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Cell-based expression cloning for identification of polypeptides that hypersensitize mammalian cells to mitotic arrest

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ABSTRACT

Microtubule inhibitors such as Vinblastine and Paclitaxel are chemotherapy agents that activate the mitotic spindle checkpoint, arresting cells in mitosis and leading to cell death. The pathways that connect mitotic arrest to cell death are not well characterized. We developed a mammalian cell-based cDNA cloning method to isolate proteins and protein fragments whose expression inhibits colony formation in the presence of microtubule inhibitors. Understanding how these proteins impact cellular responses to microtubule drugs will lead to better understanding of the biochemical pathways connecting mitotic arrest and cell death in mammalian cells and may provide novel targets that can enhance microtubule inhibitor-mediated chemotherapy.

INTRODUCTION

Microtubule inhibitors such as Taxanes (e.g. Taxol) and Vinca Alkaloids (e.g. Vinblastine, Vincristine) are commonly used chemotherapy drugs (1-3). In clinically relevant doses (e.g. 5-200nM for Taxol (4)), they perturb normal microtubule dynamics by hyperstabilization or by destabilization of microtubules and activate the mitotic spindle checkpoint (5-7). The spindle checkpoint causes extended mitotic arrest. Cell death ensues either during the mitotic arrest or after cells exit mitosis without normal chromosome segregation (sometimes called adaptation or mitotic slippage) (4, 8). The signal transduction pathways by which microtubule inhibitors and other mitotic inhibitors lead to cell death remain to be clarified (8, 9).

In spite of the apparent linkage, correlation between the function of the spindle checkpoint and microtubule inhibitor-mediated cell death remains elusive. Spindle checkpoint proteins are essential for survival in mammalian cells (10-12), thus researchers have used either cancer cells with weakened checkpoint function or cultured cells with conditional expression of mutant checkpoint proteins or siRNA to investigate the correlation. Some cancer cells with weakened spindle checkpoint function were reported to show elevated sensitivity to microtubule inhibitors (13). Other studies have shown that cells with partial loss of the checkpoint function are resistant to microtubule destabilizing drugs such as nocodazole but not to microtubule stabilizing drugs such as Taxol. These data suggest that a partial loss of spindle checkpoint may decrease cell death, and there are differences in cellular response dependent on the type of microtubule challenge (14).

Most of the proteins that participate in the central pathway of the spindle checkpoint were identified in genetic screens in budding yeast. Recent studies with mammalian cells suggest that proteins typically associated with the spindle checkpoint signalling pathway also have additional functions or are regulated

via distinct pathways. Mice haploinsufficient in BubR1 (15) or in both Bub3 and Rae1 (16) show premature aging-associated phenotypes, associated with cellular senescence involving the p53 and p16 pathways. After prolonged mitotic arrest of certain mammalian cell lines by microtubule drugs, Bub1 and BubR1 are degraded by caspases thus inducing exit from M phase without mitosis (17, 18). These examples reveal signalling pathways that may not be represented in yeast. Our goal was to identify additional proteins and biochemical pathways that modulate cellular responses to activation of the mitotic spindle checkpoint in mammalian cells. With this, we designed a mammalian cell-based cDNA screening method to identify modulators of the spindle checkpoint and subsequent apoptosis and/or senescence pathways. In particular we hoped to identify proteins that participated in pathways connecting cell death with microtubule drug challenge. The identification of these proteins would fill a significant gap in our current knowledge relating the mitotic spindle checkpoint and cell death, and potentially point to novel targets for cancer chemotherapy.

RESULTS AND DISCUSSION

The protocol consists of two separate steps; cDNA selection and validation. The first half of the process is summarized in Figure 1. We transfected cells with a cDNA library and selected for cells that rapidly initiate apoptosis or escape mitotic arrest (mitotic slippage) when treated with microtubule inhibitor. Although mitotic slippage itself does not necessarily mean the escaped cell will die, it does suggest that expression of the exogenous cDNA has compromised normal spindle checkpoint function. To be detected in our screen, expression of the cDNA might affect the microtubule inhibitor-mediated cell death/senescence pathway either in a dominant or dominant-negative manner.

