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

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Dear Editor:

Enclosed please find our manuscript entitled “*Establishment of DR3 Overexpression Cell Line to Assess the Apoptotic Response to Anti-mitotic Therapeutics*” that we are now submitting for your consideration for publication in **JOVE** as a method article.

Gain-of-function is one of the direct and important manipulations to study gene function. Transient and stable transfection 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 and continuously expressed, thereby reserved for research on long-term genetic regulation and extended pharmacology research.

Here we describe a simple protocol to generate a stable cell line overexpressing tagged death receptor 3 (DR3) to explore DR3 function. We picked up single clones after retroviral infection in order to maintain homogeneity and purity of the stable cell line. The stable cell line generated using this protocol rendered DR3-deficient HT29 cells sensitive to anti-mitotic drugs, thus reconstituted the apoptotic response in HT29 cells. Moreover, the Flag-tag on DR3 compensated the unavailability of good DR3 antibody and facilitated the biochemical study of the molecular mechanism by which anti-mitotic agents induce apoptosis.

We hope that our manuscript will make it interesting to general readers of **JOVE**,

and your kind consideration will be appreciated.

Sincerely yours,

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TITLE:

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

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KEYWORDS:

Stable cell line, single colony, retroviral infection, gain-of-function, paclitaxel, antimitotic, colon cancer, apoptosis, death receptor 3

SUMMARY:

Establishing a stable cell line overexpressing a gene of interest to study gene function can be done by stable transfection—picking single clones after transfecting them *via* retroviral infection. Here we show that HT29-DR3 cell lines generated in this way elucidate the mechanisms by which death receptor 3 (DR3) contributes to antimitotics-induced apoptosis.

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 antimitotic 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 antimitotic agents induce apoptosis.

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 efficiency⁴⁻⁷. 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, antimitotic agents are the primary treatments for many types of tumors⁸. However, some significant limitations of antimitotic 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 effects⁸. 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 antimitotics differently in an unpredictable manner. There may be a key effector molecule that affects the sensitivity of different patients to antimitotic agents, resulting in the diverse outcomes seen in clinical treatment¹². Thus, the potential differential sensitivities of patients to antimitotic 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 antimitotic 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 systems¹⁵⁻¹⁸. Thus, we set out to establish stable cell lines overexpressing DR3 to study the molecular mechanisms by which antimitotic 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 antimitotic 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 antimitotic 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

1.1 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.

1.2 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.

1.3 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.

1.4 Grow HT29 cells in DMEM in a 60 mm dish and incubate the cells at 37 °C with 5% CO₂.

1.5 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.

1.6 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.

1.7 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).

1.8 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.

1.9 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.

1.10 Aspirate the medium and wash the cells with 3 mL of prewarmed PBS. Pick up autoclaved sterile cloning cylinders (diameter x height : 8 x 8 mm) with sterile forceps and gently place them over the marked colonies. Make sure every cloning cylinder only contains one colony and avoid contamination with nearby colonies.

1.11 Add 30 µL of trypsin-EDTA to each cloning cylinder and put the dish back in the incubator for 3 min.

1.12 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.

1.13 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

2.1 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.

2.2 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.

2.3 Transfer the protein to a polyvinylidene fluoride (PVDF) membrane by wet transfer at a constant current of 400 mA for 2 h.

2.4 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.

2.5 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.

2.6 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.

2.7 Wash the membrane again as described in step 2.5.

2.8 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.

2.9 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.

3.1 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**).

3.2 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.

3.3 After 48 h of treatment, measure the cell viability using a luminescence-based cell viability assay kit according to the manufacturer's manual.

3.3.1 Take out the 96-well plate from the incubator and let it stand at RT for approximately 30 min. Then, add 50 µL 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.

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

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

3.4.1 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.

3.4.2 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.

3.4.3 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.

3.4.4 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).

3.4.5 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 LEGENDS:

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.

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 μ m.

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.

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 antimitotic 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 effects²⁴⁻²⁶.

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.

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DISCLOSURES:

The authors have nothing to declare.

