A General Method for Conditional Regulation of Protein Stability in Living Animals

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INTRODUCTION

The ability to rapidly and reversibly perturb protein levels in living animals is a powerful tool for researchers to determine protein function in complex systems. We recently designed a small protein domain based on the 12-kDa FKBP (FK506 binding protein) that can be fused at either the carboxyl or amino terminus of a protein of interest. This destabilization domain (DD) confers instability to fusion protein partners, allowing targeted degradation of the protein of interest. A small molecule called Shield-1 binds to the DD and protects the fusion protein from degradation. Small-molecule-mediated post-translational regulation of protein stability affords this system rapid, reversible, and tunable control of protein levels and functions in a variety of model systems. Theoretically, a number of transgene delivery methods (e.g., viral, liposomal, or stem cell) can be used for the analysis of a DD fusion protein in an animal model. This protocol uses tumor xenografts in mice as one such mechanism for delivering the fusion protein and presents a method for delivering Shield-1 to regulate the fusion proteins in vivo.

RELATED INFORMATION

This protocol was adapted from Banaszynski et al. (2008). Central to the technique is the creation of a stable cell line carrying the DD fused to a protein of interest (POI), as described in Regulating Protein Stability in Mammalian Cells Using Small Molecules (Hagan et al. 2009). Before use in an animal model system, the ligand-dependent stability of the DD-POI should be validated in heterogeneous or clonal cell populations in tissue culture (Hagan et al. 2009). Always confirm that the protein of interest can retain its function as either an amino- or carboxy-terminal fusion. The cell line should be tested for Shield-1 dependence of protein levels by performing dose-response and time course assays. Typically, both in vitro assays for protein levels (such as immunoblotting or ELISA) and a functional assessment of the fusion protein should be used.
In cultured cells, maximum stabilization of DD fusion proteins typically is observed using 1 μM Shield-1, with maximum protein levels achieved after 4-24 h, depending on the protein of interest. After removal of Shield-1, the protein degrades to background levels within 2-4 h. For example, Shield-1 stabilization resulted in a >50-fold increase in mean fluorescence intensity of yellow fluorescent protein (Banaszynski et al. 2006) and a sixfold increase in luminescence of a thermostable luciferase (tsLuc; Tisi et al. 2002) in vitro. Although Shield-1 stabilization displays similar dynamic ranges in living mice, it is reasonable to expect that larger doses might be necessary to achieve sufficient levels of Shield-1 at the xenograft site. For animal experiments, a range of Shield-1 dosages (e.g., 1-10 mg/kg) should be tested for optimal stabilization of destabilized transgene products. As an example, maximum stabilization of DD-tsLuc is typically seen 8-24 h after Shield-1 injection, and destabilized proteins return to basal levels by 36-48 h after injection. It is advisable to run a pilot experiment with fewer groups and smaller numbers of animals (two to four) per group to determine a suitable Shield-1 dosage regimen and to optimize transgene detection assays.

**MATERIALS**

**Reagents**

Cell line, transfected with POI constructs (e.g., HCT 116; ATCC)

*Transfect the cell lines to be used with the DD-POI constructs according to the method described in Regulating Protein Stability in Mammalian Cells Using Small Molecules (Hagan et al. 2009). Prepare cells transfected with a POI construct alone (i.e., no DD fusion) for use as a positive control.*

- Dulbecco’s modified Eagle medium (DMEM; GIBCO)
- HCT 116 medium
- Isofluorane (2%; Webster Veterinary)
- Mice, immunodeficient (e.g., nu`/nu`)
- Mice should be age-, breed-, and sex-matched.
- N,N-Dimethylacetamide (DMA; Sigma-Aldrich)
- Phosphate-buffered saline (PBS, pH 7.2; Invitrogen)
- Poly(ethylene glycol), average m.w. 400 (PEG 400; Sigma)
- Protein detection reagents (e.g., ELISA kits, immunohistochemistry reagents, immunoblotting reagents)
- Shield-1 (Clontech)
- Trypsin, with 0.05% EDTA (GIBCO)
- Tween 80 (Sigma)

**Equipment**

- Calipers
Centrifuge, equipped with swinging-bucket rotor
Dishes, tissue culture (Fisher)
Dissection tools
Flasks, culture, 175-mm³
Hemacytometer
Incubator, equilibrated with 5% CO₂, preset to 37°C
Syringes and needles
Tissue homogenizer

**METHOD**

**Cell Plating**

1. Culture cells containing a stably integrated destabilized transgene in a culture flask (175 mm³). Grow to 80% confluence.

