tail) and the N terminus of cortical cytoskeleton-associated protein of ~23 kD (CAP23). We also assayed the kinase-associated 1 (KA1) domain from microtubule-associated protein-microtubule affinity-regulating kinase 1 (MARK1), which interacts nonspecifically with acidic lipids (23). In all cases, combined removal of PI4P and PI(4,5)P2 caused depletion of the proteins from the PM (Fig. 4A and fig. S7), with little effect when either lipid was depleted alone (13). Proteins that retained a secondary membrane targeting motif, such as prenylated K-Ras tail, were still found in the PM but were no longer enriched there compared with the amounts in other [presumably negatively charged (22)] membranes (Fig. 4A and fig. S8A). These effects were due to nonspecific electrostatic interactions, because no effect was seen on the PS-specific lactadherin C2 domain (22) or the C terminus of H-Ras, which interacts with the membrane solely through its hydrophobic lipid moieties (Fig. 4A and fig. S7). Measuring K-Ras tail’s PM dissociation rate by fluorescence recovery after photobleaching (24) after PI4P and/or PI(4,5)P2 depletion revealed that the two lipids made similar contributions to the protein’s electrostatic interactions with the PM in vivo (fig. S8).

PI(4,5)P2 has been proposed to be a molecular switch that restricts the activity of several ion channels to the PM (25), a phenomenon that can be highly specific for PI(4,5)P2 (13). We wondered whether this is typical for all channels or whether some have a more general polyamionic lipid requirement, which can also be fulfilled by PI4P. For example, the heat and capsaicin-activated transient receptor potential vanilloid 1 (TRPV1) (fig. S8). The concept that tumors are maintained by dedicated stem cells, the so-called cancer stem cell hypothesis, has attracted great interest but remains controversial. Studying mouse models, we provide direct, functional evidence for the presence of stem cell activity within primary intestinal malignancies (1). Furthermore, G protein-coupled receptor 5 (leucine-rich repeat containing heterotrimeric guanine nucleotide-binding protein–coupled receptor 5) also marks a subpopulation of normal tissue stem cells in stomach, small intestine, colon, and hair follicles (3). This allele allows visualization of Lgr5+ stem cells using the multicolor Cre-reporter R26R-Confetti, we demonstrate that the crypt stem cell marker Lgr5 (leucine-rich repeat–containing heterotrimeric guanine nucleotide–binding protein–coupled receptor 5) also marks a subpopulation of adenoma cells that fuel the growth of established intestinal adenomas. These Lgr5+ cells, which represent about 5 to 10% of the cells in the adenomas, generate additional Lgr5+ cells as well as other adenoma cell types. The Lgr5+ cells are intermingled with Paneth cells near the adenoma base, a pattern reminiscent of the architecture of the normal crypt niche.

**Lineage Tracing Reveals Lgr5+ Stem Cell Activity in Mouse Intestinal Adenomas**

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The concept that tumors are maintained by dedicated stem cells, the so-called cancer stem cell hypothesis, has attracted great interest but remains controversial. Studying mouse models, we provide direct, functional evidence for the presence of stem cell activity within primary intestinal malignancies, a precursor to intestinal cancer. By “lineage tracing” using the multicolor Cre-reporter R26R-Confetti, we demonstrate that the crypt stem cell marker Lgr5 (leucine-rich repeat–containing heterotrimeric guanine nucleotide–binding protein–coupled receptor 5) also marks a subpopulation of adenoma cells that fuel the growth of established intestinal adenomas. These Lgr5+ cells, which represent about 5 to 10% of the cells in the adenomas, generate additional Lgr5+ cells as well as other adenoma cell types. The Lgr5+ cells are intermingled with Paneth cells near the adenoma base, a pattern reminiscent of the architecture of the normal crypt niche.

Intestinal tumorigenesis is thought to result from sequentially acquired mutations in specific genes, driving progression from premalignant

**References and Notes**


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**Supplementary Materials**

www.sciencemag.org/cgi/content/full/science.1222483/DC1 Materials and Methods Figs. S1 to S9 References (30–37)

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the coexpressed Cre fusion protein can be activated at will in these stem cells by tamoxifen injection and will then recombine two adjacent LoxP sites. When a so-called Cre reporter allele is crossed into the Lgr5 knock-in mouse strain, tamoxifen injection will allow single Lgr5+ stem cells to randomly adopt one of the four fluorescent colors encoded in the R26R-Confetti allele. Of importance for the current study, two of the four colors remain in the R26R-Confetti allele after tamoxifen injection (blue with red or green with yellow). One of these is active, the other silent (Fig. 1A). In theory, a second tamoxifen injection can induce “flipping” from the active color to the silent color (here termed retracing). To test this, we globally activated R26R-Confetti in the intestinal epithelium by using the β-naphthoflavone-inducible Ah-Cre mouse strain (7). As hypothesized, the already rearranged R26R-Confetti reporter could be flipped to the remaining color by a second tamoxifen injection (Fig. S1). Spontaneous color conversions were never observed.

