Translational assessment of primary tumor-derived cells

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Thesis

TRANSLATIONAL ASSESSMENT OF
PRIMARY TUMOR-DERIVED CELLS

by

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B.A., Harvard University, 2011

Submitted in partial fulfillment of the
requirements for the degree of
Master of Arts

2014
DEDICATION

This work is dedicated to my loving mother, whose battle and victory over breast cancer has inspired my enthusiasm for cancer research and pursuit of medicine.
ACKNOWLEDGMENTS

I would like to acknowledge and thank a number of people who were instrumental in the completion of my thesis. To the Boston University MAMS faculty and staff, thank you for giving me the opportunity to be a part of such a wonderful program.

To my advisor Dr. Hee-Young Park, your guidance has been invaluable throughout the MAMS program, medical school application process, and research year.

To Dr. James Rocco, thank you for allowing me to work on such a meaningful project in your lab. You have been a fantastic mentor, and your enthusiasm for translational cancer research has furthered my own interest in oncology.

To Dr. Bill Michaud, thank you all the time and effort you have put in to teaching me, helping me with my experiments, and answering my countless questions.

To Dr. Ed Mroz, thank you for your excellent suggestions for my experiments and thesis and help with data interpretation.

To my fellow lab members Rebecca, Stephanie, and Armida, thank you for your feedback, discussions, and making every day in lab an enjoyable one.

To Samantha Houston, my unbelievable girlfriend, I can’t thank you enough for supporting me through my obstacles and celebrating with me in my successes. I can’t express how much I value your love and friendship.

To Shan, Nick, and Charles, you have been the best roommates and friends anyone could ever ask for.

And finally, to my parents and twin sister Kristin, thank you for being a continuous source of love and inspiration throughout all my academic endeavors.
TRANSLATIONAL ASSESSMENT OF PRIMARY TUMOR-DERIVED CELLS

ERIC L. WU

ABSTRACT

Only a few individual cells within less than 5% of all primary tumors form the cell lines commonly used in cancer research. These growth bottlenecks result in cell lines that are often poor models of primary tumors. Co-culture of primary tumor-derived cells with an irradiated mouse fibroblast feeder layer and ROCK inhibitor, known as the Georgetown Method, offers a way to culture over 80% of tumor-derived cells in vitro to create more representative tumor cell models. In our studies, we optimized the Georgetown Method to culture head and neck cancer cells, including oropharyngeal squamous cell carcinoma, and investigated its mechanism of conditionally immortalizing cells in culture. Differential trypsinization and regular feeder layer replacement were found to significantly improve the efficacy of immortalizing co-cultured cells at both atmospheric and physiological oxygen levels. Medium conditioned by irradiated fibroblasts can also substitute for direct co-culture with a feeder layer. The Georgetown Method was found to maintain low levels of p16 in co-cultured cells, suggesting a potential mechanism by which the Georgetown Method prevents differentiation and senescence. Our ability to culture over 80% of primary tumor-derived cells allows us to test the translational value of tumor-derived cell cultures and xenografts using BH3 profiling. Conditioned medium simplifies maintenance of cell cultures and will also allow us to perform high-throughput screens without the need to separate tumor-derived cells.
from the fibroblast feeder layer. The Georgetown Method provides opportunities to expand small tissue specimens for future diagnostics, therapeutics, and biobanking.
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LIST OF ABBREVIATIONS

ChIP ................................................................................. Chromatin immunoprecipitation
CM .................................................................................. Conditioned medium
CML ............................................................................... Chronic Myelogenous Leukemia
CRC ............................................................................... Conditionally reprogrammed cell
CtBP ................................................................................ C-terminal binding protein
H&N................................................................................... Head and neck
HEC ................................................................................... Human ectocervical cell
hES .................................................................................... Human embryonic stem
HFK .................................................................................... Human foreskin keratinocytes
HPV .................................................................................... Human papillomavirus
iPS ...................................................................................... Induced pluripotent stem
MEF .................................................................................... Mouse embryonic fibroblast
NT ....................................................................................... Normal tonsilar epithelium
OPSCC .............................................................................. Oropharyngeal squamous cell carcinoma
PD ....................................................................................... Population doublings
PDX .................................................................................... Patient-derived xenograft
PTDC .................................................................................. Primary tumor-derived cells
ROCK .................................................................................. Rho-associated kinase
ROCKi ............................................................................... Rho-associated kinase inhibitor
STR ..................................................................................... Short tandem repeat
SV40 ................................................................................... Simian virus 40
T/B ........................................................................................................ Tumor/Blood (OPSCC)

TDCL .................................................................................................... Tumor-derived cell line

WHO .................................................................................................... World Health Organization
INTRODUCTION

Cancer remains a leading cause of death worldwide despite our increased understanding of cancer biology and continued advances in diagnostics and therapies. It is estimated that 2 out of every 5 people will be diagnosed with cancer at some point in their lifetime, and rates of cancer are rising as people live longer. While our increased understanding and treatment of cancer has significantly reduced mortality in some cancers like those of the breast and prostate, the prognoses for others like pancreatic cancer, which has a five-year survival rate of about 6%, remain poor (“WHO | Cancer” 2013; “Cancer of the Pancreas - SEER Stat Fact Sheets” 2013).

Cancer occurs when normal cells in the body, which regularly undergo mitosis to replace older cells, accumulate genetic mutations over time in a stepwise fashion as a result of errors during DNA replication, external mutagens such as radiation, or viral and bacterial infections that lead to unregulated growth and immortalization (Nowell 1976). Mutations that impart fitness advantages to subclones within a tissue population continue to be passed on because they expand and outcompete other cells for limited space and resources in a Darwinian-like system of natural selection (Merlo et al. 2006). If enough driver mutations, mutations that confer growth advantages and promote tumor progression, accumulate within particular subclones to achieve what Douglas Hanahan and Robert Weinberg call “the hallmarks of cancer,” these subclones can become neoplastic and form tumorigenic masses (Hanahan and Weinberg 2011). Tumors can
obstruct and impede normal organ function or metastasize to distant organs to form secondary tumors elsewhere in the body (Greaves and Maley 2012).

**CURRENT CANCER THERAPY**

Chemotherapy and radiotherapy are commonly used in treatment regimens for many types of cancers. Chemotherapeutics target rapidly dividing cells like cancer but do so with a variety of mechanisms including alkylating agents that damage DNA directly and antimetabolites that act as nucleoside analogs to substitute for the building blocks of DNA. Radiotherapy, like chemotherapy, can directly damage DNA or create free radicals that in turn damage cells and induce apoptosis. However, these treatment regimens, while killing off highly proliferative cancer cells, will also damage normal rapidly dividing cells like those that line the intestines, blood-producing cells, or hair follicles, resulting in adverse side effects such as nausea, anemia, and hair loss.

Genetic variations between tumors from the same tissue of origin can result in markedly different responses to the same therapy regimen (Gottesman 2002). Known as intertumor heterogeneity, differences between tumors often result in some tumors displaying resistance to first-line drugs. On an even smaller scale, high intratumor heterogeneity within an individual tumor can also result in a wide range of drugs responses among clones isolated from the same tumor. In highly heterogeneous multiclonal tumors, a therapy regimen will often kill off sensitive clones within a specific tumor but select for those that may be less sensitive or resistant, resulting in cancer recurrence and treatment failure (Barranco et al. 1988).
Tumor heterogeneity and adverse side effects continue to spur cancer research towards more effective and less toxic therapies. This includes advances in diagnostics to identify early-stage cancers and discoveries of new biomarkers, specific mutations that may drive the tumor or confer resistance. Once these mutations can be identified on a patient-to-patient basis, personalized therapies can avoid damaging normal cells by more precise targeting of neoplastic cells. Biomarkers can help improve efficacy of treatment by indicating disease severity and whether a therapy regimen should be intensified or deintensified.

The future of cancer treatment is racing towards personalized medicine in which each patient has an individualized treatment regimen that maximizes treatment efficacy and minimizes unwanted side effects. New discoveries in diagnostics, mechanisms, and drug targets are the steps towards realizing these goals.

CANCER CELL LINES

HISTORY

While cancer is a disease that occurs in complex organisms like humans, studying cancer within the human body is often difficult due to the inability to conduct high-throughput screens or interventional studies. In the early part of the 20th century, there was a concerted effort to develop human cell lines, a powerful tool to study cells from the body in the laboratory in a more accessible manner. There were thousands of attempts to create cell lines, but nearly all survived for only a few weeks at most (Masters 2002). In 1951 however, George Gey successfully cultured cells from a cervical tumor explant.
from a patient named Henrietta Lacks to generate HeLa cells, the first successful patient-derived cell line (Skloot 2011). Following the success of HeLa, cell lines from nearly every type of cancer have since been developed and have proven to be invaluable for cancer and molecular biology research.

From these cell lines, particularly HeLa, scientists have gained knowledge of fundamental cellular processes in both normal and abnormal tissues that have contributed to many clinical advances including a vaccine against polio as well as chemotherapeutic compounds to fight cancer (Scherer, Syverton, and Gey 1953; Eagle and Foley 1958). In the past quarter century, these cell lines have also aided in the identification of hundreds of genes that play a role in cancer development. These genes are often categorized as either oncogenes or tumor suppressor genes. Oncogenes are genes that have mutated to become permanently activated in the cell, leading to unregulated growth and division (Weinberg 2007). On the other hand, tumor suppressor genes normally prevent unregulated division by repairing DNA damage or inducing apoptosis. When mutated and inactivated, this inhibition of division is removed and contributes to unregulated growth (Weinberg 2007). A number of commonly mutated genes like TP53 and RB1 are involved in the progression of many types of cancer, and their corresponding proteins and associated downstream targets have been researched extensively (Levine, Momand, and Finlay 1991; Chinnam and Goodrich 2011).

Scientists have also begun targeting corresponding proteins of commonly mutated genes in cancers to create novel chemotherapeutics. In the 1960s, translocation of chromosomes 9 and 22, known as the Philadelphia Chromosome, was found to be
associated with Chronic Myelogenous Leukemia (CML) and was the first malignancy to be linked to a genetic abnormality (Nowell 2007). The constitutively activated tyrosine kinase BCR-ABL fusion protein generated by the Philadelphia Chromosome was subsequently targeted with a tyrosine kinase inhibitor called imatinib mesylate (Gleevec) to create the first targeted cancer therapeutic, effectively launching the future of targeted anticancer drugs (Druker et al. 2001). Gleevec serves as a model of effective and powerful cancer treatment based on genetic abnormalities or biomarkers that continues to be pursued today.

