Reg IV in their SCLC samples. Westerman et al. (2007) reported expression of Hath1 in one of three SCLC samples studied. These differences might be due to the fact that SCLC consists of a heterogenous group of tumors, a part of them displaying some intestinal features.

In the current material, the results for Reg IV and Hath1 staining were the most consistent in the samples of tumors of gastrointestinal or pancreatic origin. Pancreatic islet derived tumors stained consistently for Hath1 and remained negative for Reg IV, as did their lymph node and liver metastases. Midgut neuroendocrine neoplasms stained positive for both Reg IV and Hath1, and this staining pattern was preserved in their liver and lymph node metastases. The specific staining patterns of tumors of gastrointestinal and pancreatic origin and their stringent inheritage to the metastatic lesions derived from these tumors may be useful for tracking the site of the primary tumor when metastatic lesions of neuroendocrine neoplasms are found.

Midgut neuroendocrine neoplasms displayed strong co-expression of Reg IV and Hath1. The majority of immunoreactive protein was confined to the cytoplasm of the tumor cells directly interacting with the fibromuscular stroma tissue or vessels, thus in contact with potential cytokines or growth factors. The treatment of human colon cells SW403 with transforming growth factor-alpha (TGFα), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and hepatocyte

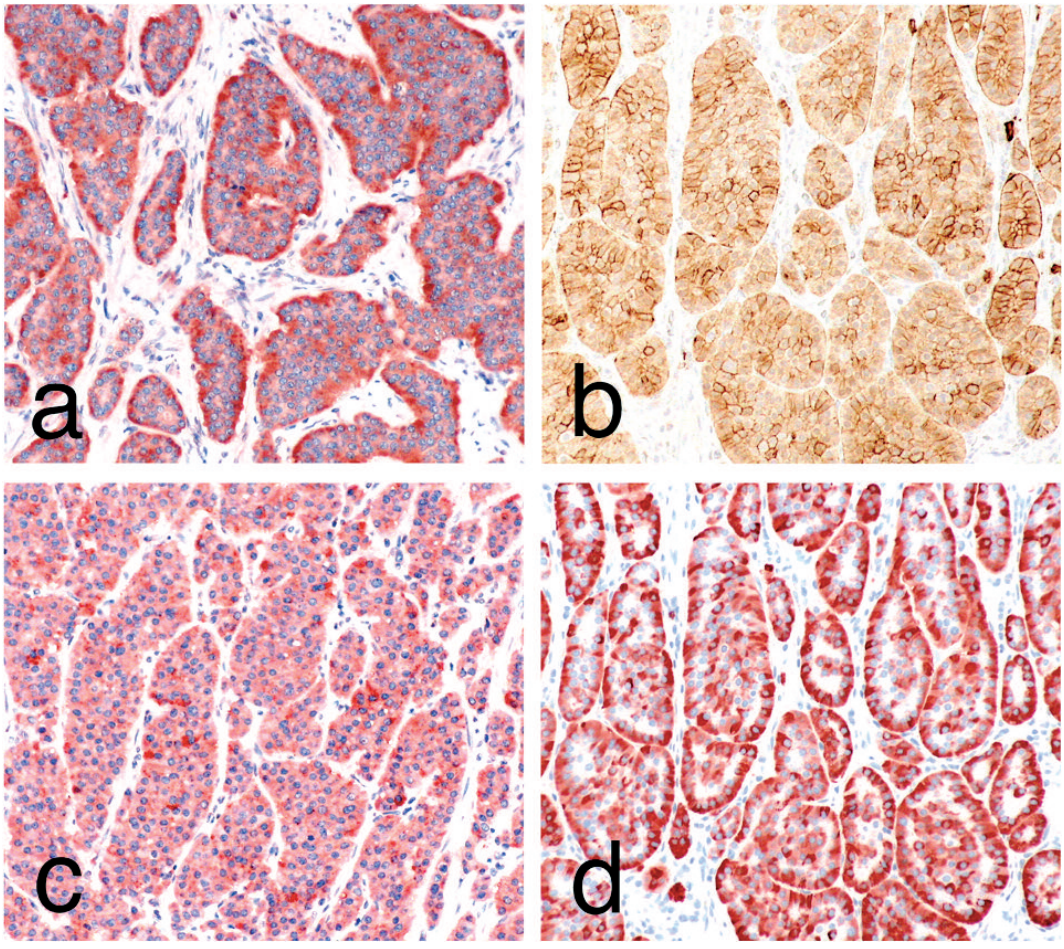

Fig. 4. In the ileal neuroendocrine tumors, positive staining for bFGF is evident in the peripheral cell layer **(a)**. The antibody to CD138, the common receptor for bFGF, stains the tumor cell membranes **(b)**. HGF staining is seen throughout the tumor, with scattered cells of higher expression **(c).** Antibodies to c-met stain the outer cell layer **(d)**, in a distribution similar to that of Reg IV and Hath1. x 100

