WZsGreen+/+: a new green fluorescent protein knock-in mouse model for the study of KIT-expressing cells in gut and cerebellum

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WZsGreen+/+: a new green fluorescent protein knock-in mouse model for the study of KIT-expressing cells in gut and cerebellum. Physiol Genomics 22: 412–421, 2005. First published June 14, 2005; 10.1124/physiolgenomics.00105.2005.—In the small intestine, interstitial cells of Cajal (ICC) surrounding the myenteric plexus generate the pacemaking slow waves that are essential for an efficient intestinal transit. The underlying molecular mechanisms of the slow wave are poorly known. KIT is currently the sole practical marker for ICC. Attempts to purify living ICC have so far largely failed, due to the loss of the KIT epitope during enzymatic dissociation. Aiming to identify and isolate living ICC, we designed a knock-in strategy to express a fluorescent tag in KIT-expressing cells by inserting the sequence of the novel green fluorescent protein ZsGreen into the first exon of the c-Kit gene, creating a null allele called WZsGreen. In the gastrointestinal tract of heterozygous WZsGreen+/+ mice, tiny ZsGreen fluorescent dots were observed in all KIT-expressing ICC populations, with exception of ICC at the deep muscular plexus in small intestine. During development of the gastrointestinal tract, ZsGreen expression followed KIT expression in a spatiotemporal way. Stellate and basket KIT-expressing cells in the molecular layer of the cerebellum also exhibited ZsGreen dots, whereas no ZsGreen was detected in skin, testis, and bone marrow. ZsGreen dot-containing intestinal cells could be isolated from jejunum and maintained alive in culture for at least 3 days. ZsGreen is a suitable alternative to EGFP in transgenic animals. The novel WZsGreen+/+ model reported here appears to be a promising tool for live studies of KIT-expressing cells in the gastrointestinal tract and cerebellum and for the further analysis of pacemaker mechanisms.

ZsGreen; interstitial cells of Cajal

IN THE GASTROINTESTINAL (GI) tract, the spatiotemporal organization of contractile activity is essential for efficient propulsion of luminal content. Besides the enteric nervous system, the role of specialized mesenchymal cells, the interstitial cells of Cajal (ICC), has been established in recent years (14, 27, 30). ICC express the proto-oncogene KIT (encoded at the W locus), a transmembrane tyrosine kinase receptor for Steel factor (encoded at the S1 locus), also known as stem cell factor (43, 45). The KIT-stem cell factor transduction pathway is required for the proper development and maintenance of various cell types, including ICC (39). Mice carrying one null allele and one wild-type (+) allele develop quite normally, whereas mice homozygous for a null mutant at either W or S1 loci are not viable, likely due to severe anemia (2, 24). Subtotal loss-offunction mutations for either the W (W/Wv) or the S1 locus (S1/SkI) are viable (24, 38). Among other features, these animals lack the ICC surrounding the myenteric plexus, a phenotype associated with the loss of slow waves and regular contraction of the intestinal musculature (5, 14, 17, 27, 30, 34, 38, 39).

Currently, KIT is the sole practical marker for ICC. Attempts to isolate living ICC with KIT antibody-based strategies have not been very successful so far, presumably due to the loss of KIT epitope during enzymatic dissociation (10). Furthermore, isolated ICC proved difficult to cultivate. Membrane-bound stem cell factor may be important for the reexpression of KIT receptors and survival of ICC in culture (31). Labeling of ICC with KIT antibodies before the enzymatic dissociation has been introduced as an alternative to enrich ICC preparations using immunomagnetic separation or fluorescence-activated cell sorting (25, 26). However, these delicate techniques are time consuming, have a low yield, and may significantly interfere with the KIT signaling pathway, as the KIT antibodies used exhibit blocking properties (21).

We report here on a novel green fluorescent transgenic mouse model designed to circumvent these problems for the study of living KIT-expressing ICC in tissues and after enzymatic dissociation.

