

Video Article

Establishing Cell Lines Overexpressing DR3 to Assess the Apoptotic Response to Anti-mitotic Therapeutics

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Abstract

Studying the function of a gene of interest can be achieved by manipulating its level of expression, such as decreasing its expression with knockdown cell lines or increasing its expression with overexpression cell lines. Transient and stable transfection are two methods that are often used for exogenous gene expression. Transient transfection is only useful for short-term expression, whereas stable transfection allows exogenous genes to be integrated into the host cell genome where it will be continuously expressed. As a result, stable transfection is usually employed for research into long-term genetic regulation. Here we describe a simple protocol to generate a stable cell line overexpressing tagged death receptor 3 (DR3) to explore DR3 function. We picked single clones after a retroviral infection in order to maintain the homogeneity and purity of the stable cell lines. The stable cell lines generated using this protocol render DR3-deficient HT29 cells sensitive to antimetabolic drugs, thus reconstituting the apoptotic response in HT29 cells. Moreover, the FLAG tag on DR3 compensates for the unavailability of good DR3 antibody and facilitates the biochemical study of the molecular mechanism by which antimetabolic agents induce apoptosis.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58705/>

Introduction

Heterologous gene expression is often employed to study the function of genes of interest. Two methods, transient and stable transfection, are prevalently used in cell and molecular biology to insert a segment of DNA or RNA into a host cell^{1,2}. Transient-transfected DNA or RNA cannot be passed on to daughter cells, so the genetic alteration can only be retained for a short period of time. On the other hand, in stably transfected cells, exogenous genes are integrated into the host cell genome, sustaining its expression in the cell line. Thus, stable transfection is a method that is usually reserved for research into long-term genetic regulation. The stable cell line can also be transplanted as xenografts into mouse models for *in vivo* study³. Stable transfection can be divided into two categories: viral and nonviral. Compared to nonviral transfection, viral infection can transfer genes into a wider variety of human cells with a higher efficiency^{4,5,6,7}. Furthermore, a stable cell line from a single clone offers the advantage of homogeneity and clonal purity.

Uncontrolled cell growth and division are the most distinguishing features of cancer. In clinical settings, antimetabolic agents are the primary treatments for many types of tumors⁸. However, some significant limitations of antimetabolic agents cannot be ignored. First, these chemotherapies can also kill normal cells along with the undesired cancerous cells and, thus, can result in severe side effects⁹. Second, antitubulin drugs are not effective against all types of tumors, despite tubulin's ubiquitous expression in a wide variety of different tissues as a cytoskeletal protein^{9,10}. It is unclear why antitubulin agents showed promising efficacy against ovarian, lung, and hematological cancers in clinical treatment, but not in kidney, colon, or pancreatic cancers¹¹. Finally, even patients with the same type of tumor can respond to the antimetabolics differently in an unpredictable manner. There may be a key effector molecule that affects the sensitivity of different patients to antimetabolic agents, resulting in the diverse outcomes seen in clinical treatment¹². Thus, the potential differential sensitivities of patients to antimetabolic treatment should be taken into consideration in order to optimize therapeutic interventions¹³.

A high-throughput whole-genome siRNA library screen demonstrated that DR3 knockdown could render sensitive cells resistant to antimetabolic drugs, implicating a role for DR3 in chemotherapy-induced apoptosis¹⁴. As a member of the tumor necrosis factor receptor (TNFR) superfamily, DR3 has been reported to mediate cell apoptosis in various systems^{15,16,17,18}. Thus, we set out to establish stable cell lines overexpressing DR3 to study the molecular mechanisms by which antimetabolic drugs induce apoptosis. An appropriate cell model for studying DR3 overexpression can be provided by the human colon cancer cell line HT29, which was found to be DR3-deficient. Additionally, in the clinic, colon cancers are insensitive to antimetabolic drugs¹⁹.