2. Count the cells. Adjust the number of cells to implant per animal depending on the growth of the cell line in animals.  
   *Typically, 0.1-5 x 10⁶ cells per animal should be sufficient for detection of the DD-POI after a few days. Normal experimental group sizes (n = 6-8 mice) should allow for statistically significant comparisons.*

**Cell Transplantation**

3. Trypsinize the cells. Quench with complete medium.

4. Pellet the cells by centrifugation at 500g for 5 min.

5. Wash the cells three times by resuspension in PBS and recentrifugation.

6. Resuspend the cells in 100 µL (i.e., 10,000 cells/µL) of DMEM without fetal bovine serum per animal.

7. Anesthetize the mice with 2% isofluorane.

8. Xenograft the cells by subcutaneous injection at desired location.  
   *Inject untransfected cells as a negative control and cells transfected with a POI construct alone as a positive control. Optionally, cells transfected with a DD-tsLuc construct can be used as an additional positive control.*

**Shield-1 Treatment**

9. Wait several days to allow cells to form stable grafts before beginning the experimental protocol.
10. Reconstitute Shield-1 in DMA at various concentrations up to 10 mg/mL. 
*The 10 mg/mL stock solution can be kept for several months at -20°C.*

11. Prepare a fresh solution of PEG 400:Tween 80 (9:1) before each injection.

12. Mix Shield-1 stocks (e.g., 3 or 10 mg/mL) 1:1 with the fresh PEG:Tween mix.

13. Inject mice intraperitoneally with Shield-1 doses of 3 and 10 mg/kg. Inject control mice with DMA/PEG/Tween vehicle alone. 
*Shield-1 can be injected intravenously, but intraperitoneal injections often produce more reliable results.*

**Experimental Analysis**

14. Assay for experimental DD-POI stabilization:

   *For direct protein measurement:*  
   i. Remove tumors. Dissect the tumor tissue to a standard amount (typically 1 g per mouse).

   ii. Homogenize the tissue.

   iii. Assay for levels of the POI by ELISA or immunoblotting.

   See Troubleshooting.  
   *For determination of long-term effects:*  
   iv. Maintain high levels of the DD-POI by injecting Shield-1 every 48 h.

   v. Periodically assay directly for DD-POI stabilization and/or for the phenotypic or functional effects of protein stabilization. 
   *The precise type of assay will depend on the protein to be examined. For example, tumor xenograft regression based on Shield-1 stabilization can be determined by measuring levels of the secreted IL-2 protein. Also, subcutaneous tumor size can be measured directly with calipers.*

**Interpretation of Results**

15. Compare the results obtained between the various control and treatment groups:

   i. A negative control group receiving xenographed cells that do not contain the DD-POI transgene but are dosed with Shield-1 will help attribute observed results to stabilization of the transgene, and not any nonspecific effects of the ligand, vehicle, or xenograft procedure.

   ii. A negative control group receiving xenographed cells containing DD-POI that are given vehicle alone will show the background level of destabilized protein activity and provides a comparison for groups in which the DD-POI is stabilized by Shield-1.

   iii. A positive control group in which mice receive cells containing unregulated POI will allow observation of transgene effects without temporal and tunable ligand control.
iv. Different doses of Shield-1 (3 vs. 10 mg/kg) can be used to determine any concentration-dependent activities of the protein of interest. Also, different dosages can affect the systemic diffusion of a secreted transgene. 

