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# Combined Conditional Knockdown and Adapted Sphere Formation Assay to Study a stemness-associated gene of Patient-derived Gastric Cancer Stem Cells

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**TITLE:**

# Combined Conditional Knockdown and Adapted Sphere Formation Assay to Study a Stemness-Associated Gene of Patient-derived Gastric Cancer Stem Cells

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**KEYWORDS:**

gastric cancer stem cell, conditional knockdown, sphere formation assay, clusterin, stemness

**ABSTRACT:**

Cancer stem cells (CSCs) are implicated in tumor initiation, development and recurrence after treatment, and have become the center of attention of many studies in the last decades. Therefore, it is important to develop methods to investigate the role of key genes involved in cancer cell stemness. Gastric cancer (GC) is one of the most common and mortal types of cancers. Gastric cancer stem cells (GCSCs) are thought to be the root of gastric cancer relapse, metastasis and drug resistance. Understanding GCSC biology is needed to advance the development of targeted therapies and eventually to reduce mortality among patients. In this protocol, we present an experimental design using a conditional knockdown system and an adapted sphere formation assay to study the effect of clusterin on the stemness of patient-derived GCSCs. The protocol can be easily adapted to study both in vitro and in vivo function of stemness-associated genes in different types of CSCs.

**INTRODUCTION:**

Gastric cancer (GC) is one of the most common and mortal types of cancers[1]. Despite advances in combined surgery, chemotherapy and radiotherapy in GC therapy, prognosis remains poor and the five-year survival rate is still very low[2]. Recurrence and metastasis are the main reasons cause the post-treatment deaths.

Cancer stem cells (CSCs) are a subset of cancer cells that possess the ability to self-renew and generate the different cell lineages that reconstitute the tumor[3]. CSCs are believed to be responsible for cancer relapse and metastasis because of their capabilities of self-renewal and seeding new tumors, as well as their resistance to traditional chemo- and radiotherapies[4]. Therefore, targeting CSCs and elimination of CSCs provide an exciting potential to improve the treatment and reduce mortality of cancer patients.

CSCs have been isolated from many types of solid tumors[5]. In 2009, gastric cancer stem cells (GCSCs) isolated from human gastric cancer cell lines were originally described by Takaishi et al.[6]. Chen and colleagues firstly identified and purified GCSCs from human gastric adenocarcinoma (GAC) tumor tissues[7]. These findings not only provide an opportunity to study the GCSC biology but also provide great clinical importance.

A particular characteristic of CSCs is their capacity to form a sphere[8]. Single cells are plated in nonadherent conditions at low density, and only the cells possessed with self-renewal can grow into a solid, spherical cluster called a sphere. Thus, the sphere formation assay has been regarded as the gold standard assay and widely used to evaluate stem cell self-renewal potential in vitro.

RNA interference (RNAi) is a powerful research tool to study gene function by the knockdown of a specific gene[9]. However, long term stable gene knockdown technologies have certain limitations, such as the challenge of exploring the function of a gene that is essential for cell survival. Conditional RNAi systems can be useful for the downregulation of desired genes in a temporal and/or special controlled manner by the administration of an inducing agent. The tetracycline (Tet)-inducible systems are one of the most widely used conditional RNAi systems[10]. The Tet-inducible systems can induce target gene silencing by controlling the expression of shRNA upon addition of an exogenous inducer (preferentially doxycycline, Dox). The Tet-inducible systems can be divided into two types: Tet-On or Tet-Off systems. The expression of shRNA can be turned on (Tet-On) or turned off (Tet-Off) in the presence of the inducer. In the Tet-ON system without an inducer, the constitutively expressed Tet repressor (TetR) binds to the Tet-responsive element (TRE) sequence containing a Tet-responsive Pol III-dependent promoter for shRNA expression, thus repressing the expression of the shRNA. While upon addition of Dox, the TetR is sequestered away from the Tet-responsive Pol III-dependent promoter. This facilitates the expression of the shRNA and leads to gene knockdown.

The protocol described here employs a functional tetracycline-inducible shRNA system and an adapted sphere formation assay to study the function of clusterin in patient-derived GCSCs. Clusterin has been identified as a novel key molecule for maintaining the stemness and survival of GCSCs in a previous study[11]. We use the described protocol to study the effects of clusterin in GCSC self-renewal. This methodology is also applicable to other types of cancer stem cells.

**PROTOCOL:**

All experimentation using patient-derived gastric cancer stem cells described herein was approved by the local ethical committee[7].

## 1. Gastric cancer stem cell culture

### 1.1. Preparation of GCSC complete culture medium

1.1.1. Prepare GCSC complete culture medium by adding fresh DME/F12 medium with the following essential ingredients: 20 ng/mL EGF, 10 ng/mL bFGF, 1% Insulin/Transferrin/Sodium selenite, 0.2% glucose, 0.5% B27, 1% Glutamax, 1% Non-essential amino acid, 10 μM 2-mercaptoethanol, 0.75 mg/mL $NaHCO_3$, 10 μM thioglycerol, 100 IU/mL penicillin and 100 μg/mL streptomycin. Filter and sterilize using a 0.22 μm filter.

NOTE: GCSC complete culture medium is recommended stored preferably no more than two weeks at 4 °C.

### 1.2. Recovery of GCSCs and culture

NOTE: GCSCs were obtained as follows: Tumor samples were subjected to mechanical and enzymatic dissociation. Single cell suspensions were obtained by filtering with nylon net from well-scattered suspension. The resulting cancer cells were cultured in GCSC Complete Culture Medium, and some cells grew to form spheres. These spheres were then subjected to enzymatic dissociation, and GCSCs can be obtained by cytofluorometric sorting of the cell population stained with CD44/CD54 markers. The detailed protocol and functional assays of the GCSCs have been reported[7].

1.2.1. Pre-warm GCSC complete culture medium at 37 °C for no more than 30 min.

1.2.2. Defrost GCSCs from liquid nitrogen storage and rapidly thaw cryovials in a 37 °C water bath. Keep swirling the vials until the entire content melts totally.

NOTE: Thaw frozen cells rapidly (<1 min) in a 37 °C water bath.

1.2.3. Transfer the entire contents of the cryovials into a 15 mL centrifuge tube containing 10 mL of GCSC complete culture medium. Centrifuge at 800 x *g* for 5 min at RT.