To study the behavior of Lgr5+ cells within Apc-mutant adenomas, we then crossed Lgr5EGFP-Ires-CreERT2/Apcfl/fl mice with the R26R-Confetti strain. Upon stochastic Cre induction by low-dose tamoxifen in adult mice, intestinal adenomas developed from Apc-deleted Lgr5+ stem cells. After 4 weeks, we observed large adenomas (Fig. S2, A to C) that either uniformly expressed a single Confetti color or consisted of anatomically separate segments that were each marked uniformly in a single Confetti color (Fig. 1B). The latter result indicates that such large adenomas were derived from several independent Apc-mutant stem cells, most likely located in adjacent crypts. Indeed, such segmental oligo-clonality of adenomas has been described (8, 9). Of note, recombination of the floxed Apc allele was more efficient than the activation of R26R-Confetti in

Fig. 1. Large adenomas consist of individually colored segments with stem cells located adjacent to Paneth cells. (A) Possible outcomes of the R26R-Confetti locus after the first Cre recombination (tracing) and second Cre recombination (retracing). (B) Confocal imaging of an adenoma in a Lgr5EGFP-Ires-CreERT2/Apcfl/fl/R26R-Confetti mouse after 4 weeks of tracing. The adenoma consists of multiple segments (white dashed lines), which are marked uniformly by a single color (or are unmarked). RFP (red fluorescent protein) and YFP (yellow fluorescent protein) tracings are shown in red and yellow, respectively. Lgr5+ stem cells (green) are located toward the base of adenoma segments. Colors are overlaid on a differential interference contrast (DIC) image in gray. (C) Distribution of stem cells marked by Lgr5-GFP and Paneth cells, marked by lysozyme (purple) at the base of wild-type (WT) crypts (left). (Right) Lgr5+ stem cells (green) are located adjacent to Paneth cells (purple) toward the base of an adenoma segment (indicated by the red dashed line). (D) Schematic representation of the intermingled stem cells and Paneth cells in wild-type crypts (left) and adenomas (right). Scale bars indicate 50 μm.
the same stem cell, because not all adenomas or adenoma segments expressed a Confetti color.

In normal small intestinal crypts, stem cells are always adjacent to Paneth cells. These Paneth cells serve as niche cells to the stem cells by producing signaling molecules such as Wnt, epidermal growth factor (EGF), and Notch ligands (10). The Apo-mutant adenomas contained Paneth cells (Fig. 1C) but typically lacked mature goblet cells and enterocytes (fig. S2, D and E). Lgr5+ adenoma cells were often located adjacent to adenoma Paneth cells, suggesting the existence of an adenoma stem cell niche (Fig. 1C). Indeed, these clusters of Lgr5+ cells and Paneth cells tended to be located toward the base of the wedge-shaped adenoma segments, a situation reminiscent of normal crypt architecture (Fig. 1D). We noted a similar arrangement in colon adenomas, in which areas of Lgr5+ cells coincided with the location of the colon-counterpart of the Paneth cell, the deep crypt secretory cell, marked by Reg4 (10, 11) (fig. S3). To mimic the next step in the tumorigenic process, we additionally crossed in an allele of K-ras that can be oncogenically activated via Cre (K-rasLSL-G12D) (12). We observed a dramatic acceleration of the growth of these Apc/Kras mutant tumors compared with Apc-mutant adenomas. The mice became moribund within 7 to 10 days, yet the crypt-like architecture was preserved in the Apc/Kras double-mutant tumors (fig. S4).

