Differential regulation of the ZsGreen1 reporter gene by the human cytomegalovirus promoter in 35 tissues of neonatal transgenic swine.

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ABSTRACT

The advent of genetically engineered pig production has revealed a wide array of opportunities to enhance both biomedical and agricultural industries. One powerful method to develop these models is transgenesis; however, selection of a suitable promoter to drive transgene expression is critical. The cytomegalovirus (CMV) promoter is the most commonly used viral promoter as it robustly drives transgene expression in a ubiquitous nature. However, recent reports suggest that the level of CMV promoter activity is tissue-dependent in the pig. Therefore, the objective of this study was to quantify the activity of the CMV promoter in a wide range of porcine tissues. Swine harboring a CMV-ZsGreen1 transgene were utilized for this study. Thirty five tissue samples were collected from neonatal hemizygous (n = 3) and homozygous (n = 3) transgenic piglets and analyzed for ZsGreen1 abundance via immunoblot. ZsGreen1 was detected in all tissues examined; however, quantification revealed that the level of ZsGreen1 expression was tissue-specific. For example, ZsGreen1 was most abundantly produced in the salivary gland, moderately produced in the esophagus and lowly expressed in the stomach. Interestingly, expression of ZsGreen1 also differed within organ. For instance, levels were highest in the right ventricle compared with other chambers of the heart. The expression patterns of ZsGreen1 were similar between homozygous and hemizygous piglets. Ultimately, these results elucidate the tissue-specific activity of the CMV promoter in the neonatal pig.

INTRODUCTION

Use of genetically engineered pig models has grown dramatically in recent years [1]. Transgenic swine are increasingly being utilized as animal models for human disease since they are anatomically, physiologically and phylogenetically more similar to humans than mice [1]. Consequently, successful xenotransplantation of transgenic pig organs to humans is eminent [2]. In addition to biomedical applications, transgenic pigs are also being developed to better understand porcine physiology and enhance the agricultural industry [3-6].

The first genetically engineered pig was generated in an effort to enhance growth rates and harbored a transgene containing the mouse metallothionein-I promoter fused to the human growth hormone gene [7]. Since then, new methodologies (e.g., homologous recombination, gene editing) have enhanced the ease and efficiency of genetically modifying the pig genome [8]. However, incorporation of a transgene remains a prominent method to produce genetically engineered pigs [1]. The most critical aspect of transgenic animal production is promoter selection [9]. Depending on the research interest and application, promoter types include tissue-specific, cell-specific, inducible/conditional or ubiquitous [9]. Since ubiquitous promoters drive transgene expression throughout the body, they are a popular choice to address whole-body questions [9].

One ubiquitous promoter is the human cytomegalovirus (CMV) immediate early promoter. It was discovered by Boshart et al. [10] and has since been heavily utilized to drive recombinant gene expression due to its constitutive and promiscuous nature [11]. These characteristics are directly related to its role in viral infection. Cytomegalovirus is

a human herpesvirus which typically lays dormant in infected host cells [12]. Cellular infection quickly (< 1 h) leads to CMV gene expression via the interaction of host transcription factors with the CMV promoter [12]. Gene products of this initial interaction mediate the activation of other promoters within the CMV genome that facilitate virus replication and invasion [12]. Therefore, the CMV promoter has evolved to contain binding sites for many ubiquitous transcription factors in order to be able to replicate in a broad range of host cells [11]. This discovery had major impacts on the scientific community; the CMV promoter is now the most commonly used ubiquitous promoter and it is also considered to be the most robust [13].

The CMV promoter has been utilized to drive transgene expression in numerous vertebrate animal models thus far including: sheep [14-16], fish [13, 17], mice [18-21], rats [22, 23], chickens [24] and pigs [3, 25-27]. In one study, the CMV promoter was used to drive expression of green fluorescent protein (GFP) in the pig [26]. As anticipated, expression of the transgene was detected in a wide range of organs. Expression of GFP was visually detected in organs derived from each of the 3 germ layers: skin (ectoderm), pancreas (endoderm), and kidney (mesoderm). Despite apparent universal transgene expression, however, some tissues appeared to express more GFP than others [26]. Upon further investigation, it became apparent that the CMV promoter is preferentially active in exocrine cells of the pig [25]. Within non-exocrine cells (e.g., hepatocytes), GFP was expressed to a lesser extent [25]. These data suggest that the CMV promoter may have limited applications for the study of non-exocrine porcine organs. Given the growing interest in the development of transgenic swine models, the

objective of this study was to quantify activity of the CMV promoter in a wide range of porcine tissues.

MATERIALS AND METHODS

Animals

All animal procedures were conducted in accordance with the University of Nebraska-Lincoln (UNL) Institutional Animal Care and Use Committee (IACUC). Experiments were performed in the UNL Animal Science Building (Lincoln, NE) and utilized transgenic swine that express ZsGreen1 controlled by the human CMV immediate early promoter; these animals were generated as previously described [3]. In hemizygous animals, one copy of the CMV-ZsGreen1 transgene is stably integrated on chromosome 14, and has remained transcriptionally active throughout the lifetime of the founder animal and throughout numerous generations [3].

Adult animals were housed individually with *ad libitum* access to water and fed approximately 2.5 kg of feed daily. Hemizygous CMV-ZsGreen1 transgenic sows were artificially inseminated with semen from non-littermate hemizygous transgenic boars and allowed to gestate to term. Following Mendelian inheritance, these matings yielded hemizygous, homozygous (2 copies of the transgene), and non-transgenic (control) littermates. After farrowing, piglets remained with their dam to suckle *ad libitum*.

At 1 d of age, 3 littermate female piglets (control, hemizygous transgenic and homozygous transgenic) were selected from 3 different litters; each litter was produced from a different dam and sire mating to maximize genetic diversity. Initially, transgene

status was assessed via evaluation of ZsGreen1 expression using an ultraviolet (UV) light and a Roscolux #15 filter (deep straw; Rosco, Port Chester, NY).

Tissue collection

Piglets were euthanized via intracardiac injection with Euthasol (1 mL/4.53 kg BW; Delmarva Laboratories, Midlothian, VA) and stored at 4°C overnight prior to dissection. The following morning, 35 organ samples representing 5 anatomical regions (brain, thoracic, digestive, renal and reproductive) were collected from each transgenic piglet including: cerebrum, cerebellum, spinal cord, anterior pituitary gland, posterior pituitary gland, hypothalamus, thymus, lymph node, the four chambers of the heart, lung, muscle, thyroid, salivary gland, esophagus, stomach, duodenum, jejunum, ileum, colon, gall bladder, spleen, pancreas, liver, kidney (medulla and cortex), bladder, adrenal gland (medulla and cortex), ovary, uterus, oviduct, and skin. Only skin samples were collected from control littermate piglets to serve as a negative control for ZsGreen1 immunoblotting. In addition, tail samples were collected from all piglets for genotyping. Samples were frozen and stored at -80°C until analysis.

Deoxyribonucleic acid extraction

Genomic deoxyribonucleic acid (DNA) was isolated from tail samples using an enzymatic digestion and phenol/chloroform extraction. Briefly, a tail slice (approximately 1 mm) was incubated overnight in a lysing solution [50 mM Tris pH 8.0, 150 mM NaCl, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), 0.1 mg/ml Proteinase K (Fisher Bioreagents, Fairlawn, NJ), 1% β-mercaptoethanol] at 55°C. The samples were extracted

twice with a 25:24:1 phenol:chloroform:isoamyl alcohol solution and once with a 24:1 chloroform:isoamyl alcohol solution before precipitating the DNA with isopropanol. DNA was subsequently resuspended in nuclease-free water.