1.2.4. Aspirate the supernatant carefully and suspend the cell pellet in 10 mL of fresh GCSC complete medium. Plate the cell suspension in a 100 mm Petri dish. Incubate the plate at 37 °C in a 5% $CO_2$ incubator and add 5 mL of fresh complete medium on the third day.

### 1.3. Subculture of GCSC tumorspheres

NOTE: GCSC cells of the tumorspheres center only have sufficient nutrients before the spheres size growing up to 80-100 μm in diameter. Once dark and low refractivity spheres appear (about 6 days of culture), it is necessary to subculture the tumorspheres.

1.3.1. Shake the dish gently and transfer the GCSC tumorsphere culture medium (the medium and the non-adherent tumorspheres) into a sterile 15 mL centrifuge tube. For larger medium volumes, larger centrifuge tubes may be needed.

1.3.2. Centrifuge at 600 x *g* for 5 min and carefully dispose of the supernatant. After centrifugation, an off-white pellet will be visible.

1.3.3. Add 2 mL of cell dissociation solution to resuspend the pellet for mechanical and enzymatic dissociation at 37 °C. Gently pipet up and down 10 times every 2-3 min in the digestion procedure to break the spheres apart until the tumorspheres are dispersed into single cell suspension. This total dissociation process is recommended to be less than 15 min.

NOTE: Perform a visual check under the microscope to confirm that no large spheres or cell aggregates remain.

1.3.4. Add 10 mL of fresh pre-warmed GCSC complete culture medium (5x the volume of the cell detachment solution) to terminate digestion procedure and centrifuge at 800 x *g* at RT for 5 min.

1.3.5. Discard the supernatant and resuspend the cells with 1 mL of fresh pre-warmed GCSC complete culture medium. Seed an appropriate number of cells into a new 100 mm Petri dish with 10 mL of fresh pre-warmed GCSC complete culture medium and incubate at 37 °C, 5% $CO_2$.

1.3.6. Refeed tumorspheres cultures after 3 days by adding 5 mL of fresh pre-warmed complete medium. After 6 days, passage cells when tumorspheres grow up to 80-100 μm in diameter.

### 1.4. Cryopreservation of GCSCs

NOTE: Do not cryopreserve GCSC cells by adding medium to tumorspheres directly. GCSC tumorspheres should be digested into single cells so that cell protective agent could enter every cell to ensure the long-term stable storage of cells. Make sure the cells are in healthy situation and without contamination.

1.4.1. Harvest GCSC tumorspheres. Centrifuge at 600 x *g* for 5 min.

1.4.2. Discard the supernatant and add 2 mL of cell dissociation solution to dissociate GCSC tumorspheres at 37 °C. Terminate the digestion procedure by adding 10 mL of GCSC complete culture medium.

1.4.3. Centrifuge at 800 x *g* for 5 min and collect GCSC single cells.

1.4.4. Gently suspend GCSC cells with serum-free cryopreservative medium. The recommended final concentration is $5 \times 10^5$ - $5 \times 10^6$ cells/mL.

1.4.5. Dispense the cell suspension in 1 mL aliquots into marked cryogenic vials.

1.4.6. Immediately place the cryovials containing the cells in an isopropanol chamber and store them at –80 °C. Transfer the vials to liquid nitrogen the following day for long-term storage.

## 2. Generation of inducible knockdown GCSC lines

CAUTION: Recombinant lentiviruses have been designated as Level 2 organisms by the National Institute of Health and Center for Disease Control. Work involving lentivirus requires the maintenance of a Biosafety Level 2 facility, considering that the viral supernatants produced by these lentiviral systems could contain potentially hazardous recombinant virus.

### 2.1. Generation of lentivirus particles

2.1.1. Synthesize 2 lentiviral vectors carrying inducible shRNA targeting human clusterin and a non-targeting control lentiviral vector (GV307) from GeneChem based on the design of **Table 1** (GV307 vector contains: TetIIP-TurboRFP-MCS(MIR30)-Ubi-TetR-IRES-Puromycin).

2.1.2. Seed 4 x $10^6$ 293T lenti-viral packaging cells into a 100 mm Petri dish with 10 mL of DMEM supplemented with 10% fetal bovine serum.

2.1.3. Incubate 293T cells overnight at 37 °C, 5% $CO_2$. Make sure that 293T cell density is about 50-80% confluent the day of transfection.

2.1.4. Bring the reduced serum medium to room temperature and prepare Tube A and Tube B as described in **Table 2**.

2.1.5. Transfer Tube A into Tube B, mix well, and incubate the complexes for 20 min at room temperature to prepare lipid-DNA complexes.

2.1.6. Remove 5 mL of medium, before adding lipid-DNA complex, leaving a total of 5 mL.

2.1.7. Add 5 mL of lipid-DNA complex into the culture dish dropwise and gently swirl the dish to distribute the complex.

NOTE: Carefully dispense liquid against the dish wall to avoid disturbing 293T cells.

2.1.8. Incubate culture dish for 24 h at 37 °C, 5% $CO_2$.

2.1.9. After 24 hours post-transfection, carefully remove the transfection medium and gently replace with 10 mL of pre-warmed DMEM supplemented with 10% FBS. Incubate for 24 h at 37 °C, 5% $CO_2$.

NOTE: All the supernatant and tips should be treated with 10% bleach prior to disposal.

2.1.10. Approximately after 48 hours post-transfection, harvest 10 mL of lentivirus-containing supernatants.

NOTE: All the cell culture vessels and tips should be treated with 10% bleach prior to disposal.

2.1.11. Filter the lentiviral supernatant using a 0.45 µm pore filter to remove cellular debris.

NOTE: All filters and syringes should be treated with 10% bleach prior to disposal.

2.1.12. Transfer clarified supernatant to a sterile container, add Lenti-X Concentrator (1/3 volume of clarified supernatant) to mix by gentle inversion.

2.1.13. Incubate mixture at 4 °C overnight.

2.1.14. Centrifuge samples at 1,500 x *g* for 45 min at 4 °C. After centrifugation, and off-white pellet will be visible. Carefully remove supernatant, taking care not to disturb the pellet.

NOTE: All the supernatant and tips should be treated with 10% bleach prior to disposal.

