# *ECRG4* expression in normal rat tissues: expression study and literature review

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## Abstract

The *Esophageal Cancer Related Gene 4* (*ECRG4*) is a highly conserved tumour suppressor gene encoding various peptides (augurin, CΔ16 augurin, ecilin, argilin, CΔ16 argilin) which can be processed and secreted. In the present work, we examined *ECRG4* expression and location in a wide range of rat organs and reviewed the available literature. *ECRG4* mRNA was identified in all examined tissues by quantitative PCR (qPCR). *ECRG4* immunoreaction was mainly cytoplasmic, and was detected in heart and skeletal muscles, smooth muscle cells showing only weak reactions. In the digestive system, *ECRG4* immunostaining was stronger in the esophageal epithelium, bases of gastric glands, hepatocytes and pancreatic acinar epithelium. In the lymphatic system, immunoreactive cells were detectable in the thymus cortex, lymph node medulla and splenic red pulp. In the central and peripheral nervous systems, different neuronal groups showed different reaction intensities. In the endocrine system, *ECRG4* immunoreaction was detected in the hypothalamic paraventricular and supraoptic nuclei, hypophysis, thyroid and parathyroid glands, adrenal *zona glomerularis* and medulla and Leydig cells, as well as in follicular and luteal cells of the ovary. In the literature, *ECRG4* has been reported to inhibit cell proliferation and increase apoptosis in various cell types. It is down-regulated, frequently due to hypermethylation, in esophageal, prostate, breast and colon cancers, together with glioma (oncosuppressor function), although it is up-regulated in papillary thyroid cancer (oncogenic role). *ECRG4* expression is also higher in non-proliferating cells of the lymphatic system. In conclusion, our identification of *ECRG4* in many structures suggests the involvement of *ECRG4* in the tumorigenesis of other organs and also the need for further research. In addition, on the basis of the location of *ECRG4* in neurons and endocrine cells and the fact that it can be secreted, its role as a neurotransmitter/neuromodulator and endocrine factor must be examined in depth in the future.

## Introduction

The *Esophageal Cancer Related Gene 4* (*ECRG4*) is a tumour suppressor gene originally cloned and identified by Su *et al*.[1] from normal human esophageal epithelium. It is located in chromosome 2q14.1-14.3 and contains 4 exons, spanning about 13 kb and having a corresponding cDNA of 772 bp. An initial bioinformatics analysis followed by biochemical characterisation demonstrated that *ECRG4* encodes a peptide hormone which is processed and secreted.[2] Further studies showed that the *ECRG4* open reading frame encodes a 148 amino acid protein which can be cleaved into multiple peptides.[3-6] Apart from the full-length protein, the cleavage of a leader peptide yields the shorter protein called augurin, and further cleavage of the last carboxyl terminal 16 amino acids produces the so-called CΔ16 augurin. The protein augurin is also cleaved by a furin-like process into two different peptides called ecilin and argilin, and argilin is cleaved into a CΔ16 form.[6] The sequences of human, mouse and rat *ECRG4* are shown in Figure 1. *ECRG4* contains a signal peptide sequence and a single putative pro-hormone cleavage site, followed by a long putative peptide which is highly conserved in mammals and fish. It also contains many aromatic amino acids and shows significant conservation in the sequence of the N-terminal putative pro-hormone cleavage site.

*ECRG4* processing by furin has been reported essential for inhibition of tumour cell (AtT-20, SF767, 400-14) proliferation.[5] *ECRG4* also contains a porin/VDAC homology domain, a cell division cycle 45 homology domain, and an anaphase-promoting complex subunit 10 homology domain, indicating involvement in mitochondrial membrane permeability and the cell cycle.[7] Anti-Flag immunocytochemistry following transfection of Flag-*ECRG4* into rat pancreatic β-TC3 cells revealed co-location into dense core granules of the secretory pathway, and Western blotting of supernatant demonstrated secretion of *ECRG4*-derived peptides.[2] Analysis of the subcellular location of *ECRG4* by immunofluorescence in transfected cells has also revealed it in the mitochondria, Golgi apparatus and endoplasmic reticulum.[3,7] In particular, *ECRG4* is first O-tyrosine-sulphated in the Golgi and then cleaved by furin just before or immediately after secretion along the constitutive secretory pathway.[5]

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Recent studies have also shown that, after secretion, *ECRG4* remains tethered in the cell surface of prostate (PC3) and kidney (HEK) epithelial cells[8] and choroid plexus cells.[6,9] Cell tethering is mediated by an NH2-terminus hydrophobic leader sequence, which enables trafficking to the surface. In some cells, such as PC3 and those of the choroid plexus, but not HEK, *ECRG4* may be further processed, with release of cell surface proteins, to generate soluble peptides.[6,8-10] In this sense, *ECRG4* has been proposed to be more similar to cytokines like the tumour necrosis factor and growth factors like epidermal/transforming growth factors, *i.e.*, cell membrane proteins which can be released by cell surface processing.[8] Besides normal esophagus, *ECRG4* has also been found by Northern blotting, RT-PCR and/or quantitative PCR (qPCR) to be expressed in many other tissues (brain, liver, kidney, heart, placenta, lung, pancreas, spleen, thymus, prostate, testis, bone marrow, skeletal muscle, cartilage, bladder, breast, corneal endothelium, and small and large intestine).[1,7,11-16] Microarray analysis indicates the highest expression in the thyroid, pituitary, testis and adrenals in humans, and in adrenals, ovaries, digits, retina and trachea in mice.[17] In the brain, its expression is highest in the olfactory bulbs, cerebellum, hypothalamus

and amygdala,[17] although further evaluation by qPCR showed the highest expression in the choroid plexus.[6] *In situ* hybridisation in mice revealed prominent ECRG4 expression in the intermediate lobe of the pituitary, glomerular layer of the adrenal cortex, choroid plexus, and atrio-ventricular node of the heart, and on embryonic day 18.5 mouse adrenal cortex, choroid plexus and bone.[2,6] Immunohistochemical analyses of *ECRG4* expression have mainly been performed in the brain. In particular, it has been identified in neurons of the paraventricular and supraoptic nuclei and axonal projections into the neurohypophysis and median eminence.[9,18] In rodents and humans, *ECRG4* has been immunohistochemically identified in the ventricular side and secretory vesicles of choroid plexus cells, and immunoblotting has revealed it in cerebrospinal fluid as a 14-kDa band.[6,9] In the developing brain of mouse embryo, the expression of *ECRG4* has been found mainly located in the choroid plexus and, in rat, in ventricular ependymal cells.[6] Other authors have also recently reported *ECRG4* full-length 14 kDa protein in mouse lung by immunoblotting and in type I alveolar cells by immunohistochemistry.[16] The aim of the present study was to analyse *ECRG4* expression in a wide series of normal rat tissues by qPCR and immunohistochemistry, in order to establish its expression profiles in the rat. A detailed review of the available literature about *ECRG4* functions has also been performed.

## Materials and Methods

### Materials

Rat tissues were obtained from six 7-week-old intact rats of Wistar strain, three males and three females, kept under a 12-h light:12-h dark cycle (illumination onset at 8.00 a.m.) at 23°C, and maintained on a standard diet and tap water *ad libitum*. Animals were sacrificed by decapitation. The study protocol was approved by the local Ethics Committee for Animal Studies. Sampled tissues are shown in Figure 2. For mRNA expression studies, tissues were immediately placed in RNA later (Qiagen, Hilden, Germany) and frozen at -80°C. Tissues for immunohistochemistry were

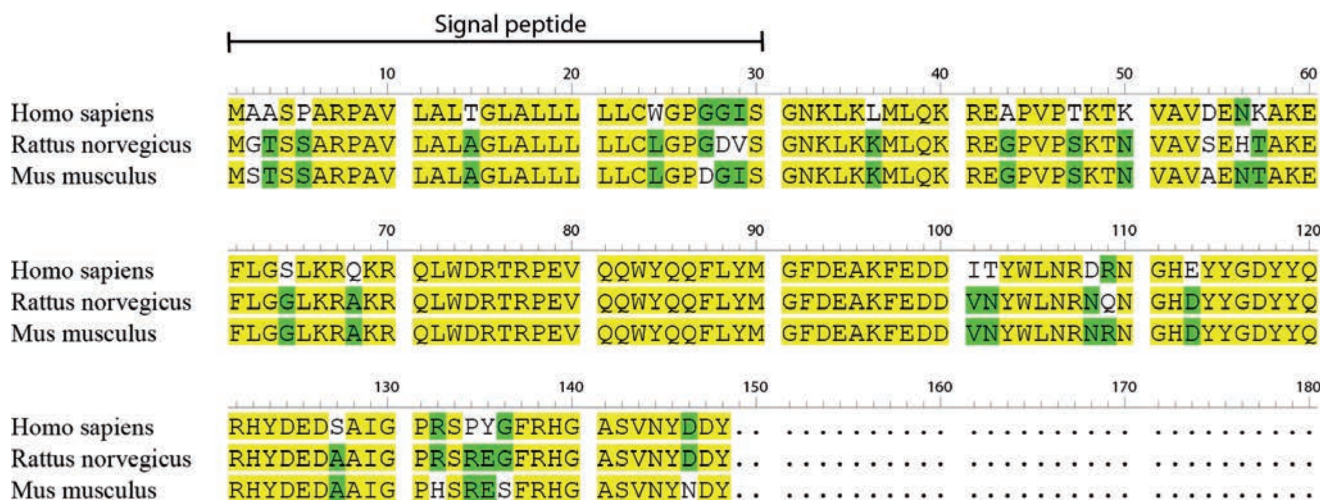


**Figure 1. Amino acid sequences of human, mouse and rat ECRG4. Signal peptide is marked. Yellow, 100% homology. Green, homology more than 50% but less than 100%.**

