Generation and characterization of *iUBC-KikGR* photoconvertible transgenic mice for live time lapse imaging during development

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Summary

A transgenic mouse line named *iUBC-KikGR* was generated which expresses the photoconvertible fluorescent protein Kikume Green-Red (KikGR) under the control of the human *Ubiquitin C* promoter. KikGR is natively a green fluorophore which can be converted into a red fluorophore upon exposure to UV light. KikGR is expressed broadly throughout transgenic embryos from the 2-cell stage onward, and in the adult. Specificity of photoconversion can range from the entire embryo, to a region of an organ, to a few individual cells, depending on the needs of the experimenter. Cell movements, tissue reorganization, and migration can then be observed in real time by culturing the tissue of interest as an explant on the microscope stage. The *iUBC-KikGR* transgenic line represents a singular genetic reagent which can be used for fate mapping, lineage tracing, and live visualization of cell behaviors and tissue movements in multiple organs at multiple time points.

Keywords

fate mapping; lineage tracing; photoconvertible fluorescent protein; KikGR; explant culture

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The complex body plan of multicellular organisms is shaped by myriad events in early development, including cell division and cell death, morphogenetic movements, cell migration, differentiation, and pattern formation. All of these processes involve multiple cell populations and dynamic interactions between them. Insight into these events is best procured by observation of interactions between cells and tissues in real time. To date, one of the most powerful tools for tracing cells over time is by expression of fluorescent proteins in living tissue. This allows for observation of cellular behaviors by wide field fluorescence or confocal imaging. With the assistance of *ex vivo* culture technology such as integrated environmental chambers to control temperature, CO_2 levels, and humidity on the microscope stage, real time observation of cellular events during development is possible.

To gain specificity in labeling particular cell types or populations with fluorescent markers, current techniques include site-specific injection, electroporation, and dye placement, or genetic techniques such as transgenes which express fluorescent proteins in a spatially- or time-dependent manner. For lineage tracing, fate mapping, and simple observation of

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specific populations in the developing embryo, the most popular technique in mice has been a bigenic approach involving a transgene expressing Cre recombinase in a time-or tissue-specific manner, and a reporter allele which activates a visual marker, such as lacZ or YFP, in cells that express the Cre transgene (Soriano, 1999; Srinivas *et al.*, 2001). This *Cre-lox* technique requires that the cell population of interest be defined by a genetic marker specific to that population, which can then be exploited by using *cis*-regulatory elements from that endogenous gene to drive Cre expression in the transgene. While this is a very powerful technique, it also has limitations. For many cell populations of interest, a useful genetic marker is not known, making it difficult or impossible to express Cre in such a way as to mark the population of interest. Even if good genetic markers are available, time and expense must be invested in creating new transgenic lines for every new cell population for which there is not a pre-existing Cre transgene. Finally, Cre transgenes usually mark entire populations of cells rather than individual cells or small subpopulations. Labeling specificity at this level requires different technology.

To circumvent these limitations, we have generated a new transgenic mouse which ubiquitously expresses the photoconvertible fluorescent protein Kikume Green-Red (KikGR) under control of the human *ubiquitin C* (*UBC*) promoter. KikGR is derived from a naturally occurring fluorescent protein which undergoes a conformational change induced by UV light. This conformational change alters the excitation and emission properties of the native protein such that it is converted from a green fluorophore (ex_{max}=507 nm, em_{max}=517 nm) to a red fluorophore (ex_{max}=583 nm, em_{max}=593 nm) (Tsutsui *et al.*, 2005). We have introduced KikGR to the mouse genome as a transgene so that we can exploit this property to specifically label small groups or even individual cells and observe their behaviors in real time. Several photoconvertible fluorescent proteins have been tested in the mouse and KikGR has been determined to be most suitable for live imaging in the mouse embryo since it is bright, developmentally neutral, and undergoes rapid and complete conversion upon exposure to UV light (360–410 nm) while remaining stably unconverted in broad-spectrum white light necessary for embryo dissection and manipulation (Nowotschin and Hadjantonakis, 2009). KikGR has been used in lineage tracing, fate mapping, and live imaging of zebrafish, chick, and mouse development to date (Hatta *et al.*, 2006; Kulesa *et al.*, 2008; Kurotaki *et al.*, 2007; Nowotschin and Hadjantonakis, 2009; Stark and Kulesa, 2007). CAGG promoter driven transgenes are sometimes subject to transgene silencing and/ or non-ubiquitous expression, especially during development (unpublished work). Previously generated KikGR transgenes have used the CAGG promoter to drive expression in a variety of tissues, but we have used the human *ubiquitin C (UBC)* promoter, as well as genomic insulators, to generate this transgene in an attempt to alleviate position effects on transgene expression, thus resulting in robust and near-ubiquitous expression of KikGR throughout the embryo and adult tissue. Here we present a new transgenic mouse which broadly expresses KikGR throughout the embryo, making it suitable for individual cell or subpopulation labeling and live imaging from the zygote to the adult. Furthermore, we demonstrate its utility for tracing cells during live imaging of *ex vivo* embryo and organ cultures.

