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Non-Selective Primary Human Tumor Cell Line Generation from Surgical Resections to be Paired with Flash Frozen and Paraffin Embedded Tissue: Advancements in Democratizing Translational Research Materials to Rural Institutions

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ABSTRACT

Translational cancer research relies on the availability of human patient tissue demonstrating the specific disease process under investigation. Biobanks of human tissue have historically been and remain to date the primary access point for cancer research samples. Biorepositories routinely supply researchers with varying sample types for use in biomedical studies, most commonly formalin-fixed and paraffin-embedded (FFPE) tissue or fresh snap-frozen tissue. In conjunction with preserved tissue samples, viable tumor cell lines derived from patient tissue have emerged to be a new gold standard in cancer research, particularly in drug discovery and functional prognostic assays. Tissue banks providing these samples are being termed “next-generation” and are adapting to directly assist researchers by performing high throughput technical studies, such as routine histology and immunostaining of donor tissue. These high-quality, next-generation biorepositories are a relatively scarce resource in the broader research community in the United States and have traditionally been associated with large centralized and very well-established university medical centers. This article describes the next-generation resources now available at the Edwards Comprehensive Cancer Center with its association with the Marshall University Joan C. Edwards School of Medicine in Huntington, West Virginia. This manuscript details the procedures, protocols, and success rates of the Tissue Procurement Program (TPP) to generate a growing cohort of viable primary human tumor cell lines representing varying malignancies to be used in conjunction with matched formalin-fixed and paraffin-embedded (FFPE) and snap-frozen tissue samples for comprehensive translational research.

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INTRODUCTION

To facilitate translational cancer research and to further improve the treatment of the disease, biorepositories have emerged to provide for the need for representative samples for study and testing. From these libraries of preserved human tissue, scientists have made significant advancements in understanding and subsequently treating these diseases in the last few decades.¹ However, as the body of knowledge has continued to grow on the subject, it has revealed even more questions about cancer biology that have yet to be answered. Now the field is shifting to not only more in-depth studies of tumor composition, molecular makeup, and druggable targets, but also into tumor behavior and

its interaction with surrounding normal tissues and other body systems.^{2,3} Traditional, first-generation biobanks have focused primarily on the acquisition and storage of formalin-fixed and paraffin-embedded (FFPE) or fresh frozen tissue. This source of patient material is an integral part of routine pathologic studies in the clinical setting and has played a significant role in cancer research to date.⁴ However, in the emerging era of personalized medicine, non-viable tissue samples have limited research potential when compared to viable patient-derived cell cultures. Viable primary tumor cell cultures allow for the discovery and validation of therapeutic targets and treatment modalities in addition to prognostic studies and tumor biomarker discovery via functional assays on living cell populations.⁵



Recently, the tissue procurement biorepository at the Edwards Comprehensive Cancer Center developed a non-selective protocol for the generation of viable primary tumor cell cultures to better meet the current needs of translational research. The protocol has been successful in generating primary cell cultures representing varying tissue types suitable for in-vitro studies, which can be paired with snap-frozen tissue samples and formalin-fixed and paraffin-embedded (FFPE) samples to provide comprehensive data for rapid advancement in the field. The protocols outlined for primary cell line generation and storage have been standardized across varying solid tumor types and have been tailored to allow for rapid, minimally selective in-vitro tumor expansion. The expanded diverse tumor populations are then assessed for quality and integrity by direct immunohistochemical comparison to the corresponding tissue of origin by a board-certified pathologist.