Genotyping

Transgene integration and copy number were evaluated via conventional polymerase chain reaction (PCR) using 3 primers. The forward (F) and reverse (R) primers were designed to flank the insertion site of the transgene (T), and an additional reverse primer (RT) aligning the transgene was included (F - GCA ACC TCT TCG ACA CTC CA; R - AGC TAC CAG GGA ACA AAG CC; RT - GGT TTC CCT TTC GCT TTC AAG T). An MJ Research PTC-200 Thermocycler (Waltham, MA) was used for PCR reactions with the following conditions: 1 X *Taq* Buffer A (Fisher Bioreagents) with a final concentration of 2.0 mM $MgCl_2$, 200 μM dNTPs, 300 nM of each primer and 1.25 U of *Taq* DNA Polymerase (Fisher Bioreagents). Cycling conditions were 95ºC for 2 min, followed by 35 cycles of 95ºC for 30 sec, 56ºC for 30 sec and 72ºC for 60 sec and a final extension of 72ºC for 10 min. The resultant PCR products were subjected to electrophoresis on a 1% agarose gel. Products generated from the F and R primer pair were 627 bp in length and reflected a wild type chromosome 14. Products from the F and RT primer pair resulted in an 847 bp product, and indicating that the transgene was present.

Protein preparation

Protein extraction and sample preparation was performed as described previously [28]. Protein was extracted by homogenization of tissue samples in radioimmunoprecipitation assay (RIPA) buffer (1 ml/100 mg; 20 mM Tris, 137 mM NaCl, 10% glycerol, 1% NP40, 0.1% SDS, 0.5% deoxycholic acid, 2 mM EDTA, 1mM PMSF, 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail) using a Biospec Tissue Tearor (Bartlesville, OK). Protein concentrations were quantified using a bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Extracted protein was mixed with 4X loading dye [2% Tris (pH 6.8), 28% glycerol, 20% SDS and Orange G] containing dithiothreitol (DTT; 100 mM) and frozen at -80°C until western blotting.

Immunoblotting

Immunoblotting was performed as described previously [28]. Tissue samples were segregated by anatomical region (brain, thoracic, digestive, renal and reproductive) and protein (10 or 20 µg) was separated using SDS-PAGE (15%) before transfer to an Immobilon-FL polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) pre-soaked in methanol. After electrophoretic transfer, nonspecific binding was blocked by incubation of blots in Odyssey Blocking Buffer (cat #: 927-40100; LI-COR Biosciences, Lincoln, NE) for approximately 3 h at room temperature. Membranes were then incubated with a mouse monoclonal primary antibody directed against ZsGreen1 (1:1000; cat #: 632598; Clontech, Mountain View, CA), diluted in Odyssey Blocking Buffer with 0.05% Tween-20 and shaking at 4ºC overnight. The following morning, membranes were rinsed in Tris-buffered saline with 0.05% Tween-20 (TBS-T) prior to

incubation with a goat anti-mouse secondary antibody (1:15000; IRDye 680; LI-COR Biosciences) in Odyssey Blocking Buffer plus 0.05% Tween-20 and 0.025% SDS for 1 h at room temperature. Blots were rinsed with TBS-T to remove excess secondary antibody and then briefly rinsed in TBS to remove excess Tween-20 prior to imaging.

Quantification of ZsGreen1 abundance

Immunoreactive ZsGreen1 was visualized with an Odyssey Infrared Imaging System (LI-COR Biosciences). Blots were scanned on an Odyssey Infrared Imager (700 channel; intensity 5.0; 169 micron resolution; LI-COR Biosciences) and converted to greyscale for analysis. Band density was quantitated with Odyssey imaging software (version 2.1; LI-COR Biosciences). Since the abundance of common loading controls (e.g., β-actin) differs across tissue types, total protein levels within each lane were used as the loading control according to Eaton et al. [29]. After ZsGreen1 imaging, membranes were stained with GelCode Blue Safe Protein Stain (ThermoScientific, Rockford, IL) for approximately 1 h and then rinsed with a 50% methanol and 1% acetic acid solution for 1 h. Blots were then reimaged (700 channel; intensity 1.0; 169 micron resolution; LI-COR Biosciences), converted to greyscale and quantitated using Odyssey imaging software (version 2.1; LI-COR Biosciences) to determine the concentration of protein present in each sample. Data are presented as the relative band density of ZsGreen1 over the total protein concentration within the entire lane.

Statistical Analysis

Analyses were performed using the Statistical Analysis System (SAS; Cary, NC; version 9.4). Analysis of variance (ANOVA) was performed via the MIXED procedure of SAS with tissue type as the fixed effect. Blot and litter were random effects. A P-value < 0.05 was considered significant, whereas a P-value > 0.05 but < 0.10 was considered a tendency. Results are presented as least squares means (LSMEANS) ± the standard error of the mean (SEM). No effect of genotype (hemizygous versus homozygous) on ZsGreen1 abundance was observed ($P > 0.10$).

RESULTS

Identification of hemizygous and homozygous CMV-ZsGreen1 transgenic piglets

Expression of ZsGreen1 in the skin of neonatal transgenic piglets was detected visually (Figure 1A) and via immunoblot (Figure 1B), indicative of CMV promoter activity. Even without the UV light, transgenic animals were identifiable via the yellow hue of their skin (data not shown), which has been previously noted in GFP pigs [25]. The genotype of the animals was confirmed by conventional PCR (Figure 1D); a schematic depicting the genotyping method is available in Figure 1C. Zsgreen1 was not detected in the skin of control piglets (Figure 1A & 1B).

Visualization of ZsGreen1 expression within tissues of CMV-ZsGreen1 transgenic piglets

Expression of ZsGreen1 was visually detectable within numerous internal organs of transgenic pigs under UV light; florescence was absent in wild-type pig organs (Figure 2;

right panel). Expression appeared robust in the intestines (Figure 2; middle panel), heart (Figure 2B; right panel), cerebellum (Figure 2B; left panel), and anterior pituitary gland (Figure 2B; middle panel). Expression was modest within the stomach (Figure 2A; middle panel), cerebrum (Figure 2B; left panel), and posterior pituitary gland (Figure 2B; middle panel). ZsGreen1 was not visually detectable in the liver or spleen (Figure 2A; middle panel). Differential expression patterns were observed within organs as well. For example, distinct segments of the intestines displayed bright ZsGreen1 expression (Figure 2A; middle panel). Moreover, expression of ZsGreen1 appeared greatest in the right ventricle compared with other regions of the heart (Figure 2B; right panel). The brain also displayed differential ZsGreen1 abundance; expression was bright within the cerebellum and modest within other regions (Figure 2B; left panel).

Expression of ZsGreen1 driven by the CMV promoter in hemizygous and homozygous porcine organs

Neural and endocrine tissues of the brain

There was an effect of tissue type on ZsGreen1 expression within the brain and nervous system of hemizygous piglets (P = 0.0002; Figure 3A). Expression of ZsGreen1 was greatest within the anterior pituitary gland, followed by the cerebellum ($P < 0.05$; Figure 3A). ZsGreen1 levels were lowest in the cerebrum, spinal cord, hypothalamus and posterior pituitary gland, which were not different from each other ($P > 0.05$; Figure 3A). In homozygous piglets, there was also an effect of tissue type on ZsGreen1 abundance (P = 0.0396). The anterior pituitary gland expressed more ZsGreen1 than the cerebrum, spinal cord, hypothalamus or posterior pituitary gland ($P < 0.05$; Figure 3B), but was not

different from the cerebellum ($P > 0.05$; Figure 3B). ZsGreen1 levels were similar between the cerebellum, cerebrum, spinal cord, hypothalamus and posterior pituitary gland ($P > 0.05$; Figure 3B).