The cDNA expression cloning method took advantage of the fact that mammalian cultured cell lines (e.g. HeLa, COS7) change their morphology and adhesiveness during mitosis and apoptosis. During interphase the cells adhere tightly to culture substrate. In mitosis, cells of most lines round up and can be detached easily by mechanical agitation. At mitotic exit, cells reattach and flatten on the substrate. We noted that mitotic cells undergoing apoptosis also exhibited increased adherence to the culture substrate compared with healthy mitotic cells, although the adherence is weaker than that of healthy interphase cells. This allowed us to select for cells containing plasmids whose expression caused increased mitotic exit and/or apoptosis in cells arrested in mitosis with microtubule inhibitors (Fig. 1). Weaver and Cleveland (2005) categorized possible outcomes after antimitotic drug treatment to five phenotypes; (a) chronic mitotic arrest, (b) mitotic death, (c) survival after mitotic exit without growth (senescence) , (d) survival after mitotic exit with continuing growth, and (e) cell death after mitotic exit (8). Our selection step would capture phenotypes (b), (c), (d) and (e), whereas our validation step (described later) would select against cDNAs that cause phenotype (d), leaving cDNAs that may be involved in the microtubule inhibitor-mediated cell death or senescence pathways.

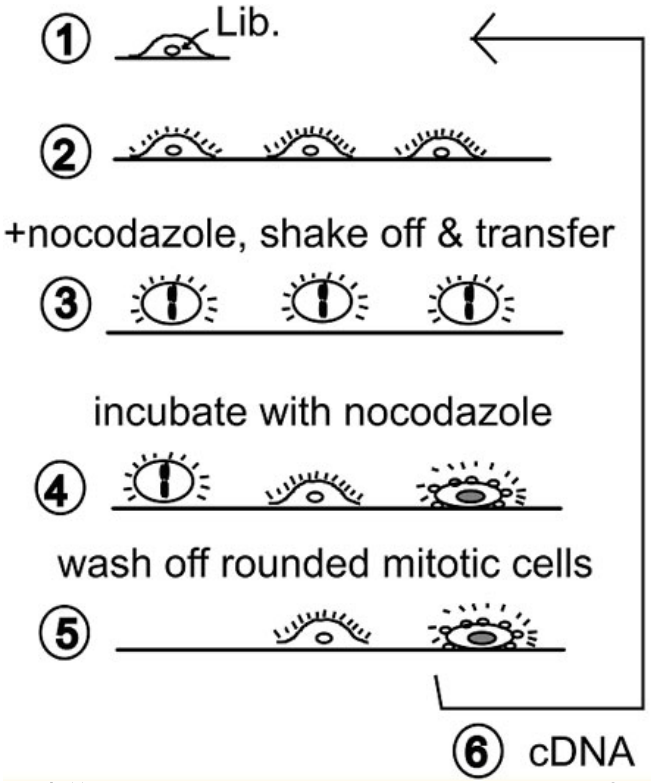

Fig. 1: Mammalian cell-based cDNA expression cloning protocol for cells that adhere to the substrate after overriding the mitotic spindle checkpoint or induction of apoptosis in the continued presence of microtubule inhibitor. See text for detailed description.

We transfected COS7 cells with a commercial human cDNA library contructed with pEXP1 plasmid (Clontech) (Step 1). We then allowed expression for 12-24 hours (Step 2). This time was determined to be optimal for reducing integration of the plasmid DNA into the host

genome thus facilitating subsequent recovery of the plasmid from the mammalian cells. The cells were rinsed extensively to remove untransfected plasmid and dead or dying cells. The cultures were then treated with microtubule inhibitor nocodazole (100ng/ml) to induce mitotic arrest. COS7 cells have a robust spindle checkpoint and, in the presence of microtubule inhibitor, remain arrested in mitosis for at least 18 hours. One hour after the initial treatment with nocodazole and every hour afterward for 13 additional hours we gently dislodged the rounded mitotic cells and incubated them on new plates in medium with nocodazole for 2 to 14 hours (Step 3). In this manner we collected cells as they entered mitosis thus avoiding cells in which expression of the transfected insert led to cell cycle arrest elsewhere during the cell cycle. One of three outcomes occurs during continued incubation in nocodazole: (i) cells remain arrested in mitosis and stay rounded, (ii) cells exit mitosis, flatten, and attach to the plate, and (iii) cells initiate apoptosis and adhere to the plate (Step 4). We washed the plates with PBS to remove rounded mitotic cells (Step 5). We added DNAzol reagent (Invitrogen) to the plates to recover the plasmids from the remaining adherent cells. Smaller sized DNA including episomic plasmid was recovered after ethanol precipitation. We used the recovered DNA to transform high-efficiency competent bacteria and recovered the plasmids as bacterial colonies (Step 6). In the first round of all six steps, starting from approximately three million COS7 transfectants, we obtained several hundred bacterial colonies and recovered plasmids from the bacterial colonies. We pooled the recovered plasmids and repeated the transfection of COS7 cells and selection protocol once more to enrich for plasmids encoding proteins that reliably induce mitotic exit or apoptosis in the presence of nocodazole (modified "cycle cloning": 19-21). In the second round, starting from approximately one million COS7 transfectants, we obtained 58 bacterial colonies. We recovered plasmids from individual colonies, and sequenced the inserts. Of these, 34 returned interpretable data. Seven plasmids appeared to have an altered structure, possibly because of recombination or deletion events that occurred during the process.

