Controlled gene expression in primary *Lgr5* organoid cultures

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The study of gene function in endodermal epithelia such as of stomach, small intestine and colon relies heavily on transgenic approaches. Establishing such animal models is laborious, expensive and time-consuming. We present here a method based on Cre recombinase–inducible retrovirus vectors that allows the conditional manipulation of gene expression in primary mouse organoid culture systems.

Transgenic approaches have become the method of choice to study gene function in primary mammalian tissues. The laboratory mouse is the most widely used vertebrate model owing to the ease of genetic manipulation and the fact that ~99% of mouse genes have homologs in the human genome[1]. Drawbacks of transgenic mouse models include the long generation time (1–2 years), low throughput and high cost. There is therefore a need for fast, cost-effective, high-throughput methods to functionally evaluate candidate genes identified by whole-genome approaches. Viral delivery of transgenes, either for overexpression or knockdown, is an established method for *ex vivo* and *in vivo* experiments and is an excellent method for genetic manipulation[2].

We have recently established a culture system, based on defined growth factors and Matrigel (BD Biosciences), that faithfully recapitulates in three dimensions the crypt-villus architecture of the intestinal epithelium[3]. These epithelial organoids retain critical *in vivo* characteristics such as lineage composition and self-renewal kinetics. The organoids contain normal numbers of Lgr5-positive (Lgr5$^+$) stem cells, Paneth cells and transit-amplifying cells in the crypt domain as well as the three differentiated cell lineages (enterocytes, goblet and enteroendocrine cells) of the villus domain. Organoids can be established from mice of any age and can be expanded for many months without genetic alterations. Small changes in the growth-factor composition of the medium have allowed us to subsequently define culture conditions for organoids derived from mouse colon[4] and stomach[5]. To make these organoid cultures accessible for genetic manipulation, we now describe a retroviral transduction system. In analogy to conditional mouse models[6], we designed the system so that gene knockdown or overexpression can be controlled.

We first explored methods to efficiently infect organoids from small intestine with a murine stem cell virus (MSCV)-based, enhanced (e)GFP-expressing, puromycin-selectable retrovirus (**Supplementary Fig. 1a**,**b**). As in the *in vivo* situation[7], organoids are continuously renewed from their stem cells, and differentiated cells are shed into the lumen. Only the stable infection of

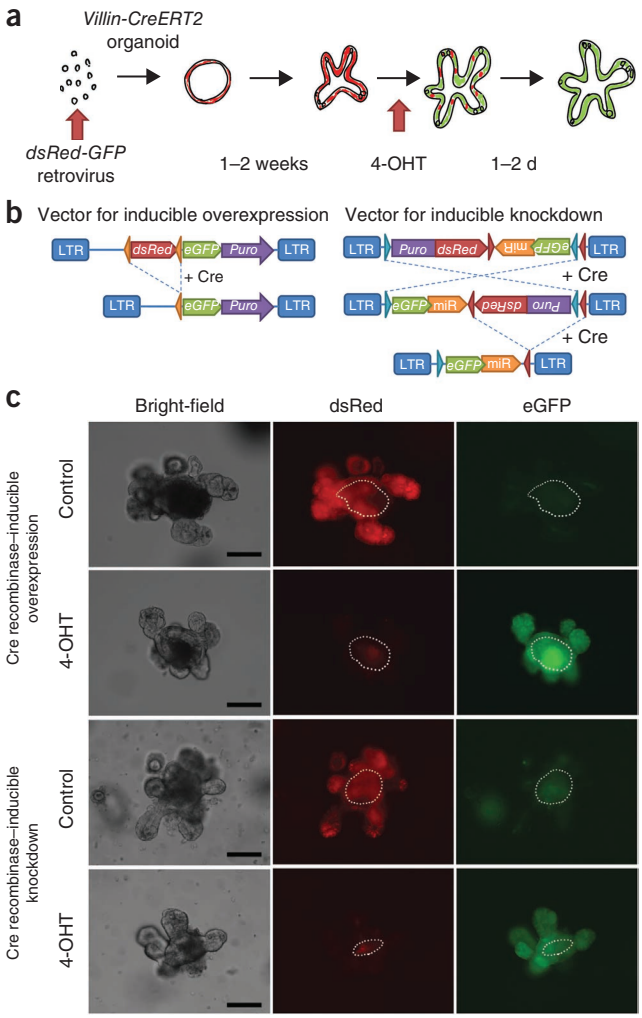

Figure 1 | An inducible organoid transduction system. (**a**) General outline. (**b**) Vector scheme for inducible overexpression and knockdown cassettes. LTR, long terminal repeat; *puro*, puromycin-resistance gene *pac*. (**c**) The micrographs show intestinal organoids transduced with the *dsRed-eGFP*–containing retrovirus before and after 4-OHT treatment. The dotted line outlines the central lumen with autofluorescence. Scale bars, 100 µm.