**LIMITATIONS**

For the most part, cancer cell lines retain the driver mutations that cause primary tumors and thus have value in helping us understand major mutations responsible for cancers in vivo. However, there has been only incremental progress in translating basic knowledge from the laboratory to the clinic, and this may be due to subtle differences between cell lines and the primary tumors they attempt to model (Gillet et al. 2011). The difficulty and low success rate in creating cancer cell lines from primary tumors suggests significant selection when trying to derive cell lines from primary tumor specimens. In past attempts, Giard et al created 13 cell lines from 200 solid tumor specimens (6% success rate) (Giard et al. 1973), Sugaya et al created 15 cell lines from 570 lung cancer samples (2.6% success rate) (Sugaya et al. 2002), and Dangles-Marie et al created 3 cell lines directly from 31 colon cancer tumors (9.7% success rate) (Dangles-Marie et al. 2007). Across all cancer types, success rates in establishing cell lines directly from
primary tumors have continued to remain at about 5% and usually lie on the lower end of the spectrum.

Intertumor heterogeneity, or differences between tumors of the same cancer type, contributes significantly to the low success rate. Most cell lines seem to be derived from only the most highly proliferative, metastatic tumors, which fail to represent the majority of tumor specimens (Sugaya et al. 2002; Mehta et al. 2007). In one study examining 15 stable cell lines derived from 570 lung cancer specimens, 11 (73%) were derived from stage III and IV cancers, and 10 (67%) of those patients had already died, indicating that a significant majority of those specimens that formed cell lines derived from the most aggressive, deadly tumors (Sugaya et al. 2002). Cell lines may thus be more representative of high-grade tumors, especially tumors composed of very proliferative subclones, but fail to effectively represent low and medium-grade tumors, which make up the majority of cancers (Dairkee et al. 2004).

In addition to intertumor heterogeneity, intratumor heterogeneity or the variety of cell types within a single tumor, may also be altered by selective pressures seen during the creation of cell lines. Culture conditions such as monolayer culture, growing on plastic, and high oxygen tension select for subpopulations of cells within the tumor that can proliferate well in the specific culture conditions. Cell lines often result from only a small percentage of viable cells that are plated, and varying culture conditions would likely generate different cell lines. Cell lines also often differ significantly from the cell types that are a part of the tumor microenvironment (Gillet et al. 2011; Gillet, Varma, and Gottesman 2013).
For example, cell culture is traditionally done at atmospheric oxygen levels (21%), and early cell culture was often conducted on plates left on the bench top. Physiological oxygen levels however are lower, ranging from below 1% in the brain to 12% in arterial blood (Panchision 2009). Research has shown that culturing cells at physiological oxygen levels (3%) increases plating efficiency and lifespan relative to cells cultured at atmospheric oxygen (Richter, Sanford, and Evans 1972; Packer and Fuehr 1977; Parrinello et al. 2003). Culture conditions like varying oxygen levels can have a significant effect on the survival of particular cells within the culture, potentially creating a cell line that differs greatly from the primary tumor.

As cells are cultured long-term for many passages, the culture becomes increasingly homogenous as clones with the most advantageous mutations proliferate faster and outcompete other cells within the culture (Merlo et al. 2006). Several studies have analyzed similarities and differences between cell lines and the primary tumors they attempt to model, looking at both genomic and transcriptomic changes. Gene expression profiles and clustering reveal that cells lines actually resemble each other more than the original clinical samples they are intended to represent (Virtanen et al. 2002). These results were shown in several solid tumors including ovarian carcinoma (Domcke et al. 2013), breast cancer (Mehta et al. 2007), head and neck squamous cell carcinoma (Chung et al. 2004), colorectal cancer (Gillet, Varma, and Gottesman 2013), and two types of leukemia (Gillet, Varma, and Gottesman 2013). It has also been shown that during their establishment, cancer cell lines highly select for genes associated with multi-drug resistance, which may contribute to the discrepancy seen between prognostic markers and
clinical results (Gillet et al. 2011). The results suggest selection and induction of genes in cell lines by common culture conditions, skewing their ability to represent primary tumors.

These differences highlight the importance of recognizing the limitations of cell lines. Because selection pressures and culture conditions limit both intertumor and intratumor heterogeneity, the cell lines isolated from patient tumors are forced into bottlenecks of growth. Of the few excised primary tumors that can form a cell line, only a few individual cells within the entirety of the tumor ecosystem ever develop the ability to proliferate and create a successful cell line. These cell lines, which represent a small fraction of all cancer cells, are currently used to make discoveries that are applied to the entire spectrum of cancers. It is no surprise that they often fail to model the in vivo character of most tumors and often lead to failures when trying to translate findings therapeutically from the laboratory to patients in the clinic. Thus, there is a need to modify the methods we use to approach cancer research and a need to study primary tumor-derived cells and low-passage cell lines.

**PRIMARY TUMOR-DERIVED CELLS**

The key to developing a panel of cell lines that can model primary tumors appropriately is to maximize both intertumor heterogeneity, increasing the success rate of culturing primary tumors in vitro, and intratumor heterogeneity, maintaining the variety of cell types within the original tumor. As evidenced by the attempts to develop human cell lines over the past century, the majority of primary tumor-derived cells fail to
proliferate in vitro, differentiating and senescing after a few passages. Normal cells can only divide a limited number of times, which is regulated by the length of their telomeres, and will stop dividing, also known as replicative senescence, after this number is reached. DNA-damage, irradiation, or toxins can also cause cells to stop replicative division. Long-term cell culture depends on continuing the proliferative ability of the cell by preventing senescence and limiting cellular differentiation. There have been several methods attempted to overcome these barriers.

One of the most common ways to immortalize primary cells has been transformation with viral oncogenes, such as the simian virus 40 large tumor antigen (SV40) or E6/E7 proteins of the oncogenic human papillomaviruses (HPV) (Bartek et al. 1991; Hawley-Nelson et al. 1989). A common theme of cellular immortalization is the inactivation of the p53 and p16/Rb pathways and activation of hTERT (Rheinwald et al. 2002). The SV40 Large T antigen and E6/E7 HPV proteins are known to activate both of these pathways. However, use of viral oncogenes bypasses tumor suppression mechanisms by inactivating the exact pathways of interest. By altering the characteristics of the cultured, immortalized cell, viral oncogenes also cause problems when trying to create an accurate in vitro model of the primary tumor (Meisner et al. 1988).

HPV immortalizes cells partly by inducing hTERT, the catalytic subunit of human telomerase (Liu et al. 2008; Liu et al. 2009). Telomeres, portions of genetic code at the end of chromosomes, act as a buffer to prevent the chromosomal ends from deteriorating and have also been shown to be vital in determining the lifespan of a cell. As cells divide, telomeres become slightly shorter with each DNA replication, limiting the number of
cellular divisions to a number known as the Hayflick Limit. Many cancers, in order to prevent cellular senescence, induce production of telomerase, an endogenous enzyme usually silenced in postnatal somatic cells that extends the length of telomeres. Exogenous expression of hTERT has been shown to increase the lifespan and number of cellular divisions (Counter et al. 1998). However, similar to the use of viral oncogenes, induction of hTERT can cause karyotypic variations, aberrant differentiation, and tumorigenicity over extended passaging (Toouli et al. 2002).

Besides viral transformation and induction of hTERT, irradiated mouse fibroblasts have been used as early as the 1970s as a feeder layer to support growth of epithelial cells, including keratinocytes (Rheinwald and Green 1975; Taylor-Papadimitriou, Shearer, and Stoker 1977). Normal keratinocytes cultured without a feeder layer exhibit a bloated phenotype, positive senescence-associated β-galactosidase staining, and senescence-associated heterochromatin foci after just a few passages. The feeder layer increases the number of epidermal cell doublings in culture significantly from about 20 to 40-60 population doublings (PD) and helps prevent the cultured cell population from being overgrown with human fibroblasts (Rheinwald and Green 1975; Green, Rheinwald, and Sun 1977; Ramirez et al. 2001). A feeder layer was also shown to support colony formation from single keratinocytes (Barrandon and Green 1987). Although the mechanism of how irradiated fibroblasts can extend keratinocyte proliferation is still unknown, findings suggest that feeder cells may enhance lifespan partly by inducing telomerase (Fu, Quintero, and Baker 2003; Rheinwald et al. 2002). Besides keratinocytes, primary tumor cultures derived from breast carcinoma were also
supported by irradiated fibroblast feeder layers (Wang et al. 2001). Feeder layers are now also commonly used to produce epithelial cell layers appropriate for skin grafting for severely burned patients (Pellegrini et al. 1999). Although promising, cells grown on feeder layers are not completely immortalized and still exhibit senescence, though after significantly more population doublings (Bisson et al. 2013).

Another important aspect of long-term cell culture is the prevention or limitation of cellular differentiation. Rho kinase (ROCK) has many roles within the cell including regulation of cytokinesis and cellular differentiation (Kosako et al. 2000; Sordella et al. 2003). Large-scale death of cultured human embryonic stem (hES) cells caused by anoikis, or death of anchorage-dependent cells induced by loss of contact with the extracellular matrix, was recently prevented with the use of ROCK inhibitors (Watanabe et al. 2007). The use of ROCK inhibitors not only prevents apoptosis of hES cells during passaging but also improves recovery and colony formation after thawing from a cryopreserved state (Claassen, Desler, and Rizzino 2009). ROCK inhibition has also been effective in maintaining embryonic and induced pluripotent stem (iPS) cells in an undifferentiated state in culture, potentially prolonging the proliferative ability of the cell (Pakzad et al. 2010).

Although some of the previous methods have proven to have some efficacy in primary cell culture, most of them continue to pose problems for long-term culture: selection of the most proliferative cell clones, eventual senescence, relying on viral transformation, or changing properties of the cultured cells.
GEORGETOWN METHOD: CONDITIONALLY REPROGRAMMED CELLS

FINDINGS

In 2010, the Schlegel group at Georgetown showed that a combination of an irradiated Swiss 3T3-J2 mouse fibroblast feeder cell layer and the ROCK inhibitor Y-27632 in cell culture, now known as the Georgetown Method (Figure 1), greatly increases the proliferative capacity of primary human keratinocytes, conditionally reprogramming cells to effectively immortalize them in vitro (Chapman et al. 2010). While both fibroblast feeder layers and ROCK inhibitors have been used in the past to increase the proliferative ability of cells in culture, the combination of the two allows both normal and neoplastic tissues to proliferate indefinitely beyond the number of population doublings achieved with either method independently or with other methods that use exogenous viral or cellular gene expression. The Schlegel group refers to cells immortalized by this system as conditionally reprogrammed cells (CRCs) due to the fact that normal differentiation is seen upon removal of the ROCK inhibitor and feeder cells, even after human ectocervical cells (HECs) were cultured for over 200 population doublings under this system (Suprynowicz et al. 2012).
Figure 1: Georgetown Method supports growth and immortalization of human epithelial cells. Following mincing and digestion, co-culture of human tissue with an irradiated feeder layer of Swiss 3T3-J2 fibroblasts and 10 µM ROCK inhibitor (Y-27632) allows cells to propagate. Small colonies of cells can be observed after 1 day, and large colonies of cells compress adjacent feeder cells after 5 days (white arrows) (Figure from Liu et al. 2012).

