ESTABLISHING, CHARACTERIZING AND DEPLOYING NOVEL INSECT CELL LINES

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ESTABLISHING, CHARACTERIZING AND DEPLOYING NOVEL INSECT CELL LINES

Presented by Kaile Zhou

A candidate for the degree of Master of Science of Plant, Insect and Microbial Sciences

And hereby certify that, in their opinion, it is worthy of acceptance.

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ESTABLISHING, CHARACTERIZING AND DEPLOYING NOVEL INSECT CELL LINES

Kaile Zhou

ABSTRACT

Insect cell lines are convenient and powerful tools that were initially used in viral propagation, optimization of viral pesticides, cell-virus interactions, and recombinant protein productions. The most commonly used cell lines were initiated from embryonic tissues, nervous system, and ovarian tissues. Although the midgut is known as the main entry of pathogens and pest control chemicals, few cell lines have been established from midgut tissues of economically important pests. My project focused on establishing midgut cell lines from four select species, including western corn rootworm, southern green stinkbug, green stinkbug, and fall armyworm. I in total initiated 317 cell cultures from the four insect species, of these 21 promising cell cultures are still being carefully maintained. I established two midgut cell lines from the fall armyworm that are continuously replicating. Additionally, experiences and new insights of cell culture initiations from western corn rootworm and (southern) green stinkbug were gained. Characterization of the two SfMG cell lines verified their identities belonging to *S. frugiperda*. The doubling time of the two cell lines indicate they are relatively fast replicating. I optimized the cell cytotoxicity assay based on cell sensitivity and linear relationship for use in future toxin-screening assays.

CHAPTER 1: LITERATURE REVIEW

1.1. The Early Work – A Brief History of Insect Cell Lines

The origin of cell culture dates back to the 1880s. In 1885, Wilhelm Roux removed a portion of the medullary plate of an embryonic chicken and maintained it in a warm saline solution for several days, establishing the principle of tissue culture (Roux, 1885). From 1907 to 1910, Ross Granville Harrison published the results of his "hanging drop" experiment (Harrison, 1910). His efforts in maintaining sterile growth of nerve cells lead to the first published examples of tissue culture and subsequently stimulated a research trend of studying *in vitro* cell growth and development. Most importantly, Harrison contributed to the systematic methodology of *in vitro* cell culture that has been widely applied. The first permanent cell line was developed by Earle (1943) from subcutaneous mouse tissue. A big surprise was that these cells propagated continuously and gradually developed into quite different cell morphologies, designated as "transformed cell lines". The first human "transformed" cell line, the "HeLa" cell line, has been widely used in studies of cell propagation, vaccine development, cell cloning, isolation of stem cells, as well as cancer research. The development of mammalian cell lines led to profound insights into insect cell cultures. Furthermore, techniques used in mammalian cells have been frequently used in insect cell cultures, including the use of trypsin to detach cells from culture flasks, methods of cell cryopreservation, and the optimization of cell culture media formulations.

The history of insect tissue culture is usually divided into three phases (Day and Grace, 1959). For the first phase, the majority of efforts focused on gametogenesis with simple

culture media. The cells were able to develop, but mitosis was rarely recorded in their studies. During the second phase, though media were devised by empirical testing to facilitate cell growth, insect cells were still not able to survive beyond three months *in vitro*. Along with the advancing knowledge of the composition of insect tissue fluids (Grace, 1959), culture media were gradually refined to promote further cell development. This period is considered the third phase of insect tissue culture, which resulted in successful practices in cell line establishment. There was a time when the first insect cell line was known to be established by Grace of the CSIRO Canberra, Australia in 1961. He reported the cell lines from the emperor gum moth, *Opodiphthera eucalypti* from pupal ovarian tissues (Grace, 1962). Unbeknownst to Grace, Gao and his colleagues, who were slightly disconnected from the international scientific community due to lack of communication tools at the time, established the first insect cell line from *Bombyx mori* (Vlak, 2007). A year later, Gao reported these cells were able to replicate polyhedrovirus in the Wuhan University *Journal of Nature Sciences*. In the time since Grace established the ovarian cell line from a moth, during the following 50 years, over 500 insect cell lines have been established from numerous insect orders and various tissue sources at an average rate of 130 cell lines per decade (Lynn, 2001; Figure 1-1). In the first 40 years, the field of cell lines has been quite productive. A commonly quoted insect cell line list was organized by W. Fred Hink, and embellished by Dwight Lynn based on published literature (Hink, 1972, 1976, 1980; Hink and Bezanson, 1985; Hink and Hall, 1989; Lynn, 2001).

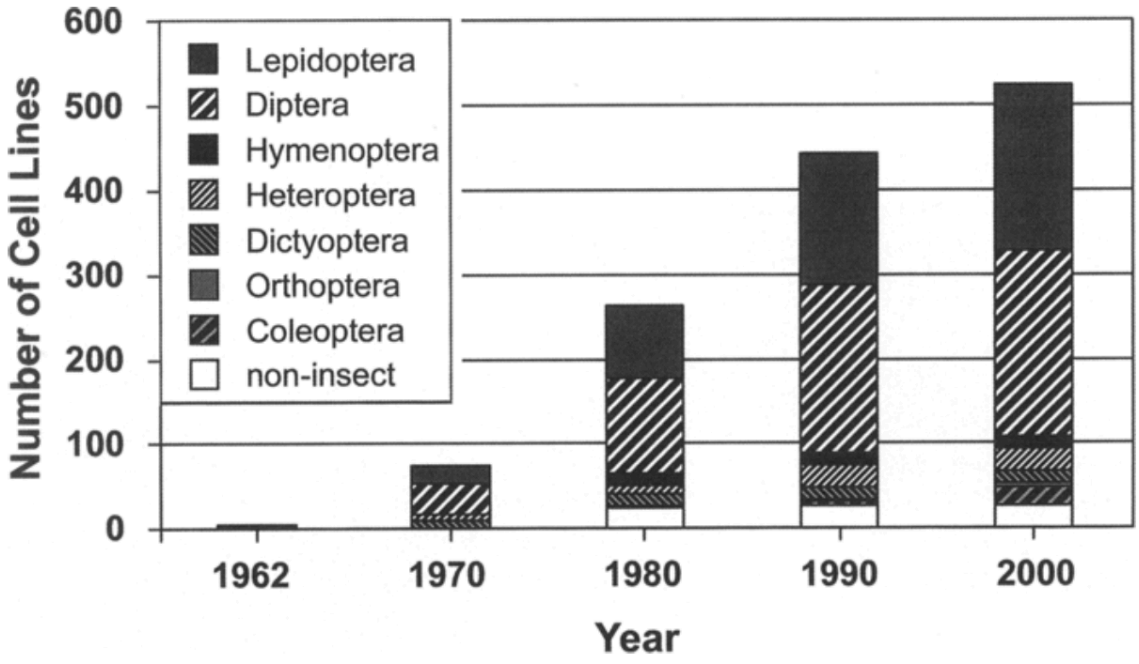

Figure 1-1. Invertebrate cell lines established since 1962 (categorized by insect orders). Each bar represents the total number of cell lines that had been reported at the indicated decades (re-drawn from Lynn, 2001).

Almost 80 percent of the established insect cell lines were initiated from Lepidoptera and Diptera. Lynn (2001) listed tissues that have been used for developing insect cell cultures. Numerous cell lines have been established over the past 15 years, but tissue sources are similar (Table 1-1). Insect cell lines are established based on tissues of interest. In past decades, the growth rate of new cell lines establishment gradually declined.

1.2. Traditional Role of Insect Cell Lines

Insect cell lines were initially used in viral propagation and in optimizing viral pesticides (Li and Bonning 2007; Lynn 2007), in research into cell-virus interactions, and for producing recombinant proteins/vaccines.

Table 1-1. Source tissues used to establish insect cell lines.

Tissue Souces	Related Functions
Ovaries	First established cell line, and commonly used with Lepidoptera
Embryos	Very commonly used tissues for cell culture
Hemocytes	Easy to obtain, but hard to grow
Imaginal discs	Important in developmental biology
Fat body	Important tissues in physiological studies, function similarly to mammalian liver
Midgut	Relevant to nutritional absorption, biological control
Neonate	Newly hatched larvae, includes a considerable number of undifferentiated cells
Cuticle /endocrine system	Relevant to pest control and pathology. Established cell lines are rare
Nervous system/muscular system	Important tissues in insect physiology studies. Now several cell lines have been established

Many researchers use insect cell lines to investigate complex virus-cell relationships (Mudiganti et al., 2006; Lennan et al., 2007). Hunter (2001) used a cell line established from whitefly and developed it into a cell line-virus system to serve future studies focused on understanding the complex relationship between the begomovirus and its whitefly vectors. Ma (2013) used a cell line derived from the small brown planthopper to study the interaction between planthopper cells and the rice stripe virus. By applying RNAi, they discovered that the viral non-structural protein NS3 of the filamentous ribonucleoprotein particles of RSV is involved in viral replication or assembly.

