Chapter 11

Rapid Screening of Gli2/3 Mutants Using the Flp-In System

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Abstract

Gli2 and Gli3 respond to the Hedgehog (Hh) signal in mammals by undergoing posttranslational modifications and moving to the nucleus. The study of Gli proteins has been hampered by the fact that their overexpression in cells prevents their proper regulation. To address this issue, we have developed a method of rapid generation of stable cell lines expressing near-endogenous and approximately equal levels of wild-type and mutant Gli proteins. This method is applicable to the study of effects of various mutations on Gli protein modifications and activity.

Key words Gli proteins, Flp-In system, Stable transfection, Flp-mediated recombination

1 Introduction

The three mammalian Gli proteins translate the Hh signal into transcriptional output of the pathway in the nucleus [1]. Gli2 and Gli3 undergo posttranslational modifications and subcellular localization changes in response to upstream signals [2–5]. Only when these proteins are expressed at endogenous or near-endogenous levels is this physiological regulation preserved. If the levels of expression are too high, Gli2 and Gli3 escape proper regulation by the Hh pathway and activate gene expression in the absence of upstream signal [4]. For these reasons, the study of the effects of mutations on Gli2/3 activity has been challenging. The two options were to either generate transgenic or knock-in mice bearing the mutation in question [6] or test multiple clones of stably transfected cell lines to select the ones with the right amount of exogenous Gli2/3 expression [7]. Both of these strategies require significant effort and are not suitable for rapid screening of a large number of mutations. Recently, we have devised a strategy to streamline the production of stable cell lines expressing near-endogenous levels of Gli2 and Gli3 based on Flp-In cells [4].

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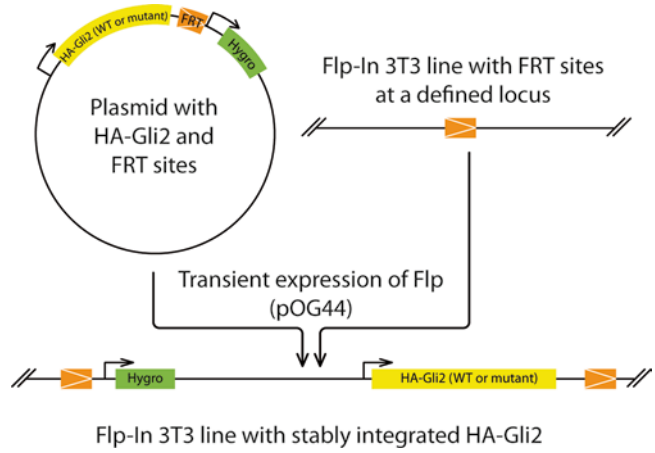

Fig. 1 Schematic representation of stable line generation using the Flp-In system. The Flp-In 3T3 cell line is co-transfected with a plasmid carrying the 3 × HA-tagged sequence of Gli2/3 (WT or mutant) and the pOG44 plasmid encoding Flp recombinase. Flp induces recombination between FRT site on the plasmid and a corresponding site in the genome of the Flp-In line. Stable line selection is accomplished by hygromycin

The Flp-In system, developed by Life Technologies, depends on Flp-mediated recombination between Flippase Recognition Target (FRT) sites [8] in the host cell and the transfected plasmid. It consists of three elements: Flp-In cell lines, the pOG44 Flp-recombinase expression vector, and a series of vectors, each containing an FRT site plus a hygromycin resistance gene (Fig. 1). Each Flp-In cell line has an FRT recombination site integrated into the genome (single copy per cell). When pOG44 is co-transfected with an FRT site-containing vector, the FRT plasmid is linearized and inserted into the defined genomic locus of the Flp-In line by the Flp recombinase. Subsequently, stable integrants are selected using hygromycin. Here, we describe technical details of the procedure in the specific case of stable lines that express near-endogenous levels of Gli2 and Gli3 mutants.