Both the ROCK inhibitor and feeder cells appear to be necessary to immortalize cells. Without a ROCK inhibitor, keratinocytes cultured with feeder cells were seen to senesce after 20 to 40 population doublings, but with the addition of the ROCK inhibitor, foreskin keratinocytes were cultured for over 100 population doublings and are considered immortal (Chapman et al. 2010). Likewise, cells cultured in only the ROCK
inhibitor without a feeder layer could not bypass senescence (Chapman et al. 2010). Cells passaged long-term with the Georgetown Method also remain karyotypically normal, an important advantage this system has over other methods used to immortalize cells (Liu et al. 2012).

Previously, less than 5% of primary tumors could be cultured outside of the body to form long-term cultures, and nonkeratinocyte epithelial cells from prostate and liver tissues were particularly difficult to culture and characterize (Castell and Gómez-Lechón 2009; Miki and Rhim 2008). Using the Georgetown Method, the Schlegel group has successfully generated cell lines from almost 90% of biopsied specimens including nonkeratinocyte epithelial cells from prostate, breast, lung, and liver tissues (Table 1) (Liu et al. 2012). Propagation of intertumor heterogeneity with the Georgetown Method is thus much higher relative to previous methods of cell line creation that selected for only the most aggressive tumors.
Table 1. Georgetown Method conditionally reprograms variety of human and animal tissues. The Schlegel group was able to culture almost 90% of all biopsy specimens tested, and cell lines generated from the samples are indicated. Success is defined as ability of cultured cells to reach 50 population doublings, or in colon and pancreas, as three times the population doublings observed with current, optimal culture conditions (Table from Liu et al. 2012).

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue type</th>
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<td>25</td>
<td>18/4B/3T</td>
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<td>16</td>
<td>16</td>
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<td>6</td>
<td>3</td>
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<td>8</td>
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<td>94</td>
<td>83</td>
<td>NA</td>
</tr>
</tbody>
</table>

B, BRCA 1/2 mutation carrier; H&N, head and neck cancer; met, metastatic; NA, not applicable; T, tumor.

In addition to maximizing intertumor heterogeneity by culturing a wide variety of both normal and neoplastic tissues, the Georgetown Method appears to also conserve intratumor heterogeneity in early passages by reprogramming the entire population of cells within an individual tumor. By using flow cytometry to measure levels of the stem
cell marker integrin α6 per cell in a population of HECs over time, the Schlegel group found expression levels indicative of a single population of cells undergoing gradual reprogramming to a stem cell-like state (Suprynowicz et al. 2012). This suggests conservation of all cells in culture. However, it has not been determined whether the Georgetown Method selects for a subset of cells during initial plating. There are also likely still selective growth advantages between clones, and we would expect the most proliferative clones to outcompete other cells in the culture after extended passaging.

The Georgetown Method thus maintains both intertumor and intratumor heterogeneity, at least in early passages, making it a powerful tool to culture and study nearly all primary cell specimens.

**MECHANISM**

Although the mechanism of the Georgetown Method is currently unknown, there are insights into how the combination of ROCK inhibition and an irradiated fibroblast feeder layer is able to immortalize co-cultured cells. Rho-associated kinases, ROCK 1 and ROCK 2, regulate many cellular processes including migration, adhesion, and differentiation (Amano, Fukata, and Kaibuchi 2000; McMullan et al. 2003). ROCK has been shown to be an important regulator of cytoskeletal changes like stress fiber formation, and its inhibition has the potential to inhibit cancer cell migration and invasion, making ROCK a target for cancer therapeutics.

ROCK inhibition with Y-26732 has been shown to prevent terminal differentiation and increase cellular proliferation in keratinocytes (McMullan et al. 2003).
High-calcium and serum in media induces terminal differentiation of cultured cells (Pillai et al. 1988), but the Schlegel group has shown that ROCK inhibition in combination with a feeder layer may suppress differentiation usually induced by these conditions (Palechor-Ceron et al. 2013). Cytoskeletal changes play a role in regulating differentiation and determining stratification of epidermal cells, specifically migration of cells within the epidermal environment and adhesive structures important for cell-to-cell interactions (Vaezi et al. 2002). Adult stem cells reside in deeper, more basal levels of the skin and migrate upwards to replenish dead cells at the surface (Blanpain and Fuchs 2006). Disruption of this pathway through ROCK inhibition in culture could allow for cells to maintain their basal-like state and proliferate indefinitely. However, the mechanism of how the ROCK inhibitor signals the cell to prevent differentiation, possibly through cytoskeletal changes, remains to be elucidated.

In addition to the ROCK inhibitor, irradiated fibroblasts are also essential for the conditional immortalization process. The Schlegel group recently showed that physical contact between CRCs and the irradiated fibroblast feeder layer is not necessary for conditional immortalization (Palechor-Ceron et al. 2013). The irradiated fibroblasts appear to release one or more diffusible factors that in combination with the ROCK inhibitor can promote keratinocyte proliferation and survival, and conditioned medium collected from cultures of irradiated fibroblasts can substitute for direct co-culture with the feeder layer. Previous research has shown that fibroblasts secrete glycoproteins such as fibronectin, laminin, and type IV collagen that could support attachment and promote proliferation of co-cultured cells (Alitalo et al. 1982). However, it is still unknown what
these diffusible factors may be or what pathways are involved for immortalization by the Georgetown Method.

Research also suggests that the DNA-damage arrest and apoptosis of the feeder cells induced by irradiation may be essential for release of the relevant factors. Release of the factors appears to correlate with the onset of apoptosis following irradiation (Palechor-Ceron et al. 2013). Apoptotic cells are known to promote wound healing in surrounding tissues \textit{in vivo} through the release of diffusible growth factors, and the process of apoptosis has been shown to be necessary to initiate regeneration in a variety of biological models including \textit{Xenopus} tadpoles and planaria (Tseng et al. 2007; Hwang et al. 2004). Caspases 3 and 7, primary regulators of apoptosis, appear to regulate the release of arachidonic acid, a precursor of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) (Li et al. 2010). PGE\textsubscript{2} is a promoter of stem cell proliferation and tissue regeneration. Whether irradiation specifically, senescence, or apoptosis of fibroblasts stimulates secretion of the factors that contribute to formation of CRCs has yet to be determined.

Many questions regarding the mechanism of the Georgetown Method still remain. Primary HECs cultured with this system appear to resemble adult stem cells, exhibiting induction of adult epithelial stem cell markers like increased expression of $\Delta$Np63$\alpha$ and CD-44 and decreased expression of Notch-1 signaling (Suprynowicz et al. 2012). This suggests that the Georgetown Method could be increasing the proliferation of cancer stem cells or turning on stem cell markers in cells within the culture. It is still unclear what diffusible factors are involved and how they, along with the ROCK inhibitor, interact with co-cultured cells to induce conditional immortalization. Following binding of these
factors, many signaling pathways regulating senescence and differentiation must also be activated or repressed to effect changes in the cell. The p16\textsuperscript{INK4A} tumor suppressor, for example, is an important regulator of the cell cycle and senescence, and p16 inactivation is seen in almost all cancers, contributing to uncontrolled cell growth (Kim and Sharpless 2006; Gil and Peters 2006). Because of p16’s key role in controlling senescence, ROCK inhibition and irradiated fibroblasts could immortalize cells through regulation of this pathway, making it a valuable point of study for elucidating both the mechanism of p16 and the Georgetown method. Answering these questions about what pathways are important to induce cellular immortalization could lead to future cancer therapeutics that target players in the pathway to induce senescence in neoplastic cells and limit their growth.

SIGNIFICANCE AND FUTURE APPLICATIONS

APPLICATIONS OF CONDITIONALLY REPROGRAMMED CELLS

The ability to create tumor-derived cell lines through the Georgetown Method represents a significant discovery for all of cancer research. Expansion of cells from primary tumors allows for the creation of tumor-derived cell lines that provide a much more representative \textit{in vitro} model than cell lines currently used in the majority of cancer research. Recent preclinical trials of new cancer therapeutics have been a poor predictor of clinical success, and the high rate of failure is attributed to testing on high-passage cell lines that can have significantly different characteristics than primary tumors (Godoy et al. 2013). Low-passage tumor-derived cells limit the selective pressures of cell culture as
well as the potential mutations and cellular contamination that can accumulate from
decades of passaging. Thus, these new models, which could encompass all types of
tissue, could provide insight into driver mutations, help elucidate mechanisms of
carcinogenesis, and offer a better model for drug discovery to increase the success rate of
new therapeutics and improve clinical outcomes.

With the ability to create cell lines from nearly 90% of all specimens, the
Georgetown Method gives laboratories the power to create viable cell biobanks, frozen
stocks of human biospecimens. These cells can be thawed and expanded when necessary,
providing an inexhaustible supply of patient-derived specimens for study and
experimentation. The applications of these cells are limitless. Because normal and
neoplastic cells can both be cultured, comparative genetic and molecular analyses could
elucidate the specific mutations that drive carcinogenesis in each individual. Addition of
clinical data to these genetic profiles and comparison of thousands of tumors from the
same tissue of origin will ultimately expand the value of biobanks even further by helping
in the identification of biomarkers to stratify patients by disease severity to determine
treatment regimen and to predict clinical outcomes, both needed for improved treatment
of oropharyngeal squamous cell carcinomas (OPSCC), a specific type of head and neck
cancer our laboratory is interested in.

In the past decade, there has been a push towards personalized therapies: creating
individualized treatment regimens for each unique tumor. More often than not, an
individual’s response to a drug differs somewhat from the expected outcome, which is an
averaging of drug responses after clinical trials. Using the Georgetown Method, cells
from a primary tumor could be isolated, expanded, and subjected to drug tests to determine drug resistance and toxicity before administering treatment. This eliminates the potential of treatment failure and limits side effects associated with most chemotherapies. Yuan et al have already shown the potential and power of the Georgetown Method in a case of recurrent respiratory papillomatosis, a disease that has no known effective treatment therapies (Yuan et al. 2013). Expanding tumor cells from a biopsy, the group used these cells to diagnose the viral infection responsible for the disease and to determine which drugs were most effective, allowing for treatment, tumor shrinkage, and cancer stabilization. It remains to be seen whether this same approach of drug screening using the Georgetown Method can be applied to other tumors.

Patient-specific in vitro drug testing combined with biomarker identification is the key to the next wave of cancer treatments, and the ability to conditionally immortalize primary tumor-derived cells provides the means to achieve that dream.