MATERIALS AND METHODS

Targeting replacement vector c-Kit-ribosomal protein L31-ZsGreen-N1 construction. A 79-nucleotide fragment of the mouse c-Kit gene (Y00864; nucleotides: 7–86; SacI/EcoRI) coupled to the nucleolar localization signal of the mouse ribosomal protein L31 (RPL31; NM_015774; nucleotide: 3322–3339; amino acid sequence: RLSRKR; EcoRI/BamHI) (29) was ligated in the pZsGreen1-N1 vector (BamHI/Sacl; Clontech, Palo Alto, CA). This construct, further referred to as c-Kit-RPL31-ZsGreen1-N1, was transformed into One Shot Top 10 chemically competent E. coli (Invitrogen, Merelbeke, Belgium) and purified using the endotoxin-free Maxiprep kit (Westburg, Leusden, The Netherlands).

Expression analysis and cytotoxicity assay in cell culture. Twenty-four hours before transfection, 2 × 105 HeLa or COS-7 cells were seeded on sterile 22-mm round glass coverslips. Cells were transfected with 1 μg of the c-Kit-RPL31-ZsGreen1-N1 plasmid using FuGene 6 (Roche Diagnostics, Vilvoorde, Belgium) in 2 ml of serum-free DMEM medium (Invitrogen), according to manufacturer’s instructions. The DMEM medium supplemented with 10% FCS (HI), 20 mM L-glutamine, 1 mM sodium pyruvate, and 0.45 mg/ml gentamicine (Invitrogen) was replaced after 16 h.

c-Kit-RPL31-ZsGreen1-N1 permanently transfected HeLa clones were selected at 400 μg/ml geneticin (GIBCO), and five clones were isolated. The Via Light high-sensitivity assay (Cambrex Bio Science, Verviers, Belgium) was performed according to manufacturer’s instructions. Briefly, 24–72 h before the assay, 25–10,000 cells were
seeded in a 96-well microplate in 100 μl of antibiotics-free medium. The 100 μl of nucleotide releasing reagent was added and incubated for 5 min at room temperature. After addition of 20 μl of ATP monitoring reagent, luminescence was measured using a luminoscan (Labsystem). The assay was performed in duplicate, and results were normalized against untransfected HeLa cells and permanently transfected HeLa cells with the nontoxic pGL2 luciferase reporter vector (Promega, Madison, WI).

Generation of WZsGreen+/+ mice. Transgenic mice were generated by Lexicon Genetics (Woodlands, TX) using a proprietary knock-in strategy. Briefly, the c-Kit-RPL31-ZsGreen1-N1 fragment was isolated by SacI-NotI digestion and cloned into the intermediate vector pKI-PLUS that allowed picking up a splice acceptor/splice donor cassette and the polyadenylation signal. Within a c-Kit gDNA contig, URA, a yeast selection marker, was inserted in place of nucleotides 5,583–5,686 by yeast recombination. The SfiI selection cassette containing the c-Kit-RPL31-ZsGreen1-N1 fusion from the pKI-PLUS vector was inserted using ligation to replace the SfiI-flanked URA cassette. The targeting was performed in embryonic stem (ES) cells containing the protamine-Cre transgene. In this ES cell line, Cre is expressed under the control of the protamine promoter during spermatogenesis. When chimeric mice from this ES cell line are bred, the targeted allele. Cycling conditions were as follows: 94°C for 3 min, 62°C annealing for 1 min, 72°C elongation for 1 min). PCR products were run on 1.5% agarose gel with ethidium bromide.

Fluorescent microscopy and immunofluorescence. Cells were grown on 22-mm round glass coverslips as described above. Whole mounts of the lamina propria were prepared from stomach, jejunum, and transverse colon by sharp dissection under a binocular. Cells were fixed for 15 min with fresh 4% paraformaldehyde (Sigma) in 0.1 M phosphate-buffered saline (PBS), whereas tissue samples and whole mounts were fixed overnight and then rinsed in PBS. Cells and whole mounts were stored at 4°C in PBS containing 0.1% sodium azide and used within a week. For sections, samples were cryopreserved in graded solutions of sucrose (10, 20, 30%; overnight each), embedded in Tissue-Tek optimal cutting temperature compound (Miles, Elkhart, IN), snap-frozen in 2-methyl butane that had been cooled on dry ice, and stored at −80°C. Sections (15 μm thick) were cut on a cryostat, mounted on glass slides coated with 0.1% poly-L-lysine, and stored at −20°C until use.

