CRISPRi Screening in Cancer Spheroids to Investigate Factors in Genome Stability

BME 400

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Section 305

Product Design Specifications

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Function: Although previous CRISPRi screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an *in vivo* environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPRi screening in order to identify sources of DNA mutations in the tumor environment. On a high scale, the team must select a viable cell line for the screen, create and optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX: a histone variant that acts as a sensitive marker for DNA damage.

Client requirements:

1. The team must choose an appropriate human cell line for the project, ensuring to maximize cell spheroid viability.
2. The team must develop a spheroid formation protocol for the chosen cell type or line that optimizes seeding density and viscosity.
3. The team must optimize the cancer spheroid characteristics and their formation protocols for CRISPR screens.
4. By May 2025, the team must develop a protocol to stain for γH2AX: an indicator for the location of maximal damage due to DNA double-strand breaks (DSBs).
5. By May 2025, the team must perform high throughput genome-wide CRISPR screening to check for the effects of different genes on the amount of γH2AX detected.
6. Future steps post-design project include performing an analysis to determine the

phenotypic differences between cells grown in monolayers (2D) versus cells grown as spheroids (3D).

Design requirements:

1. Physical and Operational Characteristics
    a. *Performance requirements:*
        i. Spheroid formation protocol should be scalable, repeatable, and optimized regarding:
            1. Seeding densities to ensure only 1 single guide (sgRNA) will enter each cell.
            2. Viscosity of growth medium (by changing the amount of methylcellulose) to ensure homogenous spheroid size.
            3. Optimizing reagent percentages of growth medium to ensure maximum cell growth.
        ii. γH2AX staining protocol should be scalable, repeatable and optimized regarding the antibody used and amount of reagents used.

    b. *Safety:*
        i. Biosafety Level 2 (BSL-2) is necessary for work with agents associated with human tissue and disease including the human lung cancer cell line which the team will be using [1]. The following universal precautions for BSL-2 are used to reduce the risk of bloodborne pathogens:
            1. Laboratory personnel receive specific training in handling pathogenic agents and are supervised by scientists competent in handling infectious agents and associated procedures [1].
            2. Access to the laboratory is restricted when work is being conducted [1].
            3. All procedures in which infectious aerosols or splashes may be created are conducted in BSCs or other physical containment equipment [1].
        ii. Cultures, tissues, specimens of body fluids, or potentially infectious wastes are to be placed in a container with a cover that prevents leakage during handling, processing, and storage [2].
        iii. Although the team does not anticipate the need to remove our cells or cancer spheroids from Hess Lab, the following BSL-2 guideline must be followed.
            1. Biological agents prepared for transport must be placed in a secondary leak-proof, unbreakable carrier. Carriers must have the biohazard label, including the identity of the agent, affixed to the outer surface of the transport container [2].

iv. If genome editing leads to the inactivation of tumor suppressors or activation of oncogenes in human cells, then oncogenesis becomes a possibility in the event of exposure [3]. This is a concern when:
    1. Genome editing components are delivered by a method that can enter human cells (e.g., viral vectors that can infect human cells).
    2. All components required for genome editing are likely to be introduced in a single exposure event, such as a needle stick or mucosal splash.
    3. Genome editing is designed to target human oncogenes/tumor suppressors or can target human oncogenes/tumor suppressors due to homology.

v. A risk assessment and containment preparations for research with lentiviral vectors should consider (1) the nature of the vector system, (2) transgene insert, and (3) type of manipulations involved [4]. Either BL-2 or enhanced BL-2 will be appropriate for these such experiments. The major risks to be considered for research with HIV-1 based lentivirus vectors are (1) potential for generation of replication-competent lentivirus (RCL) and (2) potential for oncogenesis [4]. These risks can be mitigated by the nature of the vector system (and its safety features) or exacerbated by the nature of the transgene insert encoded by the vector [4].

c. *Accuracy and Reliability:*
    i. A starting cell seeding density of 50,000 cells/cm² will be used. Cells at these densities will be monitored for growth and death rates on a live-cell microscope for 60 hours to confirm a ~30% cell death rate during the initial growth phase of the spheroids [5].
    ii. Cell seeding densities must be such that only 1 single guide (sgRNA) enters each cell to allow for analyzable results. Therefore, a total of 100-150 million cells will be required for each genome-wide CRISPR screen [6].
    iii. A minimum of 3 γ-H2AX staining experiments will be performed to establish confidence in results.