Using insect cell lines in virology research offers advantages as compared to working with whole insects. Cell lines provide unlimited material resources because the cells can be readily maintained at relatively low costs with minimum labor input. Cell lines also provide a population of cells to generate consistent samples and reproducible results.

1.3. Long-term Value of Cell Lines

1.3.1. Baculovirus Production

Many types of viruses infect insects, some of which belong to the family Baculoviridae. Most baculoviruses have relatively narrow host-ranges with three levels of specificity: tissue-specificity, species-specificity, and specificity for hosts within the Phylum Arthropoda. Susceptible organisms are commonly found in the orders Lepidoptera, Hymenoptera, Diptera, and Decapoda. Virtually all the hosts are invertebrates with over 600 known host species. Since the early 1990s, research into identifying baculovirus host-range-related genes has been performed (Thiem, 2009), but the gene functions or mechanisms that control host-range are still not clear.

Baculoviruses are rod-shaped viruses with double-stranded, circular and supercoiled DNA. More than 500 baculovirus isolates have been identified, most of which originated from Lepidoptera. Baculoviruses are useful not only as bio-insecticides, but also as vectors facilitating the expression of foreign protein in insect cells (Davis et al., 1993; Gelernter and Federici, 1986). The two most commonly used isolates in foreign gene expression are *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) and *Bombyx mori* nuclear polyhedrosis virus (BmNPV). Given those, over the past 30

years the baculovirus-insect cell expression system (BEVS; Figure 1-3) has become one of the most popular systems for routine production of recombinant proteins. Using BEVS provides several advantages. First, foreign genes are usually expressed at high levels in chosen insect cells under the control of the strong AcMNPV polyhedron promotor of the viruses. Second, the baculoviridae consists of large groups of known viruses with the ability to infect over 500 insect species. The most commonly used lepidopteran cell lines for BEVS are the Sf9 and Sf21 lines initiated from the ovarian tissue of the fall armyworm, and the High Five cell line, designated as BTI-Tn-5B1-4 initiated from the *Trichoplusia ni* embryonic tissues.

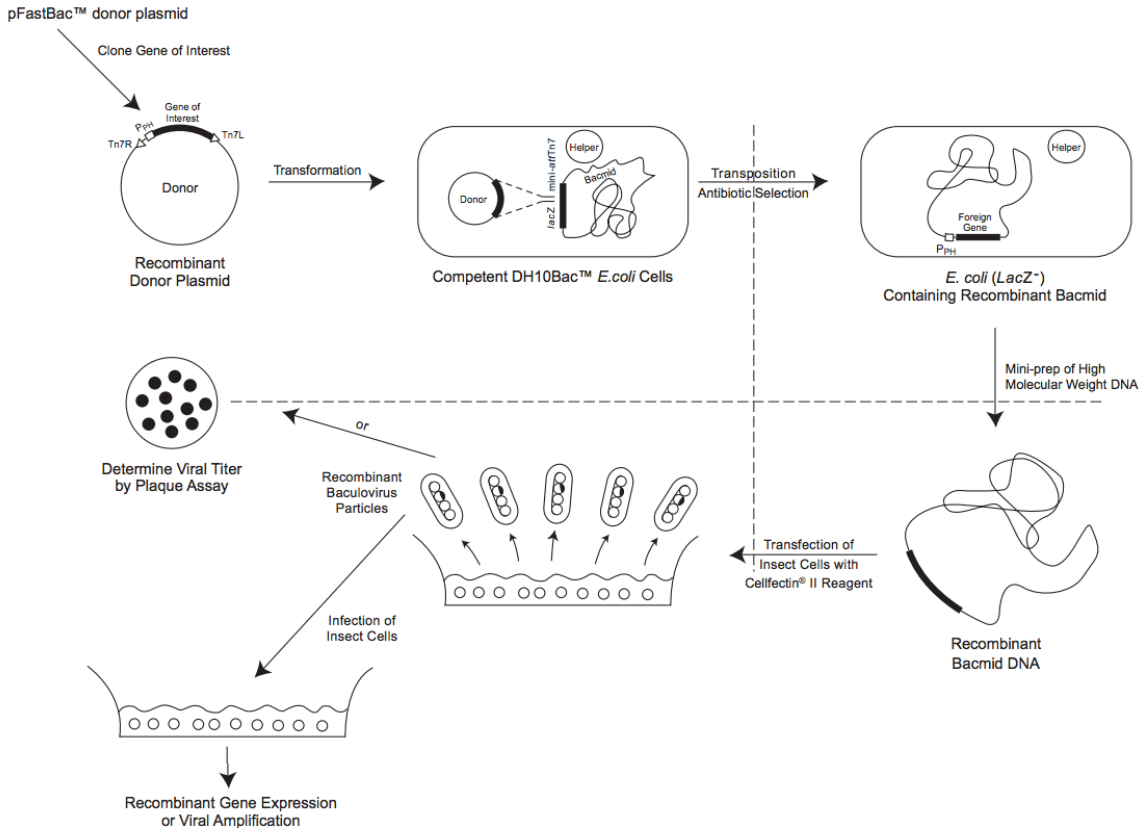

Figure 1-2. Generation of recombinant baculoviruses and gene expression with the BAC-TO-BAC expression system (Thermo Fisher Scientific).

To date, thousands of recombinant proteins, as well as health-related products, ranging from cytosolic enzymes to membrane-bound proteins, have been produced in BEVS (Kost and Condreay, 2005). These indicate that as hosts of baculoviruses, insect cell lines are standard, indispensable tools for baculovirus propagation, specific gene expression, and recombinant protein production.

1.3.2. Insecticide Discovery

During the past five decades, conventional insecticides (neuroactive chemicals) have been indiscriminately used in pest management, which led to severe environmental concerns such as insect resistance, environmental contamination with chemical residues, and pest resurgence. To date, neonicotinoid pesticides, which are replacing more harmful products, are applied with the advantages of quick breakdown and high efficiencies. However, the literature indicates that neonicotinoid pesticides impair survivorship of pollinators, reproduction of birds and small mammals. Even low concentrations of neonicotinoids and fipronil have lethal and sublethal effects on insect pollinators (Pisa et al., 2015). The loss of insect pollinators directly reduces crop yields, and significantly affects human food production and quality. Similarities are also found in aquatic invertebrate populations. Neonicotinoids are capable to enter freshwater bodies and reduce aquatic insects populations that many water-dependent birds feed on. Hallmann et al. (2014) recently discovered the negative cascading effect of low neonicotinoid concentiations on insectivorous bird populations.

More recent insect pest management technologies combine several pest management approaches into integrated pest management (IPM) programs. One aim focuses on

investigating third generation pesticides that target major insect endocrine systems, known as insect growth regulators (IGRs). Among those, insect juvenile hormones analogs (e.g., methoprene, hydroprene, pyriproxyfen), which operate by impairing insect molting/metamorphosis, have been commercialized for use in modern crop protection. Additionally, ecdysteroid receptor agonists (e.g., tebufenozide, methoxyfenozide), and chitin synthesis inhibitors (e.g., benzoylureas) have been investigated as products to impair regulation of growth, reproduction or chitin synthesis. Because of their relatively low risk to non-target organisms and the environment, high target specificity and their versatility in application methods, these important classes of new pest control products will play a greater role in the present context of environmental safety and their consequent uses in IPM and insect resistance management programs.

High throughput screening (HTS) systems are used in current pesticide discovery programs. Process-observable screening assays are able to integrate rapid detection of alternative compounds and direct function on target sites. The use of insect cell lines from specific tissues in HTS assays are much more efficient, less expensive and faster than conventional animal toxicity tests which use whole insect screening assays. Along with urgent demands for *in vitro* assay systems and very large numbers of screened compounds, HTS is becoming increasingly important in chemical discovery programs in the pharmaceutical and agricultural chemical industries.

1.3.3. Basic Insect Science

Insect cell lines also serve as important tools in basic insect science research areas, such as immunology, nutrition, and development. Stanley et al. (2008, 2012) reported that prostaglandins A_2, A_1 and E_1 influence the expression of specific genes in an established insect cell line, BCIRL-HzAM1 cells. Prostaglandins (PGs) are eicosanoids that exert important physiological actions in insects. Insect cell lines were also used to determine the requirements for nutrition, including vitamins, sugars, amino acids, and lipids (Vaughn, 1973). Similarly, Drews (1995) studied the growth of the Sf9 cells in batch and continuous culture. The results of batch culture showed that cells preferred glucose as the main energy and carbon source limiting the cell density. As for continuous culture, glucose and yeast extract concentrations limited the maximum cell density.

1.4. Next-generation Cell Lines

Based on the above knowledge, cell lines have gradually come to play an important role in almost every aspect of insect science, including both basic and applied studies. According to specific research demands, and the need to express specific properties and functions, selected cell lines are currently designated as “Next-generation Cell Lines”. Next-generation cell lines refer to insect cell lines that are initiated from specialized tissues, midgut, for example, or from insect taxa that are not represented in current insect cell line collections, such as honeybees. Most highly desired are cell lines that maintain some of the functions seen in the tissues of living insects, allowing them to be used in high-throughput screening and reprogramming.