In the second half of the procedure for validation of isolated cDNAs, we established cell lines that express the cDNA stably so that we could test their microtubule inhibitor sensitivity. This step was included because the result from transient expression experiments might be affected by experimental variables such as transfection efficiency. By establishing stable lines we could isolate proteins whose expression was important only in cellular response to microtubule drug treatment. It eliminated cDNAs that simply expressed proteins that induced apoptosis in the absence of the microtubule drugs. In this way we biased our screen to find components of the signalling pathways linking spindle checkpoint activation and mitotic cell death in mammalian cells.

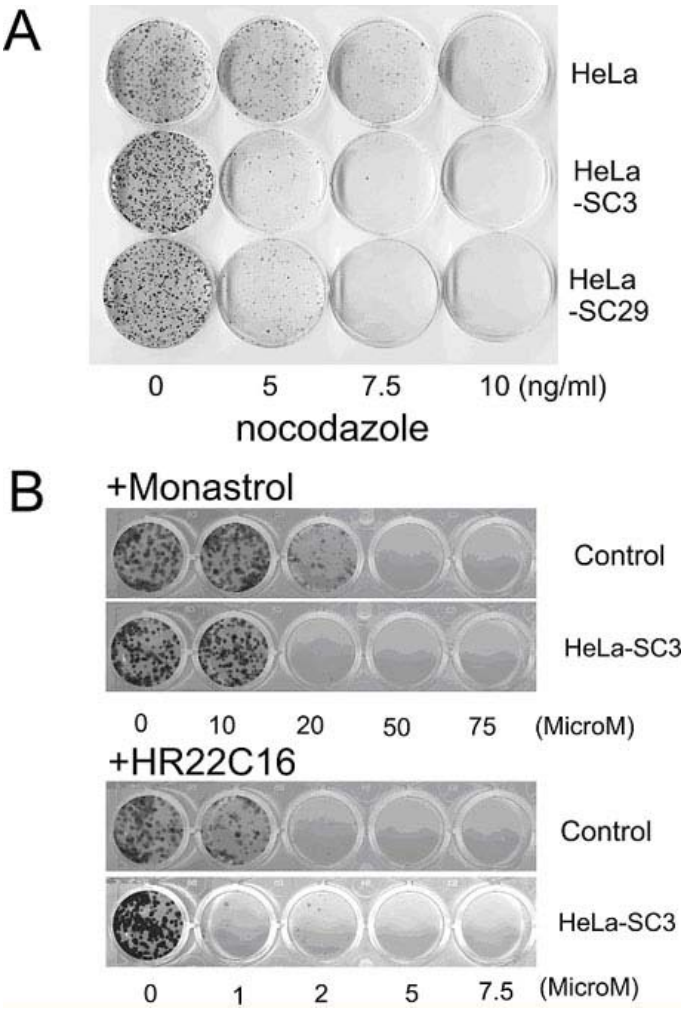

Fig. 2: (A) Elevated microtubule inhibitor sensitivity in HeLa cell derived lines stably expressing cDNA identified in the screen. The top row is the control cell line (HeLa). The second and third rows are cells stably expressing two different cDNA fragments identified in the screen (HeLa-SC3; HeLa-SC29). One thousand cells were plated on 6cm plate, treated with indicated dose of nocodazole and incubated for eight days. The medium was replenished at four days. The cells were fixed/stained with 0.5% Methylene blue in 50% Ethanol for 20 minutes, then rinsed and dried. (**B**) HeLa-SC3 cells show elevated sensitivity to Monastrol and HR22C16, kinesin inhibitors that also activate spindle checkpoint and cause mitotic arrest. Four hundred cells were incubated with indicated dose of drug for eight days and fixed/dried.