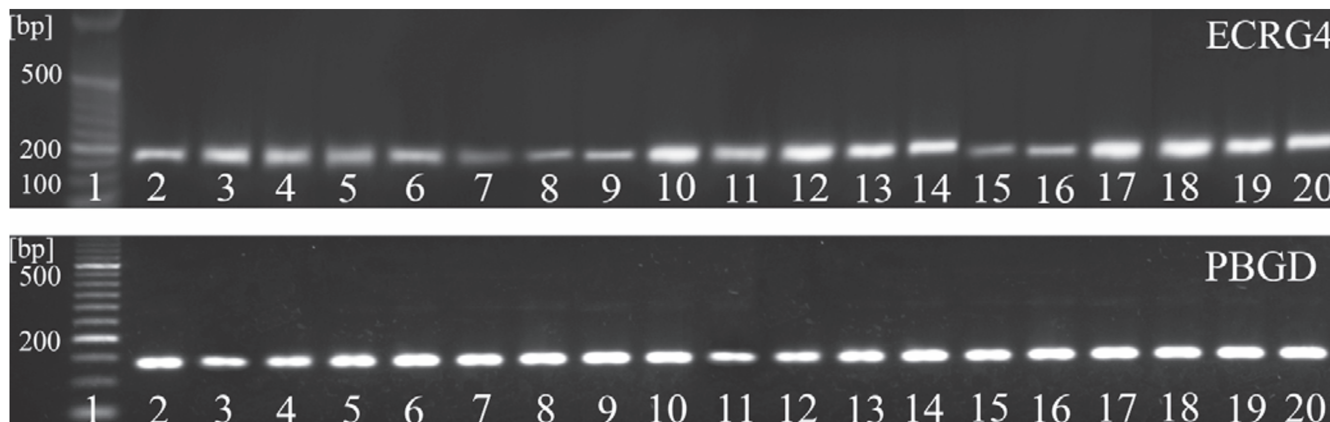


**Figure 2. Ethidium bromide-stained 2% agarose gel showing specific product for expression of *ECRG4* gene (upper panel) and reference gene (lower panel) in various tissues. 1, DNA molecular marker size; 2, esophagus; 3, stomach; 4, small intestine; 5, liver; 6, pancreas; 7, lung; 8, skeletal muscle; 9, heart; 10, uterus; 11, thymus; 12, spleen; 13, kidney; 14, urinary bladder; 15, brain; 16, hypothalamus; 17, hypophysis; 18, adrenal gland; 19, testis; 20, ovary.**

fixed in 10% buffered formalin for 48 h and embedded in paraffin.

### Quantitative PCR (qPCR)

The applied methods have already been described.[19-28] Briefly, total RNA was extracted by the method with TRI REAGENT (Sigma) and purified on columns (Rneasy Mini Kit, Qiagen). mRNA was extracted from total RNA with the PolyATract® mRNA Isolation System III (Promega, Madison, WI), the amount of total mRNA was determined by measurement of optical density at 260 nm, and purity was estimated by the 260/280 nm absorption ratio, which was consistently higher than 1.8 (NanoDrop spectrophotometer, Thermo Scientific, Milan, Italy). Reverse transcription was performed by AMV reverse transcriptase (Promega) with Oligo dT (PE Biosystems, Warrington, UK). Primers used for expression analysis experiments were designed by Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) (Table 1) and were purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw. qPCR was performed by means of the lightcycler 2.0 instrument (Roche, Milan, Italy) with ver. 4.05 software. The SYBR green detection system was used with the above-mentioned primers. Each 20-µL dose of the reaction mixture contained 4 µL template cDNA (or standards, or control), 0.5 µM of each gene-specific primer, and the previously determined optimal MgCl2 concentration (3.5 µM per reaction). The LightCycler FastStart DNA Master SYBR Green I mix (Roche) was used. The qPCR program included a 10-minute denaturation step to activate the Taq DNA polymerase, followed by three-step amplification: denaturation at 95°C for 10 s, annealing at 56°C for 5 s, and extension at 72°C for 10 s. The specificity of reaction products was checked by determination of melting points (0.1°C/s transition rate). *ECRG4* gene expression was normalised to the *PBGD* reference gene. Reaction products were separated on ethidium bromide containing 2% agarose gel and photographed in a transilluminator. qPCR efficiency was assessed by a serial dilution method. Specific bands from agarose gel were extracted by the DNA gel extraction kit (Millipore, Milan, Italy), and the amount of extracted DNA was estimated spectrophotometrically. The extracted DNA was diluted (10-fold serial dilutions) in order to obtain standard curves for efficiency calculation. The applied version of LightCycler software (4.05.) allows evaluation of amplification efficiency plots.

### Immunohistochemistry

Sections were hydrated gradually through decreasing concentrations of ethanol and then washed in deionised $H_2O$. Antigen unmasking was performed with 10 mM sodium citrate buffer, pH 6.0, in an oven at 96°C for 60 min. This treatment allowed antigen retrieval without significant damage to the tissues; only in few cases was it necessary to repeat the procedure in further samples. The sections were incubated in 0.3% hydrogen peroxide for 10 min at room temperature, to remove endogenous peroxidase activity, and then in blocking serum (2% normal goat serum) for 30 min. Sections were incubated with primary rabbit polyclonal antibody anti-*ECRG4* (HPA008546 C2orf40; Sigma Prestige Antibodies, St. Louis, MO, USA) and diluted 1:50 in PBS for 1 h at room temperature. The immunogen sequence for development of the polyclonal antibody consisted of the sequence 40-146 of the full-length human *ECRG4*. Thus, the above antibody does not distinguish between the various peptide forms which may be produced from the full-length 148 amino acid-long *ECRG4* protein. For this reason, we refer here to *ECRG4* immunoreactivity, aware that different tissues or cells may show different peptides derived from *ECRG4*. The sections were then washed in PBS three times for 5 min, incubated with anti-mouse/rabbit serum for 30 min (Cat. No. MP-7500; Vector Laboratories Inc., Burlingame, CA, USA), and developed in 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, Milan, Italy). Lastly, they were counterstained with haematoxylin. *ECRG4* immunoreaction detected in human cerebellar Purkinje cells was used as a positive control, as recommended in the datasheet for the antibody. Negative controls were performed by omission of primary antibody. In addition, in order to further verify the immunohistochemical specificity of the reaction, absorption tests with *ECRG4*-derived peptides comprising the above immunogen sequence (Prepro-Augurin 42-70 Human, Cat. no. 012-19, and Augurin Human 71-150, Cat. no. 012-25; Phoenix Pharmaceuticals, Burlingame, CA, USA) were also performed. Although competition tests were useful to confirm the specificity of *ECRG4* immunoreactivity, they did not refer to immunoreactions to specific *ECRG4*-derived peptides, due to overlaps in their sequences, the length of the immunogen sequence and the polyclonality of the antibody.

Immunohistochemical staining for *ECRG4* was evaluated by scanning whole tissue specimens under low-power magnification (X5), later confirmed under high-power magnification (X10, X20, X40). Immunohistochemical evaluation included number of positive cells, pattern of immunostaining (nuclear, cytoplasmic, nucleocytoplasmic) and staining intensity. Overall staining intensity was stratified as: –, absent; +/-, barely detectable; +, weak; ++, moderate; +++, strong.[29,30] Data for these studies are listed in Table 2.

## Results

### qPCR

Classic gel electrophoresis of the qPCR product reaction revealed the presence of *ECRG4* mRNA in all tissues and organs studied: esophagus, stomach, small intestine, liver, pancreas, lung, skeletal muscle, heart, uterus, thymus, spleen, kidney, urinary bladder, brain, hypothalamus, adenohypophysis, adrenal gland, testis and ovary (Figure 2). We then applied *qPCR* to study the relative expression levels of the *ECRG4* gene. As shown in Figure 3, the highest levels of *ECRG4* mRNA were found in esophagus, stomach, skeletal muscle, heart, uterus, urinary bladder and brain. In kidney, hypothalamus and adrenal gland, levels of *ECRG4* mRNA were moderate, and the lowest expression levels were found in small intestine, liver, pancreas, lung, thymus, spleen, adenohypophysis, testis and ovary.