For KIT immunohistochemistry, samples were rinsed three times in 10 mM Tris in 0.15 M sodium chloride, pH 7.4 (TBS), containing 1% (vol/vol) Triton X-100 (TBS-TX), incubated for 1 h in 10% normal horse serum (NHS; Hormonologie Laboratoire, Marloie, Belgium) in TBS-TX to reduce background, and incubated overnight with the primary goat anti-mouse polyclonal antiserum raised against KIT (M14, Santa Cruz Biotechnology, Santa Cruz, CA) diluted (1/900) in TBS-TX containing 2% NHS, rinsed in TBS, incubated in the dark for 2 h at room temperature (RT) in TBS containing the secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) coupled to Cy3.

The blue fluorescent Hoechst 33342 (further referred to as Hoechst) (H3570, 1/1,000, Molecular Probes, Eugene, OR) was used as nuclear counter stain. All fixed preparations were mounted with Slow Fade Light antifade (Molecular Probes).

Live observations were performed at RT. Primary cell cultures grown on coverslips were placed in DMEM high glucose containing 1/10 V fetal bovine serum and 1% antibiotic/antimicotic (see above) in the observation chamber, whereas for whole mounts the chamber was constantly perfused at 2 ml/min with Krebs solution (Sigma, Bornem, Belgium) bubbled with carbogen (95% O2-5% CO2). For KIT staining on living whole mounts, R-phycocerythrin-conjugated anti-mouse CD117 (BD Biosciences, San Diego, CA) was diluted in PBS (1/250), and small pieces of jejunum were incubated at 37°C for 30 min before visualization.

All preparations were observed on an Axiovert LSM 510 NLO META confocal microscope (Zeiss, Jena, Germany) equipped with Chroma ChromaPhysics 40×/0.8 numerical aperture and 63×/1.2 numerical aperture water-immersion objectives. For visual inspection, phase contrast transmitted light and standard fluorescence filter sets for the visualization of green (ZsGreen), red (Cy3, R-phycocerythrin), and blue (Hoechst) fluorescence were used. For confocal imaging in conventional, single-photon mode, the 488-nm excitation wavelength of the Argon 2 laser and a band-pass emission filter (500–530 nm) were used for detection of ZsGreen fluorescence, whereas the 543-nm excitation beam of the HeNe1 laser and a long-pass emission filter (560 nm) were used for detection of the red (Cy3, PE) fluorochromes.

The blue nuclear stain Hoechst was excited in two-photon (2P) mode at 740 nm with a Mai Tai tunable broad-band laser (Spectra-Physics, Darmstadt, Germany) through a short-pass main dichroic mirror (685 nm), and emitted light was detected with a band-pass emission filter (435–485 nm). ZsGreen was also excited in two-photon mode through the 685-nm main dichroic mirror and emitted light was detected through the 500–530 nm band-pass emission filter. The optimal excitation wavelength for ZsGreen in two-photon mode was determined using the Zeiss excitation fingerprinting macro (9).

ZsGreen emission spectrum was analyzed with a resolution of 11 nm using the Zeiss META spectral analyzer.

Stacks of optical sections through regions of interest in each preparation (512 × 512 pixels), 1 μm apart (Z step), were sequentially collected for each fluorochrome at each Z step.