Thoracic organs

An effect of tissue type on ZsGreen1 expression was observed among 9 thoracic organs of hemizygous piglets ($P < 0.0001$; Figure 4A). The right ventricle produced the most ZsGreen1 in the thoracic region, even when compared with the other chambers of the heart ($P < 0.05$; Figure 4A). ZsGreen1 levels in the right ventricle were greater ($P < 0.05$) than the thyroid, right atrium, left atrium, left ventricle, lung, thymus or lymph node, which were similar ($P > 0.05$; Figure 4A). ZsGreen1 levels within skeletal muscle were intermediate ($P < 0.05$; Figure 4A). In homozygous piglets, there was no effect of tissue type on expression of ZsGreen1 among thoracic organs ($P = 0.5037$; Figure 4B).

Digestive Organs

ZsGreen1 expression differed across 11 digestive organs of hemizygous piglets ($P < 0.0001$; Figure 5A). Levels were greatest in the salivary gland and lowest in the stomach, pancreas, duodenum, jejunum, ileum, colon, spleen, gall bladder, and liver, which were not different ($P > 0.10$). Expression was intermediate in the esophagus ($P < 0.05$; Figure 5A). In homozygous piglets, there was also an effect of tissue type on ZsGreen1 expression ($P = 0.0212$; Figure 5B). Expression was greatest in the salivary gland ($P < 0.05$; Figure 5B) compared with the other 10 organs, which were similar ($P > 0.05$; Figure 5B).

Renal Organs

An effect of tissue type was observed between 5 renal tissues of hemizygous piglets (P = 0.0083; Figure 6A). The adrenal medulla produced more ZsGreen1 than the adrenal cortex, kidney medulla, kidney cortex or bladder ($P < 0.05$; Figure 6A). In homozygous animals, there tended to be a difference in ZsGreen1 abundance across renal tissues (P = 0.0620; Figure 6B). Levels were highest in the adrenal medulla and lowest in the kidney medulla, kidney cortex, and bladder ($P < 0.05$; Figure 6B). ZsGreen1 levels were similar between the adrenal cortex and all other organs ($P > 0.05$; Figure 6B).

Reproductive organs

No differences in ZsGreen1 expression were detected between the ovary, oviduct, and uterus of hemizygous or homozygous piglets ($P > 0.05$; Figure 6C and 6D).

DISCUSSION

The current study is the first to provide a quantitative, spatial report on the activity of the CMV promoter in swine. As a proxy of CMV promoter activity, the abundance of ZsGreen1 protein in swine containing a CMV-ZsGreen1 transgene was quantified. In accord with the ubiquitous nature of the CMV promoter, ZsGreen1 expression was detected in every tissue examined via immunoblotting. However, our results demonstrate that the level of CMV promoter activity is highly variable across organs of the neonatal piglet. Others have observed similar results in different animal models expressing a transgene driven by the CMV promoter [13, 24, 25, 30].

Based upon these results, several hypotheses regarding the differential activity of the CMV promoter *in vivo* have arisen. The activity of the CMV promoter within a given cell-type may be influenced by integration site [30] or differentiation stage of the cell [13]; namely, CMV promoter activation may only occur in transcriptionally active regions of the genome [11]. Stochastic silencing can occur when a transgene is integrated near a heterochromatic region, such as a centromere [31], rendering the promoter inaccessible [32]. Stochastic silencing of transgenes can also occur when multiple copies of the transgene integrate in tandem [33], due to local formation of heterochromatin [32]. Thus, integration site may have a profound effect on transgene expression.

In support of this hypothesis, Furth et al. [30] observed that two different transgenic mouse lines had marked differences in CMV promoter activity across the body, despite harboring the same transgene. Likewise, transgenic animals harboring a transgene, but lacking transgene expression, have been generated [34]. However, these results could also be due to promoter methylation, which has been shown to silence transgenes *in vitro* and *in vivo* [15, 20, 35]. In contrast, Vasey et al. (2009) hypothesized that integration site does not readily affect CMV promoter activity because the same activity profile of the CMV promoter was detected in pigs and chickens harboring the same transgene, albeit integrated in different chromosomal regions [25, 26]. The activity patterns were also similar in multiple transgenic chickens expressing different reporter genes directed by the CMV promoter [24]. Thus, it remains unclear whether integration site impacts the activity of the CMV promoter *in vivo*.

In addition to chromatin status, the repertoire of transcription factors and transcriptional co-regulators present within the cell likely dictate CMV promoter activity [11]. Mella-Alvarado et al. (2013) hypothesized that CMV promoter activity is most robust in the organs that CMV infects most voraciously (brain, eye, spinal cord, pancreas, kidney, testis, ovary, skin, cartilage, and skeletal muscle), which may be a reflection of optimal transcription factor expression within those tissues. Indeed, the human CMV promoter is more potent in human cell lines compared with equivalent mouse cell lines [36]. In addition, Furth et al. [30] suggested that certain transcription factors may only be able to interact with euchromatin, whereas others retain functionality with heterochromatin [30]. Therefore, the interaction of chromatin status with available transcription factors may be an important element to tissue-specific CMV promoter activity.

In exocrine glands, Vasey et al. [25] proposed that the transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), is a major driver of CMV promoter activity. NF-kB is highly expressed in immune cells, classically regulates the expression of proinflammatory genes [37], activates in response to cellular insult such as viral infection [37], and is a potent stimulator of CMV promoter activity [11, 38-40]. Exocrine cells are classified as an immune cell given that exocrine and mucosa cells account for ~85% of all immunoglobulin-producing cells in the body [41]. Therefore, elevated CMV promoter activity is logical in tissues which contain many exocrine cells [25]. Our findings generally support this theory as elevated ZsGreen1 expression was identified in the salivary glands of hemizygous and homozygous transgenic piglets, as previously noted in CMV-GFP pigs [25]. Others have also detected robust CMV

promoter activity in other exocrine cells and tissues of the pig, such as the glandular epithelia of the snout, sebaceous glands of the skin and Harderian gland of the eye [25]. We also detected robust CMV promoter activity in the skin of CMV-ZsGreen1 transgenic pigs.

In the exocrine pancreas, however, we did not detect elevated ZsGreen1 expression in either hemizygous or homozygous transgenic piglets. These results conflict with other reports indicating elevated activity of the CMV promoter within the porcine pancreas [25, 26]. This discrepancy could be related to age-specific activity levels of the CMV promoter, as noted in zebrafish [13]. In the current study, neonatal (1 d old) piglets were utilized, whereas others examined CMV promoter activity in the pancreases of older (2 mo to 1 yr) pigs [25, 26]. Likewise, our laboratory detected robust ZsGreen1 abundance in the pancreas of 1 mo old, weaned piglets (unpublished data). Interestingly, the exocrine pancreas is largely quiescent in the neonatal piglet [42-46]; production of pancreatic juices and enzymes increased dramatically after weaning [42-44]. Thus, CMV promoter activity in the exocrine pancreas appears to be impacted by animal age, which likely reflects a dormant versus transcriptionally active organ.

In the liver, another exocrine organ that secretes bile made by hepatocytes [47], we also detected little CMV promoter activity. Similar to our data, others have reported low CMV promoter activity in porcine hepatocytes [25] and chicken liver [24]. In contrast, GFP was robustly driven by the CMV promoter in hepatocytes of zebrafish [13]. Thus, it appears that the CMV promoter is not uniformly effective in all exocrine organs and can vary in activity across species. Ultimately, these data indicate that the CMV

promoter is highly active in certain exocrine glands, which could be due to an abundance of NF-kB in these tissues [25].