SECOND GENERATION BIOBANKS PROVIDE SUPERIOR RESOURCES FOR TRANSLATIONAL RESEARCH BY COMBINING CLINICAL GRADE SOURCE MATERIAL IN CONJUNCTION WITH PROTEOMIC-COMPATIBLE FFS TISSUE AND FUNCTIONAL LIVE CELL CULTURE SAMPLES

In the clinical setting, a large number of tissue samples are obtained in hospital pathology laboratories daily for diagnostic purposes. These samples vary widely; however, a significant portion of these samples represent surgical tumor resections. These resection specimens are dissected by certified pathologists and pathology assistants in a process called gross pathology examination. Representative sections of the tumor resection specimens are then taken for diagnostic workup and preserved in formalin-fixed and paraffin-embedded (FFPE) tissue blocks.^{4,6,7} Representative slides are then cut from these tissue blocks for routine histologic staining and diagnosis. This process is essential to clinical diagnostics and is used to evaluate a patient's condition and to determine further treatment plans.⁸ After the diagnostic workup is complete, these preserved tissue samples may be stored for many years. In most cases, however, not all of the resected tissue is needed for diagnosis. The excess tissue may be acquired with the consent of the donor by a research biobank for further translational research

studies. These samples have been the primary research source material for decades and have been used to further biomedical cancer research.^{4,5,6,7,8} FFPE samples, however, are not without their limitations, as they may not retain intact genomic and proteomic data.⁹ With the emergence of molecular pathology, in conjunction with traditional histological staining techniques, snap-frozen tissue has also become a valuable resource for furthering cancer research. At the time of gross dissection, fresh tissue samples may be flash-frozen in liquid nitrogen to retain their molecular composition. This enables researchers to extract proteomic data from the samples to map out enzymatic pathways associated with the drivers of cancer and to identify therapeutic targets for drug development.^{10,11} In the clinical setting, flash-frozen samples have not yet become a routine modality used for diagnostics; however, in translational research, these samples have proven to be invaluable. In addition to the collection of fresh frozen tissue samples (FFS), certain tumor resection specimens are selected for the generation of primary tumor cell cultures (TCC). These cultures represent non-selective total living populations of clonal tumor cells and associated neoplastic cell populations to be used in functional translational studies.^{5,12,13,14} Combining the primary human tumor cell cultures (TCC), the collected fresh-flash frozen samples (FFS), and the FFPE tissue blocks allows for researchers to have a complete comprehensive representative model of the disease of study. In drug development studies, for example, researchers can identify, quantify, and then assess the in-vitro activity of therapeutic targets for future anti-cancer drug development. Herein, we describe the routine longitudinal collection of FFPE tissue samples along with matched FFS samples and the generation of matched histopathological consistent primary tumor cell lines to be placed into our biorepository at the Edwards Comprehensive Cancer Center for increased access to research materials for biomedical research at rural institutions.

ACQUISITION OF HUMAN TISSUE FOR BIOBANKING: FROM THE BODY TO THE BENCHTOP

Possible patient donors are screened by the tissue procurement staff before consenting based upon multiple criteria. These criteria primarily include the patient's history of cancer, size of the tumor, and