2.1.15. Gently resuspend the pellet in 1 mL of DMEM supplemented with 10% FBS as virus stock, store at -80 °C.

**2.2. Generation of stable transfected cell lines**

2.2.1. Seed 6 x $10^6$ GCSC cells into a 100 mm Petri dish with 10 mL DMEM supplemented with 10% FBS for 24 h at 37 °C, 5% $CO_2$ (70-80% confluence prior to infection).

2.2.2. Aspirate the medium in the dish, add the concentrated lentiviral particles diluted with 4 mL of complete DMEM medium containing polybrene reagent (5 µg/mL) into the dish. Incubate for 18 h at 37 °C, 5% $CO_2$.

NOTE: The optimal concentration of polybrene depends on cell type and may need to be test in different concentrations to decide the effective concentrations. Otherwise, it may be empirically determined, usually in the range of 2-10 µg/mL. All the tubes and tips should be treated with 10% bleach prior to disposal.

2.2.3. Change the medium, replace with 10 mL of DMEM with 10% FBS medium and incubate for 24 h at 37 °C, 5% $CO_2$.

NOTE: All the medium and the tips should be treated with 10% bleach prior to disposal.

2.2.4. Aspirate the supernatant with cell debris, replace with fresh DMEM supplemented with 10% FBS medium containing puromycin (2.5 µg/mL) and incubate for 24 h at 37 °C, 5% $CO_2$. Then replace fresh DMEM supplemented with 10% FBS medium containing puromycin (5 µg/mL) and

incubate at 37 °C, 5% $CO_2$ for additional 24 h.

2.2.5. Rinse the adherent GCSC cells twice with 5 mL of DPBS without calcium and magnesium.

2.2.6. Dissociate GCSCs with 1 mL of pre-warmed cell dissociation solution and incubate 2-3 min at 37 °C.

2.2.7. Add 5 mL of fresh pre-warmed GCSC complete culture medium to the cell suspension.

2.2.8. Dispense 3 mL into a 15 mL centrifugal tube (tube A) for cryopreserving the cells, and the other 3 mL into a 15 mL centrifugal tube (tube B) for inducing by doxycycline.

2.2.9. Centrifuge tube A and tube B at 800 x *g* for 5 min.

2.2.10. Resuspend the pellet of tube A with 1 mL of serum-free cryopreservative medium, transfer the vial to –80 °C overnight, and remove it into liquid nitrogen storage.

2.2.11. Aspirate the supernatant and resuspend the cells of tube B in 1 mL of fresh pre-warmed GCSC complete culture medium. Seed an appropriate number of cells into a new 100 mm Petri dish of 10 mL fresh pre-warmed GCSC complete culture medium with doxycycline (Dox) (2.5 µg/mL) and incubate for 48 h at 37 °C, 5% $CO_2$.

NOTE: The optimal concentration of Dox may vary between cell lines. Each cell line should be tested in different Dox concentrations to decide the effective concentrations for KD and for toxicity on the cells.

2.2.12. Confirm stable repression of clusterin in GCSC cells by western blotting.

## 3. Sphere formation assay

3.1. Thaw the frozen inducible knockdown GCSC lines (see step 1.2).

3.2. Determine viable cell density of a 10 µL sample using an Automated Cell Counter.

3.3. Adjust the volume with pre-warmed GCSC complete culture medium to obtain a concentration of 2 x $10^4$ viable cells/mL.

3.4. Dispense into 3 new 96-well ultra-low-attachment culture plate wells (0.1 mL/well) each group.

3.5. Incubate the cells in an incubator at 37 °C with 5% $CO_2$. Sphere formation should occur within 3-10 days. Monitor and record the visualization of tumorspheres formation every 2 days.

NOTE: The medium is not recommended to be changed in case of any disturbance of the

tumorspheres formation. These tumorspheres should be easily distinguished from single and aggregated cells.

3.6. Determine tumorsphere formation results by evaluating the sizes of the formed tumorspheres using imaging software.

**REPRESENTATIVE RESULTS:**

Gastric cancer stem cells from primary human gastric adenocarcinoma were cultured in serum-free culture medium. After 6 days, cells expanded from the single cell-like phenotype (**Figure 1A**) to form large spheres (**Figure 1B**).

To assess the function of clusterin in GCSCs, shRNA sequences against clusterin and scrambled were cloned into Tet-GV307-RFP-Puro vector following the protocol described above. GCSCs stably transfected with a tetracycline-regulated shRNA-clusterin expression vector were generated and then treated with doxycycline for 48 h (**Figure 2**) (and shRNA scrambled as a control). The expression level of clusterin was verified by western blot and subsequently quantified by densitometry (**Figure 3**).

The sphere formation assay was used to test the self-renewal potential of GCSCs. We hypothesized that clusterin promotes the self-renewal potential of GCSCs, and therefore fewer spheres should be observed when clusterin is downregulated by the addition of doxycycline. We demonstrated that the presence of doxycycline and knockdown of clusterin in GCSCs inhibited tumorsphere formation (**Figure 4**). The cell/sphere sizes of GCSCs were not increasing when clusterin was reduced in GCSCs (**Figure 5**). No inhibition of tumorsphere formation was observed with GCSCs transduced with the scrambled shRNA controls, indicating that doxycycline had no inhibitory effect on the tumorsphere formation (**Figure 5**). These results suggested that after clusterin silencing, GCSCs grow slowly and cannot form tumorspheres. Based on the in vitro data, clusterin plays a critical role in promoting the self-renewal activity of GCSCs, indicating that clusterin could be a promising drug target in suppressing CSCs in GC patients.

**FIGURE LEGENDS:**

**Figure 1. Cell cultures of gastric cancer stem cells.** Single cell cultures of gastric cancer stem cells were cultured for 6 days. Phase-contrast microscopic images of these cells/spheres were taken at day 0 (**A**) and day 6 (**B**). Original magnification: 10x. Bar size: 20 μm.

**Figure 2. Conditional KD of clusterin expression in gastric cancer stem cells.** GCSC lines were established by infecting lentiviral inducible shRNA control (shCtrl) or inducible shRNA targeting clusterin (shClu1, shClu2). These cell lines were treated with (Dox+) or without Dox (2.5 μg/mL) (Dox-) for 48 h as noted. Phase contrast observation of these cells were shown in top panel. Immunofluorescent observation (red) of these cells were shown in bottom panel. Original magnification: 10x. Bar size: 20 μm.

