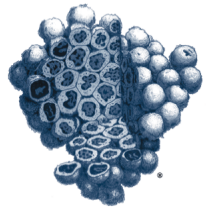



# In Vivo and In Vitro Tissue-Specific Expression of Green Fluorescent Protein Using the Cre-Lox System in Mouse Embryonic Stem Cells

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# In Vivo and In Vitro Tissue-Specific Expression of Green Fluorescent Protein Using the Cre-Lox System in Mouse Embryonic Stem Cells

JAN SCHINDEHÜTTE,[a] HIDEFUMI FUKUMITSU,[b] PATRICK COLLOMBAT,[c] GUNDULA GRIESEL,[c] CHRISTOPHER BRINK,[c] PAUL C. BAIER,[a] MARIO R. CAPECCHI,[d] AHMED MANSOURI[c]

[a]Georg-August University Göttingen, Department of Clinical Neurophysiology, Göttingen, Germany; [b]Gifu Pharmaceutical University, Laboratory of Molecular Biology, Gifu, Japan; [c]Max-Planck-Institute for Biophysical Chemistry, Department of Molecular Cell Biology, Göttingen, Germany; [d]University of Utah School of Medicine, Howard Hughes Medical Institute and Department of Human Genetics, Salt Lake City, Utah, USA

**Key Words.** Conditional green fluorescent protein expression · Embryonic stem cells · Transplantation · Mouse

## ABSTRACT

**Embryonic stem cells (ES) are pluripotent and may therefore serve as a source for the generation of specific cell types required for future therapies based on cell replacement. The isolation of defined cell populations from a certain lineage or tissue is a prerequisite for the analysis of the potential of such ES-derived cells in animal transplantation studies. Here, using the Cre/loxP system, we report the generation of murine ES cells conditionally expressing the hrGFP gene at the cell surface. Such ES cells can be differentiated in vitro into neurons displaying GFP activity in neurites. Transgenic mice derived from these ES cells permit the targeting of GFP-expression to specific tissues and provide material from the three germ layers suitable for molecular and biochemical analysis.**
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## INTRODUCTION

Mouse embryonic stem cells are derived from the inner cell mass of the blastocyst [1, 2]. Such pluripotent cells are able to generate all of the embryonic tissues, including the germ line [3]. Recently, the capacity of embryonic stem (ES) cells to differentiate in vitro has received considerable attention because of their potential in transplantation medicine [4-9]. Thus, numerous advances were made, aiming to selectively differentiate ES cells into tissues of interest. Specifically, the generation of a variety of neuronal cell types has been reported and might be of valuable use for transplantation experiments in animal models for neurodegenerative diseases. Therefore, to unambiguously detect the transferred cells in the host organ after transplantation, we introduced the humanized version of the recombinant green fluorescent protein (hrGFP) gene into undifferentiated mouse ES cells. To achieve this purpose, we used the site-specific Cre/lox recombination system of the phage P1 to conditionally express the *GFP* gene [10]. In addition, a fusion between the GFP protein and the membrane domain of the growth-associated protein 43 (GAP-43) was engineered to specifically target the GFP marker to the neurites. This strategy was adopted to detect

Correspondence: Ahmed Mansouri, Ph.D., Max-Planck Institute for Biophysical Chemistry, Department of Molecular Cell Biology, Am Fassberg 11, 37077 Göttingen, Germany. Telephone: 49-551-201-1490; Fax: 49-551-201-1504; e-mail: amansou@gwdg.de Received July 21, 2004; accepted for publication October 26, 2004.  1066-5099/2005/$12.00/0 doi: 10.1634/stemcells.2004-0163

transplanted neurons in the central nervous system and to provide a valuable tool to follow their projections. Hence, from two ES cell clones exhibiting high GFP expression levels, two transgenic lines were established by morulae aggregation. To assess GFP expression in vivo, these mice were crossed to various Cre-expressing mice. The GFP marker was thereby found in neuroectodermal derivatives using Wnt1-Cre [11] or Pax6Rel-Cre [12], in mesodermal tissues by RARβ2-Cre [13], and in the endocrine pancreas of endodermal origin using Pax4-Cre mice [14]. Furthermore, upon crossing with cytomegalovirus (CMV)-Cre–expressing mice, the *GFP* expression was ubiquitously detected [15].