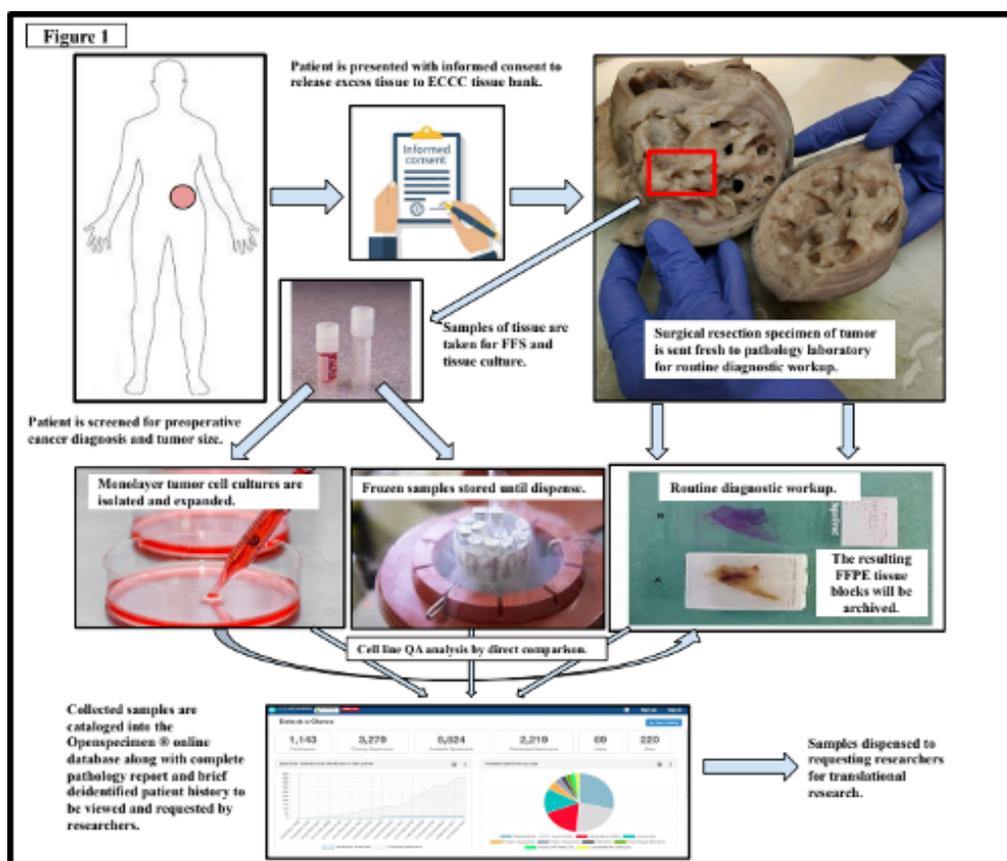
scheduled surgical procedures. Once the patient has been screened and meets acceptable criteria, they are presented with an informed consent (IRB # 112274) that details the release of excess tissue to the tissue bank to be distributed to requesting researchers. Once a patient has consented, the tissue bank staff notifies the clinical pathology laboratory. Coordination between the surgeon, operating staff, and pathology laboratory ensures that the surgical resection specimen arrives at the laboratory within minutes of surgery without any added fixatives or preservatives. The surgical specimen is then examined by certified pathology assistants under the supervision of board-certified pathologists to identify neoplastic tissue and to determine if the culture deems further processing. The remaining specimen will then be further processed by the pathology department for routine diagnostics and will result in the FFPE tissue blocks. The collected specimens will be held pending the final pathology diagnosis before formally being accepted into the biobank. Once the diagnosis is confirmed, the collected samples are cataloged on the tissue bank website powered by Openspecimen[®] to be viewed and requested by

researchers. The process from consent to specimen dispensation is outlined in Figure 1. Matched samples of grossly neoplastic tissue and corresponding non-neoplastic tissue from the surgical specimen are harvested and flash-frozen in liquid nitrogen at -196 °C for storage. The samples are de-identified and assigned a progressive recording specimen number. At this point, an additional representative sampling of the grossly neoplastic tissue is collected and placed into a tissue culture medium comprised of a basal solution of RPMI-1640 supplemented with 10% FBS (fetal bovine serum) and 2% PS (penicillin/streptomycin). The FFS tissue samples are then transferred to liquid nitrogen storage drawers and cataloged while the tissue for cell culture generation (TCC) is provided to the laboratory tissue.

CLINICAL GRADE FORMALIN-FIXED AND PARAFFIN-EMBEDDED TISSUE SAMPLES

Currently, formalin-fixed and paraffin-embedded tissue samples remain the gold standard in pathologic diagnosis in the clinical setting.^{4,6,7} Routine diagnostics are run on these samples to

detect the presence of disease on a histologic and cellular level. The utilization of FFPE tissue samples via immunohistochemical analysis with light microscopy continues to increase in scope with the inclusion of antibody and proteomic targeted special stains to subclassify various disease processes.^{15,16,17} Once properly processed and embedded, numerous representative tissue slides can be cut from the FFPE tissue samples to perform a plethora of varying diagnostic tests. In addition to standard of care diagnostics, these tissue samples can be



used as a valuable source of primary research material for translational cancer research. FFPE has been one of the primary source materials for identifying and quantifying druggable targets for cancer, including the PDL-1 (programmed death ligand-1), HER1/2 (human epidermal growth factor receptor), and ER (estrogen receptor) pathways, to name a few.^{15,17,18} The Tissue Procurement Program at the Edwards Comprehensive Cancer Center allows for researchers to request representative slides of various malignancies to further their research in hopes to continue to elucidate the mechanisms of carcinogenesis and to identify even more druggable targets to improve both cancer diagnostics and treatments. Also, with the partnership that the program has established with the pathology department of the Marshall University Joan C. Edwards School of Medicine, the entirety of the FFPE tissue samples from consented patients may be released to researchers following the 10-year clinical archival period mandated by federal regulations.