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stem cells, which will pass the transgene on to their progeny cells, will therefore maintain gene expression in the organoid in the long term. We first tested the method using retroviruses, as they are relatively safe to use, can infect cycling stem cells, are stably integrated into the genome and are therefore passed on to progeny cells, resulting in uniformly transduced organoids. We found that organoids growing in Matrigel were inaccessible to retroviral infection. We therefore mechanically separated the organoids from Matrigel by careful pipetting, briefly trypsinized them to generate smaller particles with exposed luminal (apical) domains, and subjected organoid fragments to spinoculation[8].

We have previously shown that treatment of organoids with Wnt3a results in a strong proliferative response and an increase in the number of *Lgr5*$^+$ stem cells[9]. Treatment of organoids with Wnt3a for 2–3 d before and after the spinoculation (total of 5 d) indeed resulted in an additional increase in long-term infected organoids. Two days after infection, we started puromycin selection. Not doing so resulted in the progressive loss of eGFP-expressing organoids, probably owing to a growth advantage of noninfected stem cells. Infected organoids recovered through a period of cystic growth, and within 1–2 weeks after infection started to form the characteristic crypt-villus domains of mature organoids. One week after infection, flow-cytometry analysis showed that >90% of the organoid cells were eGFP-positive (**Supplementary Fig. 1c**). We followed the infected organoids over 5 weeks, and observed no signs of loss of eGFP expression. We recently applied this protocol to rescue simultaneous deletion of *Lgr4* and *Lgr5* in intestinal organoids by retrovirus-mediated overexpression of Wnt3 using an MSCV vector[10].

Next, we tested whether the system could reproduce the loss-of-function phenotype of the Notch signaling pathway, that is, the rapid conversion of proliferative transit-amplifying and stem cells into postmitotic goblet cells[11]. Owing to the deleterious effects of this interference, we could not, with constitutive expression, propagate the organoids long enough to analyze the phenotype. We therefore modified the retroviral backbone to render gene knockdown or overexpression inducible by 4-hydroxytamoxifen (4-OHT)-dependent Cre recombinase (CreERT2) (**Fig. 1**). *CreERT2* can be either delivered to the organoids by retroviral infection or by the use of organoids derived from transgenic mice expressing the enzyme. The intestinal organoids used in this work were from *Villin-CreERT2* transgenic mice. For the conditional overexpression vector, a dsRed expression cassette flanked by *LoxP* sites (floxed) was inserted upstream of the eGFP coding sequence, effectively blocking translation of *eGFP*. Infected organoids can be readily visualized by their red fluorescence; deletion of *dsRed* by Cre recombinase results in the rapid loss of dsRed and the activation of *eGFP* expression.

For gene knockdown, we used a polymerase II–driven expression system of artificially modified microRNAs (miRNAs)[12]; expression of the miRNA of interest can be easily followed by the co-cistronic expression of *eGFP*. To achieve inducible activation of the miRNA, however, we could not use the same system as for overexpression because the translational stop signal of *dsRed* would not prevent expression of the miRNA. Therefore, we applied an irreversible Cre recombinase–driven flipping system using two different types of *LoxP* sites[13]. CreERT2 induction resulted in the flipping of the puromycin resistance gene (*pac*; here called *puro*)-*dsRed* cassette and/or the *eGFP-miRNA* cassette, followed by the excision of the *puro-dsRed* cassette. In effect, a switch from *dsRed* to *eGFP*-miRNA expression was achieved.