Besides the salivary gland, elevated levels of CMV promoter activity were also observed in non-exocrine organs like muscle (skeletal and cardiac) as well as endocrine glands (anterior pituitary) in both genotypes. In contrast, some organs had markedly low expression of ZsGreen1 in both hemizygous and homozygous animals, including the lung, liver, spleen, and kidney. It is unclear which regulatory elements are driving CMV promoter activity in these tissues. Besides NF-kB, numerous transcription factors have been implicated in the regulation of the CMV promoter, including ying yang 1 [11], activator protein 1 and its major subunits (c-Fos and c-Jun) [40], p53 [48] as well as MAP/ERK kinase kinase 1 [48], Sp1 [10], NF-1, serum response factor, Elk-1 and CCAAT/enhancer-binding protein [12]. Thus, it is apparent that the CMV promoter has adapted to respond to a variety of cell signals in order to allow for the promiscuous infection of host cells [11]. However, it remains unknown which regulatory elements are mediating CMV promoter activity in tissues of the neonatal pig.

In the current study, we compared the expression patterns of ZsGreen1 between hemizygous and homozygous piglets. Expression patterns were similar between genotypes, both hemizygous and homozygous animals had comparable high (e.g., anterior pituitary gland, skin, right ventricle) and low (e.g., lung, liver, pancreas) ZsGreen1-expressing organs. Abundance of ZsGreen1 was also compared between homozygous and hemizygous animals. Initially, we hypothesized that an additional transgene would yield more ZsGreen1 protein in the organs of homozygous animals. However, ZsGreen1 levels were highly variable in homozygous animals and no genotype

effect was detected based upon quantification of immunoblots. These results may be due to monoallelic expression, which frequently occurs in autosomes [49] and has also been described in other transgenic animal models [32, 50]. Conversely, translation of ZsGreen1 transcripts may be impaired in homozygous animals. For example, post-transcriptional silencing of high expressing transgenes is common in homozygous, but not hemizygous, plants [51-54] and appears to be mediated by short interfering RNA [51]. Many have hypothesized that doubling transcription of a transgene yields copious mRNA transcripts, which breach an unknown threshold that triggers cellular processes to suppress transgene translation [52, 55-58]. Regardless of the mechanism, transgene copy number did not significantly affect production of ZsGreen1 protein in this study.

To our knowledge, these animals represent the first pig model to express ZsGreen1. This protein is derived from Anthozoa coral reef (*Zoanthus* species), and was originally discovered by Matz et al. [59] and named zFP506. Since then, it has been has been commercialized by Clontech under the trade name ZsGreen [60]. The variant ZsGreen1 is a modification of the original protein to maximize expression, solubility and prompt chromophore maturation (Clontech, personal communication). Interestingly, it has been shown to be both structurally and functionally distinct from other fluorescent proteins, like GFP [59, 61]. ZsGreen1 displays extremely bright fluorescence and high signal-to-noise ratio; in fact, yeast cells overexpressing ZsGreen exhibited 8.6-fold more fluorescence than those expressing GFP [61]. In another study, ZsGreen1 had the greatest relative fluorescence compared with 5 other fluorescent proteins, including GFP [59]. The excitation maximum and emission maximum for ZsGreen1 are 493 nm 505 nm, respectively, allowing for visualization with standard microscopy filters [60].

Structurally, ZsGreen1 functions as a tetramer; in contrast, the quaternary structure of GFP is a monomer, which frequently dimerizes for solubility [60]. Each ZsGreen1 monomer is 231 amino acids in length with a predicted molecular weight of approximately 26 kDa [59]. Interestingly, ZsGreen1 is only 26% homologous to GFP, which is derived from jellyfish [59, 60, 62]. However, both fluorescent proteins are configured in a β-barrel structure [59]. Ultimately, the distinctions of ZsGreen1 could be advantageous for researchers seeking porcine organs expressing a novel fluorescent protein.

To conclude, this study demonstrates that the CMV promoter is ubiquitously active in all porcine tissues types examined; however, the level of activity is tissue-specific. The CMV promoter potently drives transgene expression in some exocrine organs (e.g., salivary gland) of the neonatal pig; however, it is also effective in other non-exocrine organs (e.g., skin, anterior pituitary gland and muscle) as well. In certain organs (e.g., liver, kidney), however, activity of the CMV promoter is less robust. Ultimately, this report elucidates the tissue-specific activity of the human CMV promoter in swine and provides critical information for researchers seeking an effective promoter to drive transgene expression in the neonatal pig. Moreover, these animals represent a novel resource for scientists in need of ZsGreen1-labeled porcine organs.

FIGURE LEDGENDS

Figure 1. Production of CMV-ZsGreen1 hemizygous and homozygous transgenic pigs. **(A)** Representative photograph of control piglet (left) with homozygous (middle) and hemizygous (right) transgenic siblings. ZsGreen1 was visualized with an ultraviolet light and a Roscolux #15 filter (deep straw; Rosco, Port Chester, NY). (**B)** Representative immunoblot of ZsGreen1 in the skin of control piglets (n = 3) as well as hemizygous (n = 3) and homozygous (n = 3) transgenic littermates. Lower panel represents total protein levels within each lane. (**C)** Schematic demonstrating the genotyping method utilized to distinguish between control, homozygous and hemizygous piglets. Detection of monoallelic or biallelic transgene integration was performed by conventional polymerase chain reaction (PCR) using 3 primers. The forward primer, denoted by the black arrow, amplifies a non-coding region upstream of the transgene integration site. A reverse primer (grey arrow) aligns a non-coding region downstream of the transgene and a second reverse primer, indicated in green, detects the transgene. If a transgene is present, the resulting PCR product will be 847 basepair (bp); however, in the absence of the transgene, the PCR product will be 627 bp. **(D)** PCR detection of transgene integration and copy number. The presence of only a 847 bp PCR product indicates a homozygous transgenic animal whereas a 627 bp product indicates a control animal. If both 847 and 627 bp products are detected, the genotype is hemizygous transgenic.

Figure 2. Visualization of ZsGreen1 expression within internal organs of transgenic piglets. **(A)** Representative image of ZsGreen1 expression in the internal organs of transgenic pigs. Left panel represents a photograph of internal organs from a transgenic piglet. Note the yellow color of some organs (e.g., intestines). Left panel represents a fluorescent image of internal organs from a transgenic piglet whereas right panel is a UV image of wild-type piglet organs. **(B)** Representative image of a transgenic piglet brain (left panel), the anterior and posterior pituitary glands within the sella turcica of the skull (middle panel) and a transgenic piglet heart (right panel). Cerebellum, anterior pituitary gland and posterior pituitary gland are indicated by a black, white, and grey arrow, respectively. ZsGreen1 expression was abundant within the right ventricle of the heart (right panel). Abbreviations: L, liver; St, stomach, Sp, spleen; I, intestines; RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle. ZsGreen1 was detected with ultraviolet light and a Roscolux #15 filter (deep straw; Rosco, Port Chester, NY).

Figure 3. Expression patterns of ZsGreen1 within neural and endocrine tissues of CMV-ZsGreen1 pigs. **(A)** Representative immunoblot of neural and endocrine tissue from hemizygous transgenic piglets (n = 3), using an antibody directed against ZsGreen1 (upper panel). Middle panel represents total protein levels that served as the loading control. Quantification of immunoblots revealed differences in relative ZsGreen1 protein levels between tissue types ($P = 0.0002$; lower panel). (**B**) Representative immunoblot of neural and endocrine tissue from homozygous transgenic piglets (n = 3), using an antibody directed against ZsGreen1 (upper panel). Middle panel represents total protein levels that served as the loading control. Quantification of immunoblots revealed a difference in relative ZsGreen1 protein levels between tissue types ($P = 0.0396$; lower panel). [a,b,c] Bars with alternate letters differ ($P < 0.05$).