All images were analyzed with the Zeiss LSM 510 Image Examiner software, and pictures were exported at maximal resolution in jpg format. Figures were prepared with Illustrator (Adobe, San Francisco, CA).
Primary cell cultures from jejunum. WZsGreen+/+ mice (10 days old) of either sex were killed by cervical dislocation. The jejunum was removed and placed in cold Ca²⁺/H₁₁₀₀₁-free Hanks solution containing (in mM) 125 NaCl, 15.5 NaOH, 5.36 KCl, 0.336 Na₃PO₄, 0.44 KH₂PO₄, 10 glucose, 2.9 sucrose, and 11 HEPES (pH 7.4). The mesentery was carefully removed. The muscle layers were peeled off using fine tweezers, rinsed, and placed in Ca²⁺/H₁₁₀₀₁-free Hanks’ solution, 0.8 mg/ml collagenase (type II; Worthington), 2 mg/ml bovine serum albumin (Sigma), 2 mg/ml trypsin inhibitor (Sigma), and 0.55 mg/ml adenosine triphosphate at 32°C during 15 min according to Ref. 31. Tissue pieces were triturated every 5 min to disperse cells and washed repeatedly with Hanks’ solution. Cells were resuspended in 2 ml of culture medium (DMEM high glucose, 1/10 V fetal bovine serum, and 1% antibiotic/antimicotic, Invitrogen) and plated on 0.1% coated poly-L-lysine coverslips.

RESULTS

Subcellular targeting and cytotoxicity of the c-Kit-RPL31-ZsGreen1-N1 gene product in vitro. Expression of the c-Kit-RPL31-ZsGreen1-N1 chimeric protein in HeLa cells was readily detected by fluorescence microscopy. Surprisingly, the green fluorescence was not localized into the nucleolus, in
Expression of ZsGreen in WZsGreen/+ mice. In WZsGreen/+ mice, ZsGreen expression appeared as very bright but tiny (i.e., <1-μm apparent diameter), green fluorescent dots, only one or a few per cell, whereas in +/+ animals no green fluorescence was observed.

Using standard fluorescence filter sets, the green fluorescence of ZsGreen could readily be discriminated by eye from tissue autofluorescence that appeared yellowish. The emission spectrum of the green dots in living tissues from WZsGreen/+ mice, with a maximum of ~520 nm and a hump between 540 and 560 nm, closely matched the ZsGreen emission spectrum recorded in living cells mock-transfected with the pZsGreen1-N1 vector. Considering the resolution (11 nm) of the measurement, the shift between the two spectra appeared sharp contrast with results reported for RPL31-LacZ (29). At all time points investigated (8–48 h), ZsGreen remained adjacent to, but clearly outside, the nucleus (Fig. 2, A–D). Cells transfected with the control pZsGreen1-N1 vector showed uniform expression of ZsGreen in cytosol and nucleus (Fig. 2, E–F).

The proliferation rates of untransfected, nontoxic pGL2-transfected and of five clones of HeLa cells stably transfected with c-Kit-RPL31-ZsGreen1-N1 were similar, suggesting the lack of toxicity of the c-Kit-RPL31-ZsGreen1-N1 gene product (Fig. 3).

Generation of WZsGreen/+ mice. The c-Kit-RPL31-ZsGreen1-N1 fragment integrated into the mouse genome in 400 ES clones. Southern blot analysis confirmed integration by revealing a 13-kb band for the + allele and an 8-kb band for the targeted allele after neo excision (data not shown). A male 5% chimera transmitted the WZsGreen allele to his offspring.

PCR genotyping revealed a 238-bp amplicon for the + allele and a 300-bp amplicon for the WZsGreen allele (Fig. 4). The transgene was inherited in a Mendelian way (data not shown). Although WZsGreen/WZsGreen mice died shortly after birth, WZsGreen/+ animals appeared healthy and lived normally. They exhibited only a very mild phenotype (i.e., white tail tip, white feet, and white belly spot), similar to W/+ or WlacZ/+ animals.

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negligible. Spectral analysis clearly discriminated ZsGreen fluorescence from the abundant autofluorescence in living tissues, which has a much broader emission spectrum (Fig. 5). The emission spectrum of ZsGreen, with a maximum of ~520 nm and a 540- to 560-nm hump, was not altered in fixed tissue (data not shown).