The biggest challenge for knockdown experiments was finding an effective miRNA (Online Methods). We usually tested knockdown efficiencies of miRNAs in HEK293T cells before infecting organoids. On average, we needed to test five different miRNAs to find at least two that worked (**Supplementary Fig. 2a**,**b**). On average, we found good correlation between knockdown efficiencies in HEK293T cells and in organoids (**Supplementary Fig. 2c**). Nevertheless, maximal knockdown efficiencies in organoids varied for target genes. For instance, we achieved a maximum knockdown of 50–60% for *Hes1*, but of 70% and 80% for *Cdk6*

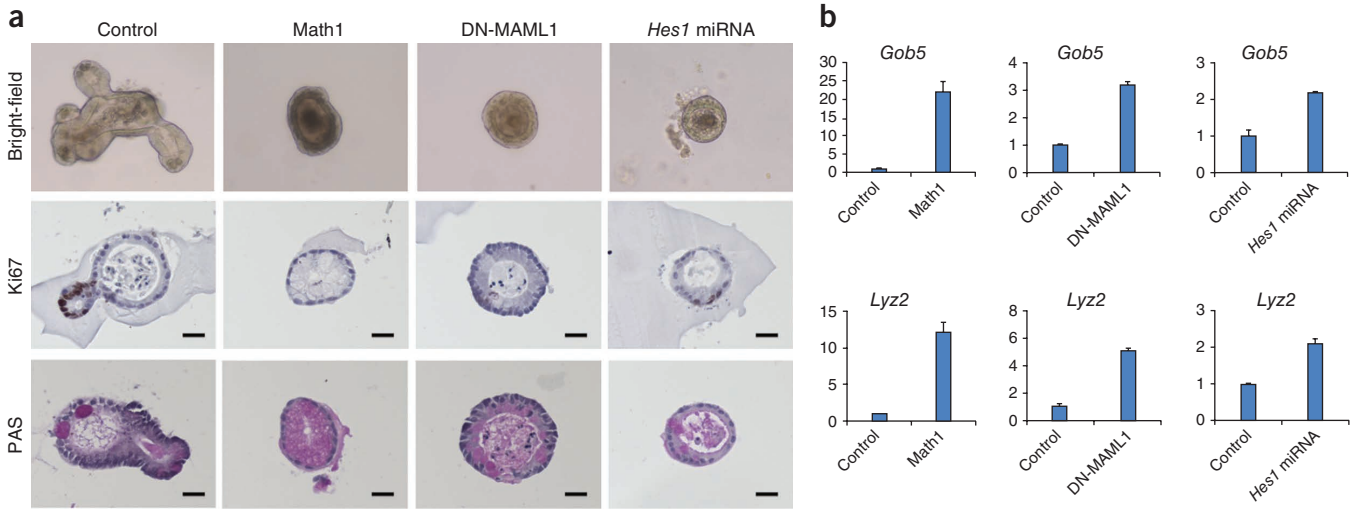

Figure 2 | Notch pathway inhibition in intestinal organoids. (**a**) The micrographs show representative organoids with Math1 or DN-MAML1 overexpression, or cells after *Hes1* knockdown. Shown are bright-field images (top), staining for the proliferation marker Ki67 (middle) and staining for PAS, a marker of the goblet cell lineage (bottom). The control sample was a tamoxifen-induced organoid transduced with the *dsRed-eGFP* overexpression vector. Scale bars, 20 µm. (**b**) Results of quantitiative PCR analysis of the indicated markers of secretory cell lineages (*Gob5* for goblet cells and *Lyz2* for Paneth cells) in control and infected organoids. Error bars, s.d. (n = 3).

and *Bmp1*, respectively (**Supplementary Fig. 2d**). For both overexpression and knockdown, the time span from infection to transgene activation in mature organoids was about 2–3 weeks.

The Notch pathway controls goblet-cell differentiation through a simple circuit in which Notch1 activates expression of the transcription factor Hes1, which in turn represses the transcription factor Math1 (encoded by *Atoh1*). Expression of Math1 drives proliferative cells into the postmitotic goblet cell fate[14]. Having established an inducible retroviral expression system, we modified the Notch signaling pathway in three ways. First, we suppressed Notch signaling by overexpression of a dominant-negative form of the transcriptional co-activator Mastermind-like 1 (DN-MAML1)[15]. Second, we bypassed Notch-Hes1–mediated repression by direct overexpression of *Atoh1*[14]. Third, we knocked down the transcription factor gene *Hes1* using a miRNA. All three approaches resulted in the same phenotype: after induction, the organoids rapidly lost their budding structures and rounded up (**Fig. 2a**). Proliferation ceased as monitored by Ki67 staining, cells differentiated toward the goblet cell lineage as visualized by periodic acid–Schiff (PAS) staining, and markers for the goblet (*Clca3*, also known as *Gob5*) and Paneth (*Lyz2*) cell lineages were upregulated (**Fig. 2b**). Loss of Ki67 and increase of goblet cell numbers very closely resembles the phenotype of Notch pathway suppression in mice[11]. Noninduced organoids did not have a phenotype different from wild-type organoids, and we detected transgene expression only upon tamoxifen induction (**Supplementary Fig. 2e**).