Our data clearly demonstrate that our approach allows the targeting of the GFP gene into any cell type in vivo. Importantly, such transgenic mice may further serve to isolate GFP-positive cells by FACS analysis from specific tissues of interest and provide a valuable material for molecular and biochemical studies. The engineered embryonic stem cells also permit the derivation of in vitro differentiated cells expressing GFP in a defined cell type by the appropriate promoter-driven Cre recombinase activity.

## Materials and Methods

### Generation of Membrane-Tagged *hrGFP*

Two synthesized complementary oligonucleotides (see below for sequences) encompassing the amino-terminus palmitoylation sites of GAP-43 were hybridized and cloned into the MscI site of pIRES-hrGFP1a (Stratagene, La Jolla, CA). The tagged *hrGFP* fragment encoding mGFP was subcloned into the pCALNLw vector (kindly provided by Dr. Miyazaki [16]) for the purpose of gene expression under the control of the CAG promoter (Fig. 1A). The pCALNL5 vector does not contain the β-galactosidase gene described previously [16]. The synthesized sequences for GAP-43 tag were 5'-CCACCATGCTGTGCTGTATGAGAAGAACCAAACAGGTTGAAAAGAATGATGAGGACCAAAAGATTG-3' and 5'GGTGGTACGACACGACATACTCTTCTTGGTTTGTCCAACTTTTCTTACTACTCCTGGTTTTCTAAC-3'.

### Genetic Manipulation of ES Cells and Generation of Transgenic Mice

Genetic manipulation of ES cells and the generation of transgenic mice by morulae aggregation were performed as previously described by Mansouri [17]. Briefly, ES cells (MPI-II; derived from 129Sv mice) were electroporated using 25 μg of the pCALNL5-derived mhrGFP construct (250 V at 500 μF in phosphate-buffered saline [PBS]). Eight days after G418 selection (250 μg/ml), 20 clones were picked and cultured onto embryonic fibroblasts before freezing. From these, 10 clones were further cultured and subjected to a second electroporation using the plasmid phosphoglycerate kinase (PGK)-Cre (kindly provided by K. Rajewski and W. Müller) and plated at very low density (1,000 cells per 8.5-cm plate). Five days later, growing single ES cell colonies were checked for high GFP expression.

The clones displaying high GFP expression after induction by PGK-Cre were chosen for morulae aggregation with eight cell embryos derived from CD1 mice. From both lines (JS-3 and JS-12), transgenic mouse lines were established and kept on 129XCD-1 mixed background. Transgenic Cre-expressing mouse strains were CMV-Cre (129Sv), Pax4-Cre and Pax6Re-Cre (FVB), RARβ2-Cre, and Wnt1-Cre (C57BL/6).

Animal experiments were conducted according to the German animal law regulations and guidelines.

### ES Cell Culture and In Vitro Differentiation

Undifferentiated mouse ES cells (ES JS-3 and ES JS-12) were maintained and expanded on gelatin-coated cell culture dishes in Glasgow minimal essential medium (G-MEM) (GIBCO-Invitrogen, Karlsruhe, Germany) containing 1% fetal calf serum (GIBCO), 10% knockout serum replacement (GIBCO), 2 mM glutamine (GIBCO), 0.1 mM nonessential amino acids (GIBCO), 1 mM sodium pyruvate (GIBCO), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, Munich, Germany), and 2,000 U/ml LIF (GIBCO). The induction of neuronal in vitro differentiation of ES cells was performed as previously described by Kawasaki et al. [18].