This approach can be used to gain insights into how mutations in Gli2 and Gli3 regulate their intrinsic transcriptional activity, subcellular localization, posttranscriptional modifications, and stability. However, it is important to note that Flp-In NIH/3T3 cells continue to express endogenous Gli2 and Gli3 proteins. Thus, if the goal is to analyze how mutations impact ligand-induced activation of Hh target genes, these wild-type endogenous proteins must be depleted, using either RNAi against the untranslated regions of the endogenous transcript or genetic deletion, for example, using the CRISPR/Cas9 system.

2 Materials

2.1 Cell Culture Materials

1. Cells: Flp-In 3T3 cells (Life Technologies).
2. Antibiotics-free media: DMEM containing 10 % fetal bovine serum (FBS), 1× GlutaMAX, 1× nonessential amino acids, 1× sodium pyruvate (all from Life Technologies).
3. High-serum culture media: antibiotics-free media with 1× penicillin/streptomycin solution (Life Technologies).
4. 0.05 % trypsin-EDTA solution (Life Technologies or equivalent).
5. Sterile phosphate-buffered saline (PBS), calcium- and magnesium-free.
6. Cell culture dishes: Nunc Nunclon or equivalent tissue culture-treated 6 cm and 10 cm round dishes and well plates (12- and 6-well).
7. Cell counter.

2.2 Materials for Stable Transfection and Cell Line Testing

1. FRT plasmid: plasmid containing an FRT site, hygromycin resistance gene, and the coding sequence for Gli2 or Gli3 tagged with the triple hemagglutinin (3×HA) tag on the N-terminus (*see* **Note 1**), stored at 100 ng/μL in water or TE buffer.
2. pOG44 plasmid stored at 1 μg/μL in water or TE buffer.
3. Cell transfection reagent: jetPRIME (Polyplus) or equivalent (*see* **Note 2**).
4. Hygromycin selection media: high-serum culture media containing 200 μg/mL hygromycin (Life Technologies or equivalent).
5. SDS-PAGE electrophoresis and western blotting equipment and materials.
6. Anti-HA antibody (HA-11 clone 16b12, Covance cat. # MMS-101P, used at 1:1,000).

3 Methods

3.1 Cell Culture

1. Culture Flp-In-3T3 cells in high-serum culture media. Split cell every 2–3 days to prevent cells from reaching over 80 % confluence.
2. To passage cells, wash once with PBS and cover with pre-warmed trypsin-EDTA solution for 3–5 min at 37 °C. Tap to dislodge cells and neutralize trypsin with high-serum culture media (for normal passage) or antibiotics-free media (before transfections). Count cells and plate at desired density.

3.2 Stable Transfection

1. Plate 5×10^5 cells on a 6 cm culture dish in 4 mL of antibiotics-free media. Immediately proceed to preparation of transfection mix (day 1, *see* **Note 3**).
2. Prepare transfection mix as recommended by the manufacturer of the transfection reagent. For each dish, use 300 ng of the FRT plasmid and 2.7 μg of the pOG44 plasmid.
3. Transfect cells as recommended by the transfection reagent manufacturer.
4. Leave cells overnight in a cell culture incubator.
5. On day 2, change media to fresh antibiotics-free media.
6. On day 3, rinse cells with PBS and trypsinize. Neutralize trypsin with high-serum culture media and split cells from each 6 cm dish into 3 × 10 cm dishes.
7. On day 4, change media to hygromycin selection media.
8. On subsequent days, split cells as needed to prevent overgrowth (keep cells below 80 % confluence; *see* **Note 4**).
9. Change media every 2–3 days to fresh hygromycin selection media.
10. Once most nonresistant cells die (after approximately 1 week), search for resistant cell clones. When the clones have grown to approximately 20–50 cells each, trypsinize cells and pool them. Depending on how many clones there are, plate cells into a single well in a 12-well or a 6-well plate in hygromycin selection media (*see* **Note 5**).
11. On subsequent days, split cells as needed. Cells no longer need to be maintained in hygromycin selection media all the time, but using hygromycin every 2–3 passages helps prevent downregulation of HA-Gli2/Gli3 expression (*see* **Note 6**).
12. Test expression and activity of HA-tagged Gli2 or Gli3 by anti-HA and anti-Gli1 immunoblot. *See* Fig. 2 for sample blots.