**Table 1. qPCR primers of *ECRG4* and *PBGD* (porphobilinogen deaminase). Oligonucleotide sequences for sense (S) and antisense (A) primers are shown.**

| cDNA | GenBank Accession number | Primer | Primer sequence (5'-3') | Position | PCR product size (bp) |
|---|---|---|---|---|---|
| *ECRG4* | XM_343562 | S | GACCTGTTCCATCAAAGAC | 169-187 | 187 |
| | | A | AGCCAATAGTTGACATCATC | 336-355 | |
| *PBGD* | NM 013168 | S | GAAAGACCCTGGAAACCTTG | 397-416 | 148 |
| | | A | TGCTCATCCAGCTTCCGTA | 526-544 | |

## Immunohistochemistry

We found *ECRG4* immunoreactivity in many tissues and organs, although expression levels differed among cell populations. As regards its intracellular location, *ECRG4* immunoreaction was mainly found in the cytoplasm. Absorption tests eliminated immunostaining in all tissues (some examples are given in Supplementary Figure 1), except for some very faint diffused stains still visible in some sections.

### Respiratory system

In tracheal epithelium, a few probably infiltrating cells with nuclear immunostaining were seen; bronchial and bronchiolar epithelial cells were negative (Figure 4A). No staining was seen in tracheal or bronchial chondrocytes. Bronchiolar smooth muscle cells (Reissesen membrane) were weakly immunopositive. In lung parenchyma, the great majority of pneumocytes were negative, although rare alveolar lining cells with weak/moderate cytoplasmic immunoreaction were found (Figure 4B).

### Muscles

Skeletal (Figure 4C) and heart muscles (Figure 4D) showed moderate immunostaining. Smooth muscle cells of the gastrointestinal tract, urinary system and vessels were rather negative or showed only weak cytoplasmic immunostaining.

### Bone

Osteoblasts, osteocytes and osteoclasts were mainly negative. Cells in the bone marrow were also negative.

**Table 2. Expression of *ECRG4*-like immunoreactivity in normal rat tissues.**

| Tissues and organs | Cell types | Nuclear positivity | Cytoplasmic positivity |
|---|---|---|---|
| Muscular tissues | | | |
| Heart | Muscle cells | - | ++ |
| Skeletal muscle | Muscle cells | - | ++ |
| Smooth muscle | Muscle cells | - | +/- |
| Bone | Osteoblasts | - | - |
| | Osteoclasts | - | - |
| | Marrow | - | - |
| | Megakaryocytes | - | - |
| Digestive system | | | |
| Esophagus | Epithelium | - | ++ |
| Stomach | Superficial epithelium | - | - |
| | Base of glands | - | + |
| Small intestine | Epithelium | - | - |
| Colon | Epithelium | - | - |
| Liver | Hepatocytes | - | ++ |
| Exocrine pancreas | Acinar epithelium | - | + |
| | Ductal epithelium | - | - |
| Respiratory system | | | |
| | Alveolar epithelium | - | - |
| | Bronchial epithelium | - | - |
| Urinary system | | | |
| Kidney | Renal corpuscles | - | - |
| | Tubular epithelium | - | - |
| Bladder | Epithelium | - | - |
| Male reproductive system | | | |
| Testicle | Spermatogonias | - | - |
| | Spermatocytes | - | - |
| | Sertoli cells | - | - |
| | Leydig cells | - | ++ |
| | Residual bodies | - | ++ |
| Epididymis | Epithelium | - | - |
| Seminal vesicles | Epithelium | - | - |
| Prostate ventral lobe | Epithelium | +/- | +/- |

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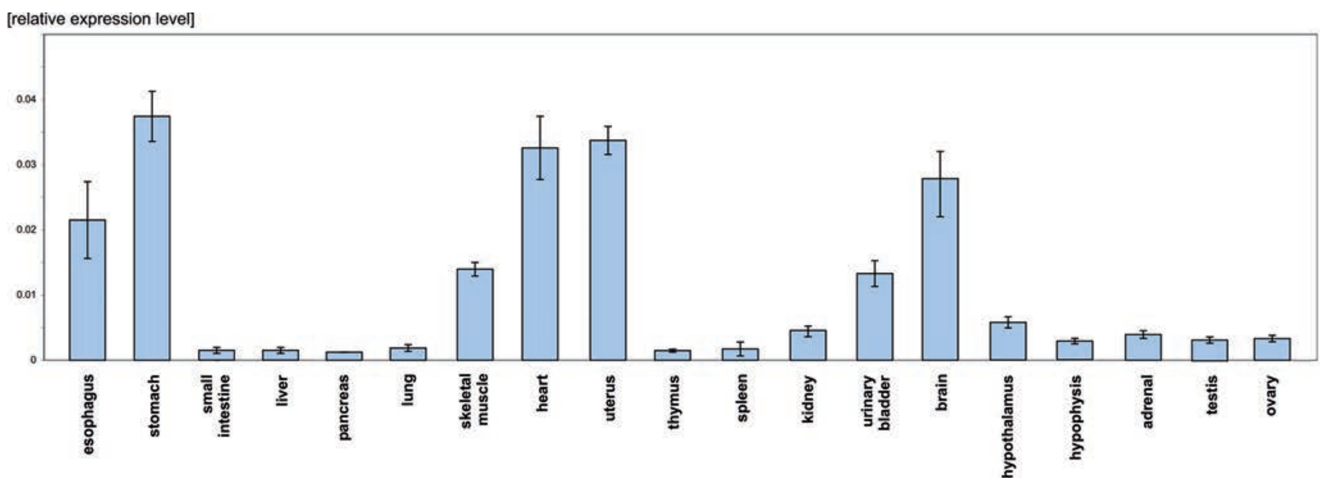


**Figure 3. Relative mRNA expression level of *ECRG4 i*n various tissues. Bar plots: expression ± SE. Expression profile calculated in relation to *PBGD* reference gene. In each group, n=3.**

### Cartilage

Chondrocytes were mainly negative.

### Lymphatic tissues

In lymph nodes (Figure 5 A-B), most of the cortical cells were negative, whereas the medulla cells showed moderate/strong cytoplasmic immunoreactivity.The epithelial cells of the thymus were negative in both medullary and cortical compartments, Hassal's corpuscles were also negative. Lymphatic cells were mainly negative, but some quite numerous groups of cells showing moderate/strong cytoplasmic immunoreactivity were also visible, mainly in the cortical compartment (Figure 5C). In the spleen, white pulp cells were mainly negative, whereas those in the red pulp showed moderate or strong cytoplasmic immunostaining (Figure 5D). Aggregates of lymphocytes along the alimentary canal were mainly negative, with a few cells showing moderate cytoplasmic staining.

### Digestive system

In the salivary glands, ductal and acinar epithelia were mainly negative, as were the connective septa (Figure 6A). The esophageal epithelium showed moderate cytoplasmic immunostaining, mainly in the superficial layers (Figure 6B). The *lamina propria* and submucosal layer were negative. Smooth muscle cells of the muscularis mucosae and muscle layer showed weak cytoplasmic staining. In the stomach, epithelial cells were mainly negative, although some cells in the gastric gland bases showed moderate cytoplasmic staining (Figure 6C). In the small intestine, epithelial cells were negative, but some cells showing strong cytoplasmic immunostaining were visible in the *lamina propria* (Figure 6D). Hepatocytes showed moderate cytoplasmic staining without nuclear immunoreaction. Connective tissue in the portal spaces was negative. Sinusoidal lining cells, portal tracts, terminal hepatic venules and hepatic arterioles were negative. Bile ducts were usually negative (Figure 6E). In the exocrine pancreas, weak cytoplasmic immunoreaction was found in some acinar secretory cells. Connective septa were negative (Figure 6F).

### Urinary system

Renal corpuscles and tubular epithelium were usually negative or weakly positive. The surrounding fibrous stroma was negative, although rare cells showing sometimes strong cytoplasmic immunoreaction were detectable (Figure 7A). Ureter and bladder epithelium (Figure 7B) was mainly negative, whereas muscle layers showed weak/moderate positivity.

### Female germinal system

In the ovary (Figure 7C-D), granulosa cells frequently showed moderate cytoplasmic immunostaining. Thecal cells showed weak cytoplasmic staining. The *corpora lutea* sometimes showed quite strong cytoplasmic immunostaining, mainly in their central parts. Interstitial gland cells showed weak cytoplasmic immunostaining. Stroma was mainly negative. The epithelial cells of the oviducts (Figure 7E) showed moderate or strong cytoplasmic immunoreactivity, whereas stroma was negative and muscle layers only showed weak cytoplasmic staining. Endometrial and vaginal epithelium showed weak cytoplasmic immunostaining. The *lamina propria* was negative.