### Immunostaining of Cultured Cells

For immunohistochemical analysis purposes, the cells were plated onto flask slides (GIBCO-Invitrogen). Following appropriate culture, the cells were washed twice in PBS and then fixed with 4% paraformaldehyde (in PBS) for 30 minutes. They were next rinsed with PBS, and unspecific antibody binding sites were blocked with 10% fetal calf serum (Invitrogen) and 0.2% Triton X-100 (Merck, Darmstadt, Germany) in PBS. The incubation with the following primary antibodies in blocking solution was performed overnight at 4ºC. For the detection of class III β-tubulin, the monoclonal primary antibody Tuj1 (Babco; 1:300; Princeton, NJ) was used. Tyrosine-hydroxylase, as a marker for dopaminergic neurons, was detected by the polyclonal primary antibody anti–tyrosine hydroxylase (TH) (Chemicon; 1:300; Temecula, CA). After overnight incubation, the cells were washed three times for 10 minutes each in PBS containing serum and Triton X-100 and next incubated with fluorescence-labeled secondary antibodies (Alexa 594 goat anti-rabbit IgG, Alexa 350 goat anti-mouse IgG; Molecular Probes; Leiden, The Netherlands) for 1 hour in the dark at room temperature.

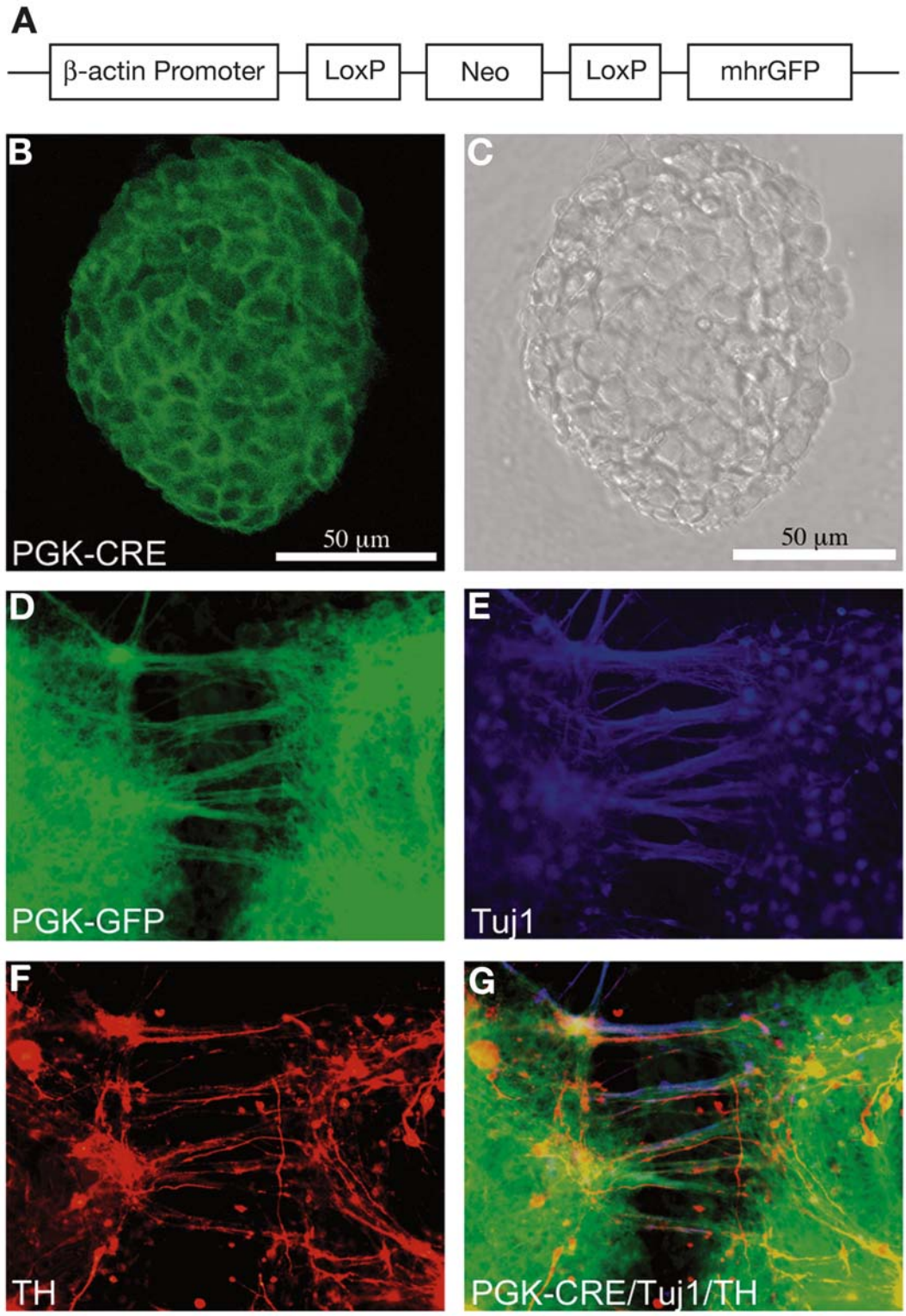


**Figure 1.** Conditional expression GFP in ES cells using PGK-Cre. ES cells carrying the construct shown in **(A)** were electroporated with PGK-Cre to induce the expression of GFP in all cells. A confocal microscopy analysis of these doubly electroporated cell clones demonstrates an ubiquitous expression of the GFP in undifferentiated ES cell colonies **(B, C)**. To assess the expression of the GFP after in vitro differentiation into neurons, ES cells were subjected to neuronal cultures and analyzed for the expression of TH **(F)** and class III β-tubulin detected by the Tuj antibody **(E)**. Differentiated neurons display GFP expression in the neurites **(D, G)**. A codetection of Tuj1, TH, and GFP labeling is presented in (G). Pictures are shown at different magnifications (scale bars: B and C, 50 μm; D through G, 1:20). Abbreviations: ES, embryonic stem; GFP, green fluorescent protein; NEO, neomycin; PGK, phosphoglycerate kinase; TH, tyrosine hydroxylase.

## Results and Discussion

To target the expression of the GFP to the cell surface and label neurites, the amino-terminus palmitoylation sites of GAP-43 gene were fused in frame with the *hrGFP* gene. This peptide has been previously shown to direct the expression of the GFP to the membrane of mature neurons as well as other cell types [19]. The tagged *GFP* was next inserted into the pCALNL5 