In this article, we describe a method which allows the generation of an HT29-DR3 stable cell line through retrovirus infection. We further show how to validate the DR3 expression in these cells using western blotting and how to assess the sensitivity to antimetabolic agents using a cell viability assay and morphological observation. The cells are amplified from single clones and, thus, have the advantage of a homogeneous

genetic background. In addition, the FLAG tag on DR3 allowed for the visualization of cellular DR3 by microscopy and analysis of gene expression and protein interaction by biochemical approaches. Furthermore, the cells can be xenografted in mice to further analyze tumor progression in response to antimitotics *in vivo*¹⁴.

The HT29-DR3 cell system presented here offered an effective tool for studying the molecular mechanisms of how antimitotics kill cancer cells¹⁴. Since overexpression and knockdown cell lines are common tools for studying gene function, this protocol can be adapted easily to other genes of interest or other cell lines and, thus, turned into an extensively used approach.

Protocol

Please note that all the mouse experiments described here were approved and performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at Tsinghua University.

1. Generation of DR3 Overexpression Cell Lines

1. Construct the plasmid for DR3 expression (pMXs-IRES-DR3-FLAG) by inserting full-length DR3 cDNA with 3x FLAG at the C-terminus into retroviral vector pMXs-IRES-Blasticidin at the restriction sites of BamHI/XhoI.
2. Grow Plat-A cells in 60 mm dishes with 4 mL of Dulbecco's modified Eagle's medium (DMEM). The next day, when the cells reach 80% - 90% confluence, transfect the cells with 2 µg of plasmid pMXs-IRES-DR3-FLAG using transfection reagent (see **Table of Materials**) according to the manufacturer's manual²⁰.
3. After 72 h, collect the supernatant, filtrate the media using a 0.45 µm sterile filter, and keep the viral suspension in the dark at 4 °C.
4. Grow HT29 cells in DMEM in a 60 mm dish and incubate the cells at 37 °C with 5% CO₂.
5. The next day, when the cells reach 30% - 50% confluence, infect the cells with 2 mL of viral suspension in the presence of 8 µg of 1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide per milliliter of viral suspension. After 4 - 6 h of incubation at 37 °C, aspirate the viral suspension, add 4 mL of fresh DMEM, and return the dish to the incubator.
6. Discard the media from the dishes 24 h postinfection and carefully wash cells with 2 mL of prewarmed PBS. Add 1 mL of trypsin-EDTA to the cells and incubate at 37 °C for 3 min.
7. After observing under a microscope at 10X magnification that most of the cells have detached, stop the trypsinization with 2 mL of complete DMEM containing 10% fetal bovine serum (FBS). Collect the cell suspension in a 15 mL tube and centrifuge the cells at 200 x g for 5 min at room temperature (RT).
8. Remove the supernatant and resuspend the cell pellet in 10 mL of DMEM. Mix well by gently pipetting up and down. Dilute the cells by factors of 30, 100, and 300. Seed the cells in 150 mm dishes with 20 mL of DMEM containing 1 µg/mL blasticidin. Incubate at a 37 °C incubator with 5% CO₂ for ~1 - 2 weeks.
9. During this period, observe the colonies every day with an inverted microscope at 10X magnification. Mark the well-isolated colonies at the bottom of the dish when the colony diameter is about 1 - 2 mm.
10. Aspirate the medium and wash the cells with 3 mL of pre-warmed PBS. Add 2 milliliters of trypsin-EDTA in a 60 mm dish. Use sterile forceps to pick up autoclaved sterile cloning cylinders, and place them in the 60 mm dish. Pick up the cylinders containing trypsin-EDTA and gently place them over the marked colonies. Make sure every cloning cylinder only contains one colony and avoid contamination with nearby colonies.
11. Return the dish to the incubator for 3 minutes.
12. After 3 min, check the cells under the microscope at 10X magnification to see whether they have detached. When the cells have lifted up, add 70 µL of culture medium to each cylinder to inactivate the trypsin. Gently mix the cell suspension with a 200 µL pipette. Make sure not to move the cylinder during this process.
13. Transfer the cell suspension from each colony to two 24-well plates containing 1 mL of DMEM per well. Add 30 µL of cell suspension per well in plate A and 70 µL in the corresponding wells in plate B. Label the plates properly and put them back in the incubator.