### Male germinal system

In the testis, spermatogonias, spermatocytes and spermatids, together with Sertoli cells, did not immunostain. Conversely, Leydig cells showed moderate/strong cytoplasmic positivity (Figure 7F). The epithelia of epididymis

**Table 2. Continued from previous page.**

| Tissues and organs | Cell types | Nuclear positivity | Cytoplasmic positivity |
|---|---|---|---|
| Female reproductive system | | | |
| Ovary | Oocytes | - | - |
| | Follicular epithelium | - | ++ |
| | Thecal cells | - | +/- |
| | Luteal cells (central) | - | ++ |
| | Luteal cells (peripheral) | - | +/- |
| Oviduct | Epithelium | - | ++ |
| Uterus | Endometrial cells | - | + |
| Vagina | Epithelium | - | + |
| Endocrine glands | | | |
| Hypophysis | Anterior lobe | - | + |
| | Pars intermedia | - | + |
| | Posterior lobe | - | + |
| Thyroid | Follicular epithelium | +/- | + |
| | Parafollicular cells | - | + |
| Parathyroid | Principal cells | - | + |
| Adrenal gland | ZG | - | ++ |
| | ZF/R | - | - |
| | Medulla | - | ++ |
| | Ganglionic cells | - | ++ |
| Islets of Langerhans | Langerhans cells | - | - |
| Lymphatic system | | | |
| Thymus | Epithelial cells | - | - |
| | Lymphatic cells, Cortex | - | ++ |
| | Lymphatic cells, Medulla | - | - |
| Lymph node | Lymphatic nodules – germinal centre | - | - |
| | Deep cortex | - | - |
| | Medullary region | - | ++ |
| Spleen | Red pulp | - | ++ |
| | White pulp | - | - |
| Nervous system | | | |
| Brain cortex | Neurons | - | +/- |
| | Glia | - | - |
| Cerebellum | Purkinje cells | - | ++ |
| | Granular and molecular layers | - | +/- |
| | Glia | - | - |
| Brainstem | Neurons | - | ++ |
| | Glia | - | - |
| Trigeminal ganglia | Neurons | - | ++ |
| Superior cervical ganglia | Neurons | - | ++ |
| Eye | | | |
| Retina | | - | - |
| Cornea | | - | - |
| Ciliary corpus | | - | - |

ZG, zona glomerularis; ZF/R, zona fasciculata-reticularis; immunostaining intensity graded as -, negative; -/+, barely detectable; +, weak; ++, moderate; +++, strong.


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and seminal vesicles were mainly negative. Prostate also showed quite variable immunoreaction.

### Endocrine glands

In the hypothalamus, neurons of the supraoptic (Figure 8A) and paraventricular nuclei showed moderate cytoplasmic immunoreactivity; the other hypothalamic nuclei were mainly negative. In the adenohypophysis, some cells showed weak cytoplasmic immunostaining; others did not immunostain. In the neurohypophysis, fibres and pituicytes showed weak/moderate immunostaining. The cells of the pars intermedia showed weak cytoplasmic immunoreaction. In the thyroid, follicular epithelial cells showed heterogeneous staining. In some follicles, epithelial cells showed weak/moderate cytoplasmic staining; others did not immunostain. Parafollicular cells also showed weak cytoplasmic immunoreactivity (Figure 8B). The principal cells of parathyroid glands showed weak cytoplasmic immunostaining. Adrenal cortex cells showed moderate *ECRG4* cytoplasmic immunostaining in the *zona glomerularis* (ZG), while *zona fasciculata-reticularis* (ZF/R) was mainly negative. Adrenal medullary and ganglionic cells showed moderate cytoplasmic positivity (Figure 8 C-D). The cells of the islets of Langerhans did not show *ECRG4* immunostaining (Figure 6F).

### Nervous system and eye

In the central nervous system, some neuronal groups showed moderate cytoplasmic immunostaining, whereas others were negative. Glial cells were usually negative, except for ventricular ependymal cells and choroid plexus epithelial cells, which showed moderate immunoreactivity. In the brain cortex and hippocampus (CA1, CA2, CA3, CA4, dorsal and ventral blades of the dentate gyrus), neurons were negative or showed weak cytoplasmic immunoreactivity (Figure 9A). In the cerebellar cortex, Purkinje cells showed moderate cytoplasmic immunostaining. In the granular and molecular layers, most cells were negative, although some showing cytoplasmic immunostaining were also detected (Figure 9B). In the brainstem, various nuclei showed different percentages of immunostained neurons and different staining intensity, *e.g.*, the olivary complex showed quite high percentages of immunoreactivity (Figure 9C). As regards the peripheral nervous system, the ganglionic cells of both trigeminal (Figure 9D) and superior cervical ganglia showed quite strong cytoplasmic immunoreaction. The structures of the retina, cornea and ciliary body were mainly negative.

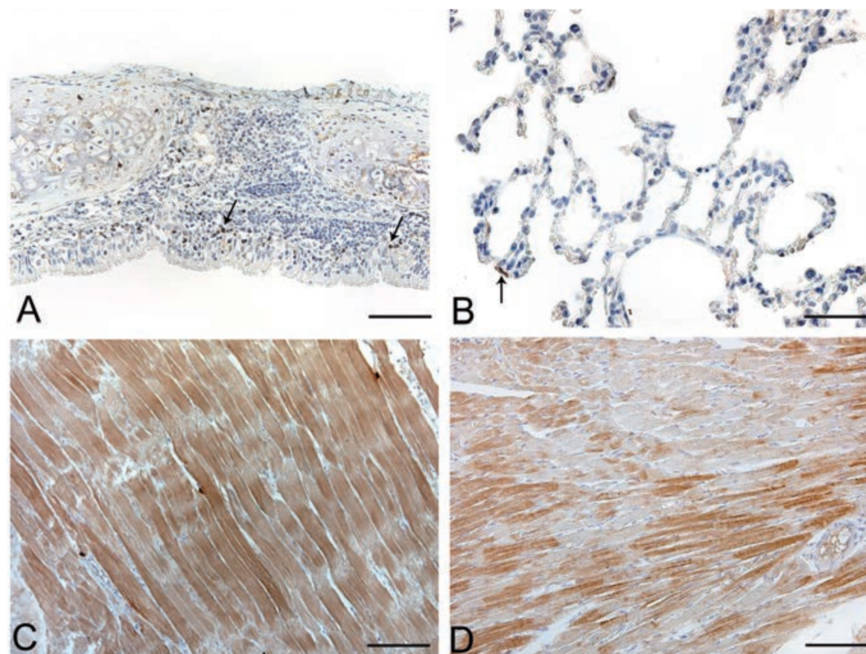


**Figure 4. Anti-*ECRG4* immunohistochemical staining of rat normal tissues. A) Trachea; positive immune cells (arrows) visible in tracheal mucosa; epithelial cells are mainly negative. B) Lung parenchyma; most cells are negative, with rare type I pneumocytes (arrow) immunostained. C) skeletal muscle. Diffuse moderate immunostaining of skeletal muscle fibers. D) Heart; groups of immunopositive heart muscle fibers interspersed with negative ones. Scale bars: A, D) 75 μm; C) 150 μm; B) 37.5 μm.**

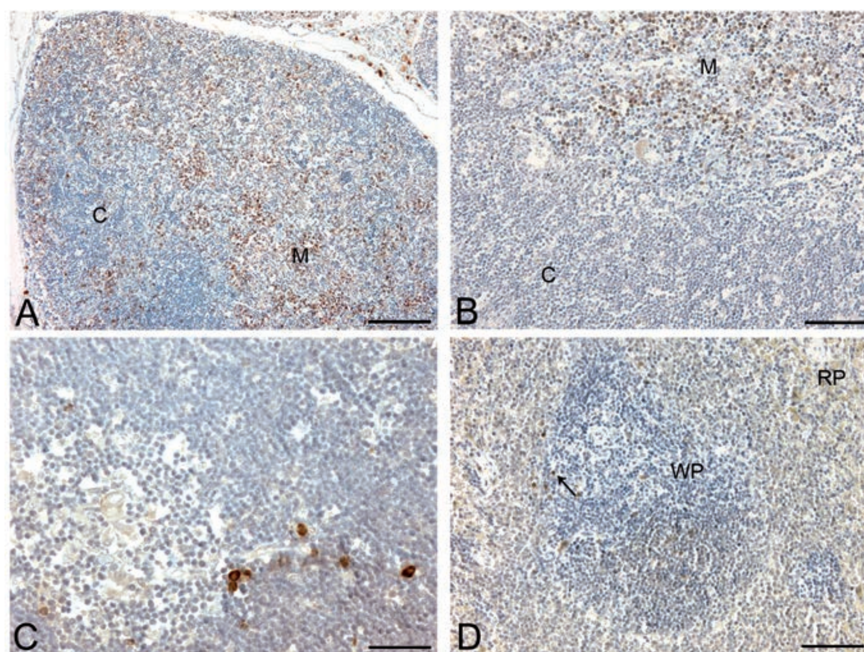


**Figure 5. Anti-*ECRG4* immunohistochemical staining of rat lymphatic system. A-B) Lymph node; in lymph node medulla (M), immunostained cells are more numerous than in cortex (C). C) Thymus; most cells are negative but some immune cells show moderate/strong immunoreactivity. D) Spleen; some positive immune cells (arrows) are visible in both white (WP) and red (RP) pulp. Scale bars: A, C, D) 75 μm; B) 37.5 μm.**

## Discussion

In this work, we carried out extensive analysis of *ECRG4* expression in many rat tissues by means of qPCR and immunohistochemistry. We discuss our results on the basis of a review of literature data on *ECRG4* expression and functions.