In two-photon mode, excitation of ZsGreen occurred between 880 and 920 nm, with a maximum at ~900 nm. The emission properties were unchanged. Bleaching was minimal in two-photon mode: on 900-nm excitation, only ~35% of fluorescence intensity was lost after 1,000 scans, compared with an ~80% loss of fluorescence intensity after 200 scans at 488 nm in single-photon mode (data not shown).

Distribution of ZsGreen in the GI tract of adult WZsGreen/+ mice. Along the GI tract, ZsGreen dots were observed only in the muscularis propria, from the distal esophagus to the distal colon. ZsGreen expression was restricted to KIT-ir ICC, which were distributed in WZsGreen/+ mice as reported in WZsGreen/+ mice (Figs. 6 and 7, C and D). In the antrum, KIT-ir ICC-intramuscular (IM) (Fig. 6A) and KIT-ir ICC-myenteric (MP) (B) contained several tiny ZsGreen dots per cell. In the jejunum, KIT-ir ICC-MP (C) expressed few tiny ZsGreen dots, whereas no ZsGreen at all was detected at the level of the KIT-ir ICC-deep muscular plexus (D). In a section of the colon, all four KIT-ir ICC populations (subserosal, myenteric, intramuscular, and submucosal) expressed ZsGreen (E). In a close-up view of the 3-dimensional reconstruction (y-z projection, 150 μm thick) of a Z stack across the entire muscularis propria highlights ZsGreen expression in the 4 distinct ICC populations of the colon (G). A y-z projection of a 3-dimensional reconstruction of ZsGreen fluorescence in the entire muscularis propria confirmed ZsGreen expression in all the colonic ICC populations (G). Maximum-intensity projections of stacks of 1-μm optical sections across regions of interest, 10 and 5 μm thick in A–D and E and F, respectively. LM and CM, longitudinal and circular muscle layers, respectively; SM, submucosa. Scale bars = 20 μm.

Fig. 6. ZsGreen expression in the gastrointestinal tract of WZsGreen/+ mice. Whole mounts (A–D and G) and sections (E and F) of fixed WZsGreen/+ tissues. Due to their very small size, ZsGreen dots (green) were often difficult to see, even at high magnification. KIT-ir appears in red. Insets in A–D show 2.5× blow ups of the boxed areas. In the antrum, KIT-ir interstitial cells of Cajal (ICC)-intramuscular (A) and KIT-ir ICC-myenteric (MP) (B) contained several tiny ZsGreen dots per cell. In the jejunum, KIT-ir ICC-MP (C) expressed few tiny ZsGreen dots, whereas no ZsGreen at all was detected at the level of the KIT-ir ICC-deep muscular plexus (D). In a section of the colon, all four KIT-ir ICC populations (subserosal, myenteric, intramuscular, and submucosal) expressed ZsGreen (E). In a close-up view of the 3-dimensional reconstruction (y-z projection, 150 μm thick) of a Z stack across the entire muscularis propria highlights ZsGreen expression in the 4 distinct ICC populations of the colon (G). A y-z projection of a 3-dimensional reconstruction of ZsGreen fluorescence in the entire muscularis propria confirmed ZsGreen expression in all the colonic ICC populations (G). Maximum-intensity projections of stacks of 1-μm optical sections across regions of interest, 10 and 5 μm thick in A–D and E and F, respectively. LM and CM, longitudinal and circular muscle layers, respectively; SM, submucosa. Scale bars = 20 μm.
Fig. 7. Detection of ZsGreen expression in living whole mounts and in dissociated cells of jejunum. A and B: ZsGreen fluorescence in ICC-MP (A) and overlay with transmitted light phase contrast image (B). C and D: ZsGreen fluorescence in ICC-MP (C) and overlay with vital labeling of KIT-ir ICC-MP with R-phyco-erythrin-coupled KIT antibodies (D). E: ZsGreen fluorescence (arrowhead) in an ICC immediately after dissociation. F and G: ZsGreen fluorescence (arrowheads) (F) and transmitted light phase contrast image (G) in a cell cluster in culture for 3 days after dissociation. Maximum intensity projections of stacks of 1-μm optical sections 10 μm thick across regions of interest in A, C, and D. Overlay of green fluorescence and transmitted light phase contrast image in B and E. Scale bars: 10 μm in A, B, and E; 20 μm in C, D, F, and G.
contained a few green dots per cell. In the jejunum, KIT-ir ICC-myenteric (MP) contained only one or no ZsGreen dot (Fig. 6C), whereas KIT-ir ICC-deep muscular plexus (DMP) were consistently negative for ZsGreen (Fig. 6D). In the transverse colon, the various populations of KIT-ir, namely subserosal, myenteric, intramuscular, and submucosal ICC expressed ZsGreen (Fig. 6, E–G). A y-z projection of a threedimensional reconstruction of Zsgreen fluorescence in the entire muscularis propria illustrated the ZsGreen expression in all four colonic ICC populations (Fig. 6G). Noteworthy, ZsGreen in submucosal ICC appeared quite bright, whereas KIT-ir was not stronger than in the other ICC populations in the colon.