FLASH-FROZEN TISSUE SAMPLES: TRANSLATIONAL RESEARCH POTENTIAL

The importance of flash-frozen tissue samples lies in the utility they have in representing the core genetic composition of tumor specimens. Genetic information, including DNA, RNA, and proteins, is well preserved in FFS. Structures from FFS can be isolated in non-denatured active forms. Research has found that the DNA extraction rate, using either laboratory methods or commercial kits in fresh-frozen samples of the human brain, is 100%.¹⁰ Furthermore, the length of time the tissue had been stored did not affect the extraction rate.¹⁰ In contrast, FFPE samples were found to have a lower DNA extraction rate using the same methods. The length of storage time and PCR measures also appeared to affect whether DNA extraction from FFPE samples was successful.¹⁰ Several studies have also shown that FFS is optimal for RNA isolation.^{11,18} When RNA from FFS and FFPE were compared, the relative level of gene expression for each gene appeared to be conserved between sample types. However, gene expression patterns did not correlate between the two types of samples due to significant RNA degradation seen exclusively in FFPE materials.¹⁹ While normalization measures can compensate for RNA fragmentation in FFPE, these extra steps are not

required at all for RNA from FFS. Furthermore, FFPE RNA that has undergone normalization measures can yield expression profiles consistent with FFS specimens, but FFS is still required to be used as a reference for the FFPE material.¹¹ In addition to DNA and RNA extraction, proteins have high efficacy of being extracted from FFS with minimal molecular breakdown. Proteomic methods including laser capture microdissection and mass spectrometry can be used to identify well-preserved proteins from FFS. This information can then be implemented to designate biomarkers and ensure samples maintain tumor heterogeneity among other uses.²⁰

