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Generation of Pluripotent Stem Cells from Adult Mouse Liver and Stomach Cells

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Induced pluripotent stem (iPS) cells have been generated from mouse and human fibroblasts by the retroviral transduction of four transcription factors. However, the cell origins and molecular mechanisms of iPS cell induction remain elusive. This report describes the generation of iPS cells from adult mouse hepatocytes and gastric epithelial cells. These iPS cell clones appear to be equivalent to ES cells in gene expression and are competent to generate germ-line chimeras. Genetic lineage tracing show that liver-derived iPS cells are derived from albumin-expressing cells. No common retroviral integration sites are found among multiple clones. These data suggest that iPS cells are generated by direct reprogramming of lineage-committed somatic cells and that retroviral integration into specific sites is not required.

By retroviral transduction of four transcription factors, Oct 3/4, Sox2, Klf4, and c-Myc, adult mouse fibroblast cells have been reprogrammed to an undifferentiated state similar to embryonic stem (ES) cells (*1, 2*), and these cells have been termed induced pluripotent (iPS) cells. Subsequently, human iPS cells were generated using two different sets of transcription factors (*3, 4*) . The generation of human iPS cells provides a method to produce patient-specific stem cells for study of the diseased state in culture (*3–5*). The mechanisms of iPS cell induction, however, are unknown. Low efficiency of iPS cell induction suggests that their origins may be of undifferentiated stem cells co-existing in fibroblast culture. In addition, retroviral integration into specific site(s) might be required for iPS cell induction (*6*).

In this study, we generated iPS cells from epithelial cells, rather than fibroblasts. Primary hepatocytes and gastric epithelial cells (Fig. 1a) were isolated from mice in which β-geo (the fusion with β-galactocidase and the neomycin-resistant gene) was knocked into *Fbx15* (*7*), which is specifically expressed in ES cells and preimplantation embryos. Fbx15-selected iPS cells from fibroblasts [mouse embryonic fibroblast (MEF) or tail tip fibroblast (TTF)] were different from ES cells in gene expression, DNA methylation and chimera formation (*2*).

Four transcription factors (Oct 3/4, Sox2, Klf4, and c-Myc) were introduced by retroviral vectors into hepatocytes or gastric epithelial cells. The efficiency of retroviral transduction was lower in epithelial cells (30~45%, fig. S1a) than in MEF (>85%, fig. S1b). When MEF were transduced with diluted retroviruses, of which transduction efficiency was ~30%, iPS cells could not be obtained (fig. S1b). Three days after epithelial cell transfection, the medium was changed to ES cell medium containing serum and G418. Two weeks later, multiple G418-resistant and ES cell-like colonies, characterized with large nuclei and scant cytoplasm, were observed from both hepatocytes and gastric epithelial cells, even with the low efficient retroviral transduction.

The G418-resistatant colonies were expanded and ~60% of them showed morphology indistinguishable from that of mouse ES cells (Fig. 1b). These cells were designated iPS-Hep (iPS-Hepatic) and iPS-Stm (iPS-Stomach). They also showed proliferation profiles similar to those of ES cells (fig. S2a). RT-PCR showed that iPS-Hep cells (Fig. 1c) and iPS-Stm cells (fig. S2b) expressed comparable levels of the endogenous *Oct3/4* and *Sox2* to those in ES cells. iPS-Hep and iPS-Stm cells also expressed ES cell marker genes, including *Nanog, Rex1, ECAT1, Rex1, Cripto, and Gdf3, at* comparable levels to those in ES cells. In contrast, Fbx15-selected iPS cells derived from TTF (iPS-TTF) showed lower

expression levels of the ES cell marker genes (Fig. 1c). We tested two culture conditions during the generation of iPS-Hep cells and iPS-TTF cells; one containing epidermal growth factor (EGF) and hepatocyte growth factor (HGF), but no serum (Fig. 1c**,** a), and the other containing 10% serum, but no EGF nor HGF (Fig. 1c, b). In both conditions, iPS-Hep cells showed higher expression levels of ES cell marker genes than did iPS-TTF cells. The promoter regions of *Oct3/4*, *Nanog*, and *Fbx15* are largely, albeit not completely, unmethylated in iPS-Hep and iPS-Stm cells (fig. S2c). This is also in contrast to iPS-TTF cells, which only showed partial demethylation (*2*). Thus, even with *Fbx15* selection, iPS-Hep and iPS-Stm cells are more similar to ES cells than are iPS-TTF cells.