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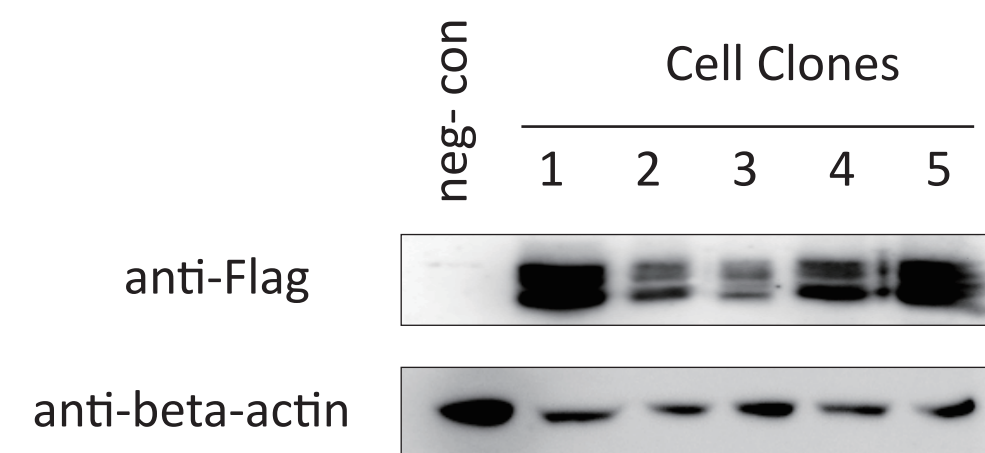
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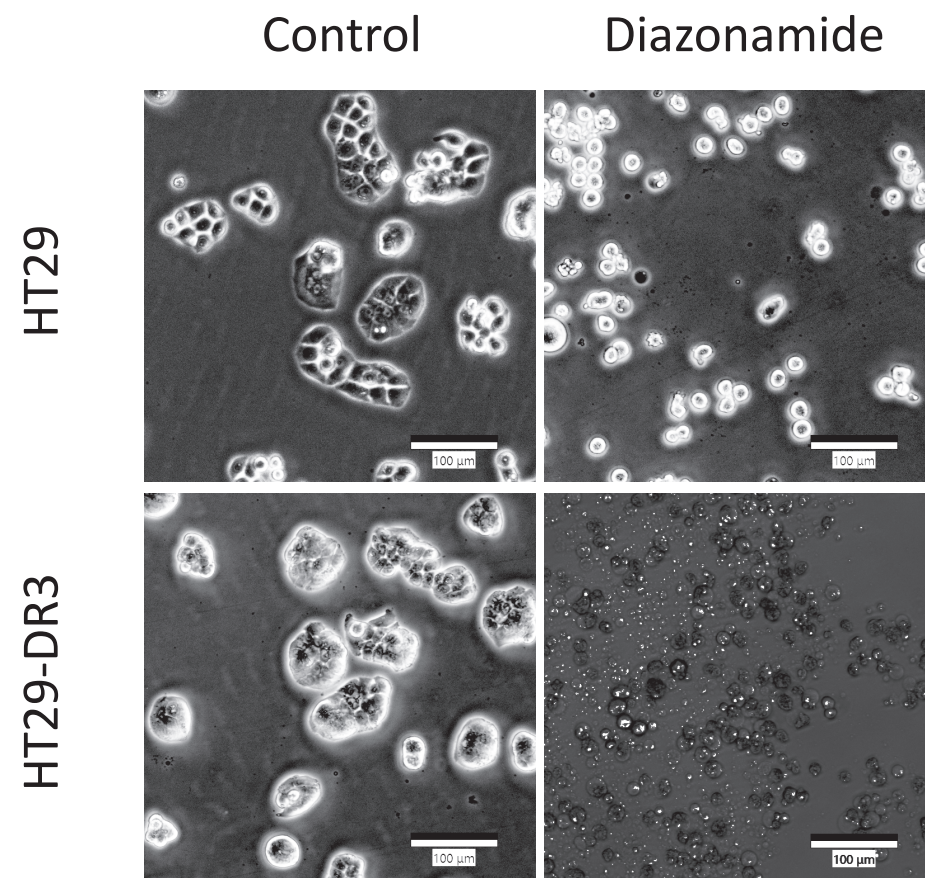
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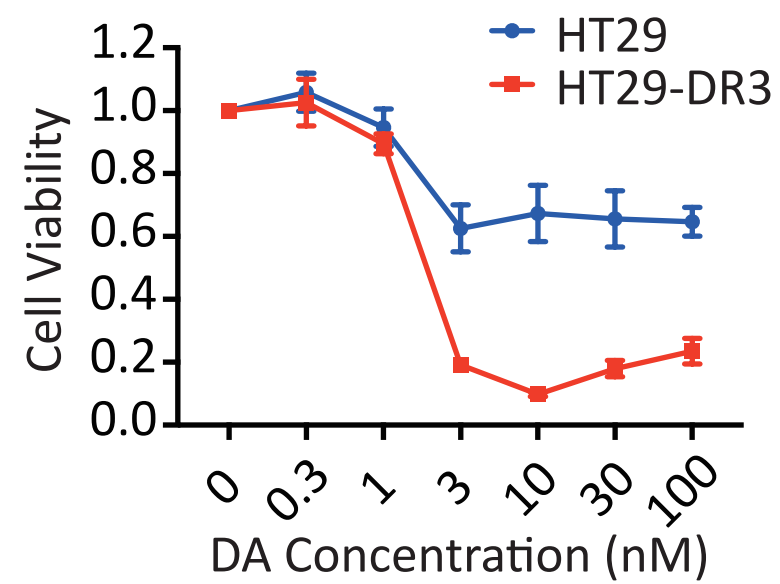
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| Name of the Reagent/Equipment | Company | Catalogue number |
|---------------------------------------|---------------|---------------------|
| DMEM | Invitrogen | C11965500BT |
| Luminescent Cell Viability Assay | Promega | G7571 |
| Paclitaxel | Selleck | S1150 |
| pMXs-IRES-Blasicidin | Cell Biolabs | RTV-016 |
| Dimethyl sulfoxide | Sigma | D2650 |
| PBS | Invitrogen | C14190500BT |
| Trypsin-EDTA (0.25%) | Invitrogen | 25200056 |
| 1,5-Dimethyl-1,5-diazaundecamethylene | Santa Cruz | sc-134220 |
| Transfection Reagent | Promega | E2311 |
| Anti-mouse secondary antibody | | |
| anti-Flag antibody | Sigma | F-3165 |
| HT29 cell line | ATCC | HTB-38 |
| plat-A cell line | Cell Biolabs | RV-102 |
| PVDF Immobilon-P | Millipore | IPVH00010 |
| Cloning Cylinder | Sigma | C1059 |
| Cell Imaging Multi-Mode Reader | Biotek | BTCYT3MV |
| CKX53 Inverted Microscope | Olympus | CKX53 |
| 12-well cell culture plates | Nest | 712001 |
| 60 mm cell culture plates | Nest | 705001 |
| 15 mL centrifuge tubes | Nest | 601052 |
| 0.22 µm steril filter | Millipore | SLGP033RB |
| Centrifuge 5810 R | Eppendorf | 5810000327 |
| 96 Well White Polystyrene Microplate | Corning | 3903 |
| Western ECL Substrate | BIO-RAD | 1705060 |
| ImageQuant LAS 4000 | GE Healthcare | ImageQuant LAS 4000 |