We generated two transgene constructs, *UBC-KikGR*, comprising the human *Ubiquitin C* promoter and first intron, followed by the KikGR cDNA and polyadenlyation sequence. A second construct, *iUBC-KikGR*, flanks the *UBC-KikGR* construct with two concatamerized genomic insulators derived from the chicken beta globin domain (cSH4x) (Figure 1). Three fluorescent founders from each construct were identified, first by PCR genotyping, then by screening fluorescence in tail snips. Two of the *iUBC-KikGR* founders and their F_1 progeny showed consistently brighter fluorescence than the third *iUBC-KikGR* line and the three *UBC-KikGR* lines; these dim lines were then discarded. All data presented here were

derived from the *iUBC-KikGR1-7* line. Similar data were obtained from a second (*iUBC-KikGR3-1*) line.

KikGR expression in transgenic embryos is observed from the 2-cell stage (in embryos derived from a transgenic dam), throughout fetal development (in embryos derived from a transgenic sire or dam), and in postnatal animals (adult tail snips). To assay functionality of photoconversion in transgenic mice, E11.5 and E15.5 embryos were exposed to UV light then imaged under both green fluorescent protein (GFP) and red fluorescent protein (RFP) filters to detect converted and unconverted protein. Furthermore, broad fluorescence of the native green KikGR at E11.5 suggested that these transgenic mice are suitable for labeling cells of interest in many embryonic organs. Several organs were dissected from E11.5 embryos, and fluorescence was observed in brain, eye, mesenchyme, limb buds, heart, lungs, somites, and gonads; grossly, all organs exhibit KikGR fluorescence at mid-gestation, though fluorescence in the liver is relatively dim. KikGR is also expressed in 2-cell embryos (when derived from transgenic dams) and in the embryonic ectoderm, with reduced expression in the visceral endoderm of E5.5 embryos (Figure 2). No expression was detected in the extraembryonic ectoderm in E5.5 embryos. Adult transgenic mice exhibit KikGR fluorescence in heart, lungs, kidney, liver (though fluorescence is dim), intestine, diaphragm, ovary, eye, brain, and stomach, but not the spleen (Figure 3). Complete photoconversion of small regions of interest via confocal microscopy in *ex vivo* organ cultures is achieved in less than 10 scans over 10–20 seconds depending on the tissue (Supplemental Video S1).

Selected organs were cultured *ex vivo*, cells of interest were photoconverted on the microscope stage, and explants were live imaged for up to 24h. Some variation over time in intensity of the photoconverted (red) signal was observed, likely owing to variation in rates of cell division and/or protein turnover in different cell types, both of which have potential to dilute the quantity of photoconverted protein in a given cell over time (Figure 4). In order to circumvent this potential problem, we have "reconverted" cells in which the intensity of red signal appears to be fading after 12h, and found that in this manner, cells can be continuously traced for as long as the explant culture thrives with no observable phototoxicity from multiple photoconversion events (data not shown).

The *iUBC-KikGR* transgenic mouse is useful for observing morphometric events during gastrulation and early body plan patterning in real time. Photoconversion of cells in the presumptive anterior visceral endoderm (AVE), an organizing tissue which directs anterior-posterior patterning of the embryos, allows for live visualization of AVE cell movements. As expected from previous studies, a distally originating region of the visceral endoderm, which may represent the AVE in E5.5 embryos (labeled red by photoconversion) migrates proximally toward the extra-embryonic ectoderm after only 3–4 h (Rodriguez *et al.*, 2005) (Figure 5, Supplemental Video S2).