**Figure 3. Expression of Clusterin in inducible knockdown GCSC lines with or without Dox treatment.** Western blotting analysis of clusterin expression in cell lines stably transfected with

tetracycline-regulated shRNA-clusterin (shClu1, shClu2) and scrambled (shCtrl) expression vector after 2 days of doxycycline treatment. The relative expression level of clusterin was quantified by densitometry and normalized against β-actin, then was indicated below the lanes of the Western blots.

**Figure 4. Phase-contrast microscopic images of inducible knockdown GCSC cells/spheres.** Single cell of inducible knockdown GCSC lines were incubated and treated without Dox (top panel) or with Dox (bottom panel) for 6 days. Phase-contrast microscopic images of these cells/spheres were taken at day 6 as indicated. Original magnification: 10x. Scale bar, 20 μm.

**Figure 5. Inducible knockdown of Clusterin inhibits GCSC self-renewal capacity.** Sphere formation assays were performed in the inducible knockdown GCSC lines. Phase-contrast microscopic images of these cells/spheres were taken at the indicated day, and the cell/sphere sizes of GCSCs were measured. n>30, ± standard error of mean (SEM).

**Table 1. Two shRNA targeting sequences against clusterin**

**Table 2. Scale of viral production using transfection**

**DISCUSSION:**

GC is the third leading cause of cancer-related death worldwide. GCSC is critical in gastric cancer relapse, metastasis and drug resistance. Using GCSCs from gastric cancer patients will allow us to explore their weak spot and develop the targeting drugs for the treatment of GC patients.

The sphere formation assay is a useful method to examine cancer stem cell self-renewal potential in vitro. Results can be presented as the percentage of spheres formed divided by the original number of single cells seeded. We adapted the original method to calculate the mean sizes of all cells/spheres at several time points to improve the results of this assay and to facilitate its reproducibility for other types of cancer stem cells. We certified that the result in this assay is highly dependent on the number of the initial seeded cells. This is a critical point of this assay to maintain initial cell isolation and make an accurate measurement of the number of the spherical colonies (excluding cellular aggregations). Additionally, it is important to optimize the counting time to clearly distinguish the spheres from cellular aggregations and single cells.

Tet-inducible systems are helpful to study the function of genes that are crucial for cell survival in vitro, just like clusterin in this protocol. They are also useful for functional exploration of genes in vivo; this can be done by adding doxycycline into the drinking water of animals. However, leakiness in the uninduced state is an often reported problem of the Tet-inducible systems[12]. In the presented experiment, a low level of leakiness is also observed with shClu2, as shown in **Figure 3**. In this case, we can carefully compare the changes of target protein in KD or scrambled control detected in the presence and absence of doxycycline to assess this effect. Another important point is the amount of doxycycline applied in the culture. As the amount of Dox may vary between cell lines, each cell line should be tested with different Dox dosages to decide the effective concentrations for KD and for toxicity on the cells.

The protocol presented here provides an efficient technique for deciphering the stemness-related genes of CSCs and studying CSCs' biology. The protocol can be easily adapted to study the functions of other critical genes in cancer stem cells, such as stemness and survival. Additionally, the conditional knockdown of gene expression in CSCs are feasible to study the biological functions of target genes not only in vitro but also in vivo. However, just some CSCs may not form solid, typical tumorspheres, this protocol should be adapted by using other methods to examine cancer stem cell self-renewal potential in vitro, for example, examining the expression of stemness-related markers.

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**DISCLOSURES:**

No conflicts of interest declared.

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Figure 1

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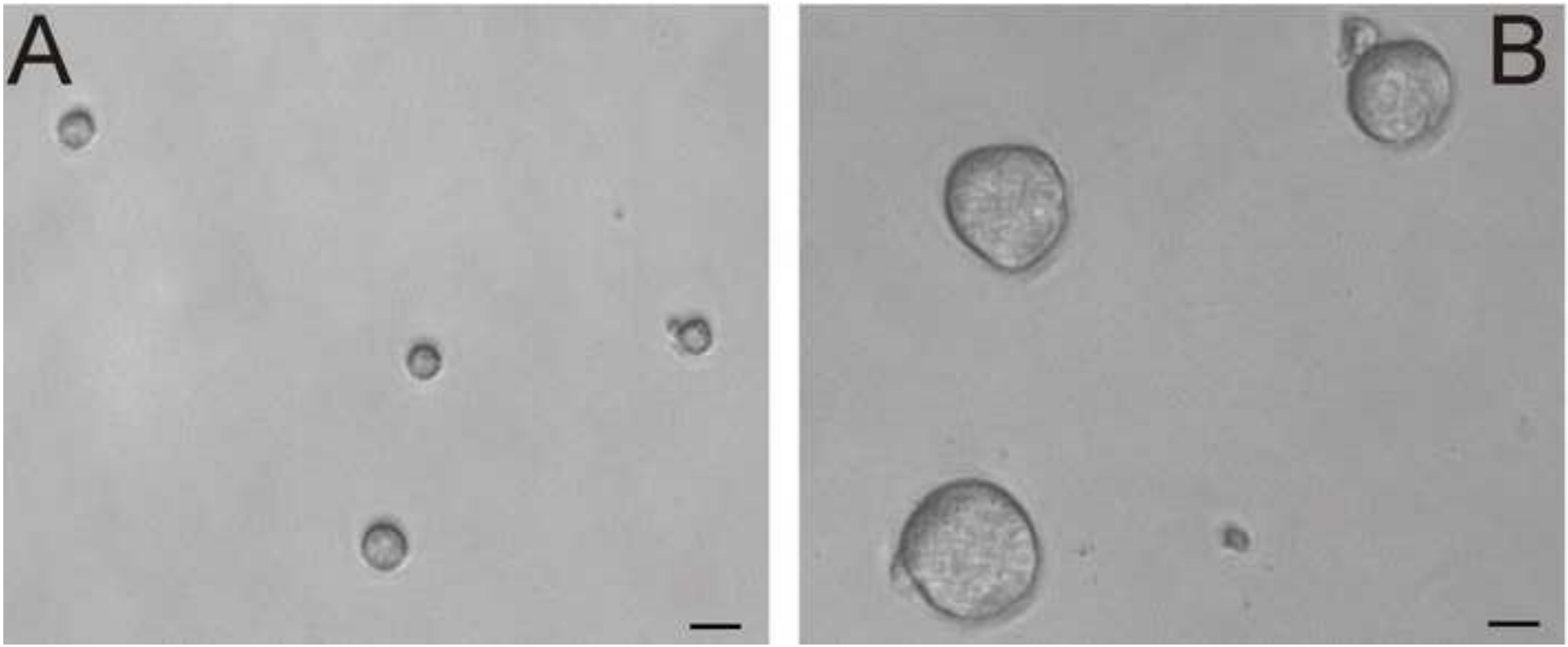