PRIMARY NON-SELECTIVE HUMAN TUMOR CELL LINE GENERATION

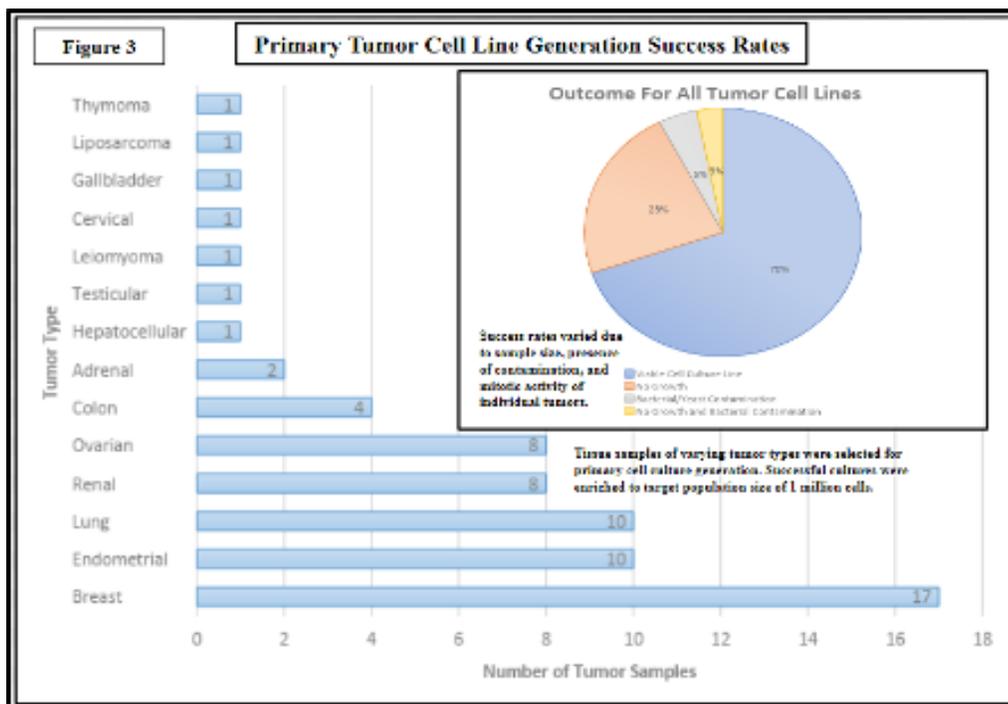
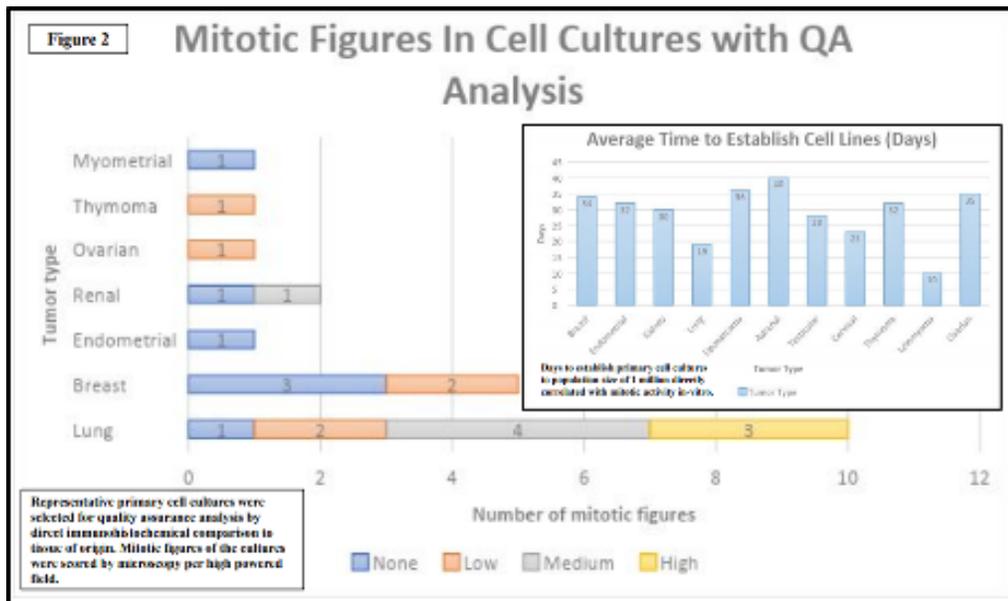
The representative fresh tissue samples from grossly identifiable neoplastic tissue were cut from selective surgical resection specimens and collected in RPMI-1640 (Thermo Fisher Scientific) tissue culture medium supplemented with 10% Fetal Bovine Serum (Thermo Fisher Scientific) and 2% penicillin/streptomycin solution. These selected tissue samples were immediately transferred to the tissue culture laboratory where mechanical dissociation with a sterile scalpel and enzymatic dissociation with .25% Trypsin[®] (Thermo Fisher Scientific) were performed to homogenize the tissue and begin the process of isolating out neoplastic cell populations. The tissue homogenates were then placed in tissue culture incubators (Heracell Thermo Fisher Scientific) to be cultured in an environment of 37 °C in a high humidity water jacket incubator in the presence of 5% atmospheric CO₂. The tissue sample homogenates were then subsequently monitored, and the culture medium was changed at average three-day intervals. Adherent tumor cells were isolated from the associated non-neoplastic connective tissue with subsequent media changes, and clonal non-selective cell populations were subcultured and passaged with the use of enzymatic dissociation using 0.25% Trypsin[®] cell detachment solution (Thermo Fisher Scientific). The monolayer cultures were grown and assessed throughout the process for cell number and viability using Trypan blue exclusion and Cellometer-Mini Automatic Cell Counter System (Nexcelom). The target population size of 1x10⁶ cells was determined to be the cutoff for sufficient cell growth and inclusion into the established primary cell culture