*For example, 10 mg/kg Shield-1 can stabilize secreted IL-2 such that it can be detected systemically, whereas at a dose of 5 mg/kg, IL-2 is only detected locally at the xenograft site.*

**TROUBLESHOOTING**

**Problem:** Transgene protein levels are not detectable after Shield-1 administration.

[Step 14.iii]

**Solution:** Consider the following:

1. Depending on the location of cell transplant, different levels of Shield-1 might be needed to reach the target tissue. Increasing the dose of Shield-1 (up to 10 mg/kg) can increase the stabilization of the transgene to locally and even systemically detectable levels. Additionally, try repeated injections of Shield-1. It is possible that Shield-1 is injected into the bowel of the animal and might not reach significant concentration in the bloodstream.

2. A good test for whether Shield-1 is reaching the targeted tissue is to express or co-express DD-tsLuc in grafted cells. This provides an optical reporter for Shield-1 stabilization at the target tissue. Briefly, 8-24 h after Shield-1 administration, inject 3 mg of D-luciferin (100 μL of a 30 mg/mL stock) intraperitoneally. Wait 5 min before imaging mice anesthetized with 2% isofluorane with a cooled charge-coupled device (CCD) camera (e.g., IVIS, Caliper). Compare the luciferase output of Shield-1-injected mice to control mice. Quantitate the signal by selecting the xenografted area as the region of interest and calculate luminescence in photons/sec/cm²/sr using image analysis software (e.g., Living Image, Caliper). If Shield-1 is reaching the tissue, the luciferase signal should be approximately sixfold above background.

3. Finally, check the maintenance of the destabilized transgene after xenograft. Harvest or biopsy implanted cells and stroma that have been passaged through the animals and plate in cell culture medium. Cells that have maintained the destabilized transgene will be responsive to Shield-1 treatment.

**DISCUSSION**

This protocol presents the steps needed to control protein stability in mice. Modifications to this protocol should be used for other model systems (e.g., rat, zebrafish) or organisms (*Armstrong and Goldberg 2007; Herm-Götz et al. 2007*). The advantages of the destabilizing domain technology are the speed of protein destabilization after drug removal, the ability to tunably regulate protein levels with Shield-1, and the extremely low basal protein levels in the absence of Shield-1. The dynamic ranges of stabilization for IL-2 and tsLuc DD-POIs in mice are similar to those seen in in vitro studies. However, the kinetics of stabilization and destabilization have only been determined for DD-tsLuc, and it may be necessary to test these
parameters for other fusion proteins, especially if a tight temporal window of stabilization is desired. If stabilization of a protein over an extended time period is desired, mice can be dosed with Shield-1 every 48 h to maintain the DD-POI levels. Prolonged treatment with Shield-1 appears to be innocuous in cells (Maynard-Smith et al. 2007) and whole animals: Nude mice treated with Shield-1 every 48 h for 2 mo showed no gross signs of toxicity (e.g., changes in feeding behavior, grooming, or activity levels). Nevertheless, researchers should consider the financial costs of long-term Shield-1 use in animals and be aware that other FKBP ligands are capable of DD stabilization (Banaszynski et al. 2006).

REFERENCES


Caution

Isofluorane (Isoflurane)

Isofluorane (Isoflurane) is an irritant and may be harmful by inhalation, ingestion, or skin absorption. Chronic exposure may be harmful. Wear appropriate gloves and safety glasses.

Caution

N,N-Dimethylacetamide (DMA)

N,N-Dimethylacetamide (DMA) is toxic and may cause harm to the unborn child. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses and use in a chemical
fume hood. Do not breathe the mist or vapor. It is also flammable. Keep away from heat, sparks, and open flame.

**Caution**

**Trypsin**

Trypsin may cause an allergic respiratory reaction. It may be harmful by inhalation, ingestion, or skin absorption. Do not breathe the dust. Wear appropriate gloves and safety goggles. Use with adequate ventilation.

**Recipe**

**HCT 116 medium**

Dulbecco’s modified Eagle medium, with 2 mM L-glutamine (GIBCO)

Fetal bovine serum (10%; GIBCO)

Penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively; GIBCO)