For decades, scientists have attempted to develop cell lines for *in vitro* studies. However, one of the greatest challenges has been generation of stable cell lines from tissues that still maintain the primary characteristics. Gao (1999) applied immunity proteins to mosquito cell line Aag-2, which in turn produced defensin A isoforms. The Schlegel group at Georgetown (Liu et al., 2012) used "conditionally reprogrammed cells" (CRCs) to develop cancer cell lines that maintained histotype specific characteristics. The next generation cell lines will become available and valuable in the near future.

CHAPTER 2: MIDGUT CELL LINES

2.1. The Four Target Insect Species

In this thesis, I report on the initiation of cell cultures from midgut tissues isolated from selected pest species. The research was conducted using four pest species, introduced in this section.

2.1.1. Western Corn Rootworm

Western corn rootworm, *Diabrotica virgifera virgifera*, is one of the most devastating corn insect pests in the American Corn Belt and more recently in Europe (Ciosi et al., 2008). In some years, corn rootworms cause up to $1 billion in yield loss and control costs, which is the reason this species captures the attention of industrial, government and academic scientists. Western corn rootworm larvae directly feed on young corn roots, leading to lethal reductions of nutrient and water uptake. The demand for novel insecticide technologies is urgent, which drives my research to establish a western corn rootworm midgut cell line.

2.1.2. (Southern) Green Stink bug

The adult southern green stink bug (*Nezara viridula*) is shield-shaped, about $1/2 \sim 3/4$ -inch in length with an overall dull green color. The development of the southern green stink bug requires about 5 weeks from egg to adult, although the time varies by ambient temperature. This pest infests a wide range of fruits, field crops, and vegetables. Crops of

economic importance include beans, citrus, cabbage, head cabbage, potatoes, tomatoes, and watercress. Similarly, the green stinkbug (*Chinavia halaris*) is a commonly encountered pest of grain, nuts, tomatoes and fruits across North America. These two pests are both polyphagous herbivores capable of long distance foraging movements (LSU AgCenter). Both nymphs and adults cause injuries by injecting digestive enzymes while feeding on crops. They are one of the most economically important agricultural pests worldwide and are distributed throughout warmer regions. Stinkbugs caused an estimated $6.5 million dollars of crop loss and control cost on soybeans in the US (Tillman, 2006). In recent years the increased use of insecticides was attributed largely to increased problems with stinkbugs, which indicates an urgent demand for corresponding novel insecticides. Little has been known on establishment of stinkbug midgut cell lines. One of the most challenging problems is the symbiotic bacterium parasitizing within the midgut. This study spent much time on cell culture medium optimization and removal of symbiotic bacteria, as well as encouraging cell proliferation.

2.1.3. Fall Armyworm

The fall armyworm (*Spodoptera frugiperda*) is an agricultural pest of the Western Hemisphere and, most recently, the pest has become a new invasive species in West and Central Africa where the first outbreak was recorded in early 2016 (Goergen et al., 2016). It overwinters in south Florida and Texas largely because it lacks a diapause period. The most significant damage is caused by larvae feeding on leaves and stems, with a wide host range of more than 80 plant species, including crop plants, such as maize, rice and

sorghum (Capinera, 1999). Hruska and Gould (1997) reported that infestation rates above 55% of the mid- to late-whorl stages of maize lead to yield losses of 15-73%.

Fall armyworm cell lines are commonly used in BEVs. A prominent example is the Sf9 cell line, originally established from ovarian tissue. It is commonly used for recombinant protein production. A midgut cell line is needed for research and HTS purposes.

2.2. Methods and Materials

2.2.1. Cell Line Initiation

2.2.1.1 Western Corn Rootworm (WCRW)

Rootworm eggs (*Rose*, non-diapausing strain) were provided by Dr. Bruce Hibbard (Plant Genetics, USDA-ARS Plant Genetics Research Unit at Columbia, MO). Eggs were surfaced sterilized by adding 10 to 20 ml dishwashing liquid (Lysol) and slightly shaking for 3 minutes. After removing the solution, the eggs were rinsed in distilled water three times and gently agitated in 10 to 20 ml formalin (buffered zinc formalin) for 3 minutes. After removal of the solution, the eggs were rinsed three times in distilled water. The egg suspension was dispensed onto filter paper using a 1 ml pipettor. The excess water was blotted away to inhibit fungal growth. Filter paper (Whatman #1) was placed inside 16 oz plastic deli containers (#127DM16BULK, WebstaurantStore, Lancaster, PA) with pinholes in each lid. Once emerged, 1st instar larvae were transferred onto artificial diets (Huynh, unpublished) in 96 well plates and incubated at 25℃, in total darkness. Larvae were transferred into new diet plates every week until they were ready for use.

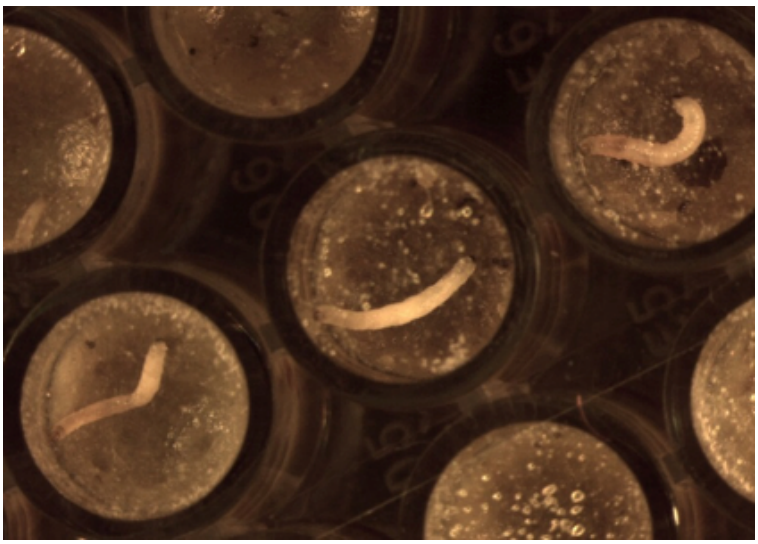

Figure 2-1. *D. virgifera* larvae reared on artificial diet, 6X magnification.

We also tested new methods for rearing *D. virgifera* larvae under sterile conditions because the availability of the artificial diet was under development. We added autoclaved agar with nutrient solution, vermiculite or potting soil to 15.5 cm-long test tubes and overlaid these substrates with surface-sterilized corn seeds. Once the seeds germinated, we pipetted surface-sterilized insect eggs into the test tubes. Test tubes were capped and maintained at 28°C.

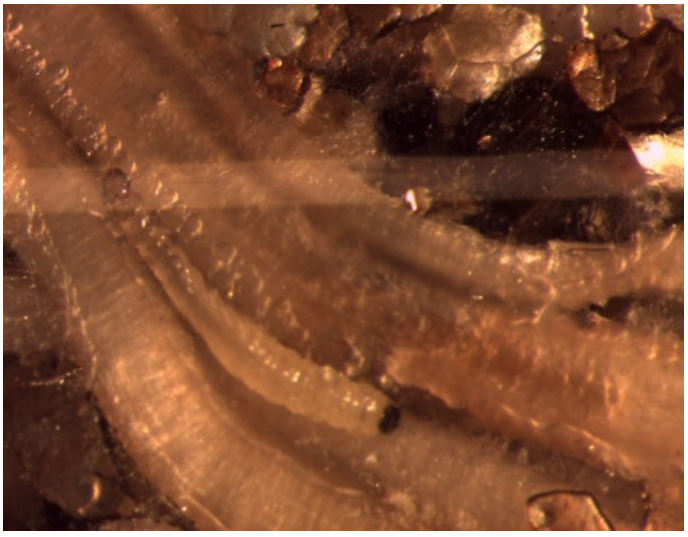

Figure 2-2. Second instar *D. virgifera* larva ingesting corn roots in the vermiculite.

Cell cultures were initiated from late second- and third instar larvae. The larvae were surfaced sterilized as follows (10 mL/solution): 3 min wash in 95 % ethanol, 3 min wash in 0.05% sodium hypochlorite, 3 min wash in 70 % ethanol, and final rinse in Calcium Magnesium free-PBS (137 nM NaCl, 4 mM KCl, 0.26 mM Na_2HPO_4, 0.15 mM KH_2PO_4, 11 mM Glucose). The larval head and anal section were removed and the gut was pulled out using forceps. The midgut tissue was washed three times in CMF-PBS solution and minced using micro scissors in 1 ml cell culture medium. The tissue suspension was transferred into $T_{12.5}$ flasks, each containing 3 ml suspension, and monitored for growth.

For embryonic cultures, eggs were gently homogenized using a plastic pestle in a small Petri dish. The homogenate was transferred to a 15 ml conical tube and centrifuged at 1,000 x g for 5 min at 4℃. Cells and tissues from the pellet were suspended in 5 ml media (containing 10% fetal bovine serum and antibiotics) and transferred to culture flasks.