ZsGreen was readily detectable in living whole mounts (Fig. 7, A–D). The combination with phase contrast transmitted illumination (Fig. 7, A and B) or with vital labeling using a fluorescent KIT antibody (Fig. 7, C and D) confirmed that KIT-ir ICC can be positively localized in the muscle coats by ZsGreen expression.

Postnatal development of ZsGreen expression in the GI tract. The appearance of ZsGreen in the postnatal GI tract followed the general proximodistal gradient of development (Table 1). The onset of ZsGreen detection lagged apparently somewhat behind the apparition of KIT-ir reported in the same regions (33).

ZsGreen in primary cell cultures. Immediately after dissociation of the jejunal muscularis propria, ZsGreen dots could be detected in very few cells (Fig. 7E), in agreement with the low number of ICC reported by Ordog et al. (25, 26). After 3 days in culture, a limited number of cells, most of them in cell clusters, contained detectable ZsGreen dots (Fig. 7F).

Expression of ZsGreen in other organs. In the cerebellum, ZsGreen was detected in the molecular layer, which harbors the stellate and basket KIT-expressing cells (35). No ZsGreen was detected in the Purkinje cells and in the granule layer (Fig. 8, A and B). In cerebellum as in the GI tract, ZsGreen was located in close vicinity of the nucleus (Fig. 8C).

Conversely, no detectable ZsGreen fluorescence was observed in skin, testis, and bone marrow, despite the fact that these organs harbor known populations of KIT-expressing cells (33).

### Table 1. Postnatal development of ZsGreen in KIT-ir ICC populations along the GI tract

<table>
<thead>
<tr>
<th>Location/Age</th>
<th>Day 0</th>
<th>Day 5</th>
<th>Day 10</th>
<th>Day 15</th>
<th>Adult</th>
</tr>
</thead>
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<tr>
<td>Antrum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICC-MP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ICC-IM</td>
<td>0</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Jejunum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>ICC-DMP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Transverse colon</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>ICC-SS</td>
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<td>0</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>ICC-MP</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>ICC-CM</td>
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<td>0</td>
<td>0</td>
<td>(+)</td>
<td>+</td>
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<tr>
<td>ICC-SMP</td>
<td>NA</td>
<td>0</td>
<td>0</td>
<td>(+)</td>
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</table>

IM, intramuscular; ICC, interstitial cells of Cajal; GI, gastrointestinal; CM, circular muscle layers; MP, myenteric plexus; SMP, submucosa plexus; DMP, deep muscular plexus; SS, subserosal; (+), clearly present; (0), scarce; 0, not detected; NA, not available.