We then tranplanted iPS-Hep and iPS-Stm cells (1×10^6 cells) subcutaneously into the hind flanks of nude mice (table S1). Four weeks after transplantation, all mice developed tumors containing various tissues of the three germ lines, including neural tissues, muscle, cartilage, and gut-like epithelial tissues (fig. S3). This demonstrated that the iPS-Hep and iPS-Stm cells are pluripotent.

These cells were also transplanted into blastocysts by microinjection (table S2). Eight iPS-Hep clones and six iPS-Stm clones, which were derived from Fbx15-reporter mice, were injected. We also injected five iPS-Hep clones that were generated with selection for *Nanog* expression. Most of these clones also contained a GFP transgene driven by the constitutively active CAG promoter (*8*). In addition, we injected one iPS-Hep clone, which we selected based on morphology without selection makers (*9–11*). Among these, we obtained adult chimeric mice from 10 iPS-Hep clones and two iPS-Stm clones, as shown by mouse coat color (fig. S4a). From one iPS-Hep clone (derived from 21-week-old mouse) and two iPS-Stm clones (derived from 12-week-old mouse), which were selected for *Fbx15* expression, germline transmission was observed, as judged from GFP expression and the presence of the transgenes (Fig. S4b). Again, this is in contrast to the iPS-fibroblast (MEF or TTF) cells in that only Nanog selection, but not Fbx15 selection, resulted in adult and germline chimeras (*2*).

Tumorigenicity was then compared between mice derived from iPS-Hep, iPS-Stm cells, or iPS-MEF cells. Forty-six adult chimeras were obtained from 10 independent iPS-MEF clones out of 12 lines injected. Of these chimeras, approximately 30% of mouse chimeras developed tumors by the age of 30 weeks (Fig. 2a). In contrast, no tumor formation was observed from 65 adult chimeras derived from 12 iPS-Hep and iPS-Stm clones in this period. In addition, approximately 20% of the F1 mice derived from eight iPS-MEF clones developed tumors by the age of 30 weeks, whereas no tumor development has been observed in F1 mice from the iPS-Hep/Stm cells (Fig. 2a). Some mice derived from iPS-Hep and iPS-Stm cells did die, especially after we relocated these mice to a conventional mouse facility, but we did not find tumors by necropsy (Fig. 2a).

There was, however, a higher incidence of perinatal death of chimeric mice derived from iPS-Hep and iPS-Stm cells than that of non-chimeras (Fig. 2b). Such higher perinatal mortality was not apparent with iPS-MEF cells. The dead mice appeared normal in gross appearance by necropsy, and the cause of death is not known. It is possible that some epigenetic abnormalities might be responsible for the perinatal death, as is believe to be the case in cloned animals (*12*). In contrast, we did not observe increased mortality in mice that survived the first day after birth (Fig. 2a).

Numbers of retroviral integration sites (RIS) were examined in iPS-Hep and iPS-Stm cells. Southern blot analyses detected 1 to 4 bands for each of the four retroviruses in each clone (Fig. 3). These are fewer than those in MEF-iPS cells, which showed 1 to 12 RIS for each retrovirus. In two iPS-Hep clones and two iPS-Stm clones, RIS were randomly distributed in multiple chromosomes, as determined with inverse PCR (fig. S5).. The integrated genes did not show preferences either in their functional categories or in their intracellular localization (fig. S6). RIS mainly reside near the transcription initiation sites (fig. S7–11).

Our data show that iPS-Hep and iPS-Stm cells are different from iPS-fibroblasts in three properties. First, iPS-Hep and iPS-Stm cells contribute to adult chimeras even with the selection for *Fbx15*. Second, no increased tumorigenicity was observed in chimera mice derived from iPS-Hep and iPS-Stm cells up to 30 weeks evaluated. These two properties are similar to iPS-fibroblast cells that we recently generated without Myc retroviruses (*11*). This suggests that Myc plays a smaller role in the generation of iPS-Hep and iPS-Stm cells than that of iPS-fibroblast cells. To test this possibility, we individually omitted each of the four factors to see the effect on the generation of iPS cells from hepatocytes. When we omitted Oct3/4, Sox2, or Klf4, no iPS cell colonies emerged (fig. S12a). By contrast, the omission of Myc decreased the colony numbers only 20 to 40% from those obtained by the four factors (fig. S12a, b). This is in contrast to the generation of iPS-fibroblasts, in which the colony numbers decreased more than 90% upon the Myc omission (*11*), supporting the minor role of Myc in the generation of iPS cells from hepatocytes.