Figure 4. Expression patterns of ZsGreen1 within thoracic tissues of CMV-ZsGreen1 pigs. **(A)** Representative immunoblot of thoracic tissue from hemizygous transgenic piglets (n = 3), using an antibody directed against ZsGreen1 (upper panel). Middle panel represents total protein levels that served as the loading control. Quantification of immunoblots revealed differences in relative ZsGreen1 protein levels between tissue types ($P < 0.0001$; lower panel). [a,b,c] Bars with alternate letters differ ($P < 0.05$). (**B**) Representative immunoblot of thoracic tissue from homozygous transgenic piglets (n = 3), using an antibody directed against ZsGreen1 (upper panel). Middle panel represents total protein levels that served as the loading control. Quantification of immunoblots indicated that there were no differences in relative ZsGreen1 abundance between tissue types ($P = 0.5037$; lower panel).

Figure 5. Expression patterns of ZsGreen1 within digestive tissues of CMV-ZsGreen1 pigs. **(A)** Representative immunoblot of digestive tissue from hemizygous transgenic piglets (n = 3), using an antibody directed against ZsGreen1 (upper panel). Middle panel represents total protein levels that served as the loading control. Quantification of immunoblots revealed differences in relative ZsGreen1 protein levels between tissue types ($P < 0.0001$; lower panel). (**B**) Representative immunoblot of digestive tissue from homozygous transgenic piglets (n = 3), using an antibody directed against ZsGreen1 (upper panel). Middle panel represents total protein levels that served as the loading control. Quantification of immunoblots revealed differences in relative ZsGreen1 protein levels between tissue types ($P = 0.0212$; lower panel). [a,b,c] Bars with alternate letters differ ($P < 0.05$).

Figure 6. Expression patterns of ZsGreen1 within renal tissues of CMV-ZsGreen1 pigs. **(A)** Representative immunoblot of renal tissue from hemizygous transgenic piglets (n = 3), using an antibody directed against ZsGreen1 (upper panel). Middle panel represents total protein levels that served as the loading control. Quantification of immunoblots revealed differences in relative ZsGreen1 protein levels between tissue types ($P = 0.0083$; lower panel). (**B**) Representative immunoblot of renal tissue from homozygous transgenic piglets (n = 3), using an antibody directed against ZsGreen1 (upper panel). Middle panel represents total protein levels that served as the loading control. Quantification of immunoblots revealed a tendency for differences in relative ZsGreen1 protein levels between tissue types ($P = 0.0620$; lower panel). [a,b,c] Bars with alternate letters differ ($P < 0.05$).

Figure 7. Expression patterns of ZsGreen1 within reproductive tissues of CMV-ZsGreen1 pigs. **(A)** Representative immunoblot of reproductive tissue from hemizygous transgenic piglets (n = 3), using an antibody directed against ZsGreen1 (upper panel). Middle panel represents total protein levels that served as the loading control. Quantification of immunoblots indicated that there were no differences in relative ZsGreen1 abundance between tissue types ($P = 0.3227$; lower panel). (**B**) Representative immunoblot of reproductive tissue from homozygous transgenic piglets (n = 3), using an antibody directed against ZsGreen1 (upper panel). Middle panel represents total protein levels that served as the loading control. Quantification of immunoblots indicated that

there were no differences in relative ZsGreen1 abundance between tissue types (P = 0.4797).

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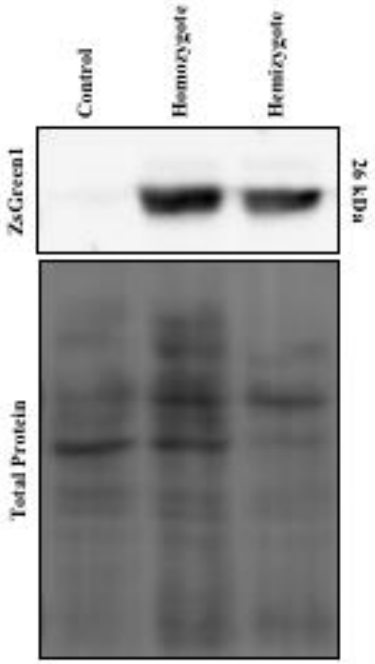
Figure 1

A

B



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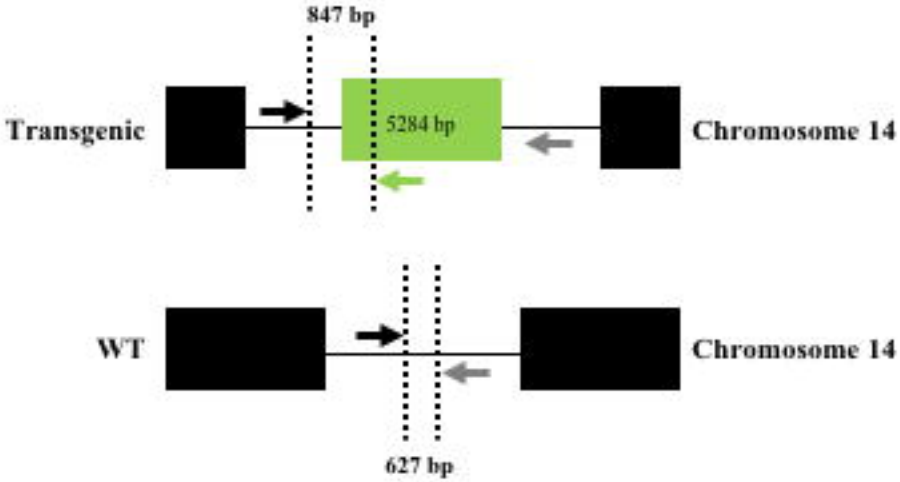

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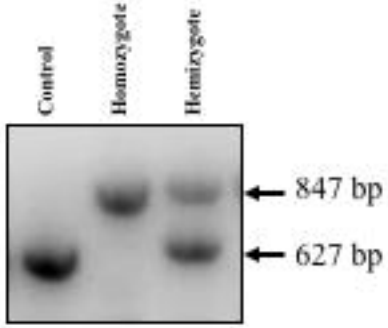

Figure 2

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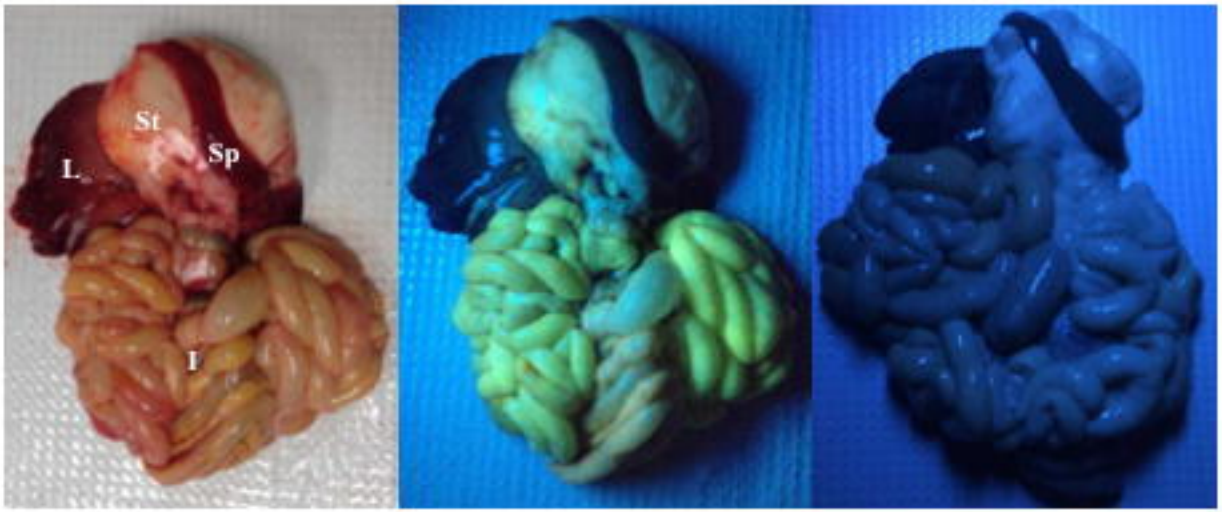