### Esophagus and *ECRG4* role in tumorigenesis

One of the first organs studied for the *ECRG4* expression and its possible role in tumorigenesis was the esophagus, which usually reveals *ECRG4* immunostaining in its epithelium. The *ECRG4* gene has been suggested to be involved in esophageal tumorigenesis, as *ECRG4* mRNA and protein are down-regulated in esophageal squamous cell carcinoma (ESCC)[1,11,31-34] and esophageal cancer cell lines (NEC, EC109 and EC9706).[31-36] No somatic mutations have been found in the *ECRG4* gene in esophageal cancer tissues,[33,35] although hypermethylation of a 5' CpG island in its promoter region is one of the main silencing mechanisms in esophageal cancer cell lines.[31,33,35] Low *ECRG4* mRNA and protein levels have been found to correlate with primary tumour size, regional lymph node metastasis and clinico-pathologic stage.[32,33] They are also associated with significantly shorter survival, *ECRG4* expression being an independent prognostic factor for ESCC patients.[32,33] Transfection of *ECRG4* gene into ESCC cells inhibits cell proliferation, colony formation and anchorage-independent growth in soft agar, and blocks cell cycle progression and reduces tumour growth *in vivo* after subcutaneous injection in nude mice.[33] As regards the molecular mechanisms involved, restoration of *ECRG4* expression in ESCC inhibits NF-kB expression and nuclear translocation and attenuates NK-kB target gene *COX-2* expression.[33] *ECRG4* has been reported to interact directly with *ECRG1*, in both binding affinity assays *in vitro* and co-immunoprecipitation assays *in vivo*. *ECRG4* and *ECRG1* co-expression further increases p21 expression, reinforces cell cycle G1 phase block and inhibits cell proliferation, strongly suggesting functional interactions between the proteins.[35] Inverse correlation has been reported between *ECRG4* expression and the Ki-67 labelling index in esophageal squamous cell carcinoma.[34]

### Expression and function of *ECRG4* in other epithelial tissues

In this study we identified *ECRG4* expression not only in esophageal epithelium but also in other epithelial cells of the digestive system, such as the cells of the bases of gastric glands, intestinal epithelium, hepatocytes and pancreatic exocrine cells. *ECRG4* immunostaining was identified in the epithelial structures of the male and female reproductive systems, such as ovary follicles, oviduct, uterus, vagina and prostate. Immunostaining in the respiratory and urinary systems was more variable. *ECRG4* has previously been identified in the epidermis, dermis and hair follicles of mouse skin.[37] Expression of *ECRG4* in many different types of epithelia (although not in all of them) obviously indicates its probable role. For some of the above epithelia, literature data indicate a role for *ECRG4* in proliferation/tumorigenesis, similar to what has already been shown for the esophagus.

*ECRG4* has been reported to play a role in cancers other than esophageal ones. It is down-regulated not only in prostate cancer,[38,39] breast cancer[40-41] and colon carcinoma,[3] but also in many human tumour epithelial cell lines (oral, esophageal, gastric, colon, lung, breast, cervical and renal cancers).[31,34,41] CpG site hypermethylation is considered to be the main mechanism of down-regulation in these neoplasms. It is significantly associated with prostate,[38,39] colorectal,[3,42] gastric[43] and breast[41] cancers. *ECRG4* hypermethylation has been reported for cell lines from colorectal, hepatocellular and breast carcinoma.[3,41] However, other regulatory mechanisms have also been suggested, as demethylating treatment was reported not to recoup *ECRG4* expression in some prostate cell lines.[39]

*ECRG4* is involved in the proliferation and

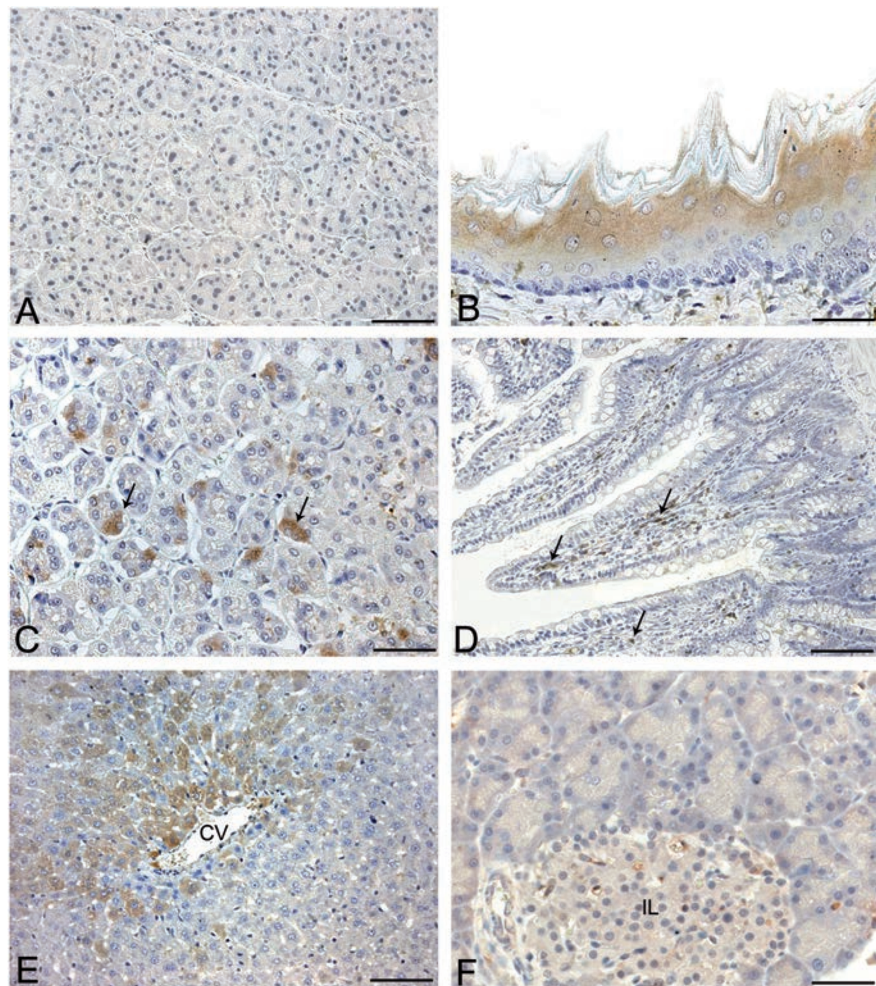


**Figure 6. Anti-*ECRG4* immunohistochemical staining of rat digestive system. A) Salivary gland; acinar and ductal epithelia are negative. B) Esophagus; moderate cytoplasmic immunostaining is appreciable in superficial layers. C) Stomach; in this slide, gastric glands are transversally cut and show moderate cytoplasmic immunoreaction in some epithelial cells (arrows). D) Small bowel; epithelial cells are mainly negative but ECRG4 positive immune cells are visible in *lamina propria*. E) Liver; groups of hepatocytes around centrilobular vein (CV) show moderate cytoplasmic immunostaining. F) Pancreas; exocrine acinar cells and cells of islets of Langerhans (IL) are mainly negative. Scale bars: A, C, F) 37.5 μm: D, E) 75 μm.**