vector to achieve a conditional expression using the β-actin promoter/CMV enhancer in combination with the site-specific Cre recombinase system [16] (Fig. 1A). This expression vector was linearized and electroporated into embryonic stem cells [17]. After 24 hours, G418 selection was initiated. Eight days later, 20 G418-resistant colonies were picked and grown onto embryonic fibroblasts. Ten of those were again electroporated using the plasmid PGK-Cre, allowing ubiquitous Cre-recombinase expression, and plated at very low density (approximately $10^3$ cells per 8.5-cm dish). Single colonies were picked after 4 to 5 days and grown as duplicates. These cells were checked for GFP expression. Clones 3 and 12 were thereby selected for their high levels of GFP expression. All of the clones examined displayed a GFP labeling restricted to the cell surface of undifferentiated ES cells (Fig. 1B). The GFP expression did not appear to be mosaic. However, we occasionally observed cells seemingly displaying lower GFP levels. This may be due to the fact that ES cells grow as aggregates and not as a monolayer. In addition, as a result of nonsynchronized growth, the amount of GFP accumulated in the cells might have not reached its maximum level. To verify whether the GFP remains localized to the cell surface after differentiation of embryonic stem cells into neurons, we generated neuron cultures from ES cells using the protocol previously described by Kawasaki et al. [18] and monitored the expression of GFP, TH, and the neuronal marker class III-tubulin after 14 days of differentiation (Figs. 1D-1G). This differentiation procedure allows the generation of neuronal cultures by a not-yet-identified stroma cell–derived factor that does not exhibit mesodermal nor endodermal derivatives [18]. The GFP labeling was thereby detected at the cell surface of all undifferentiated cells (Figs. 1B, 1C) using confocal microscopy, indicating an ubiquitous expression of GFP under the control of the PGK promoter. In ES cell–derived neuronal cultures, the GFP staining was clearly found localized to the cell surface and neurites (Figs. 1D, 1G). This latter characteristic might serve in vivo to monitor cell integration and behavior after transplantation into brain tissue, thereby omitting the use of prelabeling techniques with a lipophilic dye such as PKH-26. In addition, these conditional GFP-expressing ES cell lines represent a valuable tool to separate labeled cell types of interest by FACS following in vitro differentiation, providing the availability of the corresponding enhancer/promoter.