We tested these cell culture media for the WCRW: EX-CELL 420 (Sigma-Aldrich, St Louis, MO), Shields & Sang, CLG #2 (½ EX-CELL 420 + ½ L15), Kimura's, Schneider's, L15B, and IPL-41 (Caisson Labs, Smithfield, UT., for all but EX-CELL 420). All cell culture media contained 10% heat-treated FBS (Sigma-Aldrich, St Louis, MO) and 50 U/ml penicillin and 0.05 mg/ml streptomycin (Sigma-Aldrich).

2.2.1.2. Green Stink Bug (GSB) and Southern Green Stink Bug (SGSB)

The GSB adults were field collected by Ben Puttler (University of Missouri, Columbia) in Boone County, MO. SGSB adults were obtained from several resources, including Dr.

G. Tillman (USDA-ARS, Crop Protection and Management Research Unit), Benzon Research (Carlisle, PA), Dr. J. Davis (Louisiana State University), Dr. Bryony Bonning (Iowa State University), and Dr. Cris Oppert (Bayer CropScience, Morrisville, NC). These insects were maintained in small colonies and fed organic green beans, baby tomatoes, carrots, corns, and Japanese broccolini. Adults were surfaced sterilized as described above. The gut of both green stinkbug species was divided into 4 segments V1-V4 (Figure 2-3. Hirose et al., 2006). In practice, we usually discarded section V4 because high populations of symbiotic bacteria are reported in this region.

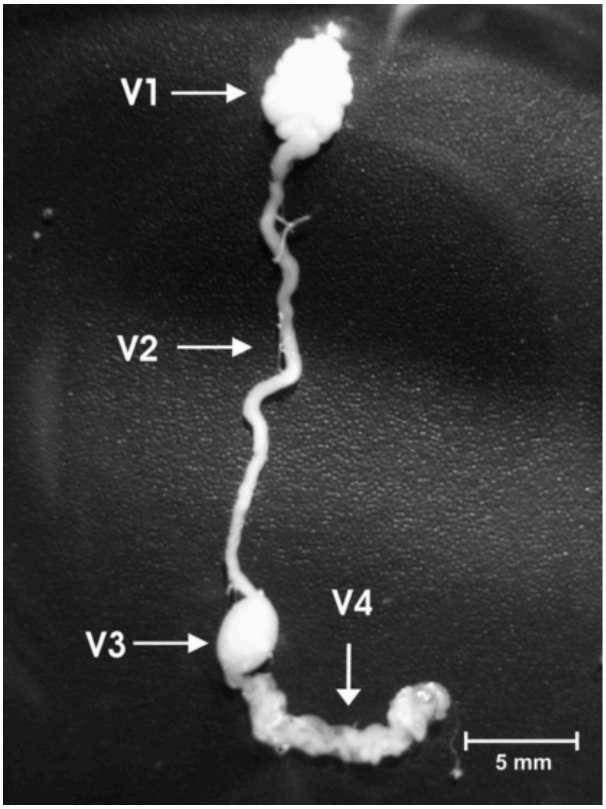

Figure 2-3. Gut sections of *N. viridula* (ventriculus 1-4; V1-V4). V1 on the anterior end; V4 on the posterior end.

Besides midguts, ovaries, testes and fat bodies were excised initially and washed twice in CMF-PBS containing antibiotics. Afterwards, we shifted our focus to midgut cell culture

initiations. In some cases, tissues were placed into flasks coated with poly-D-lysine (Fisher Scientific, Pittsburgh, PA) instead of the standard cell culture treatment.

I tested five media for the GSB and SGSB: EX-CELL 420, Shields & Sang, CLG #2, Kimura's, Schneider's, and L15B. We supplemented the media with 10% heat-treated FBS and 50 U/ml penicillin and 0.05 mg/ml streptomycin.In some cases, the cell cultures were also supplemented with 0.1 mg/ml gentamycin, 0.5 ug/ml amphotericin B, or Chloramphenicol (30, 60, 90, 120 mg/ml).

2.2.1.3. Fall Armyworm

Larvae were obtained from Benzon Research Inc., Carlisle, PA. Upon arrival, they were reared at 28℃. Fifth or sixth instar larvae were used as midgut sources. Larvae were surfaced sterilized as previously described. The peritrophic membrane, containing the food bolus, was removed. Midguts from a single insect were washed twice in CMF-PBS containing antibiotics and transferred individually into 1 ml medium containing antibiotics and minced (in 12-well plates). The tissue suspensions were transferred into a $T_{12.5}$ flask and medium was brought up to 2.5 ml.

I tested 11 media, EX-CELL 420, TnM-FH (Caisson Laboratories, Smithfield, UT), IPL-41, Kimura's, L15B, Shield's and Sang, RPMI 1640 (Sanchez et al., 2005), Sf900-II, EX-CELL 405, EX-CELL TiterHigh, DMEM/Ham's F-12. In some initiations I also used four medium additives, including fat body extract (Loeb, 2012), AlbuMAX II and KnockOut Serum, TnM-FH (a modification of Grace's medium) was the optimal medium that yielded the most viable cells. Each medium contained 10% FBS (Sigma-Aldrich, St

Louis, MO), 50 U/ml penicillin and 0.05 mg/ml streptomycin. 40 g/L AlbuMAX II (Life Technologies, Carlsbad, CA) has been used to replace the 10% FBS (Castagnola et al., 2011).

To isolate midgut epithelial cells, the procedure of Hakim et al. (2009) was followed. Briefly, midgut tissues were individually washed in Hanks Balanced Salt Solution buffer (Thermo Fisher Scientific, Waltham, MA), minced tissues were shaken on an orbital shaker (1.5 hr, 50 rpm, MIDSCI, St Louis, MO), pipetted up and down to enhance cell dissociation, and strained through a 70 um nylon sieve (Fisher Scientific, Waltham, MA). Tissue suspensions were centrifuged (800xg, 10 min, 4℃) and rinsed in flasks.

For additional enrichment, instead of transferring the sieved cells to flasks, cells in 1 ml medium were overlaid onto a 3 ml Ficoll-Paque gradient (GE Healthcare Life Sciences, Piscataway, NJ) in a 10 ml centrifuge tube and centrifuged (600 x g, 15 min, 20 ℃) (Castagnola et al., 2011). Stem cell enriched midgut cells were found in the middle fraction of the tube (Figure 2-4). They were transferred to another tube, washed twice with 5 ml medium and then were transferred into a T_{25} flask with 5 ml fresh medium. To encourage cell/tissue attachment, we added 3 ml medium (for T_{25} flasks) on the first day, and added another 2 ml on the second day. Flasks with cell attachment substrates collagen I and IV were used as well.

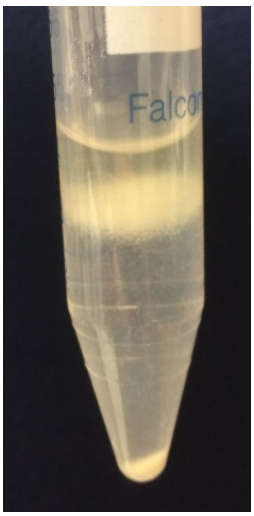

Figure 2-4. *S. frugiperda* midgut cells separated with Ficoll-Paque™ density gradient.

Cultures were maintained in 28℃ and were monitored weekly. Cultures were fed by replacing half the spent medium with fresh medium as needed. When passaging the cell cultures, we typically used trypsin and cell scrapers to remove attached cells from the flask surfaces (MIDSCI, St Louis, MO).

2.2.2. Cryopreservation of the cell lines

Sf cell lines were periodically cryopreserved while the cell lines were consistently proliferating. Cells were detached using 4 ml 90% FBS/10% fresh medium after they grew to confluency and transferred into sterile 15 ml centrifuge tubes on ice. Once the cell concentrations were adjusted to at least $3x10^6$ cells/ml, the cell suspension was diluted 1:1 with 10% DMSO solution (mixed by 4.5 ml 90%FBS/10% medium and 0.5 ml DMSO) as follows: while on ice, 4 ml 10% DMSO solution was added drop-wise to the cell solution and the tube was then gently inverted a few times. The cell suspension was aliquoted into 2 mL cryogenic vials (1 mL/vial) and was gradually frozen (~2^oC/min)

using an liquid nitrogen tank insert. Cells were stored indefinitely in a liquid nitrogen cryopreservation tank.

2.2.3. Characterization of Cell Lines

2.2.3.1. DAF-PCR

The Sf midgut cell lines (SfMG1-0611, SfMG-0617) were characterized using DNA fingerprinting (DAF-PCR) with the gene primers described by McIntosh et al. (1996): mammalian aldolase, prolactin, and interleukin. The primer sequences were aldolase-F: 5'- CCG GAG CAG AAG AAG GAG CT-3'; aldolase-R: 5'-CAC ATA CTG GCA GCG CTT CA-3'; prolactin-F: 5'-CTG GGA CAG ATG GAG GAC T-3'; prolactin-R: 5'-CTC AGG TTT TAA TCG AAT TT-3'; and interleukin-1B-F: 5'-ATG AGG ATG ACT TGT TCT TT-3'; interleukin-1B-R: 5'-GAG GTG CTG ATG TAC CAG TT-3'. In addition to the Sf cell lines, Sf larval tissue, AMCY/BCIRL-SeE-CLG1 cell line (*Spodoptera exigua*), At tissue (*Anasa tristis*), Dm tissue (*Drosophila melanogaster*), and BCIRL-TcA-CLG1 cell line (*Tribolium castaneum*) were analyzed by DAF-PCR for comparison. Cells were resuspended and pelleted in CMF-PBS solution. The cell pellet was frozen at -20℃ and was resuspended in 200µl of CMF-PBS. Genomic DNA was extracted from the cell lines and tissues using the Qiagen DNeasy Tissue Kit (Cat # 69504).