4 Notes

1. We cloned 3 × HA-tagged Gli2 and Gli3 and their mutants into the pENTR2B vector and used Gateway cloning to insert the construct into the pEF5/FRT/V5-DEST. The vectors and Gateway cloning kit were purchased from Life Technologies. In principle, different FRT-carrying vectors should also work but may result in varying expression levels. Ready-made pEF5/FRT/V5-DEST plasmids containing 3 × HA-tagged Gli2 and Gli3 WT and various mutant variants were deposited by us in the Addgene repository (*see* https://www.addgene.org/Rajat_Rohatgi/).

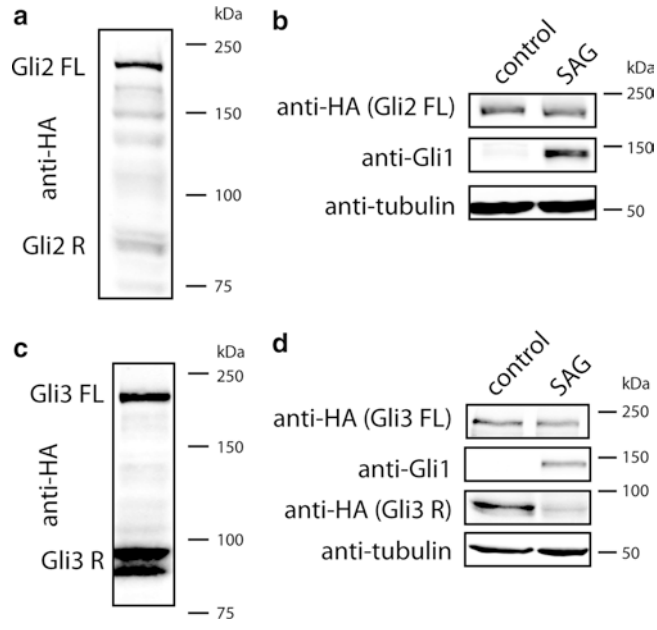

Fig. 2 Sample immunoblots of lysates from stable lines expressing wild-type 3 × HA-Gli2 (**a**, **b**) or 3 × HA-Gli3 (**c**, **d**) at near-endogenous levels. (**a**) Anti-HA immunoblotting of lysate from the cell line expressing WT 3 × HA-Gli2. FL indicates the full-length form of the protein; R indicates the proteolytically processed repressor form. (**b**) Cells expressing 3 × HA-Gli2 were left untreated or treated with 100 nM smoothened agonist (SAG) for 24 h. Full-length 3 × HA-Gli2 was detected with anti-HA antibodies. Also shown are levels of Gli1 measured by anti-Gli1 immunoblotting. Tubulin is used as loading control. (**c**) Anti-HA blot of lysate from the cell line expressing WT 3 × HA-Gli3. (**d**) Cells expressing 3 × HA-Gli3 were treated as in (**b**). Full-length and repressor forms of 3 × HA-Gli3 were detected with anti-HA antibodies. Note lack of Gli1 expression in control samples in (**b**) and (**d**), which indicates that the exogenous Gli2 and Gli3 proteins are properly regulated and do not induce pathway activation in the absence of treatment

2. We found the jetPRIME reagent to be most efficient for transfection of NIH/3T3-based cell lines, but other reagents, such as the X-tremeGENE 9 (Roche), also work reasonably well.
3. Best transfection efficiencies were achieved in our hands when the cells were transfected up to 2 h after plating, instead of 12–16 h after plating as recommended by most transfection reagent manufacturers.
4. Generally, hygromycin takes about a week to start killing non-resistant cells off. Once most cells are dead, there will be few (up to ten) clones in each culture dish, so discarding cells is not recommended. If possible, split cells into more dishes rather than throwing the extra cells away.

5. Alternatively, single clones may be picked and expanded separately, rather than pooled. Although the site of integration of the transgene is always the same in the Flp-In system, individual clones may have slightly different expression levels and/or growth characteristics, which may affect downstream assay results.
6. Stable cell lines generated using the Flp-In method tend to silence the exogenous Gli proteins over a large number of passages. It is recommended to cryopreserve early passages of stable lines and to not use these lines for longer than 15–20 passages.

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