Figure 2

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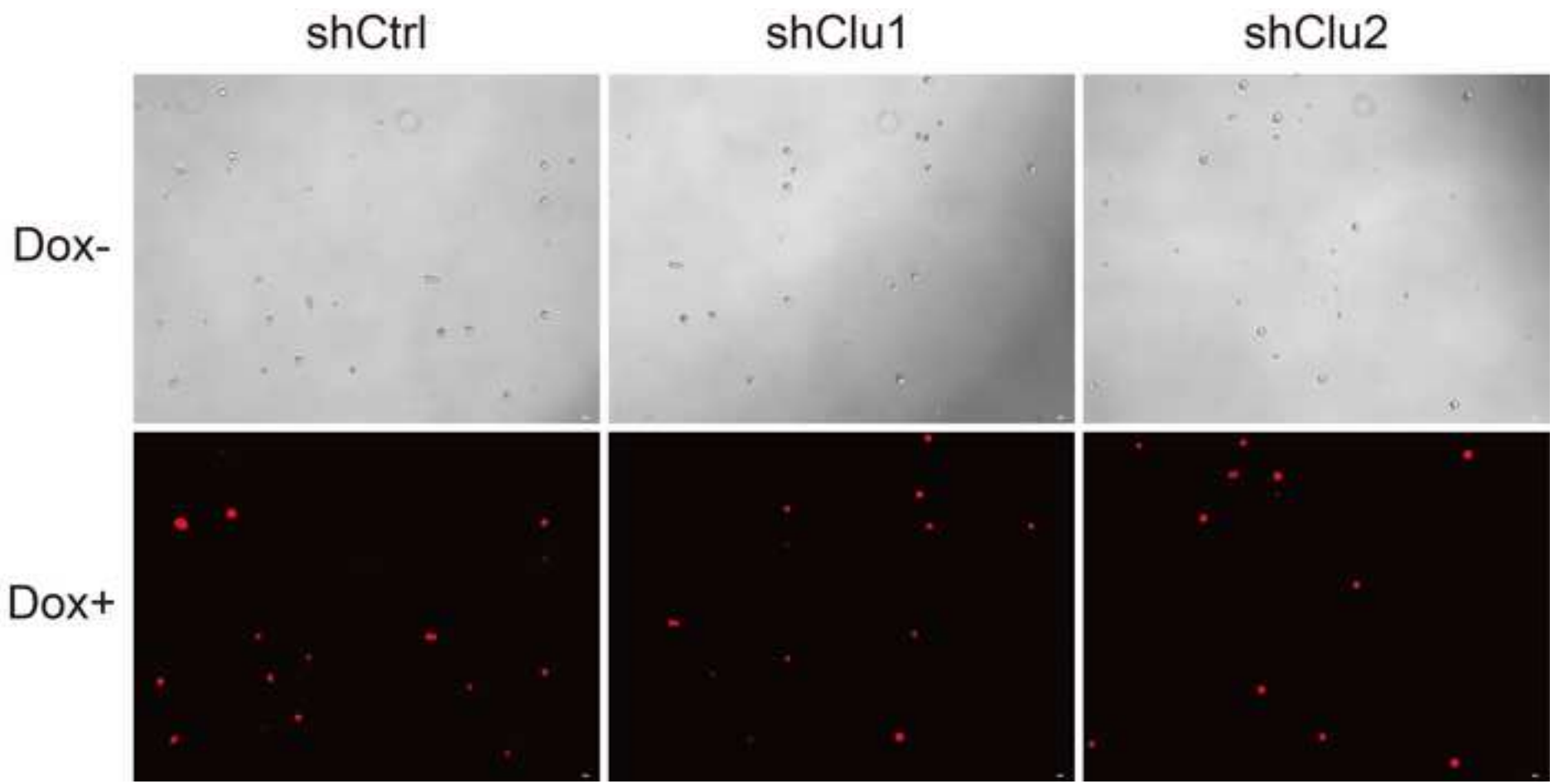

Figure 3

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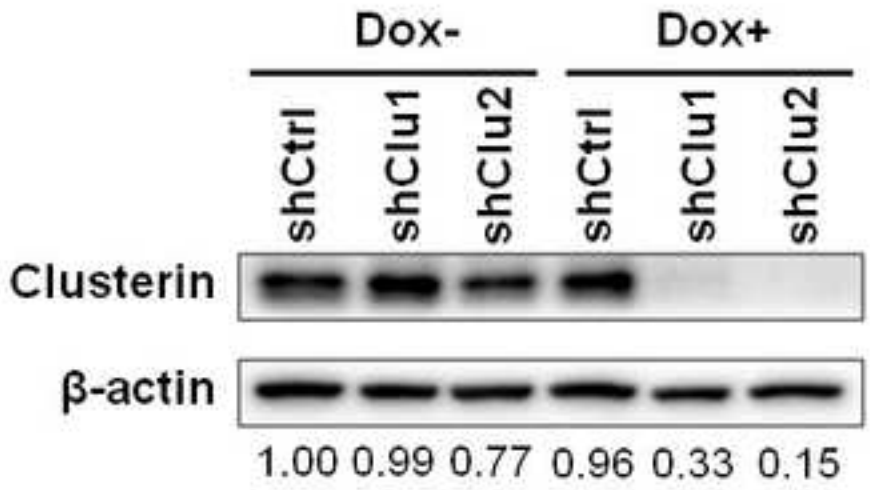