2.2.3.2. Cell Doubling Time

Cell doubling time was determined using growth curves. To create growth curves, each Sf midgut cell line was seeded into $T_{12.5}$ flasks ($2x10^5$ cells/ml, 2.5 mL/flask) Cell concentrations were determined every two days (3 flasks per timepoint) using a Cellometer® (Nexcelom Bioscience) in conjunction with 0.4% trypan blue. The linear portion of the growth curves were used to determine the doubling times (Roth V, 2006) calculated by the following equation:

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$$Doubling\ Time = \frac{duration * log2}{log(Final\ Concentration) - log(Initial\ Concentration)}$$

2.2.4. Cell Cytotoxicity Assay

I determined the sensitivity and linear range of each cell line for three commercially available cytotoxicity assays: CellTox Green Cytotoxicity Assay™ (Promega, Fitchburg, WI), Cell Counting Kit-8 Assay™ (Dojindo, Japan), and CellTiter Blue Cell Viability Assay™ (Promega, Fitchburg, WI).

2.2.4.1. CellTox Green Cytotoxicity Assay

This assay (Promega, Fitchburg, WI) measures changes in membrane integrity that occur as a result of cell death. It is based on a proprietary asymmetric cyanine dye, which is excluded from viable cells but binds to the DNA of dead cells or cells with compromised membranes (Fig. 2-5).

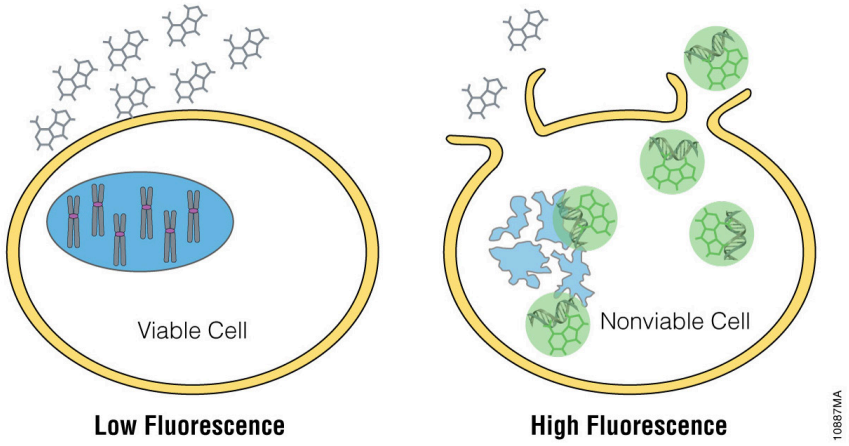

Figure 2-5. CellTox™ green dye binds DNA of cells with impaired membrane integrity (re-drawn from product manual). After the dye binds DNA, its fluorescent properties are substantially enhanced and the number of dead cells is reflected by the fluorescent signal.

This assay was set up as described in the product manual. Adherent cells were harvested and resuspended in fresh medium. The number of viable cells was determined by trypan blue with a Cellometer, and then the cell concentration was adjusted to 200,000 viable cells/ml in at least 4 ml fresh medium. Cell suspensions (2 ml each) were pipetted into two separate centrifuge tubes, one was for a "cytotoxicity control" and the other a "viability control". For the "cytotoxicity control", 80 µl "lysis solution" was added to the tube correspondingly, 80µl water was added to the "viability control". The seeded 96-well plate (as the protocol described) was incubated at 28℃ overnight before adding 100µl of the CellTox™ Green Reagent to all the wells. The plate was incubated for at least 15 min at room temperature (shielded from light) to facilitate DNA/dye binding. The fluorescent signal was measured using an excitation wavelength of 485-500 nm and emission of 520-530 nm.

2.2.4.2. Cell Counting Kit-8 Assay (CCK-8)

This assay is very straightforward and efficient, utilizing highly water-soluble tetrazolium salt that produces formazan dye upon reduction in the presence of an electron mediator. Its main advantage is that it is nonradioactive and nontoxic: CCK-8 does not harm the tested cells, which enables convenient time-course investigations. Cells were seeded into 96-well plates at concentrations ranging from 6 x 10^5 cells/ml to 5 x 10^4 cells/ml. The plates were incubated in 28℃ overnight before adding the CCK-8 dye (20 µl), and incubated for 1.5 hr and 3 hr (1-4 hr as recommended in the protocol). We also measured the absorbance (excitation wavelength: 450nm) the second day.

2.2.4.3. CellTiter Blue Cell Viability Assay™

This assay (Promega) uses the indicator dye resazurin to measure the metabolic capacity of cells as an indicator of cell viability. The fluorescent signal ($579_{Ex}/584_{Em}$) is produced by the transformation of resazurin into resorufin in viable cells.

The linear range and cell sensitivity was determined for this assay on three cell lines: CF-1 (initial cell concentration 5 x 10^5 cells/ml), SfMG1-0611 (initial cell concentration 4 x 10^5 cells/ml), and SfMG-0617 (initial cell concentration 6 x 10^5 cells/ml). The seeded plates were incubated overnight as just described. The CellTiter Blue reagent (20µl) was added to each well and the fluorescent signals ($579_{Ex}/584_{Em}$) were measured for two time points. Cell viability response curves were created according to the fluorescence data.

2.3. Results

I initially isolated midgut tissues, and later isolated eggs, fat body, testes and ovaries for specific cell culture initiations. I returned my focus to the midgut tissues to obtain the needed cell lines and tested numerous culture media for each species. The total number of initiations, number of promising cultures, and the best media for each pest species cell cultures are listed respectively in Table 2-1.

Table 2-1. A summary of insect species, specific tissues and media tested to initiate cell lines.

Insect Species	Tissue	No. Init	Promising Cultures	Best Medium
D. virgifera	larvae midgut	31	3	EX-CELL 420;
	egg	11	0	Kimura's
C. hilaris	adult midgut	27	1	CLG #2;
	fat body	11	0	Kimura's;
	testes	4	1	Shields & Sang
	ovary	4	0	
N. viridula	adult midgut	89	4	CLG #2;
	fat body	0	0	Kimura's;
	testes	17	4	Shields & Sang
	ovary	26	0	
S. frugiperda	larvae midgut	97	8	TnM-FH

For *D. virgifera*, we tested EX-CELL 420, Shields & Sang, CLG #2 IPL-41, Kimura's, L15B media; for *C. hilaris/N. viridula*, we tested EX-CELL 420, Shields & Sang, CLG #2, Kimura's, Schneider's, L15B media; for *S. frugiperda*, we tested EX-CELL 420, TnM-FH, IPL-41, Kimura's, L15B, Shields and Sang, RPMI 1640, Sf900-II, EX-CELL

405, EX-CELL TiterHigh, DMEM/Ham's F-12 media and medium additives, including fat body extract, AlbuMAX II and KnockOut Serum.

2.3.1. Establishment of Cell Lines

2.3.1.1. Western Corn Rootworm

The healthiest insect larvae were obtained using artificial diets optimized by Huynh and Meihls at BCIRL (unpublished observations). As for larvae reared on corn roots, the best corn growth occurred with the vermiculite and nutrients mixture as growth medium.

We initiated 31 midgut cell cultures and 11 embryonic cell cultures from the western corn rootworm. Among those, 3 midgut cell cultures are still viable and are being maintained (Table 2-2). The best media are Kimura's medium and EX-CELL 420 with 10% fetal bovine serum. There were once three promising egg cell cultures (DvE3-0731-CLG #2, DvE5-0804-CLG #2, DvE7-0804-SS), which had been passaged at least once. The cells were finally degraded due to insufficient nutrition supply. The cells in the midgut and embryonic primary cultures remained in suspension with few cells attached (tissue attachment is often a sign of healthy cultures).

Table 2-2. Promising cell cultures initiated from *D. virgifera* midgut.

Designation	Passage	Medium	Status
DvMG-1206	0	Kimura's + 10% FBS	viable spherical cells
DvMG-1204	0	Kimura's + 10% FBS	viable spherical cells
DvMG-1206	0	Shields & Sang + 10%FBS	viable cells, few debris

I noted fungal growth in some of the egg cell cultures. The presence of the fungicide, amphotericin B, did not adequately inhibit fungal growth in these cultures. Larvae reared under sterile or semi-sterile conditions efficiently reduced the presence of either bacterial or fungal contamination (Figs. 2-6, 2-7). Higher concentrations and various combinations of antibiotics effectively suppressed microbial growth (Fig. 2-8). As a negative side effect, this resulted in 60% - 90% cytolysis of midgut cells and tissues.