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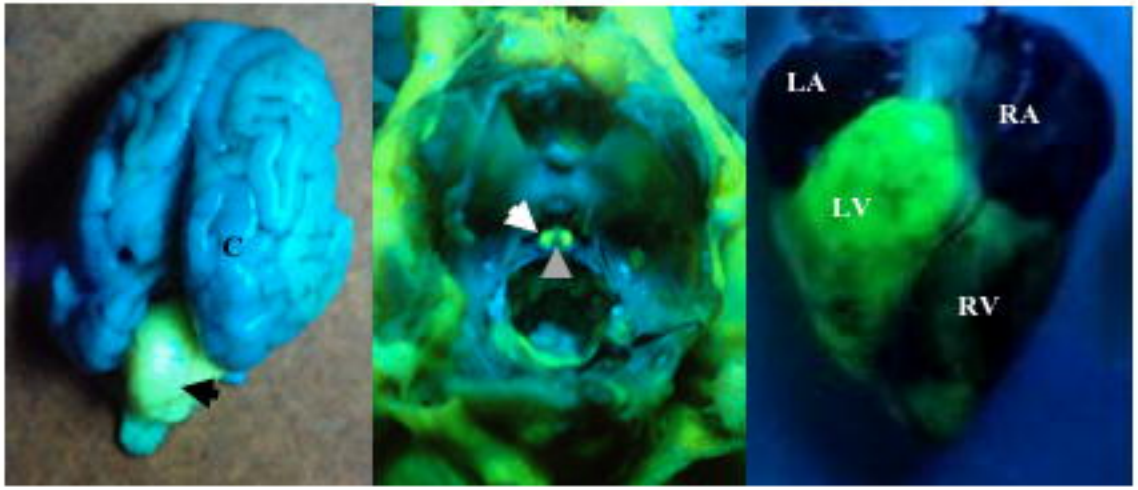


Figure 3

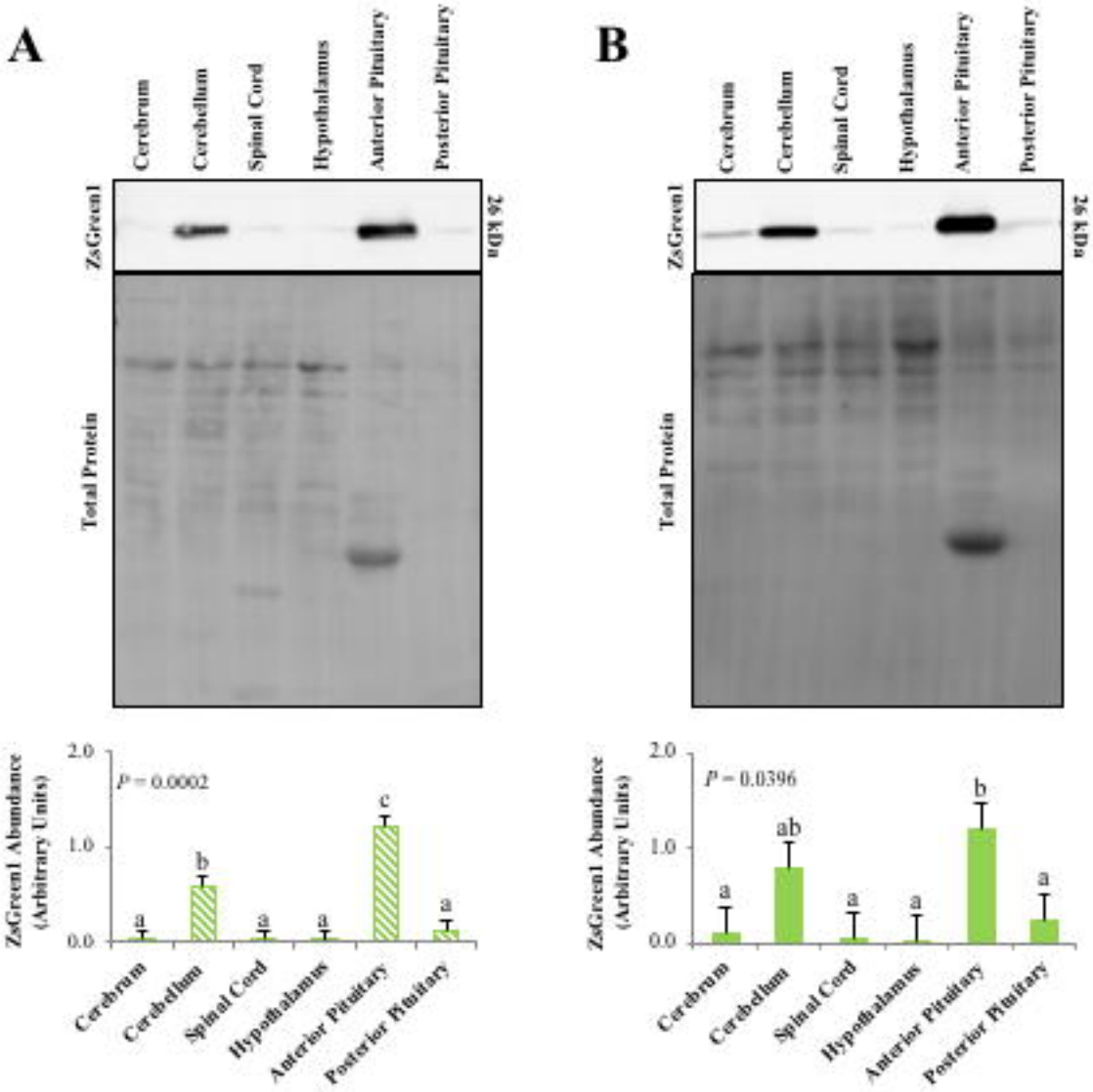
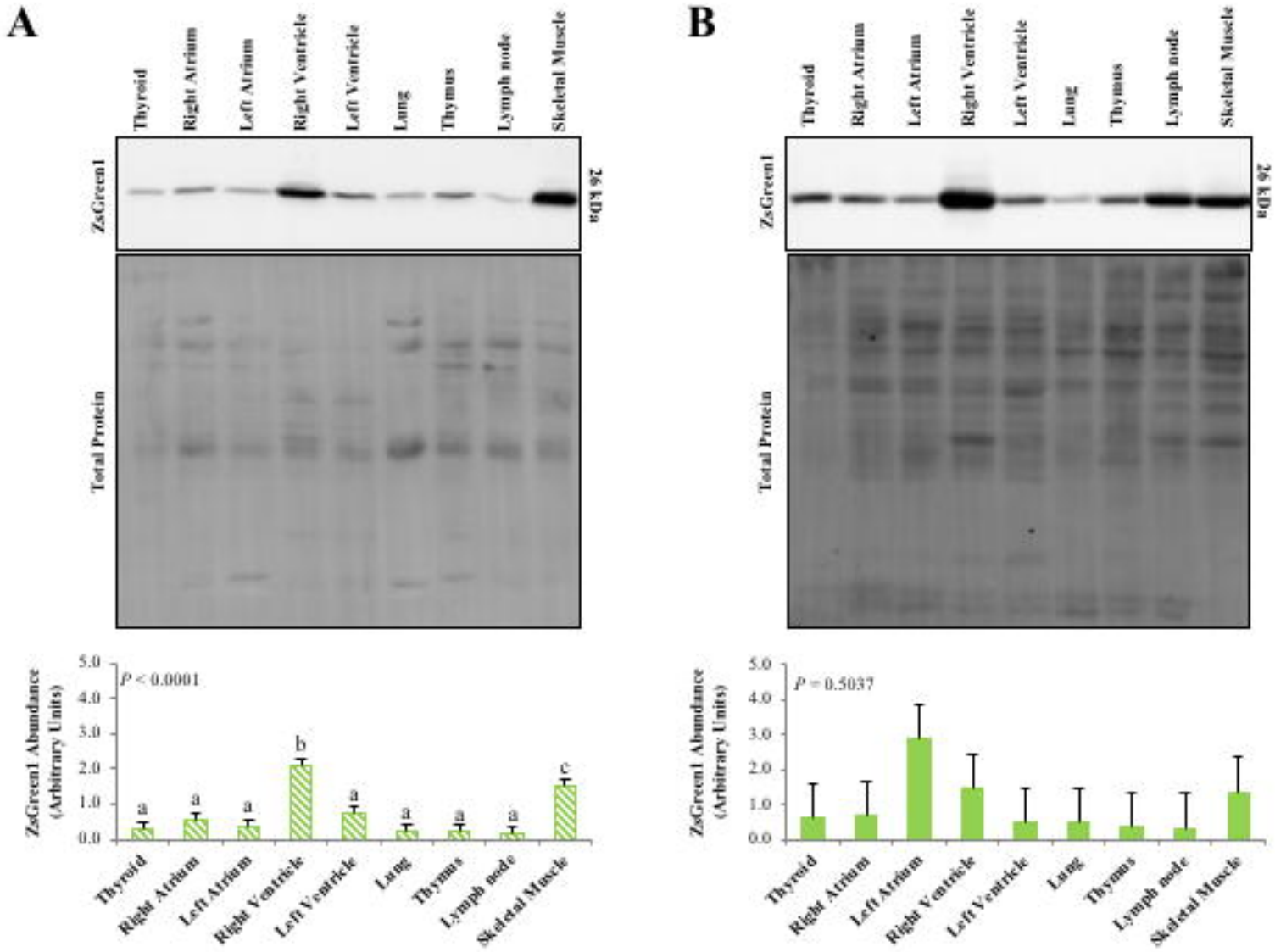