2. Verification of the DR3 Overexpression Cell Line

1. When the cells in plate B are at 90% confluence, remove the media and carefully wash the cells with 1 mL of PBS. Completely remove PBS and lyse the cells with 50 µL of 1x SDS-PAGE loading buffer. Transfer the cell lysates from the 24-well plate to 1.5 mL centrifuge tubes and boil the samples for 10 min at 100 °C.
2. Run the samples on 10% sodium dodecyl sulfate (SDS) gel at a constant voltage of 80 V for 15 min and, then, at 120 V for 1 h.
3. Transfer the protein to a polyvinylidene fluoride (PVDF) membrane by wet transfer at a constant current of 400 mA for 2 h.
4. Test the DR3 expression in different clones with anti-FLAG antibody, using the wild-type HT29 cells as a negative control (**Figure 1**). Dilute the primary antibody by a factor of 5,000 in 5% nonfat milk that is dissolved in TBST (Tris-buffered saline containing 0.1% Tween-20). Incubate the membrane with FLAG antibody at 4 °C overnight.
5. Wash the membrane with TBST by shaking it on a decoloring shaker at 200 rpm at RT. Refresh the TBST every 5 min for a total washing time of 15 min.
6. Incubate the membrane with anti-mouse secondary antibody diluted by a factor of 10,000 in 5% nonfat milk at 4 °C for 5-6 h.
7. Wash the membrane again as described in step 2.5.
8. Detect the FLAG expression using western enhanced chemiluminescence (ECL) substrate and image the membrane with a gel documentation system (see **Table of Materials**). Identify the clones expressing DR3 as the positive clones.
9. Transfer the clones in plate A that correspond to positive clones in plate B to a 6-well plate and continue to amplify the cells until growing them in 10 cm dishes. Make frozen stocks of the cells growing from positive clones and store them at -80 °C for future use.

3. Assessment of the Apoptotic Response of Cells to Antimitotic Agents

NOTE: Diazonamide is a new class of marine natural product that has remarkable activity in inhibiting cancer cell growth, which mirrors other tubulin-destabilizing agents²¹. However, its mode of action remains unclear. To test the cellular response of cells to antimitotic agents, paclitaxel and diazonamide were used to treat wild-type HT29 cells and HT29-DR3 cells. The drugs were dissolved in dimethyl sulfoxide (DMSO) to make 10 mM stocks and, then, further diluted to different concentrations: 30 nM, 100 nM, 300 nM, 1,000 nM, 3,000 nM, and 10,000 nM in DMSO.

1. Collect HT29 and HT29-DR3 cells by trypsinization. Measure the cell density using an automatic cell counter. Then, seed the cells in 12-well plates in DMEM at a density of 3×10^4 cells per well. The next day, add 10 nM diazonamide and use 1% DMSO as a negative control. After 48 h of treatment, image the cells under a microscope at 10X magnification (**Figure 2**).
2. Seed HT29 and HT29-DR3 cells in 96-well plates in DMEM at a density of 3×10^3 cells per well and allow them to grow overnight at 37 °C. Then, add dose-escalating diazonamide concentrations of 0.3 nM, 1 nM, 3 nM, 10 nM, 30 nM, and 100 nM to each well, using 1% DMSO as a negative control.
3. After 48 h of treatment, measure the cell viability using a luminescence-based cell viability assay kit according to the manufacturer's manual.
 1. Take out the 96-well plate from the incubator and let it stand at RT for approximately 30 min. Then, add 50 μ L of assay reagents to each well, shake the plate at RT for 2 min to lyse the cells, and incubate the plate at RT for another 10 min. Determine the luminescence of each well using a microplate reader (**Figure 3**).