**HEAD AND NECK CANCERS**

OPSCC, though not as prominent as some cancers like that of the breast or prostate, have seen a recent upswing in incidence, primarily due to the role of human papillomavirus (HPV) in their carcinogenesis (Chaturvedi et al. 2011). While the incidence of HPV-negative tumors, which are strongly associated with age, tobacco use, and alcohol use, has declined by 50% between 1988 and 2004 because of smoking cessation, incidence of HPV-positive tumors increased by 225% during the same time period (Applebaum et al. 2007; Chaturvedi et al. 2011). HPV-positive OPSCC cases are
seen in a largely different demographic than HPV-negative cancers and are associated with different risk factors such as decreasing age of first intercourse and increasing number of sex partners (Schwartz et al. 1998). HPV-positive OPSCC appears to be a markedly different disease due to its unique clinical presentation, lower rates of disease progression, and improved outcomes, suggesting the need for a new drug targets and de-escalated treatment regimens (Bonilla-Velez et al. 2013).

Identification of biomarkers and understanding of HPV-positive OPSCC’s unique pathogenesis are essential for proper treatment, but current available research is lacking in these areas. Because HPV-positive OPSCC is associated with improved response to therapy and better prognosis, there is a potential to lessen the degree of treatment to decrease morbidity associated with traditional treatment of OPSCC. However, the absence of reliable biomarkers to stratify patients by projected response or severity of disease highlights the need for further investigation of OPSCC and its mechanism of action. Identification of important players in the pathology of OPSCC will aid in the development of targeted therapies like cetuximab, an antibody against epidermal growth factor receptor (EGFR) shown to improve quality of life for HPV-positive OPSCC patients, to also help limit side effects and improve treatment outcomes (Bonner et al. 2010).

The Schlegel group has also suggested parallels between HPV-induced carcinogenesis and the potential mechanism for conditional immortalization, making OPSCC a unique model for studying the Georgetown Method (Liu et al. 2012). HPV codes for two oncoproteins, E6 and E7, that target and disrupt important cellular
pathways, resulting in deregulation of proliferation, apoptosis resistance, immortalization, and transformation of infected cells. E6 and E7 also induce genomic instability to make the genome more susceptible to additional mutations that contribute to increased carcinogenesis (McLaughlin-Drubin and Münger 2009). One of the major roles of the E6 oncoprotein is to induce hTERT, an important step in preventing telomere erosion and allowing a cell to proliferate indefinitely (Liu et al. 2008). The E7 oncoprotein plays a role in regulating the actin cytoskeleton and inactivating the p16/Rb pathway, a regulator of apoptosis, mitotic checkpoints, and cellular differentiation (McLaughlin-Drubin and Münger 2009; Yue et al. 2011). Conditional immortalization with the Georgetown Method could induce similar effects in CRCs including hTERT induction by feeder cells and actin/myosin disruption by the ROCK inhibitor, suggesting similar pathways of immortalization (Liu et al. 2012). Further study of both HPV-positive tumors and CRCs may provide insights into the mechanisms of both processes.

The recent epidemic of oropharyngeal cancers and the role HPV has in their pathogenesis makes them an interesting group of cancers to study. Application of the Georgetown Method to OPSCC will capitalize on the observed similarities between HPV carcinogenesis and conditional immortalization. Because OPSCC usually manifests as relatively small tumors, the Georgetown Method provides a powerful tool to expand the number of cells available for profiling, drug testing, and further experimentation needed to improve diagnostics and treatment.
FURTHER QUESTIONS

The two main problems of current cell lines that need to be addressed are how to avoid the bottlenecks during cell line creation that destroys heterogeneity and how to model the tumor microenvironment outside of the body. The tumor microenvironment has recently been discovered to be an important regulator of tumorigenesis. While tumors were previously thought to simply be a proliferation of neoplastic cells, current research suggests that the constantly evolving microenvironment within and surrounding the tumor contains a heterogeneous mixture of cells that are in constant communication with each other and their associated stroma (Quail and Joyce 2013). This microenvironment encompasses many cell types, some of which are not neoplastic, and a variety of diffusible factors like inflammatory cytokines and growth factors that influence the progression of the tumor. Rather than targeting the neoplastic cells themselves, there have been efforts to alter the tumor microenvironment instead to change promoting factors and enhance immunotherapy (Kershaw et al. 2013).

To study the tumor microenvironment, patient-derived xenograft (PDX) models have been used to recapitulate the tumor niche. PDX models were recently developed from head and neck squamous cell carcinomas by surgically implanting tissue from a patient directly into immunocompromised mice (Peng et al. 2013). The xenografts maintained the histology and gene expression patterns of the original tumor better than cells in culture and were shown to also be useful for drug testing in vivo (Peng et al. 2013). This could prove to be a powerful tool to not only expand and study primary cells
outside the body but also study them within a tumor ecosystem that plays such a large role in determining cancer progression.

However, many limiting factors prevent this technique from being widely used. The biggest problem, like in creation of cell lines, is the low xenograft success rate with a preference for aggressive tumors that are poorly differentiated, node positive, and advanced tumors from donors with poor prognoses (Peng et al. 2013). Similarities between PDX models and the original tumor still remain questionable, and further research is needed to compare them. Mechanically, xenografts require a significant number of tumor cells for implantation, a limiting factor for small OPSCC cancers and limited tissue from head and neck cancer biopsies. Unlike in cell cultures, high-throughput screens are difficult with xenografts due to the resources and time needed to inject, monitor, and care for mice. Because mice must be immunodeficient for effective xenografting, PDX models also eliminate the ability to investigate how the immune system plays a role in influencing cancer, an important point of study and therapeutic target. Finally, the cost of studying cancer through xenografts is much higher than simply studying cells in culture.

Despite the difficulties in modeling the tumor microenvironment, the Georgetown Method provides a potential solution for the first problem of cell culture: selection associated with the creation of cell lines. The Schlegel group’s 90% success rate in culturing both normal and abnormal cells from a variety of tissues represents a breakthrough for the derivation of cell lines with many potential applications (Liu et al.)
2012). However, remaining questions about the Georgetown Method still need to be addressed before it can be widely applied.

The first is to examine whether the combination of feeder cells and ROCK inhibition can be replicated to support conditional immortalization of both normal and neoplastic cells in other labs. We have chosen to apply the protocol to head and neck cancers, primarily OPSCC, to see if excised tumors can be dissociated and expanded to establish a viable biobank for our future studies. The protocol must be optimized to support our patient’s tumor cells for expansion and long-term culture.

Once the Georgetown Method can be used to create tumor-derived cell lines from our patient samples, we also hope to begin elucidating the mechanism for conditional immortalization by feeder cells and ROCK inhibition. Although p16 is known to play a role in cellular senescence and tumor suppression, the mechanism of p16 regulation is still poorly understood. Our preliminary results suggest that the combination of feeder cells and ROCK inhibition may be regulating p16 in some way to prevent senescence and/or cellular differentiation during conditional immortalization.

This study aims to apply and optimize the Georgetown Method for OPSCC and investigate the potential mechanisms of conditional immortalization. With this project, we hope to show that tumor-derived cell lines can serve as a representative model for in vivo tumors and as a valuable resource for future translational cancer research.
SPECIFIC AIMS

The goals of this study are to first understand how to culture and create tumor-derived cell lines (TDCL) from OPSCC samples and delve into the mechanism of how the Georgetown Method immortalizes cells. Coming in to the Rocco laboratory, there were difficulties applying the Georgetown Method to our own patient-derived tissue samples to culture primary tumor-derived cells and replicate the success rate of the Schlegel group. Thus, the primary objective of this study is to figure out how to apply the Georgetown Method successfully to both normal tissue and head and neck cancer samples. Specifically,

1) Growth conditions will be optimized for surgically removed oropharyngeal cancers to culture primary tumor-derived cells.

2) Efficiency and efficacy of culturing tumor-derived OPSCC will be improved to establish conditions and plans for cell biobanking.

3) Role and mechanism of feeder cells and ROCK inhibitor in preventing differentiation and senescence will be investigated.

We hope these studies show that primary tumor-derived cells can be cultured effectively in vitro and be used as a representative model for future cancer studies. Once we can culture primary tumor-derived cells, we can analyze their translational value and whether tumor-derived cells cultured in the lab and in xenografts are representative of tumors in vivo. We also hope to begin to understand how the combination of ROCK inhibition and fibroblast feeder layer induces the immortalization of primary tumor-derived cells.
METHODS

**Harvesting of Tissues: Tumor Dissociation**

Normal or tumor tissue specimens were collected with the informed consent of patients according to a protocol (11-024H) approved by the Massachusetts Eye and Ear Human Studies Committee (Boston, Massachusetts). Tissues were washed with PBS (Sigma-Aldrich) and minced with clean razor blades. The tissue was then transferred to a 15 ml conical containing 3 ml media [RPMI-1640 with L-glutamine (Lonza), 100 U/ml penicillin (Mediatech), 100 µg/ml streptomycin (Mediatech), 10 µmol/L Y-27632 (Enzo Life Sciences) in DMSO (Fisher Scientific)], 12 mg collagenase (Type 1, Worthington), and 450 U DNAse I (Sigma-Aldrich) for digestion for 1 hour at 37°C. Tissue was then spun down at 700 RPM. The pellet was resuspended in 1 ml trypsin-EDTA (Mediatech) and rocked for 5 minutes at 37°C. Viable cells were scored and the tissue was spun down and resuspended in 3 ml filter-sterilized F media [3:1 (v/v) Ham’s F-12 Nutrient Mixture (Lonza)-Dulbecco’s modified Eagle’s medium (Lonza), 5% fetal bovine serum (Sigma-Aldrich), 0.4 µg/ml hydrocortisone (Sigma-Aldrich), 5 µg/ml insulin (Sigma-Aldrich), 10 ng/ml epidermal growth factor (Invitrogen), 24 µg/ml adenine (Sigma-Aldrich), 8.4 ng/ml cholera toxin (Sigma-Aldrich), 100 U/ml penicillin (Mediatech), 100 µg/ml streptomycin (Mediatech), 292 µg/ml L-glutamine (Mediatech), 1 µg/ml amphotericin B (Mediatech)] with addition of 10 µmol/L of Y-27632 (Enzo Life Sciences). Cells were then plated on a 60 mm dish with 200,000 irradiated (3000 rads) Swiss 3T3 fibroblasts (J2 strain).
Culture and Irradiation of Fibroblasts

Swiss 3T3 mouse embryonic fibroblasts (J2 strain, ATCC CLL-92) were cultured at 37°C and 21% oxygen on 24 150 mm culture dishes and 30 ml of Dulbecco’s modified Eagle’s medium (DMEM) (Lonza) with 10% fetal bovine serum, 100 U/ml penicillin (Mediatech), and 100 µg/ml streptomycin (Mediatech) per plate. Media was changed every 2 to 3 days. When cultures were 90% confluent, each set of 8 150 mm plates was trypsinized, collected, and resuspended in 40 ml of DMEM with 10% FBS and penicillin/streptomycin. Suspensions of cells were irradiated at 3000 rads. Irradiated cells were resuspended in DMEM with 5% DMSO (Fisher Scientific), and frozen in liquid nitrogen. Prior to use, frozen cells were thawed at 37°C and washed with F media.