biobank. Average culture periods ranged greatly among the samples reported in this study (Figure 2), and time to establish viable cell cultures was influenced by various factors, including initial sample size, purity and viability of the initial sample, growth rate of the tumor, type of malignancy, and level of adherence to the monolayer culture vessel. The success of the establishment of the selected tumor

tissues varied greatly by tissue type in addition to the presence of contaminating agents, including bacteria and yeast. (Figure 3). The cell populations were then collected and transferred to a freezing medium composed of 90% Fetal Bovine Serum (FBS) and 10% Dimethyl-sulfoxide (DMSO) for frozen storage at vapor-phase liquid nitrogen. Representative cultures of the primary cell lines were grown directly onto

glass slides for quality assessment and culture purity and sample integrity. These slides were then processed and stained by rapid hematoxylin and eosin (H&E) staining for direct comparison to the tissue of origin by a board-certified pathologist. Prepared slides of the primary tumor cultures were directly compared to diagnostic slides of matched tumor samples by the pathologist for quality assurance. The cultured monolayers were assessed for phenotypic similarity to the tissue of origin and confirmation of clonal tumor cell proliferation. Metrics including retention of secondary phenotypic characteristics (i.e., intracellular mucin, cytoplasmic space, nuclear pleomorphism, intercellular-bridging, and inclusion bodies) were scored to determine the overall consistency of the derived cell lines to their corresponding tissues of origin.



These secondary phenotypic characteristics are specific to neoplastic cells of varying etiologies and lineages. Mitotic figures and nuclear pleomorphisms are important cytological indicators of cancer grade and dedifferentiation. Intracellular mucin, inclusion bodies, and intracellular bridging serve as indicators of a specific tissue of origin and may also serve in the further subclassification of a variety of neoplasms. This information was cataloged for each selected sample and was incorporated into the Openspecimen[®] biobank database for reference by requesting researchers. These living models of disease can provide researchers with valuable materials to run functional assays on specific disease models to develop new therapies and improved diagnostics.

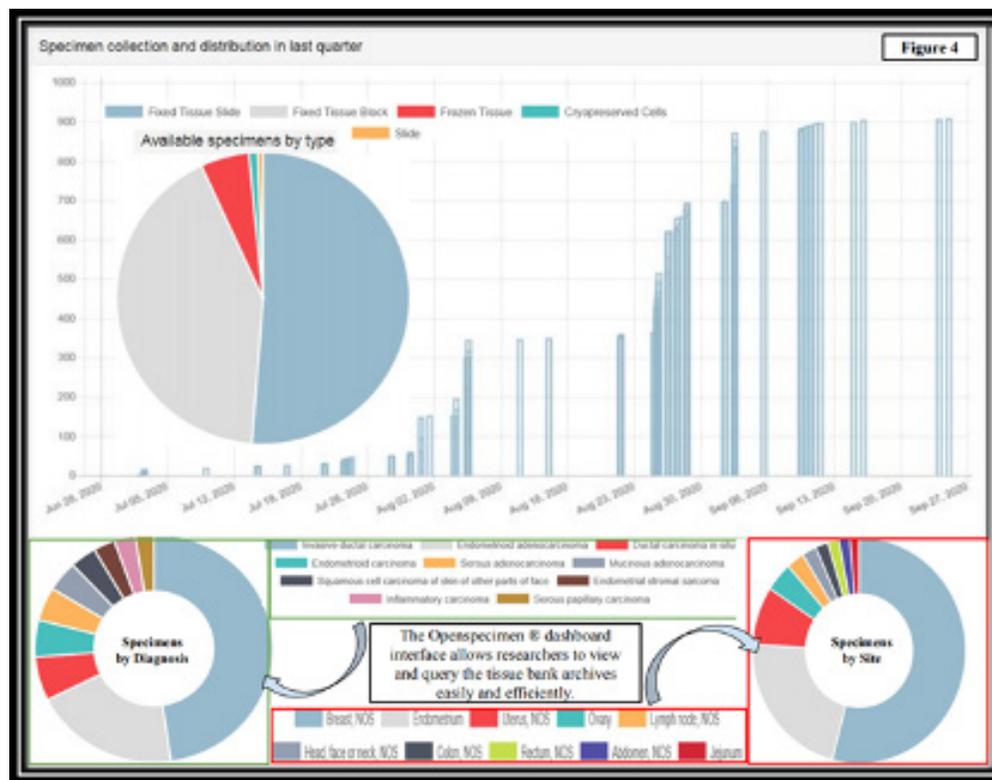
COMPREHENSIVE TRI-FOLD RESOURCES CATALOGED AND DISPLAYED IN INTERACTIVE ONLINE DATABASE OPENSPECIMEN[®]