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apoptosis of these epithelial types. Transfection of *ECRG4* or exposure to *ECRG4*-containing medium reduces the proliferation and cell viability of colorectal cell lines.[3] Jurkat and 293T cells transfected with *ECRG4* have been reported to show a significantly slower growth rate.[34] Over-expression of *ECRG4* into squamous cell carcinoma of the head and neck M2 cell line inhibits cell proliferation and promotes cell cycle arrest and apoptosis, together with Bax up-regulation and Bcl-2 and Cyclin A down-regulation. It also suppresses the growth rate and metastasis of tumor xenografts in nude mice.[44] Restoration of *ECRG4* expression in breast cancer cell lines by retroviral transduction decreases proliferation rate, migration and invasion of cells; conversely, *ECRG4* knock-down shows the opposite effect in those breast cancer cell lines which do not show suppression of *ECRG4* expression by hypermethylation.[41] Reduction of cell proliferation by *ECRG4* has been found to be due to mitotic inhibition by modulation of genes such as ubiquitin-conjugating enzyme E2C.[41] Decreased cell proliferation has been reported in human lung epithelial cells in response to *ECRG4* over-expression.[16]

Some clinical data have been published on the prognostic significance of *ECRG4* expression. In prostate cancer, for instance, it is associated with Prostate-Specific Antigen recurrence.[38] In breast cancer, *ECRG4* expression is higher in neoplasms showing earlier stage, smaller size, negative axillary lymph node status, lower grade and normal-like subtype, and less risk of distant metastasis. Down-regulation of this gene is associated with reduced disease-free and overall survival.[40,41] *ECRG4* hypermethylation is higher in stages III+IV of gastric cancer than in stages I+II.[43] The role of *ECRG4* has been investigated with reference to chemotherapeutic sensitivity. A human gastric cell line (SGC-7901) with tetracycline-inducible *ECRG4* expression has been established and *ECRG4* over-expression has been shown to increase apoptosis and inhibit growth in response to 5-fluorouracil.[45]

Our study also showed *ECRG4* expression in other epithelial types, not yet fully examined as regards their possible involvement in tumorigenesis, such as hepatocytes, pancreatic cells, and epithelial cells of the female reproductive system. Possible changes in *ECRG4* expression must also be examined in the case of inflammatory and injury responses.

## Nervous system

In the literature, *ECRG4* expression has been identified in the brain by RT-PCR,[1,11] in the choroid plexus by *in situ* hybridisation and immunohistochemistry[2,6] and in the paraventricular and supraoptic nuclei by immunohistochemistry.[9,18] Our immunohistochemical analysis confirmed the above findings, but also identified *ECRG4* expression in the neurons of many other central and peripheral nervous structures. In the central nervous system, some nuclei showed high percentages of positivity and strong immunostaining, whereas others were negative. Proper mapping of the structures of the nervous system will be necessary to evaluate exactly in which nervous structures *ECRG4* expression is higher. However, our analysis revealed higher *ECRG4* expression in some neuronal groups of the brainstem, cerebellum and peripheral ganglia, and lower percentages of positivity in the cerebral cortex. Among brainstem nuclei, the inferior olivary complex was one of the structures showing greater staining intensity. Purkinje cells showed moderate cytoplasmic immunostaining in the cerebellar cortex, but the majority of cells were negative in the granular and molecular layers.

In the central nervous system, experimental data indicate a role for *ECRG4* in proliferation/tumorigenesis, injury response and senescence mechanisms. *ECRG4* is down-regulated and hypermethylated in glioma.[3,46] Human U251 glioma cells transfected with *ECRG4* show decreased cell proliferation and inhibition of cell migration and cell cycle progression. The protein levels of NF-kB are also

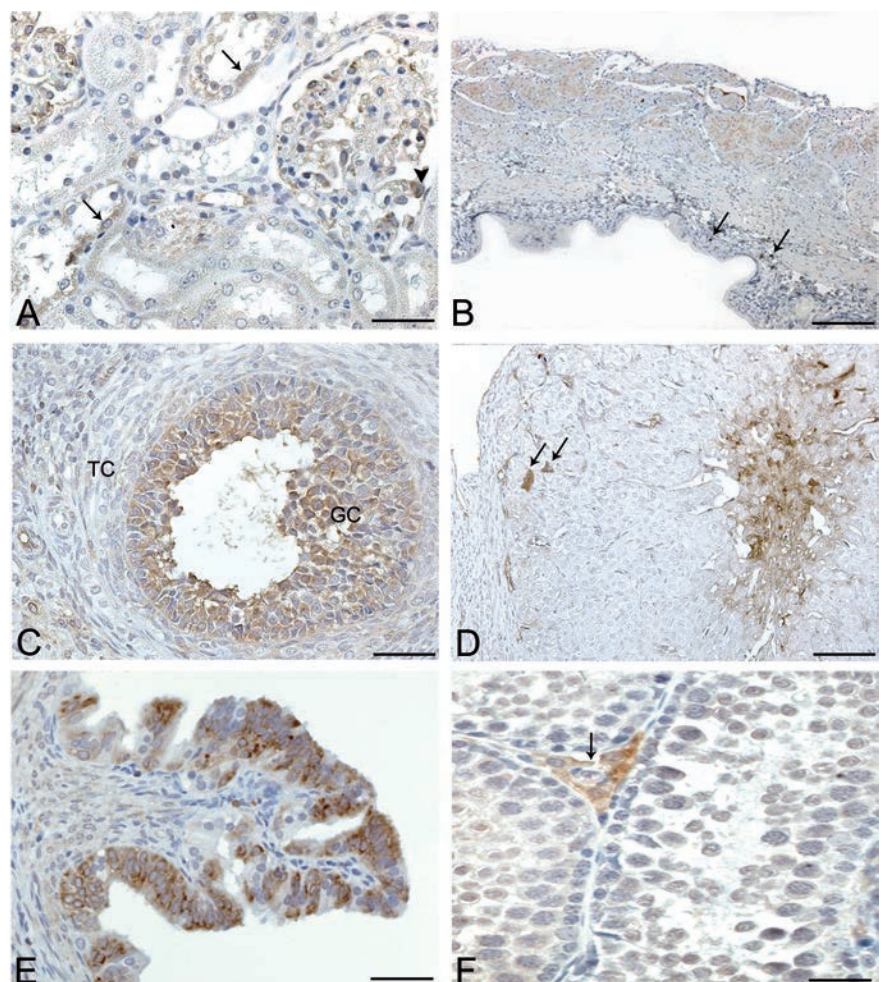


**Figure 7. Anti-*ECRG4* immunohistochemical staining of rat genitourinary system. A) Kidney; some tubular and glomerular epithelial cells show faint or moderate immunostaining, although most cells are negative. B) Urinary bladder; faint immunoreactivity of external portions of detrusor muscle; positive immune cells (arrows) also visible in mucosal layer. C) Ovarian secondary follicle; granulosa cells show diffuse cytoplasmic immunostaining; thecal cells are negative. D) *Corpus luteum*; quite strong, diffuse immunostaining is appreciable in centre, and some isolated cells (arrows) with strong *ECRG4* immunostaining also appear in periphery of *corpus luteum*. E) Oviduct; intense, selective cytoplasmic immunostaining visible in epithelium; *lamina propria* is negative. F) Testis; Leydig cells (arrow) show selective cytoplasmic positivity; germinal epithelium of adjacent seminifer tubuli is negative. Scale bars: A, D, E, F) 37.5 μm; B) 150 μm; C); 75 μm.**

inversely correlated with *ECRG4* expression.[46] *ECRG4* has been suggested to be an injury response gene in the central nervous system. A cortical lesion model in rats showed a significant decrease in the content of ECRG4 protein and mRNA (through immunohistochemistry and hybridisation *in situ*, respectively) in choroid plexus cells. This process lasted some days, and was interpreted as release of *ECRG4* from the cell surface into the cerebrospinal fluid and subsequent decrease in *ECRG4* expression throughout the proliferative phase of injury. Similar changes were not noted in the supraoptic nucleus.[6,9] The injury response in the central nervous system involves proliferation of neural stem/progenitor cells. Interestingly, *ECRG4* over-expression through intracerebroventricular injection of adenovirus vectors containing the transgene *ECRG4* significantly reduces the number of BrdU positive cells in the subventricular zone, together with suppression of nestin staining.[6] The possible role of *ECRG4* in controlling cell proliferation in the central nervous system has also been studied as regards brain development. Functional *ECRG4* knock-down in developing zebrafish increases cell proliferation of GFAP-positive cells at the ventricular surface and produces severe defects, including a ventricular hydrocephalus-like edema phenotype.[6]