**DISCUSSION**

To identify and isolate living ICC, we aimed to generate a transgenic model expressing a green fluorescent reporter gene in KIT-expressing cells, inspired by the WLacZ model (2). In this model, homologous recombination of a LacZ reporter gene into the mouse c-Kit gene led to the expression of detectable β-galactosidase activity (Xgal histochemistry) in KIT-expressing cells, including ICC of the GI tract, during embryonic development (2) and after birth (32, 36), with the nuclear targeting of the expressed β-galactosidase outlining nicely the nucleus of each KIT-expressing cell. The enzymatic activity of Xgal represents a strong amplification step that allows detection of the signal even in cells with a low expression level of KIT. On the other hand, Xgal histochemistry requires hours of incubation and cannot be performed on living tissues, two limitations for our purpose.

We selected the recently released ZsGreen1 protein derived from reef corals as green fluorescent reporter (4, 41). To maintain the fluorescent signal after enzymatic dissociation and to maximize the brightness of the fluorescent signal (since transcriptional activity of the KIT promoter and expression efficiency of the transgene could not be estimated a priori), we decided to target the expression into a subcellular compartment using the murine nucleolar localization signal RPL31. To our surprise, transfection of the c-Kit-RPL31-ZsGreen1-N1 targeting construct in COS-7 and HeLa cells revealed that the fluorescent signal accumulated adjacent to the nucleus in the Golgi apparatus (unpublished observations) instead of within the nucleolus, as previously reported for RPL31-LacZ (29). The reason of such discrepancy is unclear and was not further explored. Because expression of the c-Kit-RPL31-ZsGreen1-N1 targeting construct provided a bright ZsGreen signal, lacked cytotoxicity, and was targeted to a small subcellular compartment, we decided to use this construct for the generation of WZsGreen transgenic mice. A knock-in approach was mandatory, because only homologous recombination of the targeting vector into the first exon of the c-Kit gene ensured the specific expression of the reporter gene in KIT-expressing cells (2, 3, 8).

KIT expression has been demonstrated by in situ hybridization and/or immunohistochemistry in various cell types, including mast cells, melanocytes, hematopoietic stem cells, germinal cells in gonads, stellate, and baskets neurons in the cerebellum and ICC in the GI tract (2, 19).

In WZsGreen/+ animals, ZsGreen expression was readily detected as green dots in living whole mounts of the muscularis propria of the GI tract. These dots were quite bright but tiny, rendering mandatory the use of high magnification, high numerical aperture, and long working distance water-immersion objectives. ZsGreen expression in WZsGreen/+ tissues was verified by its spectral properties, which nicely matched the emission spectrum of ZsGreen from the pZsGreen1-N1 vector expressed in mammalian cell lines (this study) and of ZsGreen expressed in fungi (4).

At the best of our knowledge, WZsGreen represents the first application of the modified coral green fluorescent protein ZsGreen in a mammalian transgenic model. ZsGreen expression proved to be nontoxic in cell lines and in WZsGreen/+ transgenic mice, in line with reports of its excellent tolerance in plants (41) and fungi (4). The fluorescent properties of Zs-
Green appeared outstanding: brightly fluorescent in living tissues, it was unaffected by paraformaldehyde fixation and by congelation. ZsGreen can be observed with standard green fluorescence filters, it is very photo-stable and is well suited for confocal imaging. Its emission spectrum allows a clear delineation from other fluorescent signals for multiple labeling and from tissue autofluorescence. ZsGreen can be conveniently excited with the 488-nm laser line available on most confocal microscopes. It can also be excited at ~900 nm in two-photon mode. In contrast with a significant photo-bleaching of ZsGreen in two-photon mode reported in fungi (4), we observed an extremely low bleaching in two-photon mode in WZsGreen tissues, making two-photon mode best suitable for live observations over long periods of time.

In cells transfected with the c-Kit-RPL31-ZsGreen1-N1 construct and in WZsGreen/+ mice, ZsGreen expression was consistently found in the pericaryon at short distance from, but never within, the nucleus. These constructs targeted ZsGreen expression to a small juxtanuclear compartment but not into the nucleolus, raising doubts about the nucleolar specificity reported for the RPL31 localization signal (29).