Of interest, some cultures contained midgut tissues that are (or were initially) continuously contracting, from which I infer the medium encourages near-normal physiological functioning.

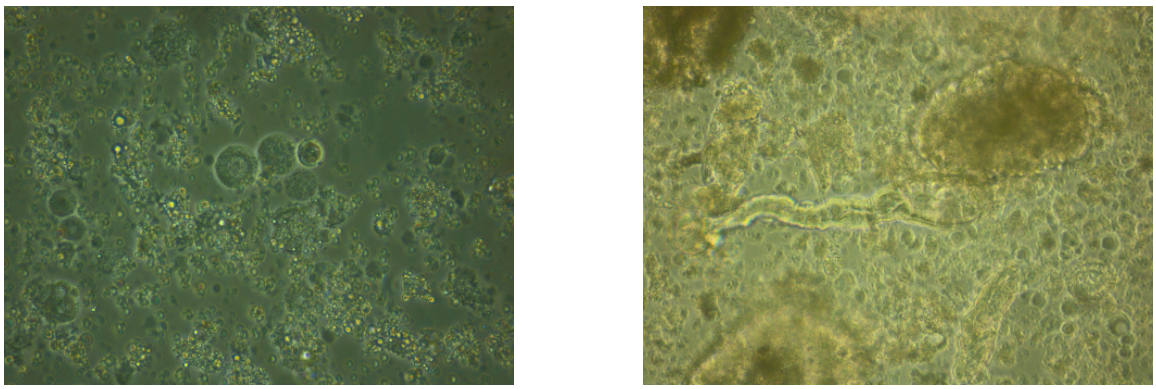

Figure 2-6. *D. virgifera* midgut cell culture DvMG1-0328-KM. Left panel: spherical cells, 400X; right panel: contracting tissues, 200X.

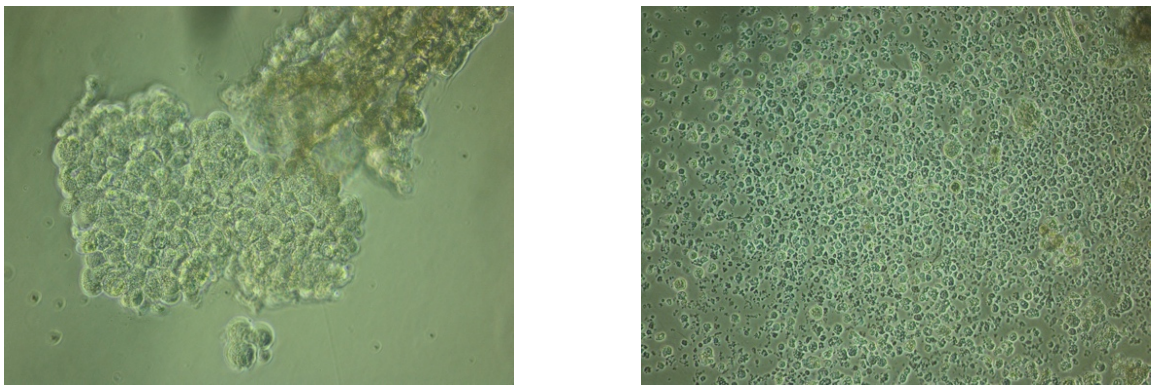

Figure 2-7. *D. virgifera* midgut cell cultures DvMG-0714-KM, 200X. Clean tissues and viable cells.

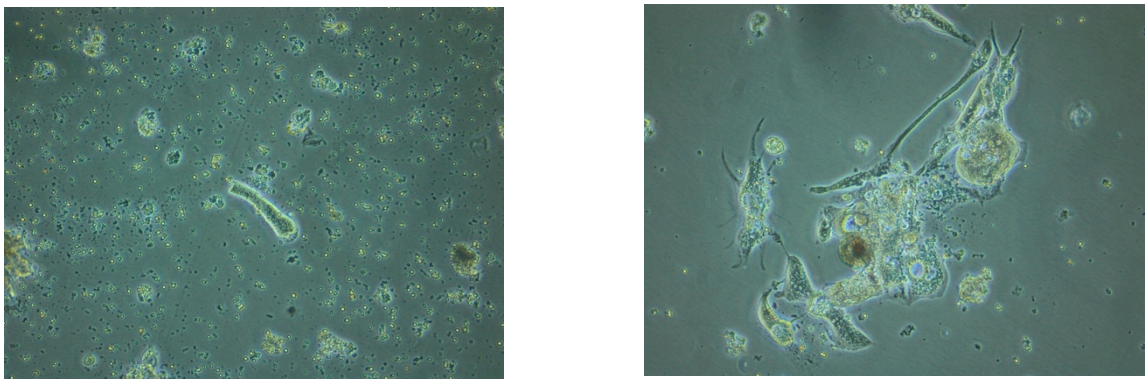

Figure 2-8. Attached midgut cell cultures from the western corn rootworm. Left: DvMG-1204-KM (400X); right: DvMG-1204-SS (200X). All cultures contain 10% FBS and antibiotics (penicillin-streptomycin, gentamycin, kanamycin, ampicillin, chloramphenicol).

2.3.1.2. Southern Green Stink Bug/Green Stink Bug

I reared the stinkbugs from eggs and fed them organic green beans, baby tomatoes, carrots, corns and broccolini. For *C. hilaris*, I initiated 27 midgut cell cultures, 11 fat body cell cultures, 4 testes cell cultures, and 4 ovarian cell cultures. For *N. viridula*, I set up 89 midgut cell cultures, 17 testes cell cultures, and 26 ovarian cell cultures. In most cultures, cells remained in suspension.

Table 2-3. Promising cell cultures initiated from *C. hilaris* and *N. viridula* midgut.

Designation	Passage	Medium	Status
ChMG2-1022	0	Kimura's	clean tissues, limited viability
NvMG3-1022	0	SS	clean tissues, limited viability
NvTS-0213	3	KM	few crystals, few cells
NvTS-0213	4	KM	many viable cells
ChTS1-100914	0	KM	many viable cells
NvTS-0213	1	KM	many viable cells
NvTS-0213	1	KM	many viable cells
NvMG-0629	1	SS	few crystals, many viable cells
NvMG-0629	0	KM	viable cells
NvMG-0629	1	SS	a few crystals

I initiated promising cell cultures from testes and midguts. Over time, the testes cells developed into sperm cells. This indicates that Kimura's medium is able to support the development of testes cells. I originally recorded clean, spherical midgut cells, but either fungi or symbiotic bacteria contaminated most cell cultures. The typical symbiotic bacteria (4-6 um) are beaded or filiform, which rapidly overwhelmed the flasks. I noted crystal formations in some cell cultures, which indicated cells with low metabolic activity.

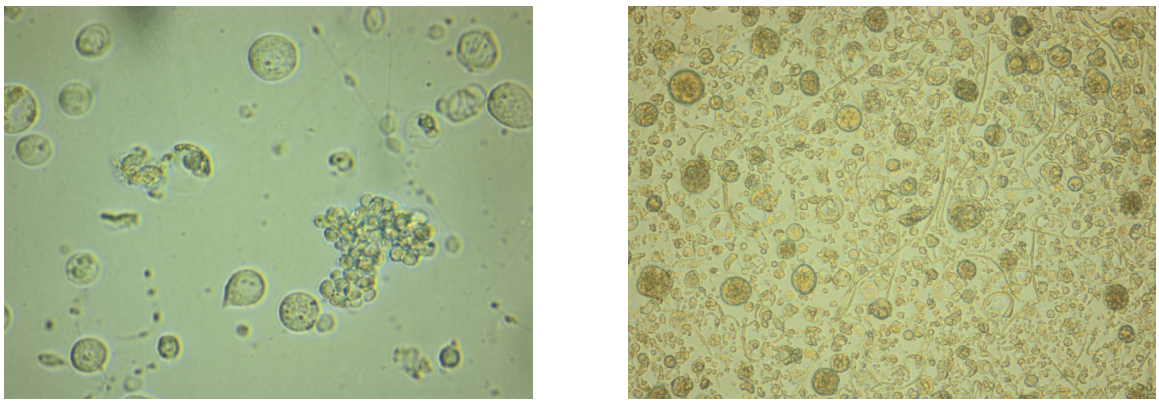

Figure 2-9. *C. hilaris* testes cell culture showing cells in suspension (left), 400X; *N. viridula* testes cell culture (right), 400X.