NOTE: The cell viability represents the relative luminescence intensity of each well to that of the control well treated with 1% DMSO.

4. Use six-weeks-old female BALB/c nude mice for an *in vivo* study¹⁴.

NOTE: The mice were maintained under specific pathogen-free conditions.

1. Trypsinize HT29 and HT29-DR3 cell and, then, stop the trypsinization using DMEM prewarmed at 37 °C. Collect the cell suspension in a 50 mL tube and centrifuge the cells at 1,000 rpm for 10 min at RT. Discard the supernatant and resuspend the cell pellet in DMEM medium without FBS. Centrifuge the cells again and resuspend the cell pellet in PBS.
2. Count the cells using an automatic cell counter and dilute the cells to a concentration of 2.5×10^7 cells/mL using PBS. Generate tumors through a subcutaneous injection of 200 μ L of the HT29 or HT29-DR3 cell suspension in the right flank of each mouse (5×10^6 cells/mouse)²².
3. Measure the tumor volume (TV) using calipers every 2 d after the transplantation. Calculate the TV using the following formula: $TV = 1/2L \times W \times W$ (here, W: tumor width, L: tumor length). When the average tumor volume reaches about 100 mm³, randomize the mice into either the control or the treated group ($n = 6$ per group) and initiate the treatment.
4. Prepare the paclitaxel solution as follows. First, dissolve paclitaxel completely in ethanol at 5% of the total volume and, then, add 5% of the final volume of polyoxyl 35 hydrogenated castor oil. Finally, make up the remaining 90% of the volume with D5W (5% dextrose in water, pH 7.4).
5. Inject the paclitaxel intravenously at a dose of 20 mg/kg 3x per week for two weeks. Measure the tumor volume and body weight 3x a week¹⁴. Terminate the experiment and euthanize the animals if the body weight decreases by 20% or the tumor volume reaches about 2,000 mm³.

Representative Results

A characterization of HT29 cells stably expressing DR3 is shown in **Figure 1**. The clones express varying levels of DR3, while the wild-type cells, which serve as a negative control, do not show exogenous gene expression. Here we only show five clones, among which clone 1 and 5 express the highest level of DR3. We chose clone 1 and 5 for the following experiments.

The morphology of HT29 and HT29-DR3 cells after the administration of diazonamide was observed by an inverted microscope at 10X magnification in **Figure 2**. After a 48 h treatment with 10 nM diazonamide, HT29-DR3 showed obvious apoptosis, with a broken cell membrane and cell debris (bottom panel). However, HT29 only showed mitotic arrest with intact cells exhibiting a round-up cell shape (top panel).

The cell viability of HT29 and HT29-DR3 cells after treatment with diazonamide is shown in **Figure 3**. After 48 h of treatment with 3 nM diazonamide, over 80% cell death was found in HT29-DR3 cells (red curve). However, the parental HT29 cells demonstrated only a slight response to diazonamide in the form of mitotic arrest (blue curve). Thus, the overexpression of DR3 reconstituted the diazonamide-induced apoptotic pathway in these cells.

The results for the *in vivo* tumor xenograft experiments have been shown in a previous paper¹⁴. The tumor implantation success rate for both HT29 and HT29-DR3 is 100%. The xenograft tumors progressed similarly in the vehicle control; however, the HT29-DR3 xenografts displayed faster tumor regression than the HT29 xenografts when treated with paclitaxel at a dose of 20 mg/kg. Our observation of a better response of HT29-DR3 tumors to paclitaxel than that of HT29 tumors *in vivo* further confirmed that an ectopic expression of DR3 renders tumor cells more sensitive to antimitotics.