Next, we applied the transduction system to organoid culture systems for mouse stomach and colon epithelium[4,5]. We established stomach and colon organoids from a *Rosa26-CreERT2* knock-in mouse (**Supplementary Fig. 3**). We transduced these organoid systems essentially using the same approach as for the small intestine. Of note, Wnt3a is a constitutive component of the culture medium of stomach and colon cultures, and it is therefore not necessary to add it around the infection day. We observed a rapid switch from dsRed to eGFP signal upon tamoxifen treatment with no leakiness of either fluorochrome both in stomach and colon organoids.

Taken together, here we presented an effective screening tool to study mammalian gene function *ex vivo*. The possibility to inducibly overexpress as well as knock down gene expression, the broad applicability to different organotypic culture systems and the short time period required are the key features of our system. Furthermore, the protocol can also be used with other virus types such as lentiviruses (**Supplementary Fig. 4**). The combination of organoid culture with an inducible transduction system is a broadly applicable tool with the potential to become a complement to classical conditional mouse models.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Accession codes. Addgene: 32702 (pMSCV-loxp-dsRed-loxp-eGFP-Puro-WPRE), 32703 (pMSCV-loxp-dsRed-loxp-3xHA-Puro-WPRE)and 32704 (pMSCV-FLIP-puro-dsRed-GFP-miRNA).

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

B.-K.K., D.E.S. and H.C. conceived the project and wrote the manuscript. B.-K.K., T.S. and M.H. developed the infection protocol and optimized the culture conditions. B.-K.K.,D.E.S. and H.F.F. designed and constructed retroviral vectors. B.-K.K. and J.H.v.E. established the organoids from *CreERT2*-transgenic mice. W.K. performed the lentiviral transduction.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Organoid culture. Organoids of small intestine, colon and stomach were established from freshly isolated glands from each organ. Incubation in PBS (pH 7.4) containing 2 mM EDTA (for small intestine) or 5 mM EDTA (for stomach and colon) was used for the isolation of glands. The culture condition of mouse small intestine, colon and stomach organoids have been described elsewhere[3–5].

Vector construction: inducible overexpression vector. A *dsRed* cassette from dsRed-express2 (Clontech) was introduced into the BglII site of MSCV-puro (Clontech) with a *loxP* site on each side. Three consecutive hemagglutinin (HA) peptides (3×HA) or eGFP from pEGFP-N1 (Clonetech) were inserted into the EcoRI site of MSCV-puro. The woodchuck hepatitis post-transcriptional regulatory element (*WPRE*) sequence was cloned into the ClaI site of MSCV-puro. Genes of interest can be cloned into the HpaI site of these vectors in frame with either an *eGFP* or a 3×HA tag sequence. The two different versions are available through Addgene (pMSCV-loxp-dsRed-loxp-eGFP-Puro-WPRE (32702) and pMSCV-loxp-dsRed-loxp-3xHA-Puro-WPRE (32703)).

Vector construction: inducible knockdown vector. The knockdown vector was generated based on the MSCV FLIPi P2G_Thy1.1 p53 plasmid (Addgene, 19748)[13]. First, we changed *eGFP* into *dsRed* from dsRed-express2 using ApaI and NsiI. Next, we replaced the *Thy1.1-p53-miR* cassette to *eGFP-miR* from the pcDNA6.2-GW vector (Invitrogen) using PmeI and AgeI. Different miRNAs can be cloned into the vector by first cloning them into pcDNA6.2-GW according to the manufacturer's protocol and then moving the cassette into the knockdown vector using PmeI and AgeI. miRNAs can be designed on the Invitrogen homepage (http://rnaidesigner.invitrogen.com/rnaiexpress/). The vector is available through Addgene (pMSCV-FLIP-puro-dsRed-GFP-miRNA (32704).