Later morphometric events in organogenesis can also be observed. Photoconversion of the rostral third of the mesonephros at E11.5, and subsequent live imaging of the gonad-mesonephros explant for 12h or more shows that the Wolffian duct, which runs through the mesonephros, continues to extend from the rostral region during this time (Joseph *et al.*, 2009). In converting the entire rostral third of the mesonephros, both mesenchymal and Wolffian duct epithelial cells are labeled red after photoconversion. After 12h *ex vivo* culture, the red labeled Wolffian duct epithelium has extended caudally beyond the photoconversion boundary of the mesonephric mesenchyme. This result suggests that the Wolffian duct continues to elongate in the rostral region even after the duct has reached its most caudal target in the urogenital sinus. Although the Wolffian duct is fully formed as a complete epithelial tube extending to the urogenital sinus at this point in development, it continues to elongate in the rostral region, presumably to accommodate later coiling in this

region which will give rise to the highly convoluted epididymis (Joseph *et al.*, 2009). Mechanisms for elongation in the rostral Wolffian duct may include convergent extension, intercalation of mesenchymal cells into the ductal epithelium, or cell division within the rostral Wolffian duct (Figure 6, Supplemental Video S3).

While the *UBC-KikGR* transgenic mouse provides an excellent platform for many lineage tracing and fate-mapping applications in development, the nearly ubiquitous expression of KikGR in this line may also be a disadvantage when considering its use in some experiments. For instance, ubiquitous expression in many organs can limit the researchers' ability to identify and track behaviors of individual cells within a population, as they become difficult to distinguish against the background of their many fluorescent neighbors. Visualizing the behaviors of individual cells in the *UBC-KikGR* transgenic mouse generally requires that red photoconverted cells migrate away from their original location in a low enough density to provide good separation between one red cell and the next, which may not be possible in all organ systems. Furthermore, researchers interested in development of the spleen or extraembryonic ectoderm will find that KikGR is expressed poorly or not at all in these organs.

Here we have characterized and described a new tool for real time analysis of mammalian development: the *iUBC-KikGR* transgenic mouse, and demonstrated its suitability for live organ explant imaging to examine events in organogenesis. The photoconvertible fluorescent protein KikGR is expressed ubiquitously in all examined organs throughout fetal development, such that a single transgenic line is suitable for fate mapping, lineage tracing, and morphogenetic movement studies in multiple organogenesis programs at multiple time points. Furthermore, the ability to label single cells or small genetically undefined groups of cells allows for a specificity and flexibility in experimental design that is unavailable with conventional *Cre-lox* bigenic systems. Transgenic embryos are easily phenotyped by fluorescence as embryos or adults, making for easy and efficient colony maintenance and screening of embryos for experiments. In addition to cell lineage and fate mapping studies, this mouse should also prove useful for observation and analysis of cellular behaviors, such as migration, in real time.

Methods

Mice

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC). For embryonic stages, noon on the day that vaginal plug was observed is considered embryonic day (E) 0.5. All experiments were performed in triplicate or greater replications.

Generation and characterization of the *iUBC-KikGR* photoconvertible transgenic mice

Transgene Design—The *UBC-KikGR* and *iUBC-KikGR* transgene constructs were generated by PCR cloning of the *KikGR* cDNA from pKikGR1-S1 plasmid (MBL Medical & Biological Laboratories Co., Ltd. Naka-ku Nagoya, Japan) with primers 5′-TATTTctcgagCCATGGTGAGTGTGATTACAT-3′ and 5′-ATATTTgggcccAGTTACTTGGCCAGCCTTGGC-3′, adding restriction sites *XhoI* and *ApaI* to the 5′ and 3′ ends of the cDNA, respectively (lowercase letters indicate engineered restriction enzyme recognition sites). PCR products were digested with restriction sites *XhoI* and *ApaI* and subcloned into the cHS2x-UBC-SH-RG-cHS2x vector (Stewart *et al.*, 2009) using the same sites, resulting in the cHS2x-UBC-KikGR-cHS2x vector. Clones were sequenced and transgene fragments were released from the vector. The 7.1kb *iUBC-KikGR* transgene fragment containing cHS2x (2 concatamerized genomic sequences derived from

chicken hypersensitive site 4 transcriptional insulators) (Potts *et al.*, 2000) flanking the human *Ubiquitin C* promoter (Schorpp *et al.*, 1996) and KikGR cDNA was released from the vector using *SalI* and *AseI*. The 2.1kb *UBC-KikGR* transgene fragment without cSH4x genomic insulators was released from the vector using *MluI* and *SphI*. Transgene fragments were purified and injected into zygotes from B6/SJLF1 crosses by standard techniques (Nagy *et al.*, 2003).