Cell Culture and Passaging

Irradiated fibroblast feeder co-culture

Two different culture conditions were used to propagate patient-derived cells in culture. The first method of culture used an irradiated mouse embryonic fibroblast feeder cell system for direct-contact co-culture. Approximately 200,000 irradiated 3T3 fibroblasts were thawed and seeded on a 60 mm dish in F medium supplemented with 10 µmol/L of the ROCK inhibitor Y-27632 unless otherwise noted. The fibroblasts were allowed to sit down on the plate for at least 1 hour at 37°C and 3% O2 before the tumor or normal cells of interest were plated on to the feeder layer. Cell cultures were maintained at 37°C and 3% O2 unless otherwise noted.
When cell cultures reached 80-90% confluence, cultures were passaged. In the Old method (Table 3), cultures were treated with 0.25% trypsin-EDTA for 5-7 minutes at 37°C. Cells were monitored by microscopy and detached by gentle tapping. Cells were collected using 3 ml of F media supplemented with 10 µmol/L of Y-27632 and a 1:30 dilution (100 µl) was plated on to a new 60 mm culture dish with an irradiated feeder layer. Because cells were passaged at a 1:30 dilution, each passage was equivalent to 5 population doublings.

In the New method (Table 3), confluent plates were first differentially trypsinized with 0.05% trypsin-EDTA [filter-sterilized 0.25% trypsin-EDTA diluted with HBSS (Mediatech)] for 5-7 minutes at 37°C to remove 3T3 fibroblasts. Cells were monitored by microscopy, and fibroblasts were detached by gentle tapping and removed by aspiration. Plates were washed with PBS and trypsinized and passaged at a 1:30 dilution with 0.25% trypsin-EDTA as in the Old method. Cells were cultured with the New method unless otherwise noted.

*Conditioned medium culture*

The second method of culture used conditioned medium from irradiated 3T3 fibroblasts instead of an irradiated feeder layer. Normal or tumor cells were passaged and maintained on plates with conditioned medium supplemented with either 10 µmol/L or 5 µmol/L of Y-27632. When cell cultures were 80-90% confluent, they were passaged the same way as in the New method, using differential trypsinization with 0.05% trypsin-
EDTA to remove any human fibroblasts and trypsin-EDTA to detach and collect cells. Cells were passaged at a 1:30 split ratio on to new 60mm plates.

Media for all cultures was changed every 2 to 3 days.

**Conditioning Media**

Two different methods were used to condition media. In early experiments, we seeded multiple 60 mm dishes with 200,000 irradiated 3T3 fibroblasts each in F media. Cultures were kept at 37°C and 3% O₂. After 48 hours, media was collected off plates and filter-sterilized with a 0.45 µm pore-size syringe filter. Conditioned medium was then diluted with fresh F media at a 1:2, 1:4, or 1:8 dilution and stored at -20°C.

Following the Schlegel group’s publication showing the use of conditioned medium in the Georgetown Method (Palechor-Ceron et al. 2013), we adopted their protocol for conditioning media. Several 150 mm culture dishes were each plated with 1.5 million irradiated 3T3 fibroblasts in 30 ml F media and maintained at 37°C and 3% O₂. Conditioned medium was collected after 48 hours, and three volumes of conditioned medium were mixed with one volume of fresh F media. The mixture was then filtered with a 0.22 µm pore-size bottle-top filter and stored at 4°C. F media was replaced on the plated fibroblasts, and conditioned medium was collected after another 48 hours. Three volumes of this conditioned medium were mixed with one volume of fresh F media, and the mixture was also filtered with a 0.22 µm pore-size bottle-top filter. This conditioned medium was combined with the conditioned medium mixture stored at 4°C, aliquoted,
and frozen down at -20°C. The conditioned medium mixture was thawed in a 37°C water bath and supplemented with 5 µmol/L Y-27632 prior to use.

**MEF Replacement**

Irradiated 3T3 fibroblasts have a limited lifespan so must be replaced regularly to support the cell culture. In the New method, the feeder layer was replaced every six to seven days. Irradiated 3T3 fibroblasts frozen in liquid nitrogen were thawed at 37°C, washed with F media, and resuspended in the appropriate media. Old fibroblasts were removed from the plate using differential trypsinization. Following the PBS wash, the resuspended fibroblasts were plated directly on to the plate. Fibroblasts attached to the plate in a few hours at 37°C.

**Cryopreservation of Cells**

Cells were frozen down at each passage. Following trypsinization and passaging of cultures, remaining cells were spun down at 1000 RPM for 5 minutes and resuspended in F media supplemented with 10 µmol/L of Y-27632 and 5% DMSO (Fisher Scientific). Cells were then aliquoted in to 1.5 ml cryogenic tubes and stored at -80°C before being transferred to liquid nitrogen. When samples were needed, cells were thawed at 37°C, washed with F media supplemented with 10 µmol/L of Y-27632, spun down at 1000 RPM, resuspended in F media supplemented with 10 µmol/L of Y-27632, and plated on to an irradiated feeder layer or into conditioned medium.
**Protein Analysis**

At each passage of human foreskin keratinocytes, proteins were extracted with RIPA lysis buffer [150 mM NaCl, 1.0% NP-40, 0.5% DOC, 50 mM Tris (pH 8.0)] supplemented with protease inhibitors Complete (Roche). Extracts were resolved on NuPAGE SDS-polyacrylamide 4-12% gradient gels (Invitrogen) in MES SDS buffer (Life Technologies), semi-dry electro-transferred to Immobilon PVDF membranes (Millipore), and incubated with indicated antibodies before detection by chemiluminescence with Western Lighting Plus ECL (PerkinElmer). Antibodies for Western include anti-p16, monoclonal antibody JC8 (a gift from James Koh, Department of Surgery, Duke University Medical Center. Durham, NC) and actin, monoclonal antibody pan Ab-5 (Neomarkers).
RESULTS

Initial attempts using Georgetown Method show need for optimization

The Schlegel group reported success in culturing 88% of tested tissues, which included normal and neoplastic cells from a variety of animal tissues (Suprynowicz et al. 2012). To test whether we could achieve similar success, the Georgetown Method was applied to cells from several patient-derived head and neck cancers collected over several months. Following dissociation, cells were plated in F medium containing 10 µmol/L Y-27632 with irradiated mouse fibroblasts. In our initial attempts, we saw limited success. Of the 109 tissues tested initially, less than 30% of cultures were passaged for 20 population doublings or more and less than 5% reached 50 population doublings, the standard the Schlegel group used to consider a culture immortalized (Table 2).

The limited proliferation seen during initial attempts of conditionally immortalizing primary tumor-derived cells indicated a problem in our application of the Georgetown Method. We considered several possibilities for our failures. First, we verified all our reagents corresponded to those of the Schlegel group including media, mouse embryonic fibroblast strain, and ROCK inhibitor. We also standardized our irradiation protocol to ensure fibroblasts were receiving an appropriate amount of radiation to induce DNA-damage arrest. Mouse fibroblasts undergoing earlier rounds of irradiation may have been overdosed with greater than 3000 rads. Once we confirmed these factors, we then sequentially tested several factors including ROCK inhibitor stability, fibroblast survival, differential trypsinization, and oxygen levels in culture to determine whether one or more of these factors was responsible for our limited successes.
Table 2. Culture efficacy of Georgetown Methods. Initial attempts of using the Georgetown Method, the “Old” protocol, resulted in less than 5% of cultures reaching 50 population doublings (PD). Differential trypsinization and feeder layer replacement in the “New” and “Rescue” protocols improved immortalization efficacy significantly. The majority of tissues tested were OPSCC, but tested samples also include normal tonsil epithelium and foreskin keratinocytes.

<table>
<thead>
<tr>
<th>Number of samples tested</th>
<th>Old</th>
<th>Rescue</th>
<th>New</th>
</tr>
</thead>
<tbody>
<tr>
<td>109</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cultured &gt;=10 PD</td>
<td>42.2%</td>
<td>100.0%</td>
<td>91.7%</td>
</tr>
<tr>
<td>Cultured &gt;=20 PD</td>
<td>28.4%</td>
<td>100.0%</td>
<td>91.7%</td>
</tr>
<tr>
<td>Cultured &gt;=30 PD</td>
<td>14.7%</td>
<td>100.0%</td>
<td>91.7%</td>
</tr>
<tr>
<td>Cultured &gt;=40 PD</td>
<td>9.2%</td>
<td>100.0%</td>
<td>83.3%</td>
</tr>
<tr>
<td>Cultured &gt;=50 PD</td>
<td>4.6%</td>
<td>76.9%</td>
<td>83.3%</td>
</tr>
</tbody>
</table>

Differential trypsinization and regular feeder layer replacement resolve senescence

Our lab regularly used a 1:30 split ratio for passaging because each passage was thus equivalent to approximately 5 population doublings. Upon review of the Schlegel group methods, we noticed that their group passaged cells at split ratios anywhere from 1:4 to 1:32, but the majority of their figures show cells being passaged every three to four days at split ratios of 1:8 or 1:16 with cells, two to four times greater than our 1:30 split ratio (Table 3). We also noted that the Schlegel group differentially trypsinized plates with versene or 0.05% trypsin to remove the fibroblast feeder layer before passaging tumor-derived cells on to fresh fibroblasts (Table 3).

Although most of our tumor samples reached the 80-90% confluence necessary for passaging within one week during early passages, our lower plating density resulted in slower-growing cultures remaining on the plate for up to two weeks before achieving appropriate confluence. After extended time on the plate, irradiated fibroblasts appeared
sickly with large vacuoles and non-uniform phenotypes, an indication of apoptosis. Replating of senescing PTDC on to a fresh fibroblast feeder layer sometimes resulted in rescue and continued growth. This led us to two hypotheses.

One hypothesis was that old irradiated fibroblasts stop producing a factor necessary for conditional immortalization. Differential trypsinization was not used during initial attempts, potentially allowing older fibroblasts to be passed on to successive cultures during passaging. A second and more likely hypothesis was that older fibroblasts are releasing factors into the media that inhibit growth. The combination of extended culturing without fibroblast replacement and passaging without differential trypsinization, referred to as our “Old” method, could contribute to the early senescence seen in our initial attempts of conditional immortalization (Table 2, Table 3).

Table 3. Comparison of Georgetown Methods. Our New method adopted differential trypsinization of the feeder layer before passaging of CRCs and weekly feeder layer replacement. Feeder layer replacement compensated for the extended time between passages due to the lower split ratio.