The third difference between iPS-Hep and iPS-Stm cells and iPS-fibroblasts is that the two former iPS have fewer RIS than did the latter. Retroviral expression levels of the four factors were higher in hepatocytes than in fibroblasts (fig. S13). This may explain, at least in part, the fewer RIS in iPS-Hep cells. In addition, it has been shown that ES cells have characteristics of the epithelium, such as tight intercellular contact and surface expression of E-cadherin (*13*). We

confirmed that the expressions of E-cadherin and β-catenin in hepatocytes were higher than those in fibroblasts and equivalent to those in ES cells (fig. S13). This similarity may also contribute to the fewer RIS in iPS-Hep and iPS-Stm cells. Further studies are required to determine the precise molecular mechanisms underlying the substantial differences between iPS-Hep and iPS-Stm cells versus iPS-fibroblasts.

To examine the origin of iPS-Hep cells, a genetic lineage tracing experiment was conducted (Fig. 4a). The Nanog-reporter mice, in which GFP and the puromycin resistance gene was knocked into *Nanog* for selection of iPS cells, were first crossed with mice expressing the Cre recombinase driven by the *albumin* promoter (*14*), and then crossed with mice expressing a loxP-CAT-loxP-β-gal cassette from the constitutively active promoter (*15*). In the triple transgenic mice, β-gal activity is turned on upon the activation of the *albumin* gene and continues even when *albumin* is turned off. Primary hepatocytes were isolated and iPS cells were generated by the four factors. Fourteen days after the transfection, puromycin selection was initiated. By thirty days after the transfection, >100 GFP-positive colonies were obtained (Fig. 4b, left and center). Most of them were also positive for β-gal (Fig. 4b, right), indicating that iPS-Hep cells were derived from hepatocytes or other albumin-expressing cells, but not from undifferentiated cells that do not express albumin. A few GFP-positive and β-gal-negative colonies were observed, which may have arisen from albumin-negative cells that co-exist in primary hepatocyte cultures, or they simply reflect incomplete excision by Cre.

In conclusion, this study demonstrates that the four transcription factors successfully reprogrammed somatic cells that had differentiated into a stage in which the *albumin* promoter is turned on. In addition, we showed that generation of iPS-Hep and iPS-Stm cells do not require retroviral integration into specific sites. This finding suggests that it might be possible to generate iPS cells with gene transfer methods free from an integration mechanism that may result in tumorigenicity after transplantation to patients (*16*).

References and Notes

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1154884/DC1
Materials and Methods
Figs. S1 to S13
Tables S1 and S2