Genotypic and phenotypic screening of *iUBC-KikGR* transgenic mice—Transgene injection generated a total of five *iUBC-KikGR* founders identified by PCR genotyping using 5′-TGAAGATCGAGCTGCGTATG-3′ and 5′-CTCCATTTGATGATCCACGA-3′ yielding a PCR product of 585 bp. Of these founders, one male and two females showed strong expression of the KikGR photoconvertible protein in tail snips as observed via imaging on a Leica MZ10 F stereofluorescent microscope (Leica Microsystems Ltd., Bannockburn, IL). Eleven *UBC-KikGR* transgenic founders were identified by PCR genotyping using the same primers, and of these one male and two female founders showed expression of the KikGR fluorescent protein in tail snips. Founders exhibiting strong fluorescence in tail snips were crossed to outbred Swiss Webster mice (Taconic, NY) to generate F_1 embryos for expression analysis and F_1 animals for line expansion. One *UBC-KikGR* founder (*UBC-KikGR-4-7*) was determined to silence transgene expression when passed on to the F_1 generation. Normal Mendelian ratios of transgenic F_1 animals were identified by PCR genotyping, but these transgenic F_1 mice exhibited no detectable fluorescence (data not shown). This line was discarded, leaving three *iUBC-KikGR* lines and two *UBC-KikGR* lines. Two *iUBC-KikGR* lines showed consistently brighter fluorescence than the third *iUBC-KikGR* line and the three *UBC-KikGR* lines; these two lines were maintained while the dimmer lines were discarded. Transgenic mice from these remaining lines are easily phenotyped, and therefore genotyped, by fluorescence.

Remaining *iUBC-KikGR* transgenic lines were assayed for fluorescent expression in midgestation embryos. Embryonic organs were dissected at the indicated timepoint and imaged by stereofluorescence as above.

Functional screen for photoconversion—Photoconversion of the KikGR protein for screening founders was achieved by placing tail snips or whole embryos on a FOTO/UV® 26 light box (Fotodyne, Inc., Hartland, WI) for 3 minutes, then imaging with both EGFP and HCRed filters to identify photoconverted and unconverted protein.

E11.5 embryos from male founder *iUBC-KikGR-1–7*, which expressed strongly in tail snips, were visually screened for KikGR fluorescence with 488nm excitation by stereofluorescence. Initial analysis of KikGR function was performed by exposing some transgenic embryos to UV wavelengths. Embryos were placed in culture dishes in PBS and these dishes were placed on a FOTO/UV® 26 light box (Fotodyne, Inc., Hartland, WI). Embryos were exposed for 3 minutes, then imaged alongside unconverted transgenic and wildtype embryos with both 488nm and 546nm excitation.

Photoconversion of KikGR fluorescent protein in embryonic organs

Photoconversion of individual cells for cell tracking was achieved via 10–15 "bleach cycles" with 405nm photonkinesis laser at duration of 100ms over the course of 10–20 seconds, using the photobleach "Track-It" function of PerkinElmer ERS UltraVIEW. A 20x extra long working distance (ELWD) lens (NA 0.45, ∞/0–2 WD 7.4) was used with a "point" region of interest (ROI) setting in an attempt to convert single cells or small groups of neighboring cells. Empirically, this configuration converted 3.75 (SEM±0.43) cells per

photoconversion event (defined here as 10–15 "bleach cycles" at 15% laser power, lasting 100μs each cycle). Full conversion of all available KikGR protein within a cell was scored by loss of signal in that cell in the green (488nm excitation) channel. At this point, photoconverted cells became visible in the red (568nm excitation) channel.