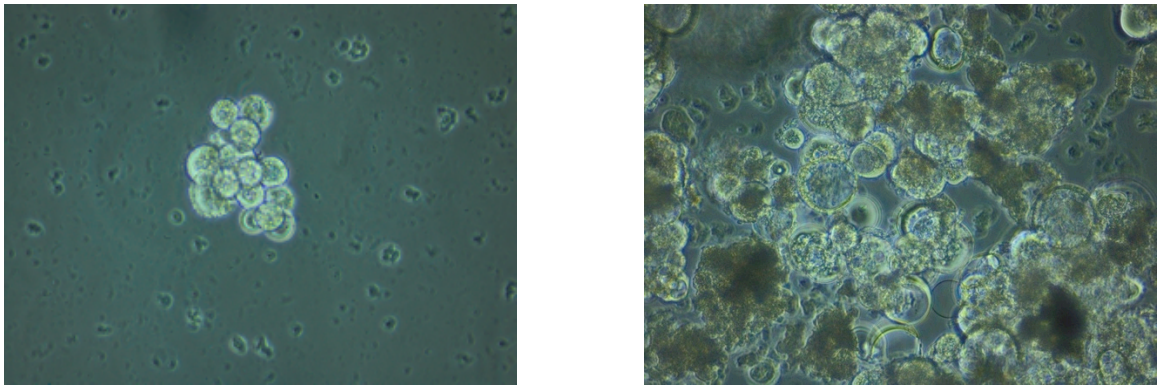

Figure 2-10. *N. viridula* midgut cultures, 200X. Left: cells in Shields & Sang medium; right: cells in EX-CELL 420 medium.

Addition of high concentrations of antibiotics suppressed the symbiotic bacteria after thoroughly washing the midgut tissues. I also launched a new approach using Ficoll-Paque^{TM} cell separation media to centrifuge the midgut cells, which led to the reduction of symbiotic bacteria.

2.3.1.3. Fall Armyworm

Fall armyworm larvae provided by Benzon were bacteria-free. 5th and 6th larvae were selected for dissection. I initiated 97 midgut cell cultures (Table 2-1) with a variety of media, of which TnM-FH was the most appropriate medium for Sf midgut cell cultures. Instead of using fetal bovine serum, AlbuMAX II (serum replacement) was found to work similarly as serum. Table 2-4 describes the most promising Sf midgut cultures that are still being maintained.

Table 2-4. Promising cell cultures initiated from *S. frugiperda* midgut.

Designation	Passage	medium	status
SfMG-0405-filterleft	1	TnM-FH	few cells; clean
SfMG1-0610	0	Tnm-FH	clean culture
SfMG-0712	2	Tnm-FH	few cells
SfMG-0706	1	Tnm-FH-AMII	many viable cells
SfMG-0706	2	Tnm-FH-AMII	many viable cells
SfMG-0706	3	Tnm-FH-AMII	many viable cells
SfMG1-0611	31	Tnm-FH	continuously growing
SfMG-0617	33	Tnm-FH	continuously growing

In some instances, cell and tissue suspensions were shaken for 30 min filtered (70 mm) and centrifuged using the Ficoll-Paque™ cell separation reagent. This facilitated the concentration of stem cells, goblet cells, and columnar cells with microvilli (Figure 2-11). None of these cultures remained viable over time, other than one culture consisting of the filtrate (SfMG-0405-filterleft).

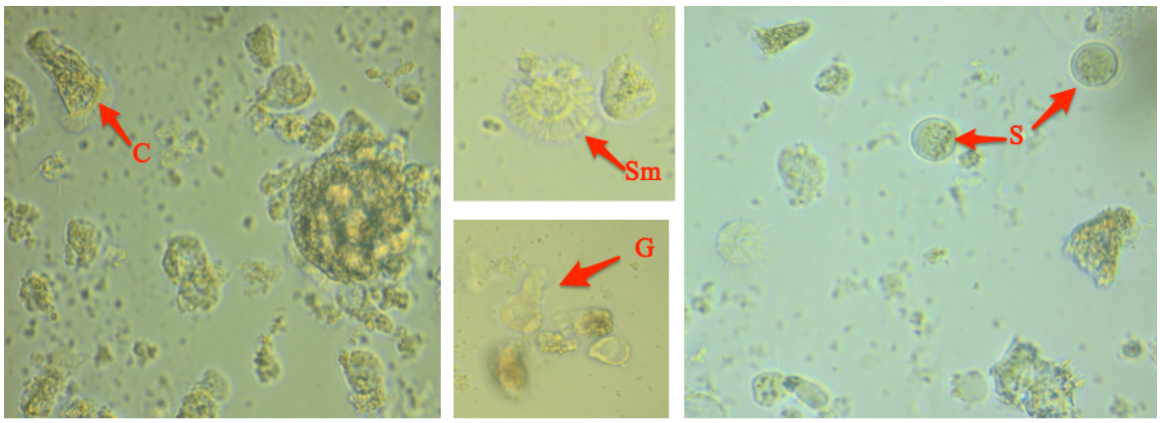

Figure 2-11. Cell morphologies in *S. frugiperda* midgut cell cultures. S = stem cells, Sm = stem cells with microvilli, C = columnar cells, G = goblet cells.

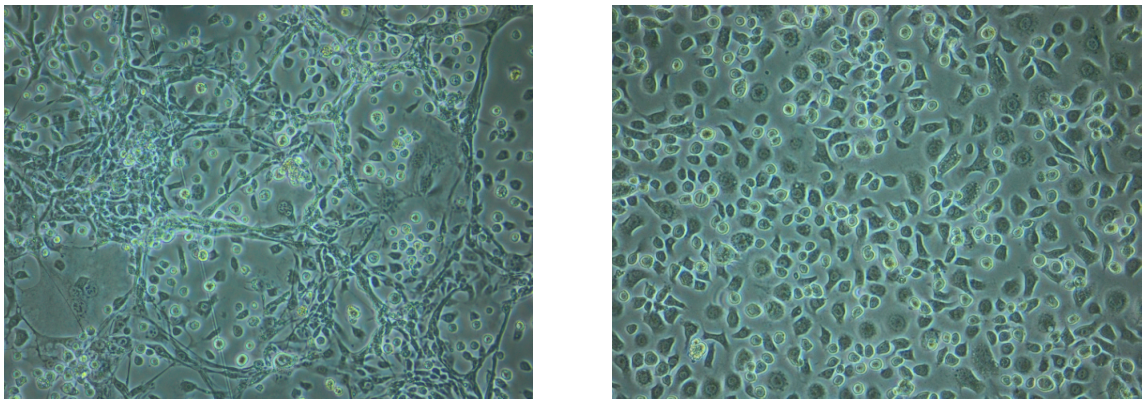

Figure 2-12. Two midgut cell lines from *S. frugiperda*. Left: SfMG1-0611, 200X; right: SfMG-0617-P33, 200X.

I have established the first two immortal midgut cell lines, SfMG1-0611 and SfMG-0617, which have been passaged numerous times. They consist of attached cells with a variety of morphologies that are consistently proliferating. When passaging these cell lines, I found that the cells are firmly attached to the flask bottom such that a conventional subculture method is infeasible. Instead of using trypsin, we used scrapers to detach the cells. Currently the SfMG1-0611 cell line is at passage 31, and the SfMG-0617 line is at passage 33.

2.3.2. Characterization of the Cell Lines

2.3.2.1. DAF-PCR

I characterized the two *S. frugiperda* midgut cell lines using DAF-PCR with three primers (Figure 2-13). PCR analyses yielded cell line DNA patterns (SfMG1-0611 and SfMG-0617) similar to their host tissues (Sf tissue), but distinctly different from other cell lines or tissues from either a different genus of the same family or other insect orders (Coleoptera, Dipetera, Hemiptera). This confirms the identity of the *S. frugiperda* cell lines.

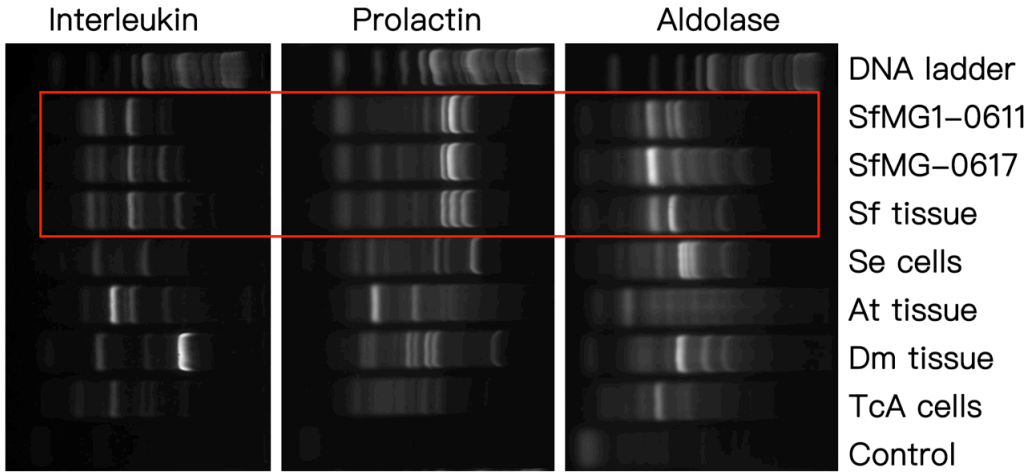

Figure 2-13. DNA authentication of two *S. frugiperda* midgut cell lines. Sf = *S. frugiperda*, Se = *S. exigua*, At = *A. tristis,* Dm = *D. melanogaster*, TcA = *T. castaneum*.