Compared with previously published transplantation studies [20-23], the GFP-labeled ES cells, described in the present report, should also permit the monitoring of ES cell-derived tissues after transplantation into living animals. Specifically, the use of such ES cell-derived dopaminergic neurons into the 6-hydroxy-dopamine lesioned rat striatum as a model for Parkinson's disease might profit from these GFP-expressing cells and provide the opportunity to follow neurite outgrowth and morphological integration of neuronal processes within the host brain. Another important advantage of these conditional GFP-expressing cell lines consists in the continuous expression of the GFP due to the ubiquitously active β-actin promoter. This improvement prevents the low expression of GFP associated with developmentally regulated activities of the promoters previously used [24]. However, the specificity of the promoter driving Cre expression is of fundamental importance, because any leakiness may hamper the isolation of the appropriate cell population.

To test whether this strategy was also effective in vivo, we have used clone 3 (ES JS-3) and clone 12 (ES JS-12) to generate germ line chimeras. Animals derived from both mouse lines were healthy and fertile. To induce the GFP expression in these mice, they were mated with transgenic mice expressing the Cre recombinase under the control of different promoters. These include the Wnt1–RAR–2, Pax6-retinal element, Pax4, and CMVβ promoters [11-15]. As depicted in Figure 2, using the CMV-Cre mice, the GFP expression was found ubiquitously detected. No mosaic GFP labeling was observed at any stage analyzed, from E10.0 to E15.5 of gestation (Fig. 2B and data not shown). After crossing with Wnt1-Cre mice, the GFP expression was found to recapitulate Wnt1 expression in the dorsal and ventral mesencephalon, the midbrain-hindbrain boundary, the dorsal spinal cord, and neural crest derivatives (Figs. 2C-2F) [11]. Furthermore, at E13.5, the GFP labeling was also detected in axons derived from dorsal root ganglia (Figs. 2C, 2D, inlet and arrows). Similarly, the GFP activity was observed in the limb bud and in the somitic mesoderm (Figs. 2G, 2H) using the RARβ2-Cre mice, whereas the use of Pax6-Cre mice promoted a labeling restricted to the retina (Figs. 2I, 2J) as previously reported [12]. Finally, in tissue of endodermal origin, using Pax4-Cre mice, we also found the GFP expressed in insulin-secreting β-cells (Figs. 2K, 2L), thereby recapitulating Pax4 expression in the endocrine pancreas [25]. We did not find any GFP signal in cell populations that were not described previously