Photoconversion of large regions of the mesonephros was achieved in a similar manner using a custom region of interest (ROI) and 15–20 bleach cycles with the 405nm laser controlled by the photokinesis accessory of the acquisition software. Photoconversion was achieved with a 10x lens (NA 0.45, ∞/0.17 WD 4.0) with 15–20 bleach cycles at 15% laser power. Three-dimensional live images in both the green and red channels were acquired by capturing a Z-stack of approximately 40μm every 45 minutes for 8–24 hours using PerkinElmer ERS UltraVIEW acquisition software. Post-acquisition analysis of cell movement was conducted with Imaris software (Bitplane, Zurich, Switzerland).

Collection and photoconversion of pre-implantation embryos

Female *iUBC-KikGR-1–7* transgenic mice were crossed to wild-type Swiss Webster outbred males. Zygotes were collected by standard techniques (Nagy *et al.*, 2003) and cultured overnight. Two-cell embryos were observed on the following day and one blastomere per embryo was photoconverted using a point-ROI as described above.

Live imaging of Wolffian duct extension

Live organ culture—Gonads with adjacent mesonephroi were dissected and placed in a Millipore culture insert (Millipore Corporation, Billerica, MA) in a 35-mm poly-d-lysine-coated glass bottom culture dish (MatTek Corporation, Ashland, MA), containing 1mL explant growth media ((Dulbecco's Modified Eagle Medium with 4.5 g/L D-glucose, without L-glutamine, sodium pyruvate and phenol red), containing 25% fetal bovine serum, 2 mM glutamate, 1 mM sodium pyruvate, 100 IU penicillin, and 0.1 mg/ml streptomycin). All reagents for making the media were purchased from Gibco (Invitrogen Corporation, Grand Island, NY). Live and still images were collected with a Perkin Elmer Spinning Disc Confocal microscope in an environmental chamber heated to 37°C and under 5% humidified CO_2.