Next, we set out to determine the transcriptional profile of Lgr5-GFPhi cells in adenomas to allow comparison to normal crypt Lgr5-GFPhi stem cells. We sorted Lgr5-GFPlo and Lgr5-GFPhi cells from 30-day-old adenomas and performed comparative gene expression analyses of the two populations. Gene set enrichment analyses demonstrated that the Lgr5-GFPhi stem cell signature determined for normal crypts (13) was strongly enriched in the Lgr5-GFPhi adenoma cells (Fig. 2). This observation suggested that the Lgr5-GFPhi adenoma cells retain stem cell properties. To substantiate this notion, we compared the clonogenic potential of Lgr5-GFPhi and Lgr5-GFPlo adenoma cells in a defined culture system developed for normal tissue Lgr5-GFPhi stem cells (14, 15). In this assay, normal Lgr5-GFPhi cells grow out into crypt-villus organoids, whereas Lgr5-GFPlo rarely grow out, consistent with the idea that Lgr5-GFPhi cells constitute multipotent stem cells. Analysis of the clonogenic potential of Lgr5-GFPhi and Lgr5-GFPlo adenoma cells in this culture system recapitulated the observations made in normal tissue: The colony-forming efficiency of Lgr5-GFPhi adenoma cells was about 20 times greater than that of Lgr5-GFPlo adenoma cells (Fig. 2, D and E).

To trace the fate of Lgr5+ adenoma cells in vivo, we injected 10-week-old Lgr5EGFP-Ires-CreERT2; Apcfl/fl/R26R-Confetti mice with tamoxifen. After allowing Confetti-marked adenomas to develop for 24 days, we induced lineage retracing by a second tamoxifen injection. Adenomas were analyzed by confocal microscopy at different time points after the second Cre pulse. We observed rare retracings within large adenomas (~six color retracings per 100 adenoma segments), which invariably involved switching to the silent color in that particular adenoma. As an example, rare blue cells were observed within red adenomas early after lineage tracing (Fig. 3). Because we observed retracing events in only ~6% of Confetti-marked adenomas and these presented typically as a single cell at one day after the second tamoxifen
injection (Fig. 3, D and E), we considered these to represent clonal retracing events. In other words, these cells were derived from a single adenoma stem cell. As expected, the blue cells were mostly located near the base of the wedge-shaped adenoma segments, the location of the Lgr5+ cells. No spontaneous color conversion was observed in control mice that were not given a second Cre pulse, in a total of 107 adenomas analyzed 27 days after one pulse of tamoxifen.

Six days after retracing, the number of blue cells within red adenomas had increased. Moreover, the growing blue clones presented as ribbonlike structures emanating from the base of the segments and projecting toward the intestinal lumen (Fig. 3B), thus resembling, in a crude and somewhat chaotic way, the more-organized tracing patterns observed in healthy intestinal tissue.

![Image](https://www.sciencemag.org/content/sci/337/6092/733/F3.large.jpg)
From day 9 to day 24 after retracing, the blue cells expanded to compose up to 30% of the volume of the adenomas (Fig. 3, C and G). A quantification of clone size over time is given in Fig. 3D. The number of Lgr5-GFP conduit stem cells within the retraced population also increased over time, indicating that Lgr5+ adenoma stem cells clonally expanded within established adenomas (Fig. 3E). The total number of Lgr5-GFP conduit cells comprised a minority of the adenoma cells. We found that around 5 to 10% of the adenoma cells were Lgr5-GFP conduit (Fig. 3F). This number is similar to that of normal crypts, which consist of 150 to 200 cells and harbor about 15 stem cells each, thus reinforcing the notion that adenomas retain characteristics of the crypt stem cell niche.

To address whether Lgr5-GFP conduit adenoma cells are able to generate all cell types of the adenoma, we studied the phenotype of individual cells within the clonal offspring of an Lgr5+ adenoma cell. As an example, Fig. 4 depicts a red adenoma after 35 days of tracing, with a subsequent retracing for 9 days, in which an Lgr5+ adenoma cell gave rise to a blue ribonucleic clone (see also fig. S5). Multiple Lgr5-GFP conduit adenoma cells were observed within the clonal ribonucleic (arrowheads), demonstrating the production of additional Lgr5+ adenoma cells within the ribonucleic. In addition, at least two blue Paneth cells (arrows) and multiple blue Lgr5- cells, resembling transit-amplifying cells (asterisks), are present. Scale bars, 50 mm.