We generated lines of HeLa cells in which the inserts were stably integrated into the host cell genome, constitutively expressed under the CMV promoter, along with the puromycin resistance gene. Among 34 candidate plasmids, 20 yield stable HeLa integrants after 3 weeks of puromycin (0.2-0.5 μg/ml) selection. We tested their sensitivity to microtubule inhibitors with a colony formation assay. Seven of the twenty HeLa-based cell lines showed enhanced microtubule inhibitor sensitivity, and results of two are shown as examples (Fig. 2A). None of the seven lines showed elevated sensitivity to VM26 (teniposide), a topoisomerase II inhibitor. For control purposes we established 48 integrant cell lines from the original cDNA library without selection. None among the 48 lines showed elevated sensitivity to microtubule inhibitors. Thus the enrichment for cDNAs coding for proteins modulating cellular responses to microtubule drug treatment was significant. Enhancement of substrate attachment in the presence of nocodazole was tested for representative cell lines HeLa-SC3 and HeLa-SC29, resulting 2.5-22 times higher substrate attachment. Detailed description and characterization of one of the candidate cDNA and the integrant, shown as HeLa-SC3, is reported in ref. 22. The cDNA encodes a dominant-negative form of TRIP1, a 19S proteasome regulatory subunit.

Although microtubule inhibitors are potent anticancer drugs, they also have significant side effects such as neurotoxicity. A new generation of mitosis-targeting drugs are being developed, including drugs such as Monastrol and HR22C16 that inhibit mitotic kinesins (8, 23). As expected, HeLa-SC3 cells are also sensitive to Monastrol and HR22C16 that activate the spindle checkpoint without microtubule disruption (Fig. 2B). Thus our selection method should prove equally useful in identifying proteins that modulate cellular responses to non-microtubule, anti-mitotic drugs.

Lastly, our protocols should be equally applicable to the screening of libraries of plasmids encoding hairpin RNAs that generate siRNA inhibitors of protein expression.

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PROTOCOLS

(1) Protocol for cDNA selection

Reagents

- HeLa or COS7 cells
- Culture medium: DMEM (Dulbecco's Modified Eagle Medium) (Gibco, cat. No 11965-092) supplemented with 10% Fetal Calf Serum, 1% MEM Nonessential Amino Acid solution (Gibco), 20mM HEPES(pH7.2), 1% Penicillin-streptomycin (Gibco cat. No 15140-122), 1mM sodium pyruvate (Sigma)
- Phosphate Buffered Saline (PBS)
- FuGene6 transfection reagent (Roche)
- cDNA expression library. We used Human Testis ClonCapture cDNA Library (ClonTech)
- 15cM tissue culture plate
- 37 degree incubator (5% CO_2)
- DNAzol reagent (BibcoBRL)
- High-efficiency competent bacteria (e.g. UltraMax DH5alpha-FT (Invitrogen))
- DNA preparation kit (e.g. Qiafilter midi kit, Qiagen)
- Other common equipment and reagents for cell culture

Note: We recommend using SV40 origin-containing library with COS7 cells to allow plasmid amplification inside the host cells and to increase the chance of plasmid recovery. It is not a requirement, however.

1. Plate HeLa (or COS7) cells to three 15cM plates
2. Transfect the cells (60-70% confluent) with cDNA library using FuGene 6. Use 10-15ug DNA/plate.
3. Incubate 12-24 hours.
4. Rinse the plate with 20ml PBS/plate five times to remove dead cells and untransfected DNA.
5. Add 15ml/plate fresh medium containing nocodazole (100ng/ml). This is hour zero.
6. At hour one, shake off rounded mitotic cells, transfer the medium with round (mitotic) cells to a 15cM plate and incubate. Add fresh nocodazole containing media to the original plates. Repeat this step every hour, up to hour 14. Then discard original three plates.
7. At hour 16, rinse plates that hold transferred round cells with PBS (30ml/plate).
8. Add 30ml DNAzol reagent. Process all 14 plates with the 30ml of DNAzol.
9. (Follow DNAzol protocol for DNA recovery) Add 15ml 100% Ethanol to DNAzol solution. Centrifuge and precipitate DNA. Rinse the DNA pellet with 75% Ethanol/water twice. Dissolve the pellet in water.
10. Use the DNA to transform high-efficiency competent bacteria. We used UltraMax DH5alpha-FT (GibcoBRL).
11. When transformed colonies emerge, collect and culture the bacteria with appropriate antibiotics, then recover plasmid. The resulting plasmid should be a mixture of candidate cDNA-containing plasmids.
12. Return to the beginning of this procedure and repeat the process. Repeating the process would enrich plasmid with "positive" cDNA and reduce false positives.
13. Once you obtain bacterial transformants after the second round, pick at least 50 colonies, recover plasmids individually and sequence. Also check the integrity of the recovered plasmids by agarose gel electrophoresis. Although the screening time is adjusted to be minimal, plasmid recombination occurs frequently within mammalian cells.