Photoconversion of cells in *ex vivo* organ cultures—Photoconversion of the rostral 1/3 of the mesonephros was achieved in explant cultures as described above (405nm laser at 15% laser power). A large region of interest (ROI) comprising all of the rostral mesonephros was defined for each explant. Photoconversion was achieved with a 10x lens (NA 0.45, ∞/0.17 WD 4.0) with 15–20 scans of the ROI at 15% laser power. A Z-stack of 4–6nm optical slices was taken through the entire explant immediately following photoconversion to confirm that photoconversion events were restricted to the defined ROI. If photoconverted cells were found outside the ROI at this point, these explants were discarded or kept as controls for culture conditions. Several unconverted explants were also cultured concurrently as controls for culture conditions and to be sure that red signal in converted explants represented photoconverted cells and not experimental artifacts. Z-stacks of converted and unconverted explants were taken immediately after photoconversion (t=0), at every 45 minute interval during the explant culture, and at the experimental endpoint (t=18h).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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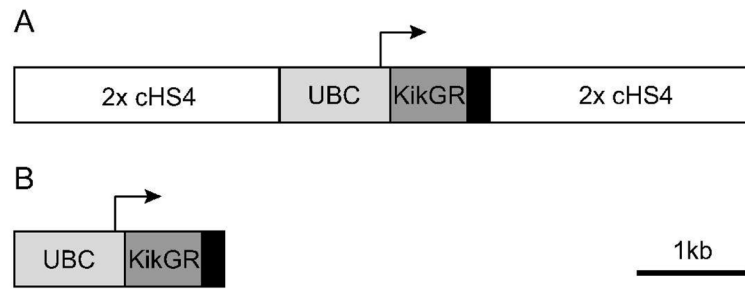
**Figure 1. *iUBC-KikGR* and *UBC-KikGR* transgene constructs**
A) *iUBC-KikGR* transgene construct, containing KikGR cDNA driven by the human *Ubiquitin C* promoter, flanked by 2 concatamerized cHS4 genomic insulator sequences. B) *UBC-KikGR* transgene construct without genomic insulators. Black box, poly-adenlyation sequence; arrows, transcriptional start site; 2x cHS4, 2 concatamerized cHS4 genomic insulator sequences; KikGR, Kikume green-red cDNA; UBC, human *Ubiquitin C* regulatory element.
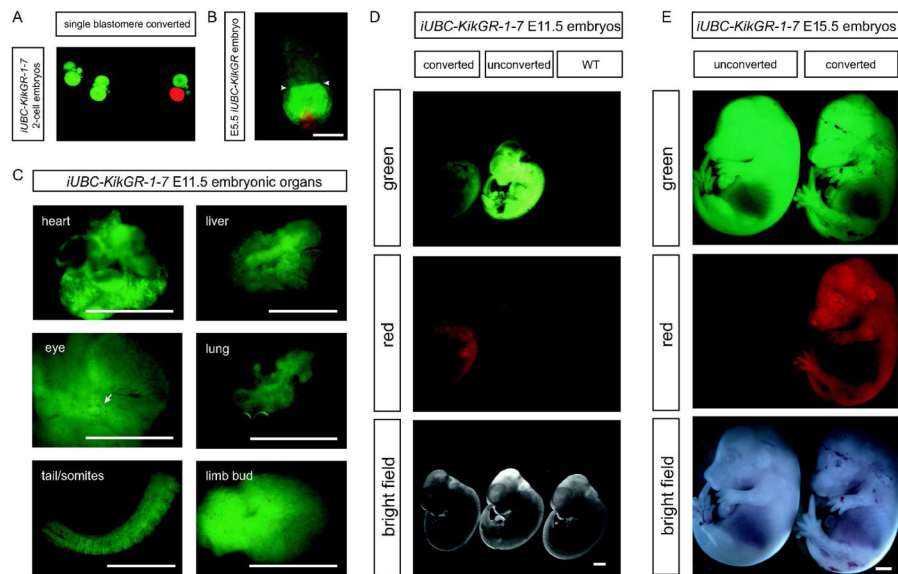