*ECRG4* has been suggested to be a secreted inducer of cell senescence in the central nervous system. Senescent mouse oligodendrocyte precursor cells show increased expression of *ECRG4* and transfection of the central glia 4 cell line with an *ECRG4* expression vector induces expression of senescence-associated acidic β-galactosidase, G1 arrest, dephosphorylation of Rb, and decreased expression of cyclins D1 and D3. The secreted form of *ECRG4* is present in the culture medium of senescent oligodendrocyte precursor cells, and the addition of recombinant mouse *ECRG4* induces senescence of mouse oligodendrocyte precursor cells and neural progenitor cells of the dentate gyrus. *ECRG4* expression is higher in some cell populations of aged mouse brain, such as oligodendrocyte precursor cells in the corpus callosum, neural progenitor cells in the subgranular zone of the dentate gyrus, hippocampal granule cells, Purkinjie cells and NeuN+ brainstem cells.[4] It is also intriguing that *ECRG4* is up-regulated in the hippocampus of a mouse model of Alzheimer's disease, *i.e.*, transgenic mice over-express human Tau23 protein.[47] Of particular interest are our findings about *ECRG4* expression in the peripheral nervous system. The structures showing higher percentages of positivity and staining intensity were the trigeminal and superior cervical ganglia. It would be interesting in the future to evaluate the possible involvement of *ECRG4* in injury responses of

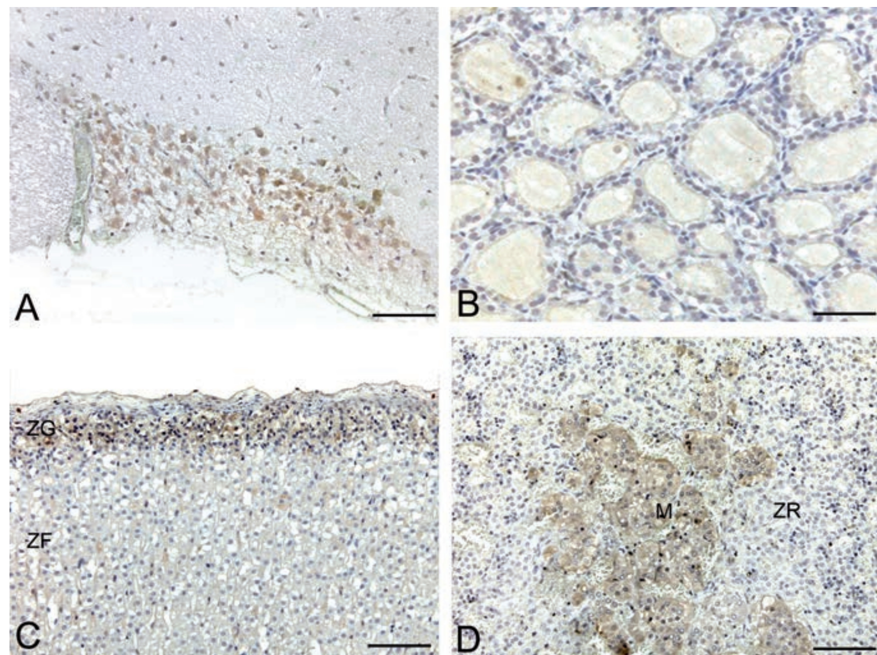


**Figure 8. Anti-*ECRG4* immunohistochemical staining of rat endocrine tissues. A) Supraoptic nucleus; note moderate cytoplasmic immunoreaction of neurons. B) Thyroid gland; negativity or faint immunoreaction of follicular epithelial cells. C) Adrenal cortex; note diffuse positivity of *zona glomerularis*, together with negativity of *zona fasciculata*. D) Adrenal medulla; appreciable and diffuse cytoplasmic positivity of medullary cells (M), together with negativity of *zona reticularis* of adrenal cortex. Scale bars: 75 μm.**

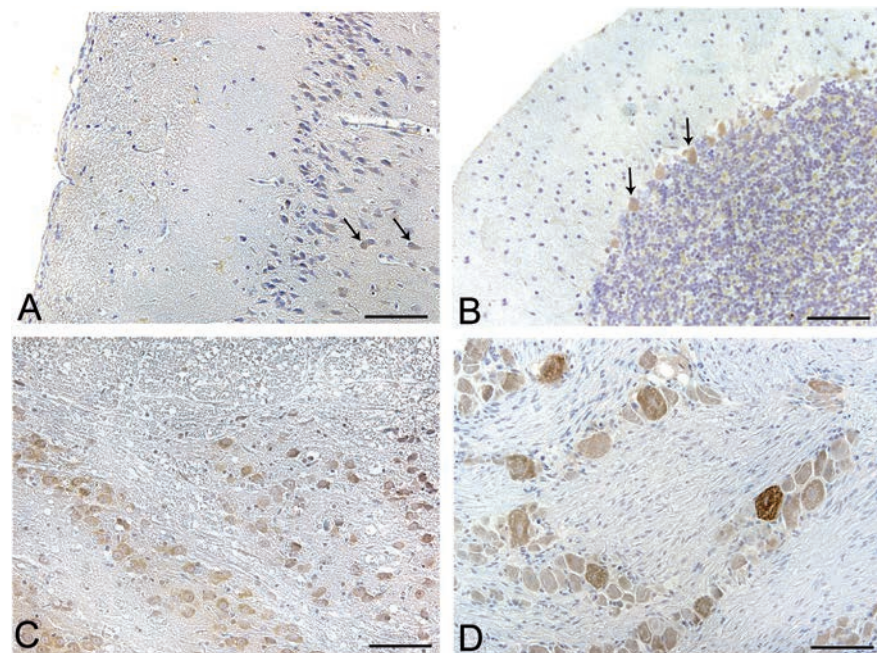


**Figure 9. Anti-*ECRG4* immunohistochemical staining of rat nervous system. A) Cerebral cortex; both positive (arrows) and negative neurons are present. B) Cerebellar cortex; note positivity of Purkinje cells (arrows). C) Inferior olivary nucleus; note *ECRG4* immunostaining of large neurons of nucleus. D) Trigeminal ganglion; ganglion neurons are positive; nerve fibres and support cells are negative. Scale bars: 75 μm.**

peripheral nerves.

In the present work, we found *ECRG4* expression in many neuronal populations of both the central and peripheral nervous systems, suggesting that *ECRG4* also plays a role as neuromodulator. This possible function has not yet been experimentally studied but will certainly be a field for further investigation, partly due to the intriguing neuroendocrine effects of *ECRG4*-derived peptides.[48]

## Endocrine system

As regards endocrine tissues, in the literature *ECRG4* expression has been identified by *in situ* hybridisation in mouse intermediate lobe of the pituitary and glomerular layer of the adrenal cortex.[2] The present analysis by qPCR confirmed previous data[1,17] on *ECRG4* expression in all endocrine glands. Our immunohistochemical analysis located *ECRG4* mainly in the hypothalamic supraoptic and paraventricular nuclei, adrenal ZG and medulla, Leydig cells, and the follicular and luteal cells of the ovary. Anti-*ECRG4* immunostaining was less intense in the hypophysis, thyroid and parathyroid glands. Although immunohistochemistry confirmed *ECRG4* expression in all endocrine tissues in which qPCR identified *ECRG4* mRNA, anti-*ECRG4* immunohistochemical intensity did not perfectly correlate with qPCR results, probably due to post-transcriptional mechanisms in the regulation of *ECRG4* expression. The presence of *ECRG4* in many different endocrine cells and its capacity to be secreted suggest a role in the modulation of various endocrine functions, as already described for other neuropeptides, with particular reference to the adrenal gland.[49-55]

There are few functional data available at present about the role of *ECRG4* in the endocrine system. Its expression in the supraoptic and paraventricular nuclei ([9,18] and our results) support the hypothesis of *ECRG4* involvement in fluid homeostasis and behaviour control. This was confirmed by a later study showing that, in the rat, injections of a synthetic fragment of human augurin (corresponding to amino acids 71-148 of ECRG4 protein) into the third cerebral ventricle or into the paraventricular nucleus increase plasma ACTH and corticosterone, without changes in the other hypothalamo-pituitary axis hormones, and these effects are blocked by pretreatment with a CRF receptor antagonist. Incubation of hypothalamic explants with augurin has also been reported to increase the release of CRF and AVP significantly.[48] Thus, augurin (and possibly other peptides derived from ECRG4 protein) could be added to the long list of neuropeptides which play a double role in modulating endocrine and nervous functions. The quite specific location of ECRG4 in ZG and adrenal medulla also suggests its involvement in modulation of the production of mineralcorticoids and catecholamines. In male and female gonads, ECRG4 immunostaining was also mainly located in steroid-secreting cells. Further analyses will be necessary to evaluate the role of this protein in the endocrine functions of these organs. Lastly, we must stress that the only report on *ECRG4* involvement in endocrine tumorigenesis is the recent paper by Chen *et al.*,[15] examining papillary thyroid carcinoma. Through qPCR and immunohistochemistry, the levels of *ECRG4* mRNA and protein are reported to be up-regulated in papillary thyroid cancer tissues with respect to matched non-tumour samples. Tumour samples also showed lower methylation frequency than paired controls. Transfection of human papillary thyroid cancer cell lines (W3 and K1) for up-regulation of *ECRG4* has been reported to promote cell proliferation activity and cell cycle transition from G1 to G2, in the absence of changes in apoptosis rate.[15] This work is particularly important, as this is the first time that an oncogenic role has been reported for *ECRG4*, unlike the oncosuppressor function reported for most cancers previously studied. This means that *ECRG4* may also play a role in the tumorigenesis of some tissues in which *ECRG4* expression is not normally evident.