Figure 4

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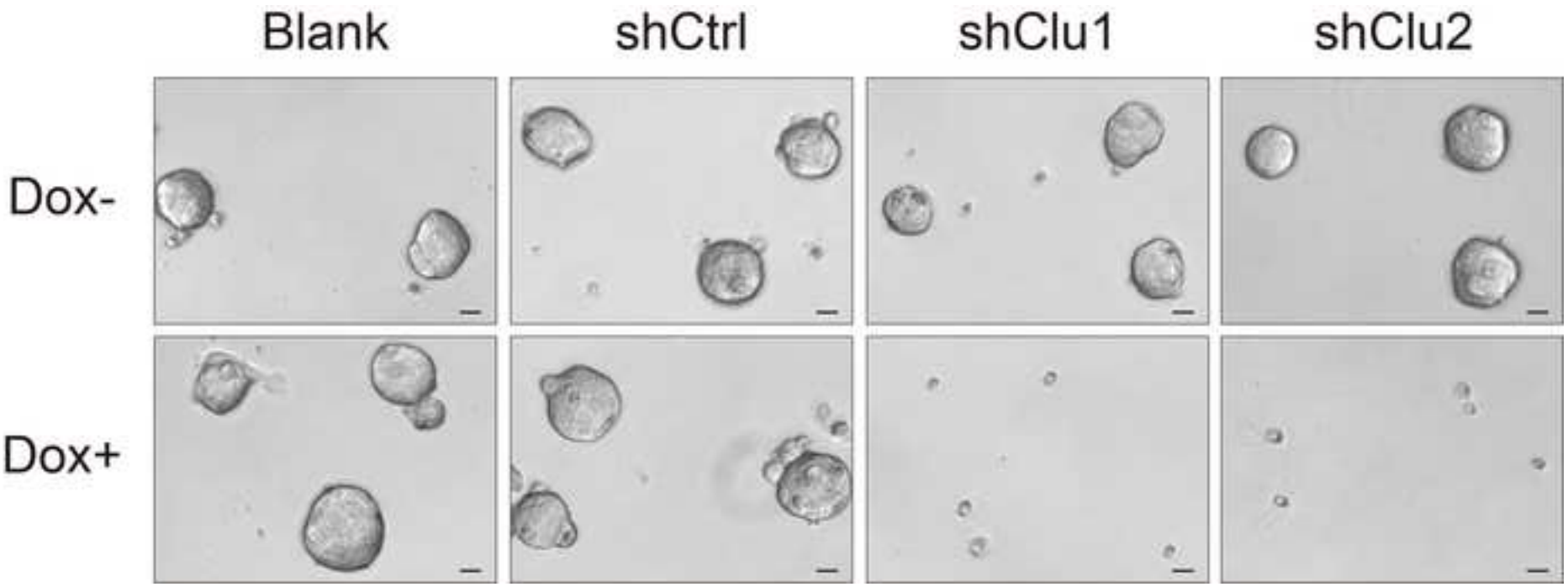

Figure 5

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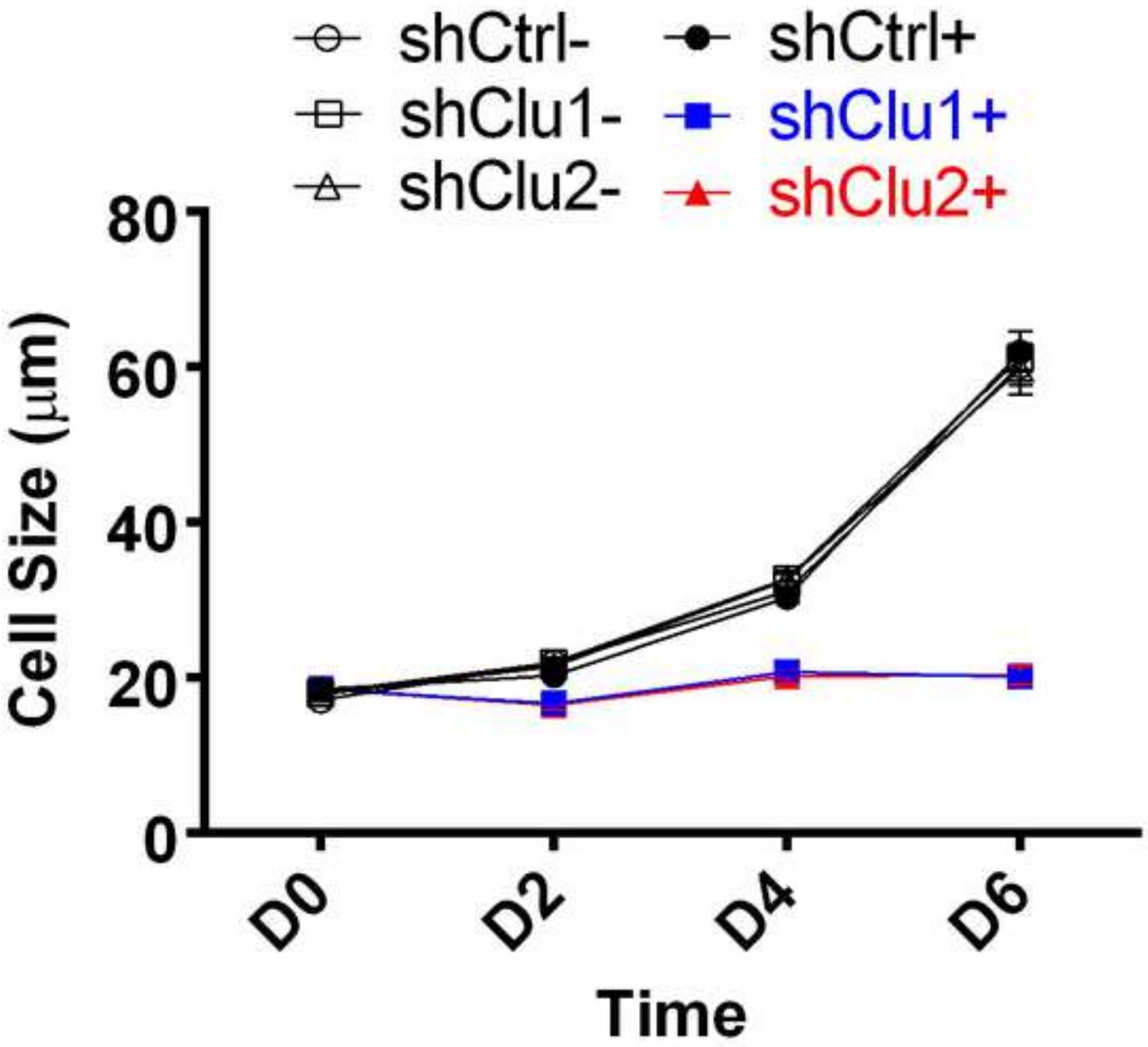

| Table 1. Two shRNA targeting sequences against clusterin | | | |
|---|---|---|---|
| Name of Gene | Species | Gene ID | Targeting Sequence |
| CLU-1 | Human | 1191 | TGAAACAGACCTGCATGAA |
| CLU-2 | Human | 1191 | GGGAAGTAAGTACGTCAAT |