The complete specimens consisting of FFS, FFPEs, and living cell cultures are cataloged into the Openspecimen[®] online database for open source access by researchers. Utilizing the Openspecimen[®] biorepository software, researchers can easily query

the tissue bank archives for the desired sample types representing their specific disease of study. The software interface displays the quantity and source type of each sample and allows rapid screening of samples in our archives for specific criteria (Figure 4). From the interface, researchers can request and view specific sample information, including brief de-identified patient history and complete pathologic diagnosis. Researchers can then submit sample requests to our biorepository staff and may also request additional clinical research services, including specialized immunohistochemical studies, pathology consultation, send-out molecular studies, and specialized sample preparation. Applications can also be filed by researchers to partner with the tissue bank for specialized sample collection for IRB-approved research protocols, which can be tailored to specific projects. The open-source software interface enables researchers to model their projects around the up-to-date tissue bank archives to ensure that they will have seamless access to the material they require to fulfill the stated research objectives in various grant-funded initiatives, in addition to allowing them to plan future endeavors around previously collected samples to expedite their work.

DISCUSSION

Advancements in cancer research, particularly in the specialties of molecular medicine and personalized therapeutic strategies, have significantly increased our understanding of cancer biology and have made great strides in the treatment of the disease. To further us down the path of personalized medicine and the promise of precision therapies that provide maximum effectiveness with minimal side-effects, the link between the clinical pathology laboratory and translational



researchers must be strengthened to allow for a streamlined path of cutting-edge, clinically relevant investigations. The comprehensive three-fold biobanking strategy of the Edwards Comprehensive Cancer Center Tissue Procurement Department provides partnered researchers at rural institutions with a complete toolkit that they can use to further their research. The next-generation resources now available enable researchers to move seamlessly through the basic science discovery phase to functional assays on living cells to test their hypothesis. The complete disease models available to rural researchers at Marshall University and other researchers throughout the country have the potential to provide vital information about cancer biology and etiology, specifically in niche populations of rural southwestern Appalachia where the majority of tissue donors to the ECCC Tissue Procurement Department live.

CONCLUSION

The Edwards Comprehensive Cancer Center biorepository is a non-profit endeavor with a clear and concise mission statement: to advance cancer research in our region for the benefit of our community. Donors to the tissue bank are aware that they will receive no direct benefit from their contribution. However, advancements derived from their donations may contribute to a high standard of care in the future treatment of cancer. The advancements in cancer biobanking at the Edwards Comprehensive Cancer Center at Cabell Huntington Hospital, with its direct partnership with the pathology practice of Marshall University's Joan C. Edwards School of Medicine, is opening new doors and providing new opportunities to rural research institutions. The program is continuing to advance in size and scope, with future collection sites and partnerships with other biorepositories in the state of West Virginia currently being investigated. Supplementary research services in clinical histopathology and research consultations are also being expanded to further the work of basic scientists in their investigations. With the growing capabilities of the tissue procurement department and the expansion of the diagnostic pathology department, we hope in the future to grow our research capacities to include other specialty areas, such as molecular pathological studies and genetic

analysis. We hope that the Tissue Procurement Program will continue to be an ever-expanding bank of valuable source material for the advancement of cancer research in Appalachia.

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