## Muscles and cartilage

As regards other non-endocrine and non-nervous tissues and cells, we found *ECRG4* expression, although quite moderate, in heart muscle cells and not only in the atrioventricular node as reported by Mirabeau *et al*.,[2] indicating the greater sensitivity of immunohistochemical analysis. Apart from heart muscle cells, ECRG4 was also identified in skeletal and, to a lesser extent, in smooth muscle cells. *ECRG4* is highly expressed in developing cartilage; its expression is low in undifferentiated mesenchymal cells, but then increases in proliferating chondrocytes and decreases during hypertrophic maturation. *ECRG4* expression is decreased in human and experimental osteoarthritic cartilages.[56] However, its ectopic expression or exogenous administration has been reported not to affect the chondrogenesis of mesenchymal cells, hypertrophic maturation of chondrocytes or dedifferentiation of differentiated chondrocytes, although its ectopic expression does reduce the proliferation of primary-culture chondrocytes.[56] Analysis of gene transcript expression has recently suggested a potential role for *ECRG4* also in chordoma tumorigenesis.[57]

## Expression and role of *ECRG4* in the immune system

In the recent literature, many data have emerged regarding the role played by *ECRG4* in inflammation and the immune response. qPCR has previously shown that *ECRG4* is highly expressed in freshly isolated polymorphonuclear leukocytes (PMNs) and peripheral blood mononucleated cells (PBMCs) (where methylation of the gene promoter is very low) and in CD4- and CD8-positive T cells and CD19-positive B cells.[7,58]

Our study is the first to analyse the distribution of ECRG4 immunostained cells in the main lymphatic tissues. *ECRG4* was found to be strongly expressed in the cytoplasm of groups of cells located in lymphatic tissues. These cells were mainly located in the medullary compartment of lymph nodes, cortical compartment of thymus and red pulp of the spleen. Rare positive cells are also sometimes found at the margins of lymphatic tissues of the respiratory and digestive systems, and are polymorphonuclear leukocytes, macrophages and lymphocytes. Conversely, positive cells were not found in bone marrow. The fact that *ECRG4* expression has not been identified in all immune cells in the lymphatic tissues, but only in some of them, may be explained with reference to their functional/proliferative state and cell sub-type. *ECRG4* expression is known to be higher in resting cells than in activated ones.[7] *ECRG4* methylation and expression is high and low, respectively, in actively proliferating cell lines in culture, including the leukocyte lineage (*e.g*., Jurkat, THP1 and HL60).[58] The *ECRG4*-positive cells in lymphatic tissues are probably resting/non-proliferating cells. The very low *ECRG4* expression in bone marrow may also be explained as due to the high proliferative rate of this tissue. Further analysis involving markers of various lymphatic cells will be necessary to identify the positive cell types in these tissues better.

In human PMNs and PBMCs, *ECRG4* is located on the cell surface and may be released, in entire or cleaved forms, in response to cell stimulation by lipopolysaccharide (LPS) and *N*-formyl peptides (fMLF).[58] In particular, recent analyses have shown that 20-50% of human CD16+ leukocytes (neutrophil granulocytes) are positive for *ECRG4* on the cell surface and about 10% of CD14+ leukocytes (monocytes) are ECRG4+. A subset of leukocytes is CD14+/CD16+/ECRG4+, in which *ECRG4* co-locates with the LPS receptor complex, made up of TLR4, MD2 and CD14, through molecular interaction with the C-terminus of *ECRG4*.[59] In addition, the C-terminal domain of *ECRG4* (*ECRG4*[133-148]) may be processed and shed in a thrombin-like consensus sequence[58-60] or internalised by cells through binding to the TLR4 innate immunity receptor complex[59]. Another intriguing fact is that *ECRG4*-derived peptides have been reported to activate the NF-kB pathway in mouse peritoneal macrophages.[58] In burn injuries and

blunt traumas, a significant reduction in *ECRG4* PMN surface expression has been reported, indicating release of protein in response to injury response.[58,59] Thus, during injury and inflammatory responses, cell surface processing of *ECRG4* by proteases may generate peptides (such as *ECRG4*$^{133-148}$) which in turn activate other cells.

It has recently been suggested that *ECRG4* inhibition of tumour growth is exerted more by immunosurveillance than classic tumour suppression.[10] *ECRG4* does show peculiar characteristics with respect to other tumour suppressor genes, as it is located on the cell surface and sheds into extracellular milieu.[10] Some authors have also pointed out that a direct anti-mitogenic effect of *ECRG4* over-expression has not always been demonstrated when tumour cells are transfected *in vitro*.[60] Conversely, *ECRG4* transfection in intracranially injected glioma cells has been reported to increase survival and decrease tumour burden. The glioma micro-environment is typically characterised by immunosuppressive phenotypes, but *ECRG4*-expressing gliomas show greater prevalence of activated amoeboid microglia/macrophages and myeloid cells chemo-attracted to the brain tumour. Further *in vitro* analyses have shown that *ECRG4*-expressing glioma tumour cells increase activation and phagocytosis in co-cultured primary microglia cells; this effect may also simply be produced by *ECRG4*-conditioned media. The intact thrombin cleavage site in *ECRG4* was found to be necessary for microglia activation and chemo-attraction of myeloid cells, *ECRG4*$^{133-148}$ being the main peptide involved.[60] In conclusion, membrane-bound *ECRG4* may be considered as a *sentinel factor* processed by proteases (thrombin) to give soluble pro-inflammatory *ECRG4* forms (*ECRG4*$^{133-148}$) which then exert anti-tumoral activity through recruitment and activation of immune cells.[10,60] Moreover, *ECRG4* expression is higher in a Fas-resistant variant of Jurkat cells. Conversely, Jurkat cells showing stable expression of *ECRG4* by gene transfection (Jurkat ECRG4 cells) show higher survival rates and lower mitochondrial membrane depolarisation (MitoCapture) in response to anti-Fas antibody. Over-expression of *ECRG4* also reduces caspase-8-dependent apoptosis induced by TNF-alpha. Instead, knock-down of endogenous *ECRG4* mRNA with siRNA in Jurkat FasR cells causes decreased survival rates and increased sensitivity to alterations in mitochondrial membrane depolarisation after Fas stimulation.[7]

A role for *ECRG4* has also been proposed for response mechanisms of mucosal and cutaneous structures to local injuries and infections. *ECRG4* has been identified in the epidermis, dermis and hair follicles of mouse skin. In a punch biopsy injury model, its expression is reduced in the granulation tissue of the injured site but is increased at the wound margins, suggesting a role as a late-injury response gene. *ECRG4* transduction of fibroblasts *in vitro* produces a significant decrease in the rate of directional migration, in the absence of changes in proliferation. A significant delay in the rate of wound closure was found *in vivo* after viral-mediated intradermal gene delivery.[37] As regards response to injury, it should also be noted that *ECGR4* gene expression is up-regulated in regenerating caudal fin of zebrafish.[61] *ECRG4* is also expressed in both epithelium and stroma of mouse middle ear mucosa. Its expression was dramatically down-regulated in an experimental model of bacterially mediated otitis media, characterised by the development of mucosal hyperplasia and leukocytic infiltration. Conversely, induction of *ECRG4* over-expression through transduction inhibited cell migration from an explant of inflamed mucosa *in vitro*, and *in vivo* induction of *ECRG4* expression with adenovirus encoding *ECRG4* attenuated mucosal thickness and inhibited inflammatory cell infiltration.[62]

More recently, *ECRG4* gene expression has been reported to be decreased in mouse lungs after inflammatory lung injury or intravenous administration of lipopolysaccharide and, conversely, up-regulated after lung preconditioning with isoflurane anaesthesia.[16]

## Conclusions

*ECRG4* has been reported to inhibit cell proliferation and increase apoptosis in various cell types. It is down-regulated, frequently due to hypermethylation, in various tumours, including esophageal, prostate, breast and colon cancers, together with glioma (oncosuppressor function). It has recently been reported to be up-regulated in papillary thyroid cancer (oncogenic role). A role for *ECRG4* has been reported in the immune system and in possible involvement in inhibition of tumor growth by immunosurveillance. Our study identified *ECRG4* expression in many other structures not yet analysed from the pathophysiological viewpoint. The possible involvement of *ECRG4* in the tumorigenesis of these structures (also with reference to immune function) must be examined in the future. In addition, due to the location of *ECRG4* in neurons and endocrine cells and its capacity to be secreted, its role as a neurotransmitter/neuromodulator and endocrine factor may be hypothesised and is still to be clarified.

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