Table 2. Scale of viral production using transfection

| Component | Volume |
|---|---|
| Tube A | |
| Reduced Serum Medium | 1.5 mL |
| Transfection Reagent | 41 µL |
| Tube B | |
| Reduced Serum Medium | 1.5 mL |
| P3000 Enhancer Reagent | 35 µL |
| pHelper 1.0 (gag/pol component) | 9 µg |
| pHelper 2.0 (VSVG component) | 6 µg |
| shCLU 1/2 or control plasmid | 12 µg |

| Name of Material/Equipment | Company | Catalog Number |
|---|---|---|
| 0.22 µm filter | Millipore | SLGP033RB |
| 1-Thioglycerol | Sigma-Aldrich | M6145 |
| 2-Mercaptoethanol | Gibco | 2068586 |
| Animal-Free Recombinant Human EGF | Peprotech | AF-100-15 |
| B-27 Supplement (50X), serum free | Gibco | 17504044 |
| Corning Costar Ultra-Low Attachment Multiple Well Plate | Sigma-Aldrich | CLS3474 |
| Countess Cell Counting Chamber Slides | Invitrogen | C10228 |
| Countess II Automated Cell Counter | Invitrogen | AMQAX1000 |
| D-(+)-Glucose | Sigma-Aldrich | G6152 |
| DMEM/F-12, HEPES | Gibco | 11330032 |
| DMEM, High Glucose, GlutaMAX, Pyruvate | Gibco | 10569044 |
| Doxycycline hyclate | Sigma-Aldrich | D9891 |
| DPBS, no calcium, no magnesium | Gibco | 14190250 |
| Fetal Bovine Serum, qualified, Australia | Gibco | 10099141 |
| GlutaMAX Supplement | Gibco | 35050061 |
| Insulin, Transferrin, Selenium Solution (ITS -G), 100X | Gibco | 41400045 |
| lentiviral vector | GeneChem | GV307 |
| Lenti-X Concentrator | Takara | 631232 |
| Lipofectamine 3000 Transfection Reagent | Invitrogen | L3000015 |
| MEM Non-Essential Amino Acids Solution, 100X | Gibco | 11140050 |
| illex-HV Syringe Filter Unit, 0.45 µm, PVDF, 33 mm, gamma steriliz | Millipore | SLHV033RB |
| Nalgene General Long-Term Storage Cryogenic Tubes | Thermo Scientific | 5000-1020 |
| Nunc Cell Culture/Petri Dishes | Thermo Scientific | 171099 |
| Opti-MEM I Reduced Serum Medium | Gibco | 31985070 |
| Penicillin-Streptomycin, Liquid | Gibco | 15140122 |
| pHelper 1.0 (gag/pol component) | GeneChem | pHelper 1.0 |
| pHelper 2.0 (VSVG component) | GeneChem | pHelper 2.0 |
| Polybrene | Sigma-Aldrich | H9268 |
| Recombinant Human FGF-basic | Peprotech | 100-18B |
| Sodium bicarbonate | Sigma-Aldrich | S5761 |
| STEM-CELLBANKER Cryopreservation Medium | ZENOAQ | 11890 |
| StemPro Accutase Cell Dissociation Solution | Gibco | A1110501 |

| | | |
|---|---|---|
| UltraPure 1 M Tris-HCI Buffer, pH 7.5 | Invitrogen | 15567027 |
| ZEISS Inverted Microscope | ZEISS | Axio Vert.A1 |

Mar 02, 2020
Dr. Nam Nguyen,
*Journal of Visualized Experiments*

Dear Dr. Nam Nguyen,

**Re: Manuscrcipt# JoVE60799R1**
**Title: Combined Conditional Knockdown and Adapted Sphere Formation Assay to Study a stemness-associated gene of Patient-derived Gastric Cancer Stem Cells**

Thank you for reviewing the above-referenced manuscript submitted earlier to your office. We would like to take this opportunity to express our appreciation to you and Reviewers. In accordance with the Editorial and Reviewers' comments and suggestions, the manuscript has been revised accordingly. We feel that this revised manuscript has been strengthened by the Editorial and Reviewers' comments and suggestions. A point-by-point response to the Editorial and Reviewers' comments and suggestions has been prepared and follows this cover letter.

I hope the changes and explanations satisfy the requirements of the Editorial Board. I thank you again for reviewing the manuscript and look forward to your favorable reply soon.

Yours sincerely,

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**A point-by-point response to the Editorial comments and suggestions**

*1. Please remove commercial language and use generic language whenever possible: Accutase, etc..*

**Our reply:**

Thanks for your kindly reminder. We used “cell dissociation solution” instead of Accutase, and checked the manuscript thoroughly.

*2. Please discuss some limitations of the protocol in the discussion.*

**Our reply:**

We added the discussion of the limitation of this protocol (Pls see Line 396 on Page 9).

*3. Figure 2: Please include scale bars.*

**Our reply:**

We added scale bars in Figure 2.

**A point-by-point response to the Reviewers’ comments and suggestions**

**Reviewer 2:**

*1. In the title and in the manuscript, the authors mentioned they used "patient-derived gastric cancer stem cells" to explore this study. However, the authors did not elucidate in the manuscript how they separate, isolate and identify gastric cancer stem cells from patients. Can the authors explain how they got GCSCs from gastric cancer patients and how they identified them instead of the statement "defrost GCSCs from liquid nitrogen storage"?*

**Our reply:**

Thanks Reviewer for pointing out this important issue. GCSCs were obtained as follows:1) Tumor samples were subjected to mechanical and enzymatic dissociation; 2) Single cell suspensions were obtained by filtering with nylon net from well-scattered suspension; 3) The cancer cells were cultured in GCSC Complete Culture Medium, and some cells grew to form spheres; 4) The spheres were subjected to enzymatic dissociation, and GCSCs can be obtained by cytofluorometric sorting of the cell population stained with CD44/CD54 markers (Pls see Line 102 on Page 3). GCSCs were identified by

in vitro tumor sphere formation and serial transplantation in immunodeficient mice. The detailed protocol and functional assays of the GCSCs have been reported previously (Pls see Reference 1 enclosed in the reply letter; Cell Research, 2012, 22:248-258).