(2) Protocol for secondary screen (validation step)

1. Establish integrants of candidate cDNA by selection with resistance to marker drug.

Reagents

- HeLa cells
- Culture medium: DMEM (Dulbecco's Modified Eagle Medium) (Gibco, cat. No 11965-092) supplemented with 10% Fetal Calf Serum, 1% MEM Nonessential Amino Acid solution (Gibco), 20mM HEPES (pH7.2), 1% Penicillin-streptomycin (Gibco cat. No 15140-122), 1mM sodium pyruvate (Sigma)
- Candidate cDNA plasmids, at least 1 ug for each transfection
- FuGene6 transfection reagent (Roche)
- 6-well tissue culture plate
- 37 degree incubator (5% CO_2)
- Puromycine (Sigma), or appropriate selection drug
- Other common equipment and reagents for cell culture

1. Transfect 30-50% confluent HeLa cells in 6-well plate with each candidate plasmid using FuGene6 (Example: if you have 50 candidates, you'll perform 50 independent transfections).
2. 48 hours after transfection, add appropriate selective reagent to the culture, which will select transfectants that stably express the cDNA. The cDNA library we used contained puromycin resistance under an internal ribosome entry site thus was coexpressed with the cDNA. We used 0.2-0.5 ug/ml puromycin. With successful selection, you should observe massive cell death of nontransfected cells in 5 days.
3. Replace culture medium with drug every 3 days. After 2-3 weeks, surviving colonies will emerge.
4. Pool the surviving colonies to average the effect of varying expression.

Optional: you can pick up colonies, verify and quantify cDNA expression by RT-PCR and select representative colonies. However, this option will significantly increase the labour. Given the number of the candidates to be processed, we suggest using simple protocol for now. Clone isolation, if desired, can be done after candidates for positive are isolated in next step.

2. Test nocodazole sensitivity of the transfectants and determine "positive."

We used the colony formation assay shown in Figure 2, which takes about 9 days.

Reagents

- HeLa cells and candidate integrants from above
- Culture medium: DMEM (Dulbecco's Modified Eagle Medium) (Gibco, cat. No 11965-092) supplemented with 10% Fetal Calf Serum, 1% MEM Nonessential Amino Acid solution (Gibco), 20mM HEPES (pH 7.2), 1% Penicillin-streptomycin(Gibco cat. No 15140-122), 1mM sodium pyruvate (Sigma)
- Phosphate Buffered Saline (PBS)
- 24-well tissue culture plate
- 37 degree incubator (5% CO_2)
- Nocodazole (stock: 1mg/ml in DMSO)
- 0.5% Methylene blue in 50% Ethanol/water
- Other common equipments and reagents for cell culture

1. Prepare culture for the candidate integrants along with controls.
2. Trypsinize cells to detach cells. Count cells and dilute to 500 cells/ml in 15ml.
3. Plate the diluted culture to 12 wells in 24-well plate, 1ml/well.

4. 24 hours later, add nocodazole to the culture in 6 concentrations (final concentrations; 0, 2.5, 5, 7.5, 10, 12.5 ng/ml). Use two wells for each concentration for duplicate. This is day one.
5. On day four, add 1ml medium with defined concentration of nocodazole to all wells.
6. On day eight, discard medium and add 200 ul of 0.5% Methylene blue solution to each well. Keep the plate on rocking shaker for 20 minutes.
7. Discard Methylene blue solution. Rinse wells by water twice, then dry.
8. Compare colony formation between control and the cells to be tested. Count cell lines with poor colony formation and the expressed cDNA as "positive." Optional: quantify the results using imaging analysis software.

To test taxol sensitivity, we suggest using following set of concentrations: 0, 0.5, 1, 1.25, 1.5, 2 nM.