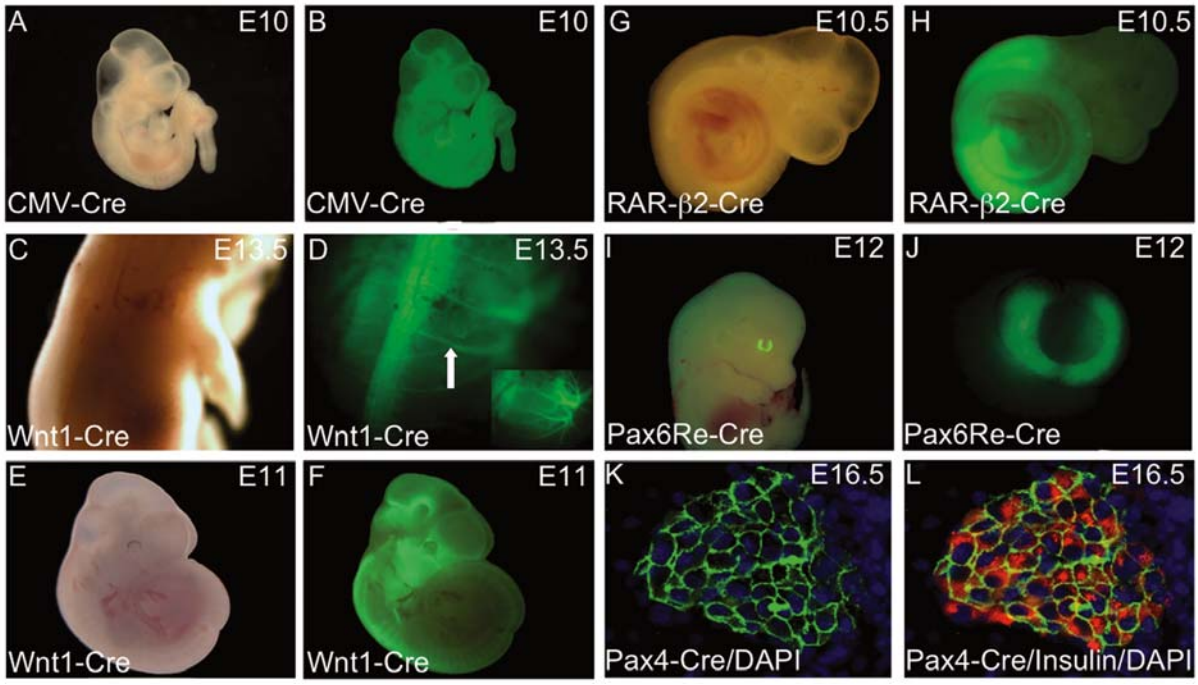


**Figure 2.** Conditional expression of the GFP in transgenic mice. To assess in vivo the conditional GFP expression, ES JS-3–derived and ES JS-12–derived mice were crossed to various Cre-expressing mouse lines. An ubiquitous GFP labeling was observed after mating ES JS-12–derived mice with CMV-Cre mice **(A, B)**. To induce GFP expression in tissues of ectodermal origin, Wnt1-Cre **(C-F)** and Pax6Re-Cre **(I, J)** mice were used. The GFP can be detected in the limb bud at E9 and E10 of gestation as well as in the somitic mesoderm after mating to RARβ2-Cre mice **(G, H)**. Similarly, Pax4-Cre mice were able to target the GFP to the endoderm. Hence, the GFP was found in the islet of Langerhans, restricted to developing beta cells (red staining) at E16.5 **(K, L)**, thereby recapitulating Pax4 expression in the endocrine pancreas. Abbreviations: CMV, cytomegalovirus; CRE, cytomegalovirus; ES, embryonic stem; GFP, green fluorescent protein.

for the tested Cre-expressing transgenic lines. This indicates that the generated GFP-expressing mouse lines did not interfere with the specificity of the tested promoters. Altogether, our data demonstrate that the two ES-derived transgenic mouse lines, JS-3 and JS-12, provide a valuable tool to mark and visualize in vivo any cell type after breeding with transgenic mice expressing the Cre recombinase under specific promoters. These transgenic lines may even serve to evaluate and test the specificity of promoters driving Cre. This issue is of crucial importance, considering the potential use of such promoters to select a specific cell type after ES cell differentiation, such as dopaminergic neurons. It should be pointed out that the use of ES JS-3 and ES JS-12 as a potential source to tag any specific tissue from differentiated ES cells, such as germ cells using Oct4 sequences [26], is determined by the specificity of the promoter driving Cre-expression. In addition, the labeled tissues may further be isolated from the embryo, and the cells may be subjected to sorting using the FACS method to provide material for molecular analysis, such as expression profiling or proteomic studies. GFP-labeled tissues may also be isolated (such as Wnt1-expressing ventral mesencephalic cells) and used for transplantation or cell lineage-tracing experiments.

Although several similar transgenic mouse lines expressing GFP are now available [27–29], the presented ES cells and transgenic mice have the unique advantage of targeting the GFP to the cell membrane.

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