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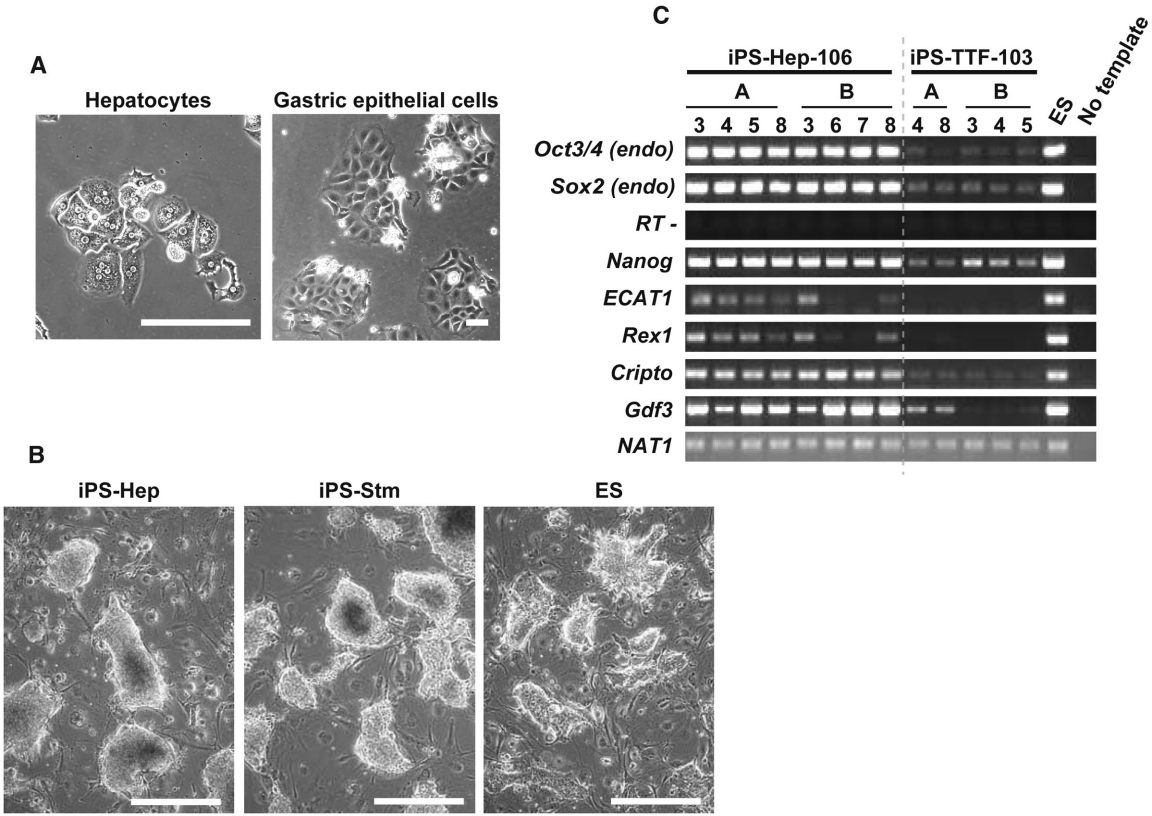
Fig. 1. Characterization of iPS cells from adult mouse hepatocytes and gastric epithelial cells. a) Morphology of a primary culture of hepatocytes and gastric epithelial cells on gelatin-coated plates. Scale bars = 50 μm. **b)** Morphology of iPS-Hep and iPS-Stm cells on STO feeder cells. Scale bars = 500 μm. **c)** RT-PCR analyses of ES marker gene expression in iPS-Hep cells, iPS-TTF cells, and ES cells. Two culture media were used during retroviral transduction; in clones labeled with A, serum-free medium with EGF and HGF was used, whereas in clones labeled with B, medium containing 10% serum, but no EGF nor HGF was used. For *Oct3/4* and *Sox2*, we used primer sets that amplified the endogenous transcript only (endo). As a loading control, *NAT1* was used (*17*). As a negative control, PCR for the *Sox2* was also carried out for the templates without reverse-transcription (RT-).

Fig. 2. Pluripotency of iPS-Hep/Stm cells. a) Tumorigenicity and mortality of iPS cell-derived mice. The cumulative mortality related with tumor (left panels) and overall mortality (right panel) of the chimeric mice (upper) or F1 mice (lower) generated from iPS-MEF or from iPS-Hep/Stm are shown. “Death” includes the cases sacrificed because of weakness. All the death cases were dissected to evaluate the cause of death. Shown at the bottom of each figure were the numbers of mice analyzed at each time point. **b**) Higher incidence of perinatal death in chimeras derived from iPS-Hep and iPS-Stm cells. Shown are the numbers of live and dead pups at the day of birth.

Fig. 3. Retroviral integration sites in iPS-Hep and iPS-Stm cells. Southern blot analyses of retroviral integration of Oct3/4, Sox, Klf4 and c-Myc in iPS-Hep and iPS-Stm clones. Genomic DNA isolated from iPS-Stm, iPS-Hep, or iPS-MEF, and wild-type ES cells were analyzed. The number of detected bands is shown at the bottom. Arrowheads indicate bands corresponding to the endogenous loci. Open arrowheads indicate bands corresponding to *Oct3/4* pseudogenes.