d. *Life in Service:*
    i. Before preparing them for CRISPRi screening, cells will need to be in culture for approximately 2 months to allow for optimization of the spheroid formation protocol, including the spheroids' size and cell quantity. This time period may consist of thawing a new vial of cells for continuing cell culture if the passage number exceeds 25 [7].

ii. After infection with the selected genome-wide library, a minimum of 6 days will be required before the cells can be further utilized [5].
iii. After generating spheroids from these cells, a minimum of 21 days will be required for the genome-wide screening [5].

e. *Shelf Life:*
   i. When not in culture or experimental use, cells will be stored using cryogenics for long-term use. Frozen cells will be kept in cryovials with medium containing a cryoprotective agent like DMSO. These vials will be immersed in liquid nitrogen at a temperature below -135℃ [8].

f. *Operating Environment:*
   i. Cells needed to generate spheroids for this project will be kept in standard incubation conditions: 37℃, 5% CO_2, and 90–95% relative humidity [9].
   ii. Cells in culture will be maintained in a cell culture medium supplemented with fetal calf/bovine serum to deliver essential growth factors. The medium will contain phenol red, which will indicate the need to replace it if it turns yellow (acidic) or purple (alkaline) [9].
   iii. For preparation for CRISPRi screening, spheroid cells will be exposed to a pooled single-guide RNA (sgRNA) library and CRISPR associated protein 9 (Cas9) via lentiviral vectors [10].

g. *Ergonomics:*
   i. Protocols should create a more efficient and accurate experimental system that better mimics *in vivo* tumor conditions, while proving the effects of γH2AX in DNA damage.
   ii. The design of cell culture methods should be optimized by selecting appropriate cell lines and developing correct spheroid formations to enhance the accuracy, relevance, and scalability of CRISPRi screening in a 3D tumor model.
   iii. The γH2AX staining protocol should be optimized for improved precision. Additionally, γH2AX foci counting should be performed via high-throughput screening for efficiency.

h. *Size:*
   i. Spheroids will be generated in 60 mm tissue culture plates [5].
   ii. Cell seeding density for spheroids will be restricted to 50,000 cells/cm² to ensure appropriate CRISPRi screening [5].
   iii. To adhere to a spheroid size suitable for γH2AX staining, spheroids will be kept to ~500 μm in diameter [11].

i. *Weight:*
    i. Although solutions have been presented for single-cell analysis, literature does not cover this aspect for 3D models such as spheroids [12]. Tools like a flow-apparatus are needed for the accurate measurements of mass density, size and weight of such spheroids [12].

j. *Materials:*
    i. In order to leave room for the engineering design element, the client did not give specific requirements for materials other than cell type to be used for the project.
    ii. The client has recommended that the team select a human lung cancer cell line that (1) adheres well to spheroids and (2) has a high percentage of viable cells.

k. *Appearance:*
    i. Since the spheroids should have an overall appearance that will accurately replicate the 3D tumor microenvironment, the spheroids must be: translucent, porous, uniformly spherical, and have a stiffness analogous to the lung cancer cell line selected [13].

2. Production Characteristics
    a. Quantity:
        i. By the end of this semester, the team will design, fabricate, and develop two appropriate protocols for the chosen cell line, one for spheroid formation and one for γH2AX.
        ii. The final protocols should be capable of accurately mimicking the 3D tumor environment for CRISPR screening, including features for effective spheroid formation and reliable γH2AX staining to assess DNA changes.
        iii. The protocols can be adapted to other similar human cancer cell lines; however, some of the characteristics may be subjected to change.

    b. *Target Product Cost:*
        i. The budget for the project is $1,000. Some of the materials and services that the team expects to use are listed below; however, many are already available in Hess Lab and will not need to be purchased.
            1. Biological materials such as reagents (for various media), antibodies (for γH2AX staining) and lentivirus for gene insertion.
            2. Obtaining new cell lines if needed.

3. Designing of CRISPR library.

ii. This project is a part of an emerging field, so similar products on the market are not available for non-clinical purchasing purposes. Therefore, the target cost of the final prototype is to remain within the budget proposed.