A
B
Cerebrum
Cerebellum
Spinal Cord
Hypothalamus
Anterior Pituitary
Posterior Pituitary
ZsGreen1
26 kDa
Total Protein
ZsGreen1 Abundance (Arbitrary Units)
2.0
1.0
0.0
P = 0.0002
P = 0.0396
a
b
c
ab

Figure 4


A
B
Thyroid
Right Atrium
Left Atrium
Right Ventricle
Left Ventricle
Lung
Thymus
Lymph node
Skeletal Muscle
ZsGreen1
Total Protein
26 kDa
ZsGreen1 Abundance (Arbitrary Units)
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4.0
3.0
2.0
1.0
0.0
a
b
c

Figure 5

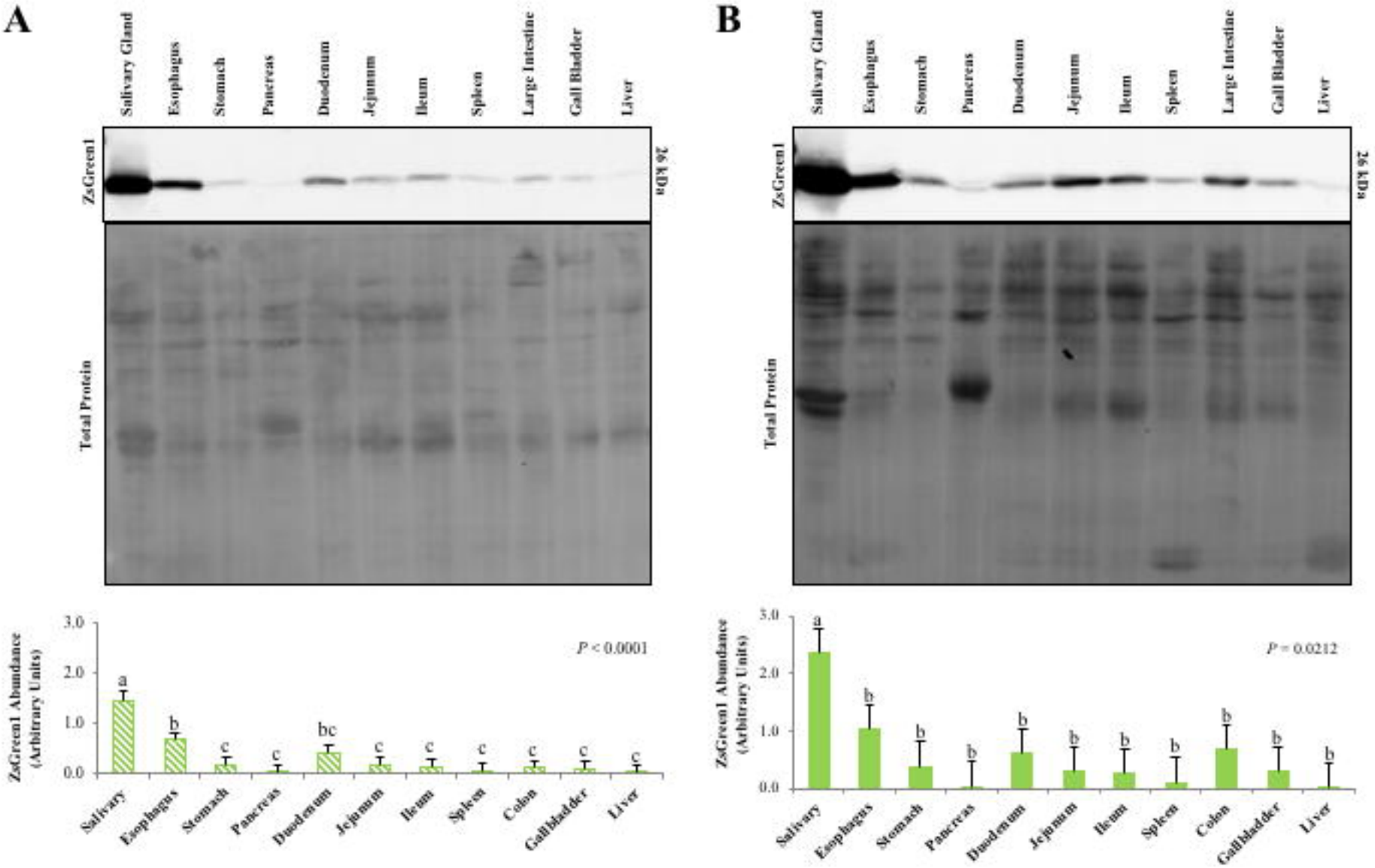
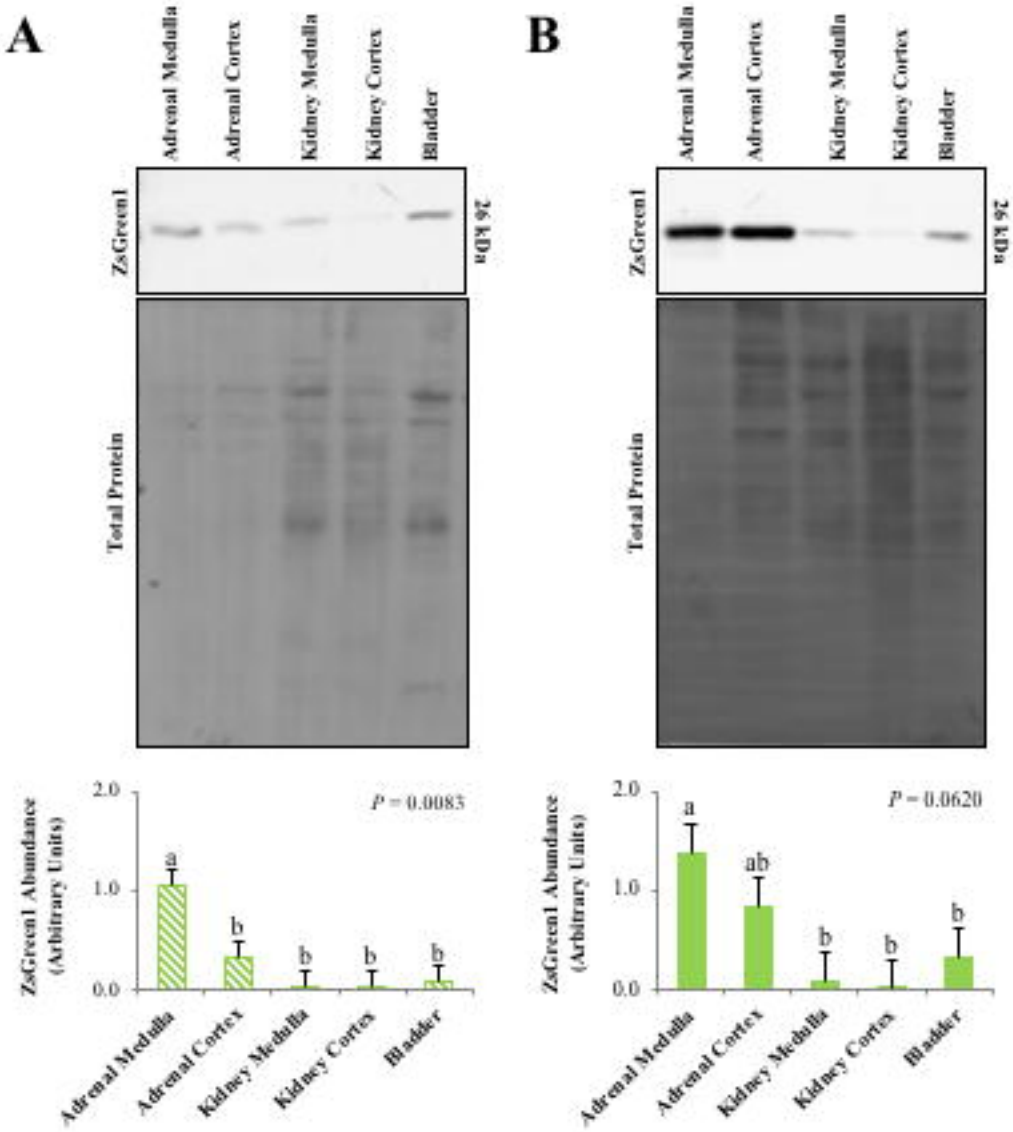