The timing of apparition of ZsGreen in the postnatal GI tract followed the proximodistal gradient of development and apparently lagged somewhat behind the reported timing of apparition of KIT-ir (33). The lower sensitivity of ZsGreen detection compared with KIT-ir likely accounts for the apparently slightly delayed apparition of ZsGreen in the GI tract during postnatal development. The distribution of ZsGreen expression along the adult GI tract was in excellent agreement with the established distribution of ICC in the muscularis propria (33), and KIT immunohistochemistry confirmed that ZsGreen expression was restricted to KIT-ir ICC.

Fig. 8. ZsGreen expression in cerebellum. ZsGreen was expressed in stellate and basket cells of the molecular layer (M). No ZsGreen was detected in Purkinje cells (P) or granular layer (G) (A and B). In the cerebellum, as in the gastrointestinal tract, ZsGreen localized beside, but not into, the nucleus delineated here with Hoechst (blue) (C). Maximum-intensity projections of stacks of 1-μm optical sections 5 μm thick across regions of interest in A and B, and single optical section in C. Scale bars: 20 μm in A and B; 5 μm in C.
The delicate, punctuate, perinuclear ZsGreen expression pattern in W/ZsGreen+/+ tissues was generally more difficult to apperhend than KIT-ir, which decorated the entire ICC network. Nevertheless, in stomach and colon, ZsGreen fluorescence readily allowed identification of the KIT-expressing ICC populations both in living and in fixed specimen. Conversely, in the jejunum, only a few green dots were present in ICC-MP, whereas at the level of the DMP, ZsGreen dots were completely lacking. This is in line with observations that KIT expression could hardly be detected by in situ hybridization in ICC-DMP in the mouse small intestine (15, 18, 33) and that KIT-ir is relatively low in ICC-DMP in the mouse and human small intestine (33, 37).

Conversely, a quite bright ZsGreen signal was observed in colonic submucosal ICC, whereas KIT-ir in these cells was not stronger than in the other colonic ICC populations. The possible significance of this remains to be elucidated. Mast cells were consistently ZsGreen negative.

Despite the low ZsGreen expression, cell dissociation was performed on jejunal samples of suckling animals, as the current dissociation protocol had been optimized for these conditions. Although the yield was fairly low, the identification of ZsGreen-positive cells, immediately after dissociation and in culture, indicated the potential of the method to enrich preparations of viable KIT-expressing ICC once improved dissociation protocols in stomach and colon will become available. Specially, the presence of KIT-expressing ICC identifiable by their ZsGreen fluorescence in cell clusters offers interesting perspectives for electrophysiological studies, as clusters optimally retain the intercellular connections and functional properties of native tissues (11, 20). Different classes of ICC can be identified and potentially sorted using fluorescent activated cell sorting based on the ZsGreen signal combined with specific ICC subpopulation markers such as NKCC1 [labels ICC-MP (42)] or NK1 [labels ICC-DMP (16)].

In the cerebellum, ZsGreen expression was observed in the molecular layer that contains the KIT-expressing stellate and basket cells (13, 22, 23, 35). Conversely, no ZsGreen expression was detected in skin, testis, and bone marrow, although these organs harbor KIT-expressing cells (1, 2, 28, 33, 40). It is unclear whether the lack of detectable fluorescence in these tissues is due to a lower KIT expression level. The KIT promoter is fairly complex and contains numerous cell- and developmental-specific regulatory elements (6, 7, 44). Insertion of the reporter gene into the first exon of c-Kit and thereby deleting the 5' end of the native c-Kit gene results in the loss of some regulatory elements. Namely, in hematopoietic and germ cell lineages, the lack of DNase I hypersensitive sites and enhancer integrating transcriptional signals, which are lacking in our W/ZsGreen model, results in a low level of KIT expression (6). The differences observed in ZsGreen expression compared with KIT expression could thus be related to the fact that the reporter could be subject to regulatory elements different from the endogenous c-Kit gene.

Despite some shortcomings, the novel W/ZsGreen model reported here nevertheless appears to be a promising tool for live studies of KIT-expressing cells in the GI tract and cerebellum.

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DISCLOSURES

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