<table>
<thead>
<tr>
<th></th>
<th>Schlegel Method</th>
<th>Old Method</th>
<th>New Method</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Culture oxygen levels</strong></td>
<td>21%</td>
<td>3%</td>
<td>3%</td>
</tr>
<tr>
<td><strong>Media change</strong></td>
<td>Every 2-3 days</td>
<td>Every 2-3 days</td>
<td>Every 2-3 days</td>
</tr>
<tr>
<td><strong>Passaging split ratio</strong></td>
<td>1:4 to 1:32 (mostly 1:8 and 1:16)</td>
<td>1:30</td>
<td>1:30</td>
</tr>
<tr>
<td><strong>Passaging frequency</strong></td>
<td>Every 3-5 days</td>
<td>Every 7-14 days</td>
<td>Every 7-14 days</td>
</tr>
<tr>
<td><strong>Differential trypsinization</strong></td>
<td>With 0.05% trypsin or versene</td>
<td>None</td>
<td>With 0.05% trypsin</td>
</tr>
<tr>
<td><strong>Feeder layer replacement</strong></td>
<td>None reported</td>
<td>None</td>
<td>Every 7 days</td>
</tr>
</tbody>
</table>
With an adoption of differential trypsinization during passaging and replacement of the fibroblast feeder layer every seven days, our modified Georgetown Method, referred to as our “New” method, was applied to early-passage (5 population doublings) primary human foreskin keratinocytes (HFK) (Table 3). The Schlegel group first showed the ability of the Georgetown Method to conditionally immortalize cells in primary keratinocytes (Chapman et al. 2010). With our new protocol, we successfully cultured the HFK cells for 100 population doublings for a period of 138 days (Figure 2). The growth rate remained stable throughout, corresponding to the results seen by the Schlegel group. Culture on the feeder layer only without the ROCK inhibitor resulted in senescence of HFK cells after 45 population doublings. Removal of the ROCK inhibitor from HFK cultures that were initially grown with both the ROCK inhibitor and feeder cells induced senescence within 15 population doublings after the switch.
Figure 2: Differential trypsinization and feeder layer replacement supports growth of foreskin keratinocytes. Human foreskin keratinocytes (HFK) were cultured for 100 population doublings using the Georgetown Method. HFKs cultured without the ROCK inhibitor senesced after 45 population doublings. Removal of the ROCK inhibitor induced senescence after 15 population doublings.

The ability of our revised protocol to support long-term cultures of HFK suggested that regular replacement of the feeder layer and removal of fibroblasts with differential trypsinization prior to passaging could improve our efficacy in culturing primary head and neck specimens. Thawing out early-passage (5 population doublings) cells that previously senesced under our Old method after 20 to 30 population doublings, we also showed that our modified system supports the conditional immortalization of
many types of specimens, including OPSCC (T/B), normal tonsilar epithelium (NT), and foreskin keratinocytes (HFK) (Figure 3). Frozen stocks of cells that were thawed and regrown using the modified protocol successfully bypassed previous points of senescence.

![Graph showing comparison between Old and New systems for OPSCC (T/B), human foreskin keratinocytes (HFK), and normal tonsilar epithelium (NT)]

**Figure 3: Modified protocol resolves senescence previously seen during initial attempts of using Georgetown Method.** Application of our modified New Georgetown Method to OPSCC (T/B), human foreskin keratinocytes (HFK), and normal tonsilar epithelium (NT) allows cultures to bypass previous points of senescence seen under the Old system by 15 population doublings or more. Arrow indicates cultures are still growing and being passaged.

While less than 5% of cultures under our Old system were able to reach 50 population doublings, greater than 80% of cultures grown under the modified system were successfully immortalized (Table 2). Some cultures were also grown with a
“Rescue” method in which they were initially grown with the Old method but switched over to the New method during their passaging. Over 75% of these cultures also reached 50 population doublings, suggesting potential of the New method to rescue senescence (Table 2).

Three late-passage OPSCC samples were also thawed 10 population doublings prior to their previous points of senescence under the Old system and cultured under the New system. Because these cultures were grown with the Old system during early passages and thawed into the New system, they are considered to be grown under the Rescue method. All three of these cultures were propagated past their previous points of senescence, but two of the three cultures, T/B 75 and T/B 81, eventually senesced (Figure 4).

Overall, there was a significant increase in survival with the New system compared to the Old system, with over 80% of tissues cultured with the New system still propagating at least 50 population doublings (Figure 5).
Figure 4: OPSCC cultures grown under the Rescue method pass previous senescence points but some eventually senesce. OPSCC culture T/B 75 (A), T/B 80 (B), and T/B 81 (C) were thawed 10 population doublings prior to their previous senescence points under the Old system. Applying differential trypsinization and regular fibroblast replacement, all passed senescence points seen under the Old system, but T/B 75 and T/B 81 eventually senesced. T/B 80 continued propagating and was effectively immortalized.
Figure 5: Differential trypsinization and MEF replacement results in significantly increased survival of cultured tissues. Less than 5% of tissues cultured under the Old method reached 50 PD, but about 80% of tissues cultured under the New system successfully did. Tissues cultured under the Rescue method had slightly lower long-term survival than the New method but still had significantly higher survival than the Old method.

**ROCK inhibitor loss is not likely cause of limited PTDC survival**

In addition to the fibroblast feeder layer, the ROCK inhibitor is an integral player in cellular immortalization. Degradation or decreased efficacy of the ROCK inhibitor
over time could hinder immortalization of cell cultures. Culture media is changed every two to three days, and the ROCK inhibitor is added to F media either the day of or day before use. Y-27632 has a half-life of 12 to 16 hours in serum, so it may lose its efficacy by the time the media is used (Ishizaki et al. 2000). However, because Y-27632 is known to be an irreversible inhibitor, the ROCK pathway will still be inhibited even if Y-27632 is removed from culture. The ROCK pathway will become activated again only after cells undergo replication.

We decided to examine the efficacy of the ROCK inhibitor in culture and whether its short half-life could affect cell culture growth. OPSCC cultures were grown either with F media supplemented with ROCK inhibitor no more than one day before use (Normal ROCKi), which duplicates our initial attempts of applying the Georgetown Method; F media supplemented with ROCK inhibitor immediately before use of the media (Fresh ROCKi); or 10 uM of ROCK inhibitor was added to the culture every day (Everyday ROCKi). We saw no significant difference in growth between the three ROCK inhibitor conditions in supporting growth of OPSCC T/B 75 and T/B 81 (Figure 6). These results suggest that the ROCK inhibitor remains stable enough in culture to support immortalization of OPSCC and/or intermittent ROCK inhibitor exposure is sufficient to induce immortalization. It also decreases the likelihood that the loss of ROCK inhibitor was the cause of early difficulties to conditionally immortalize cells using the Georgetown Method.
Figure 6: Old method ROCK inhibitor conditions are sufficient to support immortalization through Georgetown Method. OPSCCs T/B 75 (A) and T/B 81 (B) cultures were grown in either F media supplemented with Y-27632 no more than one day before use (Normal ROCKi), F media supplemented with Y-27632 immediately before use (Fresh ROCKi), or supplemented with Y-27632 directly into the culture every day (Everyday ROCKi). No significant difference in growth was observed between the ROCK inhibitor conditions.
Conditional immortalization is supported at physiological and atmospheric oxygen

Cell culture at physiological oxygen levels (3%) is known to increase plating efficiency and lifespan of cultured cells relative to cell culture at atmospheric oxygen levels (21%) (Richter, Sanford, and Evans 1972; Packer and Fuehr 1977; Parrinello et al. 2003). We questioned whether oxygen levels affected the efficacy of conditionally immortalizing co-cultured cells. Our initial attempts of applying the Georgetown Method were conducted at physiological oxygen (3%), but use of our modified Georgetown Method on early-passage HFK cultures showed similar steady growth in both physiological and atmospheric oxygen (Figure 7A).

Tumor cells frozen down after extended passaging at 3% oxygen under our Old system were thawed 10 population doublings before their previously observed senescence point and grown with our modified protocol at both physiological and atmospheric oxygen. Cultures grown at 3% oxygen surpassed the previous senescence point, but cultures grown at 21% oxygen senesced 10 population doublings after thawing and plating (Figures 7B, 7C, 7D). Because cells were grown at 3% oxygen at early passages, the early selection of cells that thrive better at physiological oxygen could impact their survival when switched over to growth in atmospheric oxygen. Keratinocytes we cultured were thawed from low-passage cultures, limiting the selection of cells by culture conditions. Support of HFK growth in both 3% and 21% oxygen with our New system suggests that the Georgetown Method is equally effective at both physiological and atmospheric oxygen levels.
Figure 7: Low-passage HFKs are immortalized in both physiological and atmospheric oxygen but high-passage OPSCCs are immortalized at only physiological oxygen. Low-passage HFK cultures (5 PD) grown at 3% and 21% oxygen showed similar rates of growth (A). Thawed high-passage OPSCC cultures of T/B 75 (B), T/B 80 (C), and T/B 81 (D) initially cultured at 3% oxygen were successfully grown at 3% oxygen but senesced when cultured at 21% oxygen.

Conditioned medium can substitute for direct co-culture with feeder layer to support immortalization

The ability of our New method, which adopts differential trypsinization and feeder layer replacement, to support immortalization of cultured cells suggest that old fibroblasts fail to support conditional immortalization and/or have a detrimental effect on co-cultured cells. The Schlegel group recently reported that direct co-culture with
irradiated fibroblasts is not necessary for the Georgetown Method to work, and media conditioned by irradiated fibroblasts can be used instead to immortalize cells (Palechor-Ceron et al. 2013). Eliminating the need to differentially trypsinize during passaging and replace fibroblasts regularly would make the process of immortalizing cells more efficient and effective. We decided to test whether conditioned medium from irradiated fibroblasts could immortalize OPSCC and normal tonsil epithelium as effectively as direct co-culture with a feeder layer. Rather than studying HFKs, we decided to study normal tonsil epithelium because it is more similar to the OPSCC we are interested in.

During our initial tests, irradiated fibroblasts were plated in F media and allowed to condition the media for 48 hours before harvesting and filter sterilizing the media. Conditioned medium was then diluted with unconditioned F media to different ratios to compare the efficacy of lower dilutions of conditioned medium to support immortalization. All media was supplemented with 10 uM of Y-27632. We observed that all concentrations of conditioned medium resulted in comparable rates of growth to direct co-culture with irradiated fibroblasts over 35 population doublings (Figure 8A). This shows that conditioned medium diluted to as low as 1:8 can substitute for direct co-culture with a feeder layer. Whether there is a difference in growth over a longer period of time between the varying concentrations of conditioned medium remains to be determined.

Following our initial conditioned medium tests, the Schlegel group recently published a paper detailing their methods of conditioning media (Palechor-Ceron et al. 2013). For later conditioned medium tests, we decided to apply their findings:
conditioning media over 96 hours rather than 48 hours, using a 3:1 dilution of conditioned medium to unconditioned F media for cell culture, and supplementing conditioned medium with 5 μM of Y-27632. We have compared the efficacy of conditioned medium to fibroblast co-culture for several tissues including 6 OPSCC samples using our initial media conditioning method and 3 OPSCC and 2 normal tonsilar epithelium samples using the Schlegel media conditioning method. We found that conditioned medium can support culture growth as well as direct co-culture in all samples tested (Figure 8B, 8C, 8D).