A
Salivary Gland
Esophagus
Stomach
Pancreas
Duodenum
Jejunum
Ileum
Spleen
Large Intestine
Gall Bladder
Liver
ZsGreen1
26 kDa
Total Protein
ZsGreen1 Abundance (Arbitrary Units)
3.0
2.0
1.0
0.0
P < 0.0001
a
b
c
c
bc
c
c
c
c
c
c
Salivary
Esophagus
Stomach
Pancreas
Duodenum
Jejunum
Ileum
Spleen
Colon
Gallbladder
Liver
B
Salivary Gland
Esophagus
Stomach
Pancreas
Duodenum
Jejunum
Ileum
Spleen
Large Intestine
Gall Bladder
Liver
ZsGreen1
26 kDa
Total Protein
ZsGreen1 Abundance (Arbitrary Units)
3.0
2.0
1.0
0.0
P = 0.0212
a
b
b
b
b
b
b
b
b
b
b
Salivary
Esophagus
Stomach
Pancreas
Duodenum
Jejunum
Ileum
Spleen
Colon
Gallbladder
Liver

Figure 6


A
B
Adrenal Medulla
Adrenal Cortex
Kidney Medulla
Kidney Cortex
Bladder
ZsGreen1
26 kDa
Total Protein
ZsGreen1 Abundance (Arbitrary Units)
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1.0
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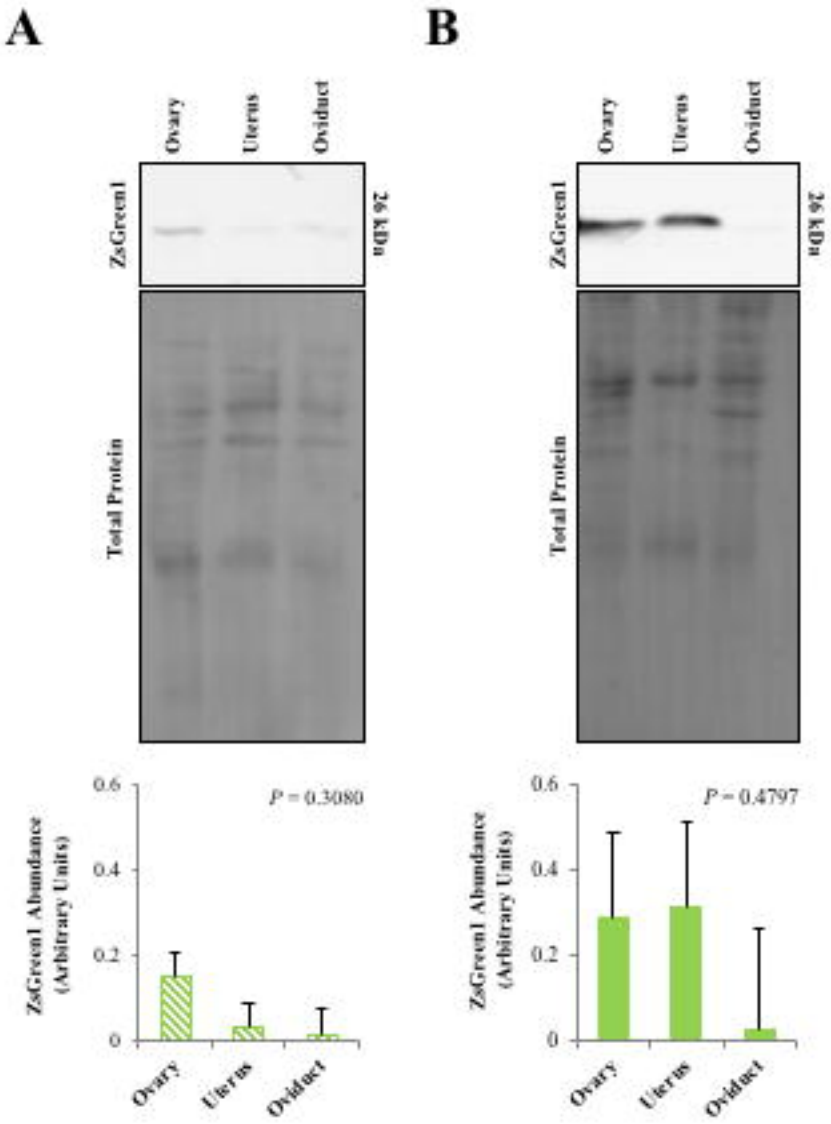
P = 0.0083
P = 0.0620
a
b
ab

Figure 7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B
Ovary
Uterus
Oviduct
ZsGreen1
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Total Protein
ZsGreen1 Abundance (Arbitrary Units)
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0.4
0.2
0
P = 0.3080
P = 0.4797