Vector construction: Math1 and DN-MAML1 vectors. Sequence encoding mouse Math1 (*Atoh1*) and a fragment encoding amino acids 13–74 of human MAML1 (dominant negative form of MAML1) were cloned into the HpaI site of the inducible overexpression vector using the In-Fusion Advantage PCR Cloning Kit (Clontech).

Vector construction: *Hes1* knockdown vector. Five DNA sequences (**Supplementary Table 1**) were annealed and ligated into linearized pcDNA6.2-GW/EmGFP-miR (Invitrogen). The whole *emGFP-miR* cassette was amplified by PCR and moved into the inducible knockdown vector using PmeI and AgeI.

Testing knockdown efficiencies. To test the knockdown efficiencies of different miRNAs, we tested their efficiency via transfections with the inducible overexpression vectors containing the gene to target. The miRNAs were flipped into their active form by transfecting the plasmid into Cre recombinase–expressing *Escherichia coli*. One microgram of miRNA vector plus 0.3 µg of target vector are mixed with 8 µl of polyethylenimine (PEI) (1 mg ml^{-1}) and added to HEK293T cells grown to 60% confluence in 12-well tissue culture plates. Two days later, the expression of dsRed was checked under a fluorescence microscope and the dsRed fluorescence was evaluated. Alternatively, cells can also be analyzed using flow cytometry.

Retroviral infection. Before infection, we grew intestinal organoids in a 24-well culture plate with standard ENR medium (50 ng ml^{-1} mouse epidermal growth factor (EGF), 100 ng ml^{-1} Noggin and 500 ng ml^{-1} mouse R-Spondin 1)[3]. One well per infection was sufficient. Two days before the infection we changed the medium to ENR medium plus 50% Wnt3a conditioned medium and 10 mM nicotinamide. For organoids of colon and stomach no change of the medium is necessary, as their standard medium already contains Wnt3a conditioned medium[4,5].

Virus production. We grew Platinum E cells (Cell Biolabs) in a 150-cm dish under selection with puromycin (1 µg ml^{-1}) and blasticidin (10 µg ml^{-1}) to a confluence of 80%. We transfected 30 µg of plasmid using 240 µg of PEI (Polysciences) and puromycin and blasticidin–free medium. We changed medium once on the next day to remove PEI. After two more days, we collected the medium, passed it through a 0.45-µm filter and centrifuged it at 8,000*g* overnight at 4 °C. We discarded the medium and resuspended the pellet in 250 µl of infection medium (ENR medium plus 50% Wnt3a conditioned medium plus 10 mM nicotinamide plus 10 µM Y27632 (p160ROCK inhibitor, Sigma) plus 8 µg ml^{-1} Polybrene (Sigma).

Preparing organoids for infection. We transferred organoids to a 15-ml Falcon tube and dissociated them with a fire-polished glass pipette. The glass pipette should have an opening of about 0.5–1 mm after fire polishing. Organoids were pipetted about ten times up and down. The size of the organoids after this procedure is shown in **Supplementary Figure 5**. We incubated organoid fragments with TripLE (Invitrogen) for 5 min at 37 °C. This broke down the fragments to small cell clusters. We added medium containing 5% serum and spun down cells at 1,000*g* for 5 min, discarded supernatant and resuspended cell clusters in a small volume of infection medium.

'Spinoculation' and plating. We combined cell clusters with 250 µl of viral suspension and transferred them into a 48-well culture plate. We centrifuged the plate at 600*g* at 32 °C for 60 min (spinoculation)[8]. The plate was placed for another 6 h in an incubator at 37 °C. After this time, we collected cells, transferred them into 1.5 ml Eppendorf tube and spun them down 1,000*g* for 5 min. Then we discarded the supernatant, resuspended the pellet in 100 µl Matrigel (BD Biosciences) and split it into two wells of a 24-well culture plate. We added 500 µl of infection medium without polybrene.

After infection. Two days after infection we changed medium to ENR plus puromycin (2 µg ml^{-1}). We split organoids 1:3 when necessary. After removal of Wnt3a (contained in infection medium), spheres changed into budding organoids within 1–2 weeks. Once the organoids have regained their crypt-villus architecture, expression of the cDNA or miRNA can be induced with 4-OHT (Sigma, H7904, working concentration 1 µM).

Immunohistochemistry. Organoids were fixed with 4% paraformaldehyde for 1 h at room temperature (18–21 °C). Next, samples were passed through an ethanol series and embedded in paraffin. Immunohistochemistry was performed using standard techniques.

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