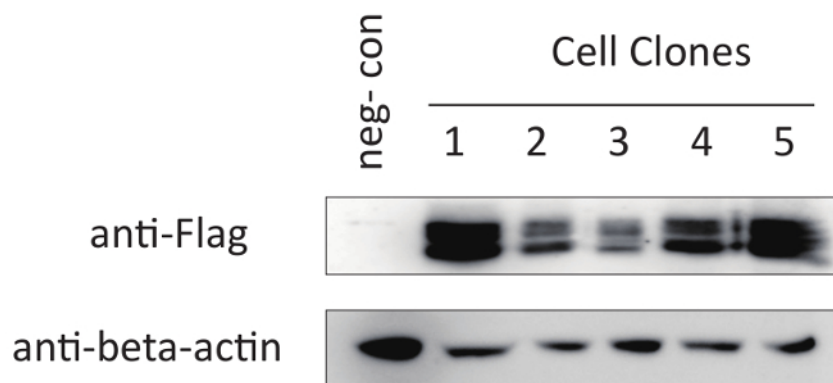


Figure 1: Analysis of DR3 expression in different clones. DR3 expression levels were analyzed by western blotting with anti-FLAG (top panel) and anti-beta-actin antibodies (bottom panel, as an internal control). Parental HT29 cells were used as a negative control. [Please click here to view a larger version of this figure.](#)

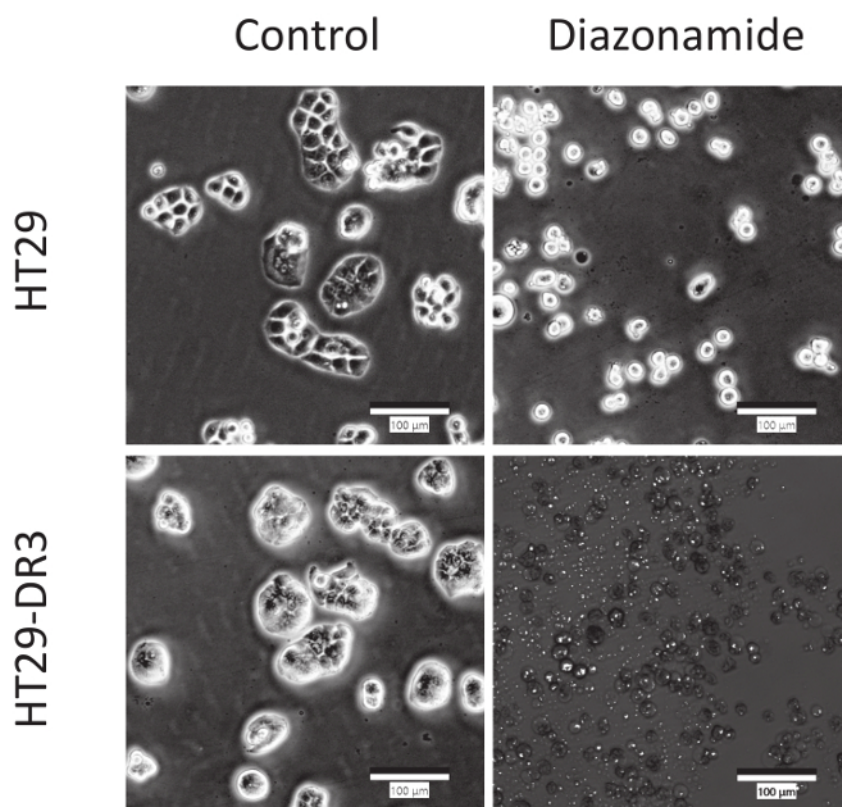


Figure 2: Morphology analysis of HT29 and HT29-DR3 cells after the treatment with diazonamide. HT29 (top panel) and HT29-DR3 (bottom panel) were treated with 10 nM diazonamide for 48 h. The scale bars = 100 μm. [Please click here to view a larger version of this figure.](#)