The ability of conditioned medium to immortalize cells without co-cultured fibroblasts, the cause of our initial limited success with the Georgetown Method, is a novel finding that eliminates the need for differential trypsinization and feeder layer replacement. Conditioned medium will allow us to more easily conduct high-throughput screens, including DNA sequencing and chromatin immunoprecipitation (ChIP), without the otherwise necessary step of separating tumor-derived cells from co-cultured fibroblasts.
Figure 8: Conditioned medium supports OPSCC and NT growth as effectively as direct co-culture with irradiated fibroblasts. Dilutions of conditioned medium (CM) as low as 1:8 were just as effective as fibroblast direct co-culture in supporting OPSCC T/B 99 growth (A). CM was able to support growth of OPSCC T/B 41 (B) and two different cultures of normal tonsilar epithelium as effectively as a fibroblast feeder layer (C, D). All CM was supplemented with Y-27632.

ROCK inhibition maintains low p16 expression at low passages

Although the mechanism of how the Georgetown Method conditionally immortalizes co-cultured cells is currently unknown, we have found evidence that the ROCK inhibitor may contribute to conditional immortalization of cells through p16 regulation. Low-passage (5 PD) HFK cells were thawed and plated on an irradiated
feeder layer in either F media or F media supplemented with Y-27632. These cells were
maintained in both 3% and 21% oxygen, and cell lysates were collected at each
passaging.

p16 is known to tightly regulate the cell cycle and cellular senescence, but the
mechanism of its regulation is still not understood. We found that at relatively low
passages (20-35 PD) with the Georgetown Method, HFK p16 expression was maintained
at low levels while HFKs cultured without the ROCK inhibitor showed a gradual increase
in p16 expression (Figure 9A). Because atmospheric oxygen levels are known to induce
stress and senescence in cell culture, we also tested whether ROCK inhibition could
maintain p16 expression at low levels in 21% oxygen in addition to 3% oxygen. Similar
patterns of p16 expression seen in cultures grown at 3% oxygen were also seen at 21%
oxygen (Figure 9A).

HFK cultures grown on an irradiated feeder layer without the ROCK inhibitor
senesced after 40 population doublings, but HFK cultures grown on an irradiated feeder
layer with the ROCK inhibitor were maintained for over 100 population doublings and
effectively immortalized at both 3% and 21% oxygen (Figures 2, 7A). At higher passages
of HFK cultures, we saw that p16 expression gradually began increasing around passage
13 (65 PD), despite being cultured with the ROCK inhibitor (Figure 9B). Even with
increasing p16, cultures continued to maintain a steady growth rate at these higher
population doublings and showed no phenotypic signs of senescence (Figure 7A).

Our preliminary data suggests that ROCK inhibition may prevent senescence by
maintaining p16 levels during low passages. However, the increase in p16 expression at
later passages suggests that ROCK inhibition may also be acting through additional pathways besides p16 such as p21 and p53 to maintain the proliferative ability of the culture. Further research is needed to understand how ROCK inhibition can maintain p16, why p16 increases during later passages, and p16’s mechanism of regulation.

Figure 9: ROCK inhibition maintains low p16 levels in low-passages but not at higher passages. At low passages (20-35 PD), HFKs grown with Y-27632 maintained low p16 levels in both 3% and 21% oxygen, but HFKs grown without Y-27632 had increasing p16 expression (A). p16 levels of HFKs cultured with Y-27632 increased after about 65 PD and remained relatively high even as HFKs continued proliferating for 100 PD (B).
DISCUSSION

This study has optimized the use of the Georgetown Method for the culture of head and neck cancers and begun to investigate its mechanism to conserve the replicative potential of cultured cells. Our findings show that differential trypsinization and regular feeder layer replacement can resolve the limited survival of tumor-derived OPSCC cells seen in our earlier attempts to conditionally immortalize tumor-derived cells with the Georgetown Method. Our application of the Georgetown Method has achieved comparable levels of success to those seen in the Schlegel lab in the immortalization of both normal and neoplastic cells.

P16 PATHWAY AND EPIGENETIC MARKS

However, a fifth of tested tissues failed to be cultured using this method, results similar to those obtained by the Schlegel group. Why particular tumor specimens of different tissues are not immortalized is currently unknown, but identifying the common characteristics of these tissues could lead to important discoveries about the mechanism of the Georgetown Method and eventual chemotherapeutics that target these pathways to prevent immortalization of cancer cells in vivo. Because the majority of our samples was OPSCC and tested for HPV, we examined HPV status of our samples, but our preliminary analysis saw no correlation between HPV status and CRC failures.

After identifying the problems of our Old method, we found that some cultures initially cultured without differential trypsinization and fibroblast replacement during early passages and switched over to our New method in later passages (labeled as Rescue
cultures) still senesced but at later passages than those seen during the Old method. While our New method supported growth of over 80% of tested tissues, a lower proportion of tissues cultured with the Rescue method were successfully immortalized.

Our findings that the ROCK inhibitor in culture maintains low p16 levels in early passages suggest a potential role of p16 in the mechanism of the Georgetown Method. The p16\textsuperscript{INK4A} pathway is known to regulate cell cycle progression and senescence, preventing damaged cells from inappropriately dividing. Our lab recently reported that the C-terminal binding protein (CtBP), a target in many stress-signaling pathways, plays an important role in p16 regulation and senescence (Mroz et al. 2008). CtBP potentially links various cellular stresses to senescence seen in primary cells via the p16 pathway. The p16 promoter is also known to be “bivalent” and shows the potential to “resolve” epigenetically to a repressed or activated state, which can be passed on to progeny to affect their sensitivity to stress signals (Bernstein et al. 2006; Mikkelsen et al. 2007). Early culture stresses caused by the Old method could set early epigenetic marks for senescence that remain irreversible even when tumor-derived cell cultures are switched to more favorable culture conditions in later passages. This could explain the decreased survival of Rescue cultures compared to New cultures, and early cellular stresses could contribute to the observed senescence of a fifth of tested tissues. Favorable conditions under the New method may not be enough to reverse these epigenetic marks. Our findings suggest that the ROCK inhibitor may help preserve the bivalency of p16 to maintain the replicative potential of tumor-derived cells. However, further research is still
needed to fully understand how the Georgetown Method prevents senescence, through p16 and possibly additional senescence pathways such as p14^{ARF}, p21, and p53.

**CONDITIONED MEDIUM AND TUMOR MICROENVIRONMENT**

The ability for conditioned medium to substitute for direct co-culture with an irradiated fibroblast feeder layer effectively eliminates the previous problems caused by old fibroblasts including the lack of a diffusible factor necessary for growth and/or an inhibitory factor released by old fibroblasts. The only problem we observed when using conditioned medium is potential outgrowth of human fibroblasts in culture since growth of human fibroblasts is suppressed by co-culture with an irradiated fibroblast feeder layer. Because human fibroblasts generally proliferate at a faster rate than keratinocytes, growth in conditioned medium may result in overgrowth of human fibroblasts. However, differential trypsination can be used to selectively remove human fibroblasts from the plate.

Culturing with a fibroblast feeder layer requires separation of primary cells from co-cultured fibroblasts before primary cells can be analyzed. The ability to culture primary cells with conditioned medium instead and without an irradiated fibroblast feeder layer would be very useful for high-throughput screens by making them faster and their results more reliable. It completely eliminates the need to separate primary cells from fibroblasts in culture before analysis is done. DNA sequencing or ChIP, for example, will be much easier and more accurate without the presence of mouse fibroblasts to contaminate samples and skew results.
The fact that irradiated fibroblasts release a diffusible factor that can conditionally immortalize cells in the presence of a ROCK inhibitor also provides clues to the mechanism of the Georgetown Method and information about how cancer may be regulated. There recently has been an increased focus on understanding the tumor microenvironment and its impact on tumor progression. Rather than just a homogenous mass of cells, tumors have high heterogeneity, and specific cells within the tumor niche could release diffusible growth factors necessary for the overall tumor to grow and proliferate. One compelling hypothesis for why less than 5% of tumors can form cell lines is that culture conditions fail to model the complexity of the tumor microenvironment. Irradiated fibroblasts in conjunction with the ROCK inhibitor may be recapitulating the tumor niche in vitro by releasing factors usually released by other cells in the tumor microenvironment necessary for tumor growth.

The Schlegel group found that only irradiated, senescent fibroblasts release diffusible factors necessary to immortalize co-cultured cells. As our cells age, our tissue niche may also change as older cells senesce, and these senescent cells may release factors that promote neoplastic cell growth, ultimately increasing the risk of cancer. If we can identify the factors that support cancer cell proliferation, we may be able to use them as future chemotherapeutic targets to regulate the tumor niche.

**ONGOING AND FUTURE WORK**

The ability to culture over 80% of our tested tissues for over 50 population doublings allows us to accomplish many of our goals using TDCL. Rather than testing
therapies or elucidating cellular pathways using established cell lines, which represent less than 5% of all cancers, we can significantly increase inter-tumor heterogeneity of our experimental models by creating a huge library of tissues from which to study. Use of these tissues has the potential to make huge strides in our understanding of immortalization and senescence, the tumor microenvironment, translational in vitro models, and viable biobanking.

**UNDERSTANDING THE TUMOR MICROENVIRONMENT**

The limited number of tumors that do form cell lines without the Georgetown Method could be doing one of two things: they could either be niche-independent, possessing mutations that allow them to grow despite the absence of diffusible growth factors in cell culture, or they could have an autocrine signaling pathway, regulating their own growth through release of growth factors from the same cell. Currently, we are testing the latter hypothesis and examining whether conditioned medium from established cell lines contain a diffusible factor similar to that released by the feeder layer that can immortalize cells in culture.

**TRANSLATIONAL ASSESSMENT OF TUMOR-DERIVED CELLS**

Besides understanding the mechanism of the Georgetown Method, we are especially interested in assessing the translational value of cultures derived this method. Through genome-wide analysis, PDX models have been shown to conserve mutations, genomic features, and relative clonal diversity (Li et al. 2013). Because most OPSCC
tumors are small and cell numbers are insufficient for creating PDX models, expansion of cells through the Georgetown Method provides a means to culture enough cells for future xenografts.