Fig. 4. iPS-Hep cells originated from albumin-expressing cells. a) Strategy for cell fate tracing experiment. **b)** Phase contrast, fluorescent, and X-gal staining photographs of iPS cell colonies derived from hepatocytes of the triple transgenic mouse. Red arrows indicate β-gal-negative colonies. Scale bars = 2 mm.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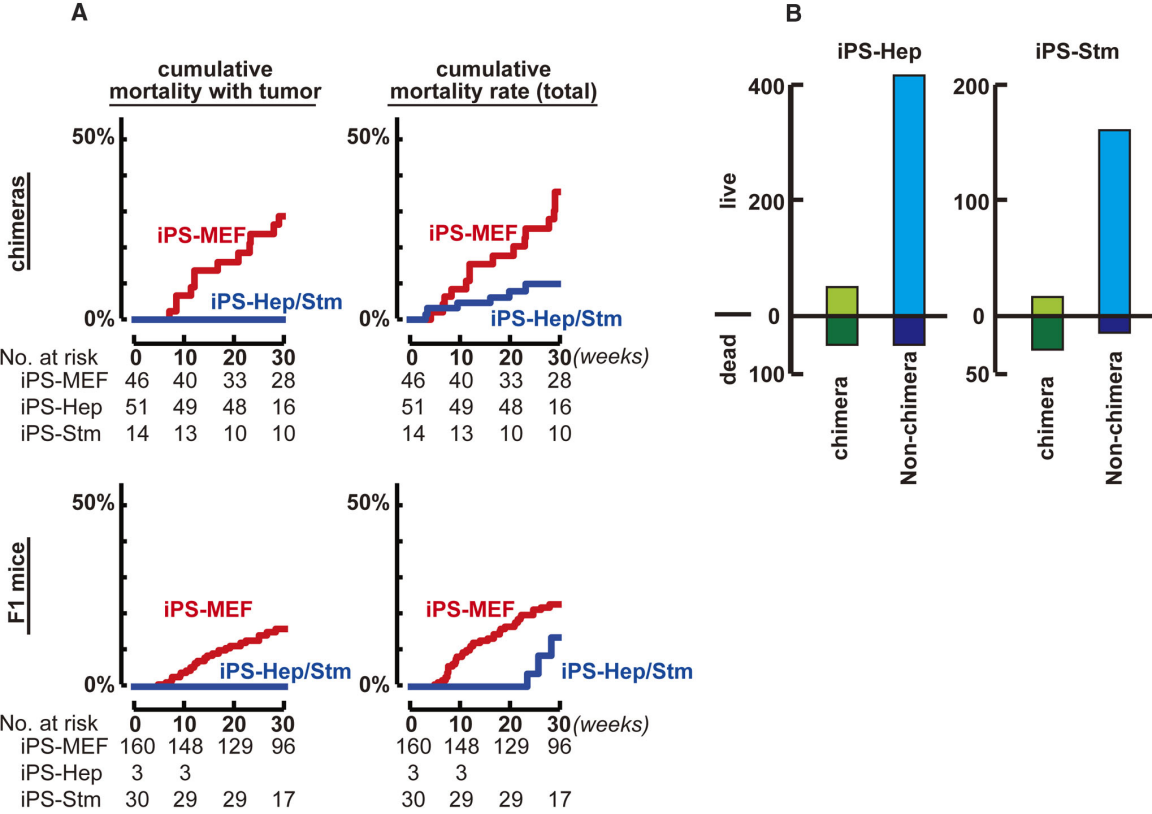