growth factor (HGF) have been shown to induce expression of *Reg IV*. Moreover, the levels of *bFGF* and *HGF* mRNA have been reported to correlate with the level of *Reg IV* in the inflamed gut mucosa in ulcerative colitis. (Nanakin et al., 2007.) Interestingly, in cell cultures of auditory progenitor/stem cells, treatment with bFGF has also been reported to induce expression of Hath1 (Chen et al., 2007). We found that the spatial expression of bFGF in the midgut neuroendocrine tumors was similar to that of Reg IV and Hath1, i.e. mostly confined to the of the outer cell layer in contact with the stroma. Staining for the HGF receptor, c-met, also revealed strong reactivity in the peripheral cell layers, while expression of the ligand, HGF or scatter factor, was seen throughout the tumor tissue, and only individual tumor cells expressed greater amounts. CD138 (syndecan), the common receptor for fibroblast growth factors (FGFs), showed distinct membrane staining throughout the tumor tissue. It is tempting to speculate that the interaction with the surrounding stroma via paracrine and/or autocrine cytokines and growth factor loops may contribute to the expression of Reg IV in the peripheral cells.

It appears that Hath1 and Reg IV do not directly regulate each other. In the developing intestinal epithelium of the mouse, Notch signaling represses Math1 expression, and the following signaling cascade leads to neuroendocrine differentiation (review in Schonhoff et al., 2004). Reg IV gene is also activated during intestinal differentiation and maturation (Schröder et al., 2006). Forced expression of either Reg IV or Hath1 by transfection of the permissive human colon cancer cell line LS174T did not induce expression of mRNA for the other protein (Heiskala et al. in preparation). This suggests that the regulatory pathways for Reg IV and Hath1 expression are either independent, or converge further upstream.

The direct mechanism of action of Reg IV, as well as its specific receptor, remain to be elucidated. Reg IV, like the other Reg proteins, is a secreted protein and can even be detected in the circulation of patients with gastric cancer (Mitani et al., 2007). Given the expression of Reg IV in the outermost cell layer of the midgut carcinoids, it is interesting that the treatment of cancer cells with Reg IV, in addition to activation of pro-survival mechanisms, also enhanced the expression of matrilysin, which facilitates tumor invasion (Bishnupuri et al., 2006). It remains to be demonstrated whether Reg IV also acts as a prosurvival factor of carcinoids in vivo. If so, expression of Reg IV may contribute to the behavior of these tumors, which, despite their very low mitotic rate, exhibit metastatic propensity and relative refractoriness to conventional therapy.

The current results are also consistent with another potential function for Reg IV, a suppressor of inflammation. We originally reported strongly upregulated expression of Reg IV in the inflamed IBD mucosa (Kämäräinen et al., 2003). We suggest that the function of Reg IV here could be to confer a protective mechanism in the diseased mucosa by suppressing inflammation. Indeed, the pancreatitis-associated protein (PAP), a member of the type III subclass Reg family that is mainly produced by Paneth cells, also accumulates in diseased IBD mucosa (Gironella et al., 2005). PAP has been shown to prevent tumor necrosis factor-alpha (TNF-alpha)-induced NFκB activation in monocytes, epithelial, and endothelial cells, leading to reduced expression of mRNA for proinflammatory cytokines and adhesion molecules, hence clearly mediating an anti-inflammatory activity in IBD mucosa. Reg IV positive, invasively growing midgut neuroendocrine tumors do not induce the inflammatory responses that are usually seen around infiltrating carcinomas, and mucocellular (signet ring) gastric cancers that contain high amounts of Reg IV also invade the stomach wall without inflammatory responses. Reg IV may thus have the same kind of inflammation attenuating ability at the sites of tissue destruction as PAP.