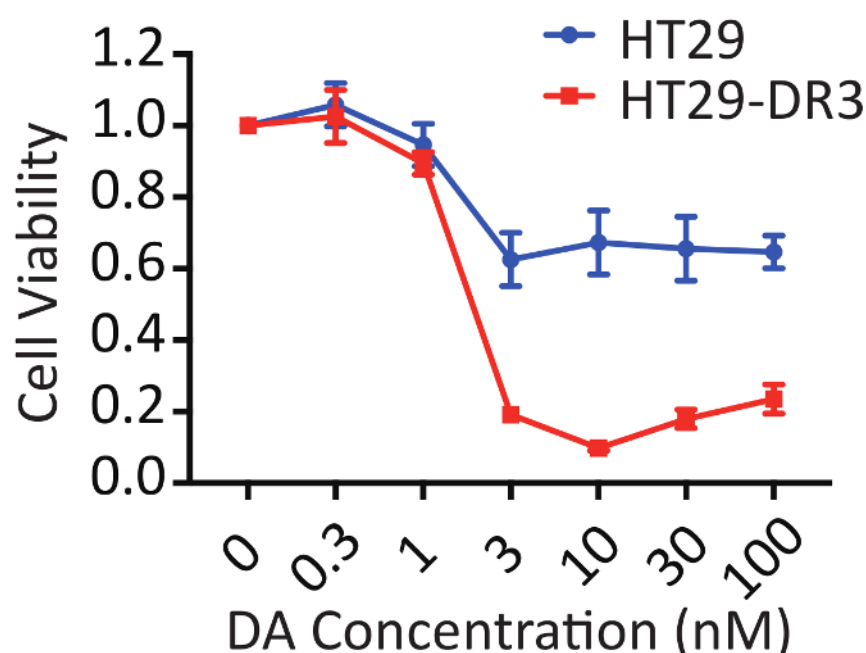


Figure 3: Dose-response curves of HT29 and HT29-DR3 cells to diazonamide. Cell viability was measured after 48 h of treatment with serial concentrations of diazonamide. The error bars = the standard deviation (SD) of experimental triplicates. DA = diazonamide. [Please click here to view a larger version of this figure.](#)

Discussion

In this manuscript, we describe a method to generate HT29-DR3 stable cell lines. HT29-DR3 cells provide a model for studying the molecular mechanisms by which DR3 contributes to apoptosis induced by antimetabolic agents. This approach is versatile and repeatable. To ensure the success of the procedure, four key steps of the protocol need to be considered. First, in order to produce a high enough virus titer, it is recommended to perform DNA transfection when the Plat-A cells are at 80% - 90% confluence. Second, the viral suspension should be stored at 4 °C for no longer than 2 weeks, covered from light. Third, when dividing the infected cells into 150 mm dishes, it is recommended to do serial dilutions to get well-separated colonies, as the infection efficiency varies in different cells. Finally, when picking up a single colony, there should not be any contamination with the surrounding colonies.

This protocol works well with most cell lines but can potentially be difficult for cells that do not tend to form single colonies. In such cases, other cell-sorting techniques, such as antibiotics combined with fluorescence-activated cell sorting (FACS), will be needed.

Stable transfection includes both viral and non-viral methods. In this protocol, we used a retroviral infection to generate cells overexpressing DR3, reasoning that physical methods, such as electroporation, are more toxic to cells²³ and chemical methods, such as lipid-mediated DNA-transfection, have a low efficiency in many cell types and have potential off-target effects^{24,25,26}.

Ectopic expression is a direct and efficient way to elucidate gene functions. Therefore, this protocol can be used to study other target molecules. Furthermore, gene knockdown is also important in functional studies, and it is possible that the protocol can be adapted to gene knockdown systems. Compared to other gene knock-in and knock-out approaches, such as CRISPR-Cas9, the protocol presented here is easy to apply and affordable for all laboratories.

Disclosures

The authors have nothing to declare.

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