Figure 2. *iUBC-KikGR* transgenic embryos exhibit KikGR fluorescence and functional photoconversion throughout embryonic development
A) 2-cell embryos showing single cell photoconversion. B) E5.5 embryo showing photoconversion of individual cells in the embryonic ectoderm and visceral endoderm. Arrowheads, boundary between embryonic and extra-embryonic ectoderm. Scale bar = 100 µm. C) Expression of KikGR photoconvertible fluorescent protein in multiple embryonic organs at E11.5. Arrow, eye. Scale bar = 1 mm. D) E11.5 whole embryos exhibit KikGR fluorescence throughout the embryo and photoconversion after exposure of the entire embryo to UV light. Wild type embryo is shown for comparison. Scale bar = 1 mm. E) E15.5 whole embryos exhibit KikGR fluorescence throughout the embryo and photoconversion after exposure to UV light. Scale bar = 1 mm.
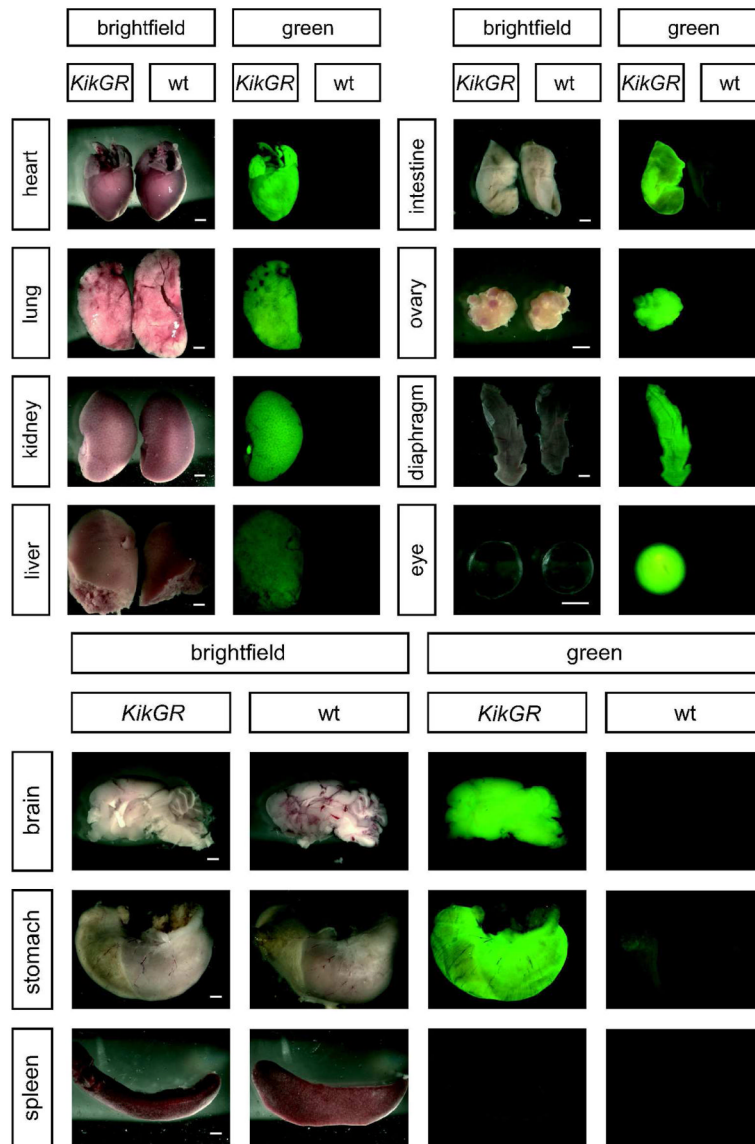
Figure 3. *iUBC-KikGR* **transgenic adult mice express KikGR in most organs**
KikGR fluorescence is observed in most organs, though it is dim in the liver. No fluorescence is observed in the spleen. Scale bar = 1 mm.
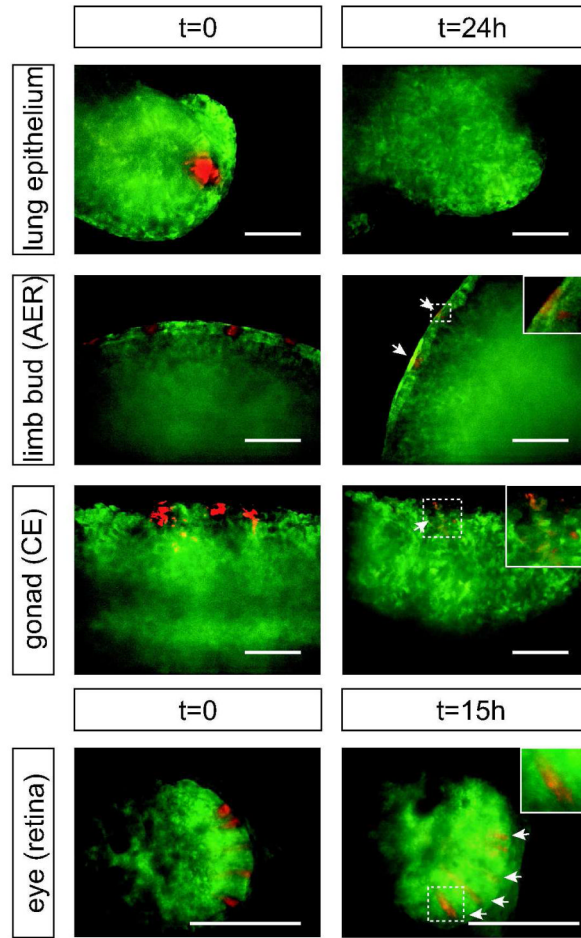
Figure 4. Photoconversion and signal stability of KikGR in multiple embryonic organs
E11.5 *UBC-KikGR-1–7* embryonic organs showing photoconversion of small regions of
lung eptithelium, limb bud, gonads, and retinal epithelium. After culturing the organs for
15–24 h the photoconverted cells can still be visualized in most organs. Loss of signal in the
lung epithelium is most likely due to dilution of the photoconverted protein during rapid cell
division. Time (t) expressed in hours after photoconversion. AER, apical ectodermal ridge;
CE, coelomic epithelium; arrows, photoconverted cells still visible after 15–24h. Scale bar =
100 µm.
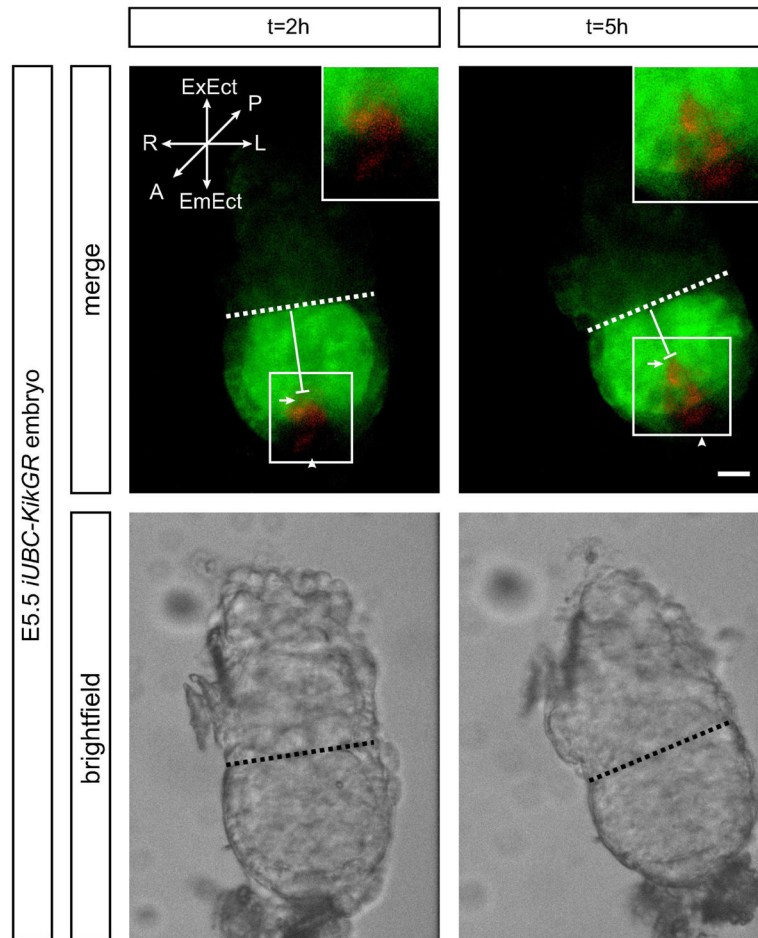
Figure 5. Photoconversion of the distal visceral endoderm at E5.5 reveals anterior migration toward the extra-embryonic ectoderm