NAT1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iPS-Hep 51 49 48 16
iPS-Stm 14 13 10 10
iPS-MEF 160 148 129 96
iPS-Hep 3 3
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iPS-Stm
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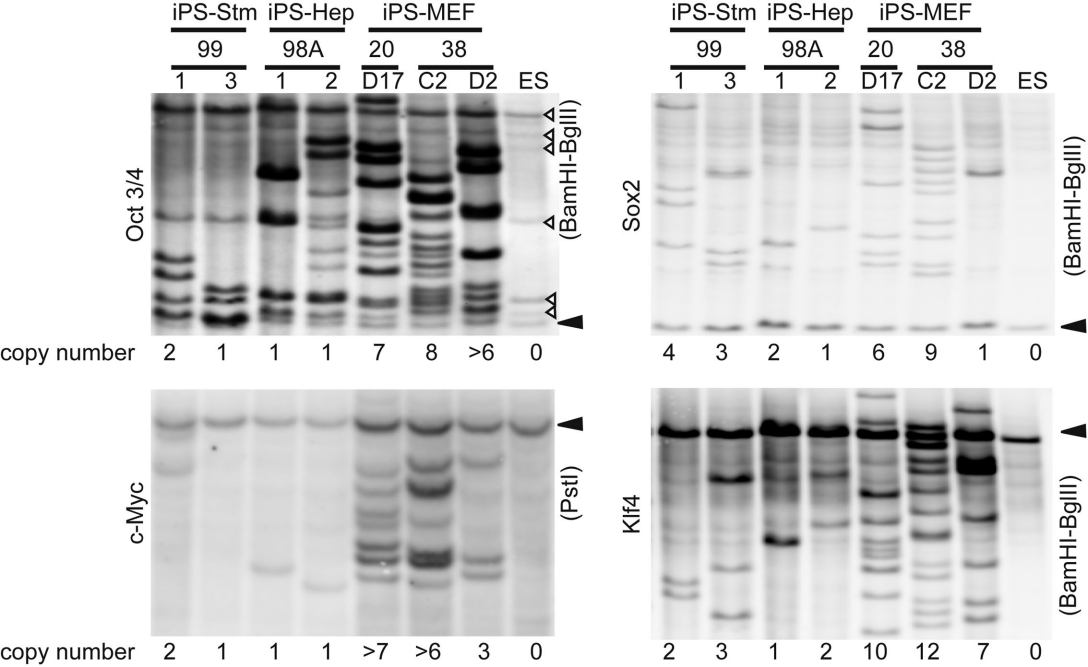
Non-chimera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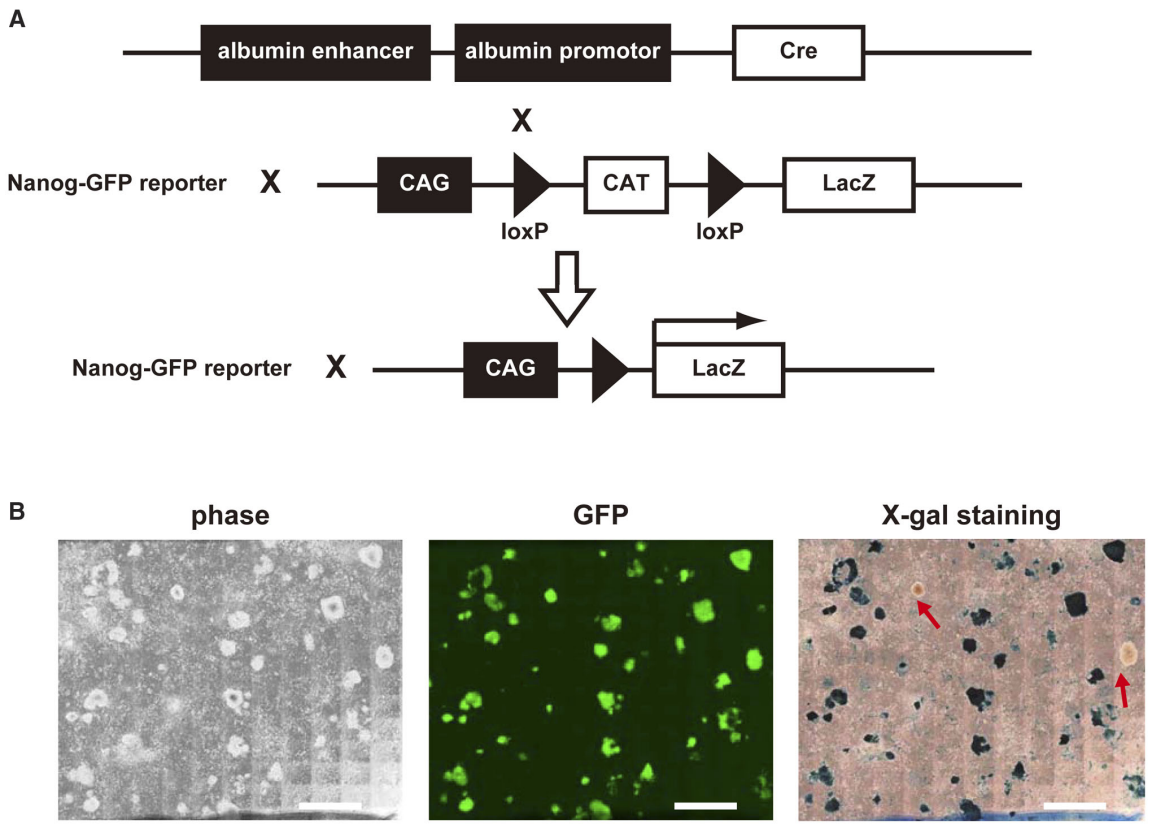
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