In this study we have shown that the expression of Reg IV and Hath1 in human neuroendocrine tumors follows a pattern strictly dependent on the origin of the tumor. Neuroendocrine tumors of the midgut express both Reg IV and Hath1, whereas pancreas-derived neuroendocrine tumors only express Hath1. We suggest that the tumor origin-specific protein expression pattern of these neuroendocrine cell growth and differentiation markers can be used as a new tool to diagnose and follow neuroendocrine malignancies of the gastrointestinal tract. Although both proteins are implicated in the maturation of the secretory neuroendocrine cells in the gastrointestinal tract, Reg IV and Hath1 do not appear to belong to the same signal transduction pathway, or their pathways converge further upstream. Based on our immunohistochemical findings, we propose potential functions for Reg IV as a prosurvival factor or as a suppressor of inflammatory responses. The mechanism of action and the direct receptor of Reg IV remain unknown, however. Given the ability of Reg IV to transactivate epidermal growth factor receptor, and epidermal growth factor receptor activation to induce the expression of Reg IV, it is likely that there is a regulatory loop between these two proteins.

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References

Arakagi M., Tsuchiya K., Okamoto R., Yoshioka S., Nakamura T., Sakamoto N., Kanai T. and Watanabe M. (2008). Proteosomal degradation of Atoh1 by aberrant Wnt signaling maintains the undifferentiated state of colon cancer. Biochem. Biophys. Res. Commun. 368, 923-929.

Ben-Arie N., Bellen H.J., Armstrong D.L., McCall A.E., Gordadze P.R.,

Guo Q., Matzuk M.M. and Zoghbi H.Y. (1997). *Math1* is essential for genesis of cerebellar granule neurons. Nature 390, 169-172.

Bermingham N.A., Hassan B.A., Price S.D. Vollrath M.A., Ben-Arie N., Eatock R.A., Bellen H.J., Lysakowski A. and Zoghbi H.Y. (1999). *Math1*: an essential gene for the generation of inner ear hair cells. Science 284, 1837-1841.

Bishnupuri K.S., Luo Q., Murmu N., Houchen C.W., Anant S. and Dieckgraefe B.K. (2006). Reg IV Activates the Epidermal Growth Factor Receptor/Akt/AP-1 Signaling Pathway in Colon Adenocarcinomas. Gastroenterology 130, 137-149.

Chen W., Cacciabue-Rivolta D.I., Moore H.D. and Rivolta M.N. (2007). The human fetal cochlea can be a source for auditory progenitors/stem cell isolation. Hear. Res. 233, 23-29.

Gironella M., Iovanna J.L., Sans M., Gil F., Peñalva M., Closa D., Miquel R., Piqué J.M. and Panés J. (2005). Anti-inflammatory effects of pancreatitis associated protein in inflammatory bowel disease. Gut 54, 1244-1253.

Hartupee J.C., Zhang H., Bonaldo M.F., Soares M.B. and Dieckgraefe B.K. (2001). Isolation and characterization of a cDNA encoding a novel member of the human regenerating protein family: Reg IV. Biochim. Biophys. Acta 1518, 287-293.

Heiskala K., Giles-Komar J., Heiskala M. and Andersson L.C. (2006). High expression of RELP (Reg IV) in neoplastic goblet cells of appendiceal mucinous cystadenoma and pseudomyxoma peritonei. Virchows Arch. 448, 295-300.

Kämäräinen M., Heiskala K., Knuutila S., Heiskala M., Winqvist O. and Andersson L.C. (2003). RELP, a novel human Reg-Like Protein with up-regulated expression in inflammatory and metaplastic gastrointestinal mucosa. Am. J. Pathol. 163, 11-20.

Leonard J.H., Cook A.L., van Gele M., Boyle G.M., Inglis K.J., Speleman F. and Sturm R.A. (2002). Proneural and proneuro-endocrine transcription factor expression in cutaneous mechanoreceptor (Merkel) cells and Merkel cell carcinoma. Int. J. Cancer 101, 103-110.

Leow C.C., Polakis P. and Gao W.Q. (2005). A role for Hath1, a bHLH transcription factor, in colon adenocarcinoma. Ann. N.Y. Acad. Sci. 1059, 174-183.

Machold R.P., Kittell D.J. and Fishell G.J. (2007). Antagonism between Notch and bone morphogenic protein receptor signalling regulates neurogenesis in the cerebellar rhombic lip. Neural. Dev. 2, 5.

Mitani Y., Oue N., Matsumura S., Yoshida K., Noguchi T., Ito M., Tanaka S., Kuniyasu H., Kamata N. and Yasui W. (2007). Reg IV is a serum biomarker for gastric cancer patients and predicts response to 5-fluorouracil-based chemotherapy. Oncogene 26, 4383-4393.