E5.5 embryos were photoconverted in a region at the distal tip of the visceral endoderm, representing the presumptive anterior visceral endoderm (AVE) prior to migration. Representative frames at 2 h and 5 h post-photoconversion are shown. After 5 h in culture, labeled cells are observed migrating anteriorly toward the extra-embryonic ectoderm. The embryo is oriented such that the presumptive anterior region is center and facing the objective. Vertical bars indicate distance from distal tip of embryo to migratory front of photoconverted cells; dotted line represents the embryonic-extraembryonic junction. The photoconverted cells have migrated approximately 25 µm toward the embryonic-extraembryonic junction from t = 2 to t = 5 h. Arrowhead, labeled cells in visceral endoderm near the node; arrow, presumptive AVE cells; A, anterior; EmEct, embryonic ectoderm; ExEct, extra embryonic ectoderm; L, left; R, right; t, time (expressed in hours). Scale bar = 50 µm.
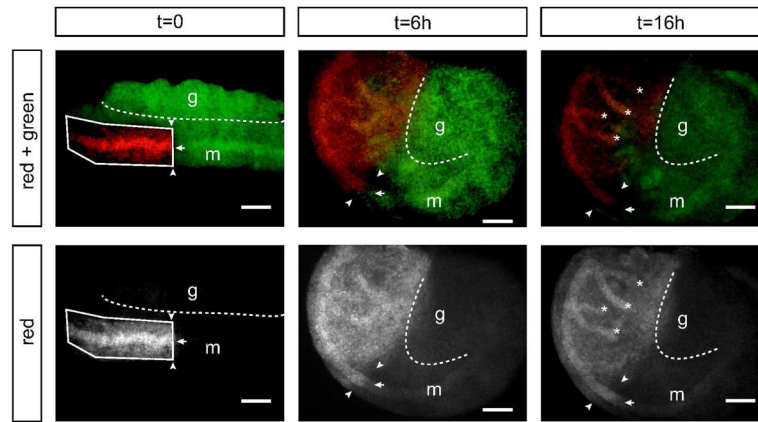
Figure 6. Photoconversion of the mesonephros at E11.5 reveals Wolffian duct extension in anterior region

Rostral third of mesonephros including mesonephric mesenchyme and Wolffian duct epithelium were converted at E11.5 (t = 0), then cultured *ex vivo*. Over time, the photoconversion boundary of the Wolffian duct epithelium (arrow) extends caudally beyond the photoconversion boundary of the surrounding tissue (arrowheads). In addition, branches from the rostral Wolffian duct are observed (asterisks). Box, ROI for photoconversion; dotted line, gonad-mesonephros boundary; g, gonad; m, mesonephros; t, time (expressed in hours). Scale bar = 200 μm.