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| Decoloring Shaker | HINOTECH | TS-2000A |
| Blasticidin | InvivoGen | ant-bl-1 |
| Automated cell counter | BIO-RAD | TC 20 |
| Opti-MEM I Reduced Serum Medium | Thermo Fisher | 31985070 |

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Article Title:

Establishment of DR3 Overexpression Cell Line to Assess the Apoptotic Response to Anti-mitotic Therapeutics

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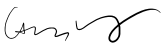
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Response Letter

Dear editor,

We would like to thank you and the reviewers for your efforts in evaluating our manuscript entitled “Establishment of DR3 Overexpression Cell Line to Assess the Apoptotic Response to Anti-mitotic Therapeutics” (JOVE-58705).

We were grateful for the constructive comments by all the reviewers. Below please see additional data and the point-by-point response to editorial and reviewers’ comments. We have also made changes in the manuscript accordingly and highlighted the major changes.

We hope that you will find the revised manuscript acceptable for publication in *JOVE*.

Sincerely yours,

Gelin Wang, Principal Investigator
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Below is the point-by-point response to editor’s and reviewers’ comments.

Editorial Comments:

We appreciate the editorial comments.

Textual Overlap: We re-wrote the indicated text in red.

Abstracts: The short abstract was added before the long abstract as following: Cell models for gene overexpression are important tools for studying gene function. Picking single clones after retroviral infection is a direct way to generate homogeneous and pure stable cell lines. In this article, HT29-DR3 cell lines generated in this way help reveal the mechanisms by which DR3 contributes to anti-mitotics-induced apoptosis.

Protocol Language: we checked the language and wrote the protocol section in imperative tense.

Protocol Detail: We added protocol details and split 3.4 to 4 sub steps to depict the animal experiments.

Protocol Highlight: We highlighted about 2 pages of text that should be visualized in yellow.

Figures: Figures have been renewed as following:

Figure 2

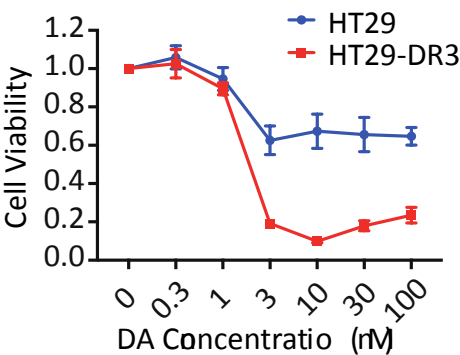
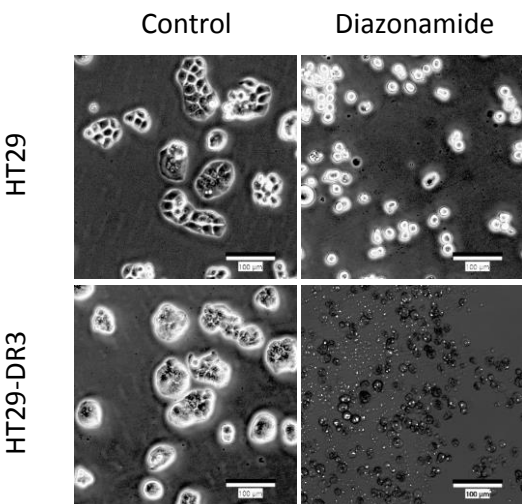


Figure 3



These data have not been published.

Commercial Language: All commercial products have been sufficiently referenced in the table of materials. We replaced the commercial sounding language with generic names.

Responses to Reviewer #1:

Reviewer #1:

Minor Concerns: Although this looks like a promising study, the authors have just briefly provided their results and they have mostly regarded their figures. I would recommend them to emphasize their important results in the text, besides figures and tables. They should compare the efficacy of their method with a conventional one.

Thanks for the constructive suggestions, we elaborate the data in Results section. As Figure 1 shows, different clones express variant levels of DR3 after infection, picking single clones ensure gene stability and homogeneity. In addition, pool of the cells probably could not reflect gene function significantly. By comparison with a conventional approach, such as limited dilution, this approach is simple and efficient.

Responses to Reviewer #2:

Reviewer #2:

Manuscript Summary:

The manuscript described a procedure on how to establish a stable cell line with DR3 overexpression. This cell line can then be used as a cell line model to study anti-mitotic drugs. Overall, the experimental procedures were clearly written and the reagents were also listed.

We are thankful for the comments from the reviewer.

Major Concerns:

1) In the introduction, more references are required to better justify the importance of establishing DR3 overexpressed cell line.

DR3 is a totally novel molecule that discovered to be involved in anti-mitotic drugs induced apoptosis. Gain-of function is classical and direct way to study gene function. Therefore, overexpression cell line is the essential model to identify the molecular mechanisms of DR3 mediated apoptosis after treatment with anti-mitotic agents. As requested, we added references (Reference 10-13) to justify the importance of establishing DR3 overexpressed cell line.

2) Step 2.3, line 132, usually the semi dry transfer wouldn't need hours for a complete transfer. The authors claimed the transfer was performed at 400 mA for 2 hrs. The authors should double check the experimental condition to ensure the accuracy.