*2. In the title, the authors mentioned they were going to study "the stemness-associated genes" of patient-derived GCSCs. However, in the manuscript the authors only used one gene--Clusterin. Also, the authors did not elucidate why they use Clusterin, "the role of which in CSCs has not been fully understood" to explore this work instead of the well-known stemness-associated genes such as Sox2, Oct-4 etc. Can the authors explain this in the manuscript?*

**Our reply:**

Thanks Reviewer for the kind suggestion. We have changed the title to "Combined Conditional Knockdown and Adapted Sphere Formation Assay to Study a stemness-associated gene of Patient-derived Gastric Cancer Stem Cells" in the revised manuscript.

We agree with Reviewer's concern that using the well-known stemness-associated genes such as Sox2, Oct-4 etc is a good choice for this protocol. The reasons why we chose Clusterin for this study are as follows:

1. The results for the well-known stemness-associated genes such as Sox2, Oct-4 would be more predictable. Actually, we aim to extend this protocol to study other unknown CSC markers which have not been fully understood so far.
2. Clusterin has been identified as a novel key molecule for maintaining the stemness and survival of GCSCs in our previous study (Pls see Reference 2 enclosed in the reply letter; Int J Biol Sci., 2019, 15(2):312-324). We thus use Clusterin to demonstrate that this protocol is applicable for studying the functions of novel candidate genes regulating cancer stem cells.

**Reviewer 4:**

*1. A more detailed explanation is needed on why was clusterin chosen? Please refer to your previous work.*

*How did you obtain the gastric cancer stem cells? This needs to be clearly explained in the protocol.*

*Did you check for the stem cell markers upon thawing the frozen vials? Did you check for CXCR4 in your cells?*

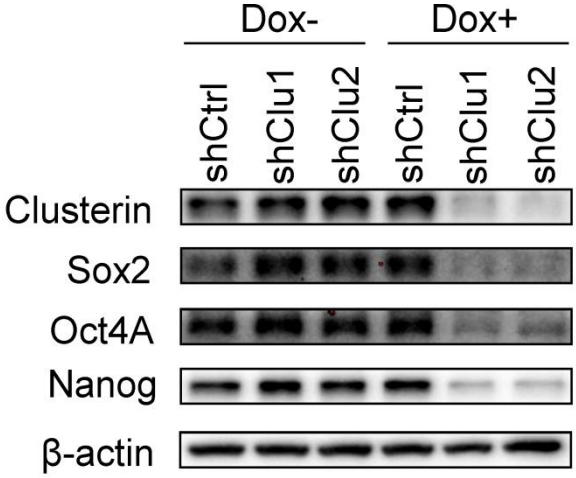
**Our reply:**

Thanks Reviewer for the kind suggestion. We added the detailed explanation on why Clusterin was chosen in the revised manuscript (Pls see Line 79 on Page 2).

Thanks Reviewer for pointing out this important issue. GCSCs were obtained

as follows:1) Tumor samples were subjected to mechanical and enzymatic dissociation; 2) Single cell suspensions were obtained by filtering with nylon net from well-scattered suspension; 3) The cancer cells were cultured in GCSC Complete Culture Medium, and some cells grew to form spheres; 4) The spheres were subjected to enzymatic dissociation, and GCSCs can be obtained by cytofluorometric sorting of the cell population stained with CD44/CD54 markers (Pls see Line 102 on Page 3). The detailed protocol and functional assays of the GCSCs have been reported previously (Pls see Reference 1 enclosed in the reply letter).

We checked the expression of several stem cell markers such as Sox2, Oct4A, and Nanog in the GCSC cells comparing to the Clusterin knock-down (shClu) GCSC cells. As shown in the below image, Sox2, Oct4A and Nanog are consistently positive in the GCSCs without Clusterin knock-down. We didn't check the expression of CXCR4 in GCSCs, and functional assays of the GCSCs have been reported previously (Pls see Reference 1 enclosed in the reply letter).




*2. Do you coat the plate prior to cell suspension? How many cells are plated in 100 mm Petri dish? How do you ensure that the cells form tumorspheres?*
*Subculture of GCSCs is not presented well - Need to bring out clarity on whether these spheres are adherent or non-adherent e.g., in 1.3.1 when you transfer the medium do you transfer the cells as well, do you need to perform trypsin treatment? Do you individually pick up individual colonies for downstream assay?*
*Did you perform a similar experiment with positive controls (e.g. Sox2) to show indeed the cells exhibit stem cell characteristics?*
*Keywords contain the name and the volume of journal.*

**Our reply:**

No, we don't coat the plate prior to cell suspension.

About 5 x $10^5$ - 5 x $10^6$ cells are plated in the 100mm petri dish.

Tumorspheres are constantly monitored under a phase-contrast microscope, and the expression of several stem cell markers such as Sox2, Oct4A, and Nanog were examined to ensure these cells to form tumorspheres.

Thanks Reviewer for the kind suggestion. The tumorspheres are non-adherent. Therefore, we transferred both tumorspheres and their culture medium to a single tube for subculture (Pls see Line 130 on Page 3). The tumorspheres were then collected by centrifugation, and subjected to enzymatic dissociation into single cells (we use accutase instead of trypsin). We don't individually pick up single colonies for downstream assay.

We checked the expression of several stem cell markers such as Sox2, Oct4A, and Nanog in GCSCs, as well as in the shClu-transfected GCSCs (as shown in the above image). Sox2, Oct4A, and Nanog are all positive in GCSCs without Clusterin knock-down.

Thanks Reviewer for the kind suggestion, and we deleted the name and the volume of journal in the keywords.

**Reference:**


1. Chen T, Yang K, Yu J, Meng W, Yuan D, Bi F, Liu F, Liu J, Dai B, Chen X, Wang F, Zeng F, Xu H, Hu J, Mo X. Identification and expansion of cancer stem cells in tumor tissues and peripheral blood derived from gastric adenocarcinoma patients. Cell Res., 2012, 22(1): 248-258.
2. Xiong J, Wang S, Chen T, Shu X, Mo X, Chang G, Chen JJ, Li C, Luo H, Lee JD. Verteporfin blocks Clusterin which is required for survival of gastric cancer stem cell by modulating HSP90 function. Int J Biol Sci*.,* 2019, 15(2):312-324.