3. Miscellaneous
   a. *Standards and Specifications:*
      i. Cell lines used for this project should comply with the requirements outlined in part 1b - *Safety* above. Specifically, cell lines used and their associated reagents and protocols will need to comply with requirements for BSL-2 labs; meaning, they are or will contain bacteria, viruses, and organisms that pose a moderate safety hazard to healthy adult humans [14].
      ii. The use of human cell lines and other organisms in research for this project is outlined in various standards and regulations including Section 29 CFR 1910.1030 *(OSHA Bloodborne Pathogens),* Section 21 CFR 876.5885 *(Tissue culture media for human ex vivo tissue and cell culture processing applications),* and a comprehensive cell line guideline by Geraughty, *et al* (2014):
         1. Bloodborne pathogens are microorganisms in the blood that can infect and cause diseases in humans [15].
         2. Human cell lines are designated by the FDA as Class II (Special Controls) [16].
         3. If applicable, genetic modification processes on the cell line must be detailedly-noted and kept track of, including but not limited to, sequence details, insertion vectors and modes, and antibiotic resistance markers [17]. Following modification using lentivirus, cell lines may be additionally tested to confirm non-infectivity [17].
      iii. Human cell lines that may be needed for this project are already available in the client's lab, or can be obtained from another lab, meaning they already comply with the requirements listed above. In the case that biological research materials are obtained from another lab or institute, an MTA (material-transfer agreements) must be signed by both the provider and recipient to define their rights regarding the materials [17].
      iv. In the unlikely case that new human cell lines must be acquired, additional ethical and authentication considerations must be taken into account:
         1. Ethical considerations are outlined in Part 3c - *Patient-related concerns* below.

2. For cell line authentication, short tandem repeat (STR) is the recommended profiling method, outlined in ASN-0002 *(Authentication of Human Cell Lines: Standardization of STR Profiling)* provided by the American National Standards Institute (ANSI) and the American Type Culture Collection (ATCC). STR is a DNA-based sequence method that allows for detection of mutations and adventitious agents, as well as confirmation of the origin of the interested cell line [18].

b. *Patient-related concerns:*
   i. As cell lines and other tissue materials needed for this project are either already available in the client's lab or in another lab, their ethical and other patient-related considerations have already been considered.
   ii. In the unlikely case that a new cell line is obtained from patients or donors, the Patient Consent Form and associated Patient Information Sheet will be needed to explain the need for the specimen and the purpose and importance of the research [17]. Patient/donor and original tissue information such as age, sex, clinical history, site of origin, nature of tissue specimen, stage and grade of cancer or other disease/ pathology, and other important information must also be recorded.
   iii. For maximal biological relevance to patient *in vivo* tissue, the team will ensure our spheroids are designed based on critical 3D parameters: stiffness analogous to lung cancer tissue, replication of the complex cell-cell interactions and cell-extracellular matrix (ECM) interactions, and increased accuracy in CRISPRi screening environment to better detect precancerous cells [19], [20].

c. *Competition:*
   i. 3D cancer spheroid models that can be utilized for CRISPR-Cas9 screens have been implemented in previous research, with and without using an exogenous extracellular matrix (ECM).
      1. Takahashi *et al*, 2020 utilized 4% Matrigel™ as a bio-scaffolding to plate A549 and H1437 cells spheroids, which can better model *in-vivo* interactions between cells and the ECM [21]. The study's CRISPRi library gene list as well as its phenotypes have a strong overlap with those from Han *et al*, 2020 (described below), suggesting that inner cells of ECM-based 3D spheroids may be similar to ECM-free 3D spheroids.
      2. Han *et al*, 2020 devised an ECM-free scalable method to propagate 3D spheroids [5]. H23 cells were seeded on pre-treated ultra-low

attachment or polyhema-covered plates in RPMI 1640 medium with 0.75% methylcellulose. Similar methods have been found to be implemented in other studies, such as by Ferrarone, *et al* (2024) and by Stiff, *et al* (2024) [22], [23]. A total of around 210,000 single guide RNAs (sgRNAs) were designed for the CRISPRi library with around 10 sgRNAs per gene [5]. Samples were used to calculate growth and/or tested for drug resistance [5].

3. 3D stem cell models have also been genomically edited via CRISPR-Cas9 to better model various disease phenotypes. Cells can be assembled with or without supporting scaffolds. Some example scaffolds include laminin-rich Matrigel, collagen matrix, or those that are naturally derived from decellularized tissues [24].

ii. γH2AX has been used in various research as a marker to monitor DNA damage and repair, such as in understanding the toxicity of cigarette smoke or in understanding the effects of chemotherapeutic treatments in cancer patients [25], [26]. High throughput systems have also been developed to aid with counting γH2AX foci [26].

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