Now that we are able to reliably expand primary tumor-derived cells with this method, one of our ongoing projects is to compare BH3 profiles of the primary tumor, tumor-derived cultures, and murine xenografts to determine how well tumor-derived cell cultures model the original tumor. BH3 profiling is a method to predict cellular fate decisions via measurements of responses to stimulation of various parts of the apoptotic pathway (Moore and Letai 2013). By looking at BH3 profile differences, we can make predictions about the chemosensitivity of a given population of cells and observe how the cell population changes throughout the culturing process (Figure 10). If populations of PTDC have similar BH3 profiles to their original corresponding tumors, this suggests the Georgetown Method conserves the cellular diversity and characteristics of the original tumor during expansion, making tumor-derived cell lines representative tumor models for future clinical applications. We can also investigate whether intratumor heterogeneity is conserved throughout the expansion process, especially after long-term passaging, or whether specific clones are selectively immortalized. Ideally, similar signals between the tumor and tumor-derived cultures would indicate a high degree of translatability for future drug screens, drug testing, and cancer studies.

Cell culture conditions often select for subpopulations of cells, and with increased passaging, heterogeneity decreases as the fastest growing cells outcompete slower growing cells. Xenografts are known to preserve the heterogeneity of the original tumor.
fairly well, but the heterogeneity of cultures grown with the Georgetown Method is currently unknown. Our finding that the Georgetown Method maintains low p16 expression suggests maintenance of p16 bivalency to keep cells in a stem cell-like state that preserves their replicative potential. Whether a large majority of cells retain the capacity to divide or whether a subpopulation of cancer stem cells is maintained has yet to be determined. Following expansion of the tumor-derived cell cultures, we are also creating xenografts examine whether heterogeneity of the original tumor is conserved in xenografts and whether xenografts differ significantly from cell cultures. We can thus begin to determine the quality and applicability of tumor-derived cell cultures and subsequent xenografts as an appropriate model for cancer research.

**Figure 10: Translational assessment of PTDC with BH3 profiling.** By comparing BH3 profiles of the primary tumor, resultant CRCs, and mouse xenograft, we can observe how the cell population changes throughout the conditional immortalization process.
**TISSUE BIOBANKING**

Because we have developed the ability to expand tumor-derived cells consistently with our New method, we can return to early-passage frozen stocks of tumor-derived cells for expansion. With currently over 120 different OPSCC tissue specimens collected, we have begun to form a biobank of tissues through systematic expansion and cryopreservation of each specimen (Figure 11). However, there are several issues we must consider as we begin biobanking of primary tumor-derived cells.

Because tumors may contain a variety of cell types including normal epithelial cells and endothelial cells in addition to the neoplastic cells, we must verify whether our expanded cultures contain the tumor cells we are trying to study. The Georgetown Method supports cultures of both normal and neoplastic cells, creating the risk that certain cell types could outcompete others because of faster growth in culture. Our initial plan of attack to distinguish between these cell types has several approaches. First, xenografts will be created from cell cultures to ensure they contain neoplastic cells. In our preliminary xenograft tests with one normal tonsil epithelium specimen and two tumor-derived cell specimens, we saw that normal epithelium did not form a tumor whereas the tumor-derived cells did as we expected. However, further samples will need to be tested to verify the reliability of xenografts to distinguish between normal and neoplastic tissues. Besides xenografts, we will also use cell markers for identification. Because the majority of our samples are HPV-positive, we will use antibodies against p16 to identify HPV-positive tumor cells and markers like VE-Cadherin and CD31 to distinguish between endothelial cells and tumor cells. PCR amplification of E6/E7 RNA
transcripts will also identify HPV-positive cells, and karyotyping will identify aneuploidy or other chromosomal abnormalities not present in normal cells.

The sheer number of patient specimens also holds the risk of cross-contamination between cultures. Before drug testing or drug screening, we must also validate that tumor cultures correspond to cells from the correct patients. Short Tandem Repeat (STR) analysis is the gold standard for cell-line validation, and we can apply this technique to the original tumor, tumor-derived cell cultures, and xenografts. Analyzing STR profiles at all three points of expansion will ensure that our tumor-derived cell lines stay consistent throughout our expansion process. Our biobank also consists of blood samples for each patient that can be used for future sequencing.

With our large number of patient samples, we must systematically expand and preserve these cells to create a reliable stock for future validation, experiments, and xenografting. Following initial dissociation of primary tumors, cells were expanded to about 5 population doublings in culture before being collected and frozen down in liquid nitrogen. Our plan is to expand these initial freezedowns to derive a stock of about 20 million cells per specimen at 10 population doublings that can be thawed and used for procedures including xenografts and validation assays. We have already begun expansion of several of our tumor-derived cell specimens, particularly ones with BH3 profiling data for the original tumor to initiate BH3 profile comparisons between the tumor, cell culture, and xenografts. With our biobank, we will ultimately be able to thaw and expand frozen cells easily with the Georgetown Method for future studies on OPSCC and other primary tumor-derived cells.
Figure 11: Biobanking and validation plan for primary tumor-derived cells. Primary tumor cells were initially dissociated, expanded 5 population doublings, and frozen down in liquid nitrogen. These stocks will be expanded to 10 population doublings, yielding a general stock of about 20 million tumor-derived cells for future validation, xenografts, and other experiments. Cells will continue to be maintained, passaged, and frozen down every 5 population doublings.

The ability to culture over 80% of primary tumors with the Georgetown Method is a powerful tool that can revolutionize future cancer research. It allows scientists to move past the limits on inter-tumor heterogeneity that current cell lines have on research and begin studying tumor biology with more representative primary tumor-derived models. The Georgetown Method also provides a technique to expand cells from small tumors like those seen in OPSCC or early-stage melanoma whose small specimen sizes have limited characterization, diagnostics, and treatments. We can expand on our
understanding of how the diversity of cell types within a tumor can impact the tumor microenvironment and take advantage of these discoveries to find targets for new chemotherapeutics. Tumor-derived cells also have the potential to be a novel clinical tool for diagnostics and testing of tumor drug resistance, already shown to be effective for a case of recurrent respiratory papillomatosis. Because p16 is inactivated at early stages of tumor development for nearly all cancers, the potential maintenance of low p16 expression by ROCK inhibition to regulate senescence is an exciting finding that could shed light on this important tumor suppressor. Further research is still needed to fully understand how the Georgetown Method conditionally immortalizes cells and to evaluate the translational value of CRCs to the clinic.
REFERENCES


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Education

Boston University School of Medicine, Boston, MA; M.A., May 2014
Masters of Arts in Medical Sciences
Coursework in Physiology, Biochemistry, Histology, Pathology, Biostatistics;
Masters thesis work conducted in Surgical Oncology Department at Massachusetts General Hospital (James W. Rocco’s laboratory).

Harvard University, Cambridge, MA; A.B., May 2011
Molecular and Cellular Biology (Major); Global Health and Health Policy (Minor)
Coursework in Mathematics, Chemistry, Physics, Molecular and Cellular Biology,
Stem Cell and Regenerative Biology, Genetics, and Global Health; Senior thesis in Molecular and Cellular Biology (Craig P. Hunter’s laboratory).

Awards and Honors: Harvard College Research Program Grant and Dean’s Summer Research Award, Summer 2010; Nominated for Senior Thesis Hoopes Prize, May 2011.

Extracurricular Activities: Phillips Brooks House Association Director, Chinese Students Association, Pianist for campus musicals, Novice Heavyweight Crew.

C. Leon King High School, Tampa, FL; International Baccalaureate Diploma, May 2007
GPA: 7.75/4.00

Awards and Honors: National Merit Finalist; AP Scholar with Distinction; Bausch & Lomb Honorary Science Award; 12-time State First Place Winner of piano competitions in the Florida Junior State Convention of National Federation of Music Clubs; Two-time soloist with the Tampa Bay Symphony (two series of six performances in 2004 and 2006).

Extracurricular Activities: National Honor Society, Piano, Co-Captain of Varsity Tennis Team, Scepter (Newspaper) Editor, Key Club, Mu Alpha Theta, Music Director of “Bye Bye Birdie”.

Experience

Research and Clinical Experiences

Graduate Researcher, July 2013-May 2014
Dr. James W. Rocco’s Laboratory, Surgical Oncology, Massachusetts General Hospital, Boston, MA
Optimized growth conditions for primary tumor-derived cells from head and neck cancers and evaluated the translational value of tumor-derived cell lines.

**Undergraduate Researcher, January 2010-May 2011; Full-time Research Assistant, July 2011-July 2012**  
Dr. Craig P. Hunter’s Laboratory, Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA  
Conducted study on the roles of the dsRNA protein channel sid-1 of *C. elegans* in coordinating stress responses, behavior, and cholesterol metabolism.

**ASB Premedical Trip Participant, Spring 2009; ASB Premedical Trip Director, Spring 2010**  
Phillips Brooks House Association Alternative Spring Break Premedical Trip, New Orleans, LA  
Volunteered in community health centers and mobile health clinics in New Orleans. Built homes for Katrina victims. Met with Tulane Medical School Dean and learned about Katrina’s impact on New Orleans healthcare.

**Undergraduate Research and Physician Shadowing, January 2008-May 2008**  
Dr. James C. Cusack’s Laboratory, Massachusetts General Hospital, Boston, MA  
Conducted experiments on oxidative stress in cancer cells and shadowed Dr. Cusack in operating room.

**Summer Research Intern (SPARK Program); Summer 2006, Summer 2007, Summer 2008**  
Dr. Jin Q. Cheng’s Laboratory, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL  
Studied cancer cell signaling, interaction between mTOR and Akt, and regulation of Src.

**Hospital Volunteer; Summer 2004, Summer 2005**  
H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL  
Interacted with patients in surgery waiting areas and in Radiation Oncology Department.

**Teaching**  
**Laboratory Teaching Fellow; March/April 2012, March/April 2013**  
Life Sciences High School Outreach Program, Harvard University, Cambridge, MA  
Taught high school students from schools around the New England area science concepts and laboratory techniques such as PCR and gel electrophoresis through hands-on laboratory sessions. Garnered interest in science and served as mentor for students interested in pursuing future careers in science.
**Leadership Roles and Other Medical-Related Activities**

**Logistics Director, May 2010-January 2011**
Harvard Undergraduate Global Health Forum: Global Medical Brigades Trip, El Paraíso, Honduras
- Launched inaugural Global Medical Brigades for Harvard students. Run logistics for trip to Honduras to provide medical care and supplies to over 500 patients in Honduras.

**ASB Program Coordinator, August 2010-March 2011**
Phillips Brooks House Association Alternative Spring Break Program, Cambridge, MA
- Organized 5 Alternative Spring Break trips across the United States for over 50 students. Initiated Winter Break volunteer programming with New Mexico Navajo Reservation public health program.

**Social Chair, January 2009-December 2009; Vice-President January 2010-December 2010**
Harvard-Radcliffe Chinese Students Association, Harvard University, Cambridge, MA
- Promoted Chinese culture on campus through weekly social, cultural, and educational-political events. Spearheaded creation of Sib Committee, pairing upperclassmen with freshmen to serve as mentors. Helped CSA become one of the largest cultural organizations on campus with nearly 1000 members.

**Publications**

**Peer-reviewed Articles**

**National Meeting Abstracts**