Nanakin A., Fukui H., Fujii S., Sekikawa A., Kanda N., Hisatsune H., Seno H., Konda Y., Fujimori T. and Chiba T.(2007). Expression of the *REG IV*gene in ulcerative colitis. Lab. Invest. 87, 304-314.

Ohara S., Oue N., Matsubara A., Mita K., Hasegawa Y., Hayashi T., Usui T., Amatya V.J., Takeshima Y., Kuniyasu H. and Yasui W. (2008). Reg IV is an independent prognostic factor for relapse in patients with clinically localized prostate cancer. Cancer Sci. 99, 1570-1577.

Oue N., Hamai Y., Mitani Y., Matsumura S., Oshimo Y., Aung P.P., Kuraoka K., Nakayama H. and Yasui W. (2004). Gene expression profile of gastric carcinoma: Identification of genes and tags potentially involved in invasion, metastasis, and carcinogenesis by serial analysis of gene expression. Cancer Res. 64, 2397-2405.

Oue N., Mitani Y., Aung P.P., Sakakura C., Takeshima Y., Kaneko M., Noguchi T., Nakayma H. and Yasui W. (2005). Expression and localization of Reg IV in human neoplastic and non-neoplastic tissues: Reg IV expression is associated with intestinal and neuroendocrine differentiation in gastric adenocarcinoma. J. Pathol. 207, 185-198.

Park E.T., Oh H.K., Gum J.R., Crawley S.C., Kakar S., Engel J., Leow C.C., Gao W.Q. and Kim Y.S. (2006). HATH1 expression in mucinous cancers of the colorectum and related lesions. Clin. Cancer Res. 12, 5403-5410.

Schonhoff S.E., Giel-Moloney M. and Leiter A.B. (2004). Minireview: Development and differentiation of gut endocrine cells. Endocrinology 145, 2639-2644.

Schröder N., Sekhar A., Geffers I., Müller J., Dittrich-Breiholz O., Kracht M., Wedemeyer J. and Gossler A.(2006). Identification of mouse genes with highly specific expression patterns in differentiated intestinal epithelium. Gastroenterology 130, 902-907.

Schroyer N.F., Helmrath M.A., Wang V.Y.C., Antalffy B., Henning S.J. and Zoghbi H.Y. (2007). Intestine-specific ablation of *Mouse atonal homolog 1 (Math1)* reveals a role in cellular homeostasis. Gastroenterology 132, 2478-2488.

Takehara A., Eguchi H., Ohigashi H., Ishikawa O., Kasugai T., Hosokawa M., Katagiri T., Nakamura Y. and Nakagawa H. (2006). Novel tumor marker REG4 detected in serum of patients with resectable pancreatic cancer and feasibility for antibody therapy targeting REG4. Cancer Sci. 97, 1191-1197.

Tsuchiya K., Nakamura T., Okamoto R., Kanai T. and Watanabe M. (2007). Reciprocal targeting of Hath1 and ß-catenin by Wnt glycogen synthase kinase 3b in human colon cancer. Gastroenterology 132, 208-220.

Violette S., Festor E., Pandrea-Vasile I., Mitchell V., Adida C., Dussaulx E., Lacorte J.M., Chambaz J., Lacasa M. and Lesuffleur T. (2003). Reg IV, a new member of the regenerating gene family, is overexpressed in colorectal carcinomas. Int. J. Cancer 103, 185-193.

Westerman B.A., Breuer R.H.J., Poutsma A., Chhatta A., Noorduyn L.A., Koolen M.G., Postmus P.E., Blankenstein M.A. and Oudejans C.B. (2007). Basic helix-loop-helix transcription factor profiling of lung tumors shows aberrant expression of the proneural gene atonal homolog 1 (ATOH1, HATH1, MATH1) in neuroendocrine tumors. Int. J. Biol. Markers 22, 114-123.

Yang Q., Bermingham N.A., Finegold M.J. and Zoghbi H.Y. (2001). Requirement of *Math1* Cell Lineage Commitment in the Mouse Intestine. Science 294, 2155-2158.

Zhang Y., Lai M., Lv B., Gu X., Wang H., Zhu Y., Zhu Y., Shao L. and Wang G. (2003a). Overexpression of Reg IV in colorectal adenoma. Cancer Lett. 200, 69-76.

Zhang Y., Ding L. and Lai M. (2003b). *Reg* gene family and human diseases. World J. Gastroenterol. 9, 2635-2641.

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