Step 2.3, To ensure sufficient transfer of protein of various sizes, we usually transfer the protein onto PVDF membrane by Wet Transfer at constant current of 400 mA for 2 h.

Minor Concerns:

- 1) In the abstract, line 22, "picked up" should be replaced with "picked".*
- 2) In table 1, "invitrogene" should be changed to "Invitrogen".*

We replaced “picked up” with “picked” in line 22, and the "invitrogene" with "Invitrogen" in table 1.

Dear Dr. Dsouza,

We would like to thank you for your effort in reviewing our manuscript entitled “Establishing of Cell Lines Overexpressing DR3 to Assess the Apoptotic Response to Anti-mitotic Therapeutics” (JOVE-58705).

We are grateful for your comments that has helped us make a better revision. Below please see the point-by-point response to your editorial comments. We have also made changes in the manuscript accordingly and highlighted the major changes.

We hope that you will find the revised manuscript acceptable for publication in *JOVE*.

Sincerely yours,

Gelin Wang, Principal Investigator
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Tsinghua University
Beijing 100084, China
Tel: (86) 10-62798919
E-mail: gelinwang@tsinghua.edu.cn

Below is the point-by-point response to the editor's comments.

1 About the grammatical errors: A native English speaker has thoroughly revised the manuscript for grammatical errors.

2 About the license agreement: The license agreement has been signed and uploaded.

3 About the reference: We added the references as requested.

4 In vivo experiment: We did test the response of HT29 and HT29-DR3 xenograft models to paclitaxel in our previous work, and the results are referred to *Qi, et al. 2018* (reference 14).

5 About the molecular mechanisms: The HT29-DR3 cells system offered us an important tool to study the mechanisms by which anti-mitotic drugs induce apoptosis.

6 About the MOI: We did not measure the MOI in this protocol.

7 The note after step 1.6 was changed to step 1.7.

8 The size of the cloning cylinder: The diameter and height of the cylinder are both 8 mm.

9 Step 1.1: The details of constructing pMXs-IRES-Blasticidin-DR3 were added.

10 Step 1.2: The transfection procedure and the reference were added.

11 Step 1.6: The information of blasticidin has been added to Table of Materials.

12 Step 1.9: To inactivate trypsin, add 70 μ L DMEM to each cylinder.

13 Step 2.1: The cell lysates are transferred from the 24-well plate to 1.5 mL tubes before boiling.

14 Step 2.6: The information of anti-mouse secondary antibody was added to Table of Materials.

15 add Step 2.9: The positive clones are frozen and stored at -80 $^{\circ}$ C.

16 The note before Step 3.1: The drugs were dissolved in DMSO to make 10 mM stocks and then further diluted to different concentrations: 30 nM, 100 nM, 300 nM, 1000 nM, 3000 nM and 10000 nM in DMSO.

17 Step 3.1: The cells were counted using an automatic cell counter (Table of Materials). The cell morphology was observed under a microscope at 10X magnification.

18 Step 3.3: The cell viability represents the relative luminescence intensity of each well to that of the control well treated with 1% DMSO.

19 Please add results to demonstrate the outcomes from the in vivo paclitaxel treatment. What was the tumor implantation success rate for HT29 and HT29-DR3? What are the observations from the paclitaxel treatment in these groups? Please discuss the significance.

Instead we only add a note for the in vivo paclitaxel treatment, as the results have already been published in our previous paper (Qi et al., 2018). The tumor implantation success rate for both HT29 and HT29-DR3 are 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 ectopic expression of DR3 renders tumor cells more sensitive to anti-mitotics.

21 Step 3.4.1: The procedures how to establish xenograft models were described in detail and the reference was added.

22 Step 3.4.2: The tumor volumes were measured by calipers.

23 Step 3.4.4: In the animal experiments, the dose of paclitaxel was 20 mg/kg. We euthanized the animals after injection of paclitaxel for two weeks, otherwise, terminate the experiment and euthanize the animals if the body weight decreases by 20% or the tumor volumes reach 2000 mm³.

24 We added our funding sources in the Acknowledgements part.