35 days + 9 days re-tracing

Fig. 4. Lgr5+ adenoma cells expand and give rise to adenoma Paneth cells. A red adenoma segment retraced for 9 days after 35 days of adenoma development in a Lgr5-EGFP-Ires-CreERT2/Apcfl/fl/R26R-Confetti mouse. The left image is an overlay of four channels; DIC is shown in gray, the original segment in red, Lgr5+ cells in green, and the retraced cells in blue. Within the red segment, a blue clone appears, originating at the Lgr5-EGFP conduit base of the cryptlike structure. Multiple Lgr5-GFP conduit adenoma cells occur within the clonal ribbon (arrowhead), demonstrating the expansion of Lgr5+ adenoma cells. In addition, at least two blue Paneth cells (arrows) and multiple blue Lgr5- cells, resembling transit-amplifying cells (asterisks), are present. Scale bars, 50 mm.

3D. The number of Lgr5-GFP conduit stem cells within the retraced population also increased over time, indicating that Lgr5+ adenoma stem cells clonally expanded within established adenomas (Fig. 3E). The total number of Lgr5-GFP conduit cells comprised a minority of the adenoma cells. We found that around 5 to 10% of the adenoma cells were Lgr5-GFP conduit (Fig. 3F). This number is similar to that of normal crypts, which consist of 150 to 200 cells and harbor about 15 stem cells each, thus reinforcing the notion that adenomas retain characteristics of the crypt stem cell niche.

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The original studies that propose the existence of cancer stem cells in human colorectal cancer have their basis in transplantation of sorted cell populations into immunodeficient mice (16–18). The manipulations required for sorting, combined with the xenotransplantation setting, impose inherent limitations to the interpretation of the outcome of this assay (19). In an alternative approach, single-cell sorting of cultured primary colorectal cancers has been used to demonstrate that about 1 in 20 cells has the capacity in vitro of multilineage differentiation, considered an attribute of stem cells (20). In a follow-up study, this culture-based assay was combined with the classic xenograft approach to demonstrate that colon cancer stem cells display high Wnt pathway activity (21). The stem cell marker used in the current study, Lgr5, is encoded by a Wnt target gene and itself constitutes a Wnt receptor component (22).

References and Notes

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**Supplementary Materials**

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Materials and Methods

Figs. S1 to S5

References (25, 26)

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**Closed-Loop Control of Epilepsy by Transcranial Electrical Stimulation**

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Many neurological and psychiatric diseases are associated with clinically detectable, altered brain dynamics. The aberrant brain activity, in principle, can be restored through electrical stimulation. In epilepsies, abnormal patterns emerge intermittently, and therefore, a closed-loop feedback brain control that leaves other aspects of brain functions unaffected is desirable. Here, we demonstrate that seizure-triggered, feedback transcranial electrical stimulation (TES) can dramatically reduce spike-and-wave episodes in a rodent model of generalized epilepsy.

Closed-loop TES can be an effective clinical tool to reduce pathological brain patterns in drug-resistant patients.

A successful, although not well-understood, therapy in drug-resistant cases of Parkinson’s disease and depression is deep brain stimulation (1–3), in which high-frequency stimulation is applied continuously. In many diseases, such as epilepsies, events recur unpredictably and often are separated by long interictal intervals (4–6). In such instances, a closed-loop, transient feedback control could abort seizure episodes without inducing detrimental side effects of continuous stimulation (7–13). We attempted to achieve seizure control by means of closed-loop transcranial electrical stimulation (TES) in a rodent model of generalized (“petit mal”) epilepsy (14, 15) because previous experiments have shown that even very weak TES can reliably entrain neurons in widespread cortical areas (16–20).

We first demonstrated the effect of TES on cortical excitability. Local field potentials (LFPs) and multiple-unit activity (MUA) were recorded by chronically implanted tripolar electrodes (Fig. 1A) and placed in the deep and superficial layers of the frontal and parietal cortical areas (21). TES was applied either between the left and right...

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**Fig. 1.** Experimental setup. (A) Artifact reduction by means of tripolar recordings of LFP and unit activity in superficial, mid-, and deep cortical layers. CSD is derived from the three signals (LFPs from channels a and c are subtracted from the 2x amplitude of channel b activity). The derived CSD signal is filtered (10 to 130 Hz), and signals exceeding the predetermined amplitude threshold are detected (spike detection). The thresholded signals are used to trigger TES, applied to the skull either in a bipolar configuration (left versus right hemispheres) or frontal midline versus parietal areas (as shown). (B) Example of LFP signal during a spontaneously occurring SW episode, CSD, and its filtered version in the absence of TES stimulation. (C) Similar SW episode in the presence of TES stimulation. The gating (red) “dead time” pulse (80 ms) was used to prevent prolonged spurious triggering of the stimulator during the wave components of SW episode.