2.3.2.2. Cell Doubling Time

I generated growth curves for the two *S. frugiperda* midgut cell lines. The doubling time of the two cell lines was estimated using the linear portion of the curves. As noted in the figures, the growth curves have distinct linear portions with low standard deviations. The

doubling time of the SfMG1-0611 midgut cell line is 73.92 h (Figure 2-14) and its mean cell diameter is 9.28 ± 4.02 mm. The doubling time of the SfMG-0617 midgut cell line is 50.4 hr (Figure 2-15) and its mean cell diameter is 9.24 ± 3.87 mm.

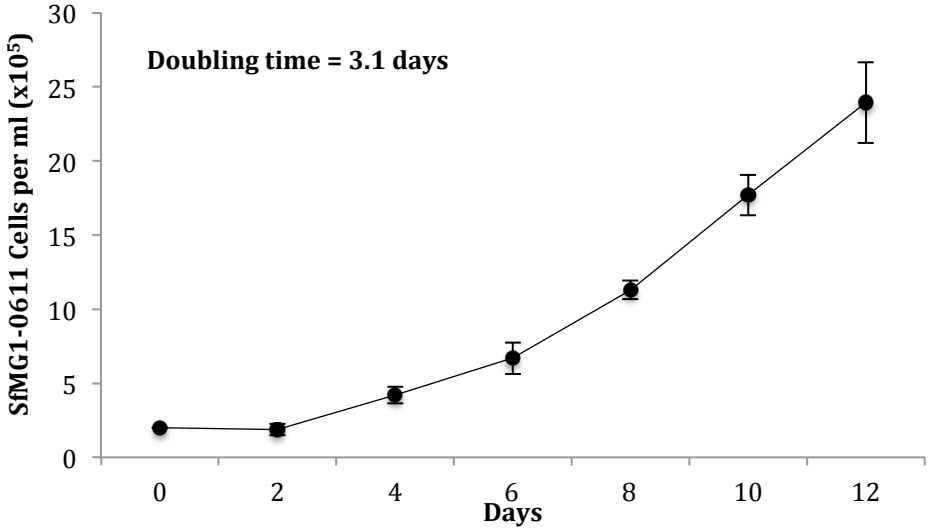

Figure 2-14. Growth curve of SfMG1-0611 cells.

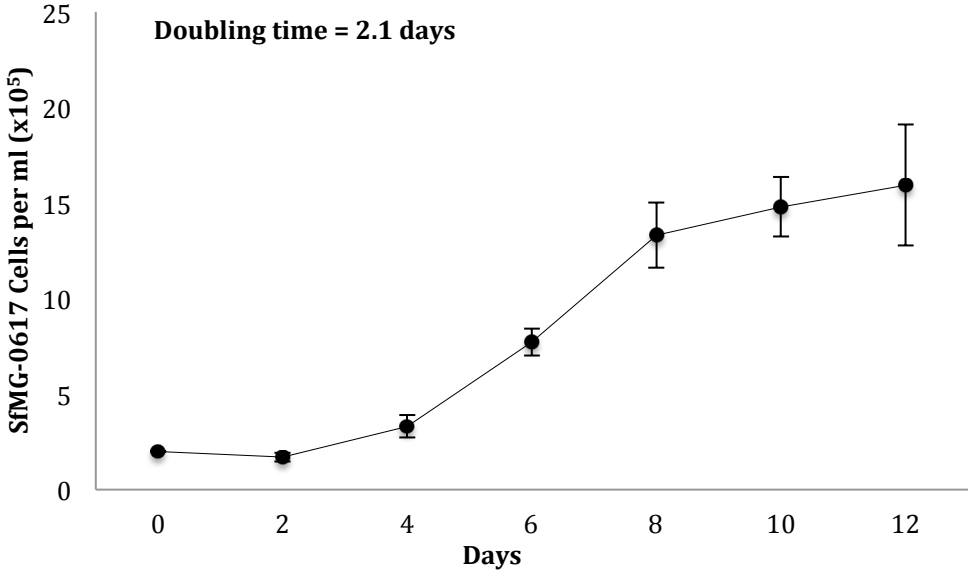

Figure 2-15. Growth curve of SfMG-0617 cells.

2.3.3. Cell Cytotoxicity Assay

I investigated three cell cytotoxicity assays for the two *S. frugiperda* midgut cell lines to determine the optimal assay conditions according to cell sensitivity and linear correlation.

2.3.3.1. CellTox Green Cytotoxicity Assay.

While the highest cell concentration used was 2 x 10^5 cell/ml, the CellTox Green Cytotoxicity assay (data not shown) showed a very weak signal after the recommended incubation time (3 h). I determined the linear range and cell sensitivity, finding that a 16 h incubation time was needed to produce a distinctive fluorescent signal shift. There was also no significant difference in fluorescence between 625 cells/well compared to media controls. Overall, the CellTox Green cytotoxicity assay was impractical for the two cell lines, especially not appropriate for a time-course study, because of the time required for the assay.

2.3.3.2. Cell Counting Kit-8

Compared to the CellTox Green Cytotoxicity assay, similar results were observed for the Cell Counting Kit-8 (CCK-8) assay. Three replicates were investigated for each treatment. For most cells, the CCK-8 assay usually needs 1-4 h incubation time with the reagent. But for the *S. frugiperda* midgut cell lines, 15-24 h was required for distinct absorbance readings (Figure 2-16), possibly due to cell membrane characteristics.

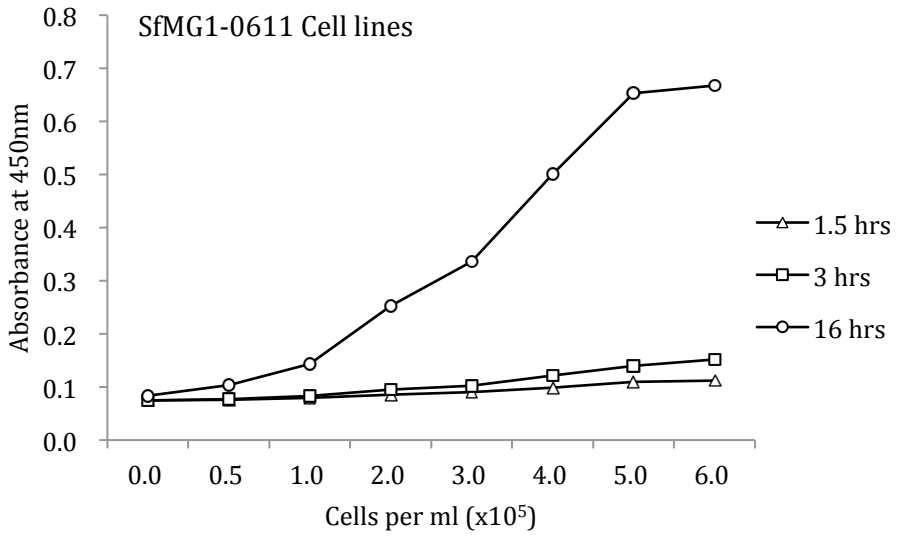

Figure 2-16. Cell sensitivity and linear range of SfMG1-0611 using the CCK-8 kit.

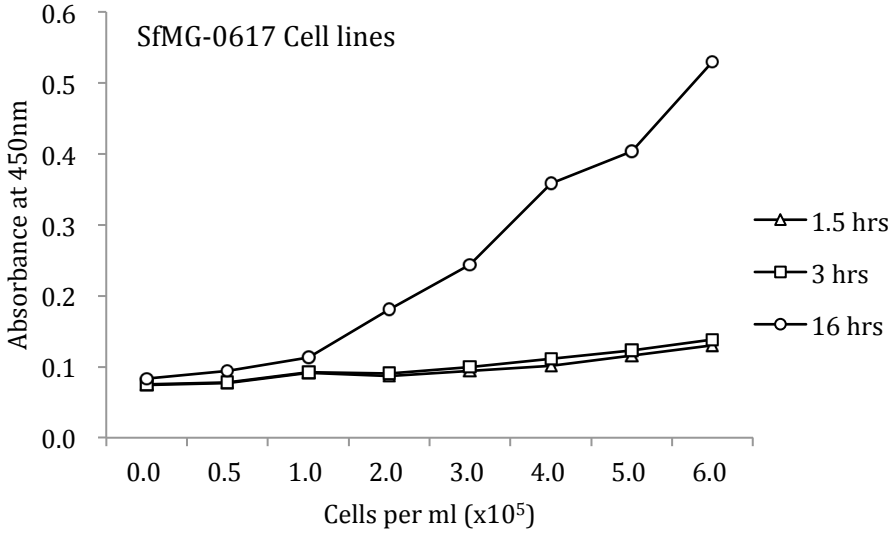

Figure 2-17. Cell sensitivity and linear range of SfMG-0617 using the CCK-8 kit.

2.3.3.3. CellTiter Blue Assay

This assay performed well for Sf9 cells according to J. Hasler. As is shown in the response graph (Figure 2-18), I tested three cell lines, including CF-1, SfMG1-0611, and SfMG-0617 cells. CF-1 cells are susceptible to some of the *Bt* Cry toxins (Baines et al., 1997), and I plan to use this line as a positive control in future *Bt* toxins studies.

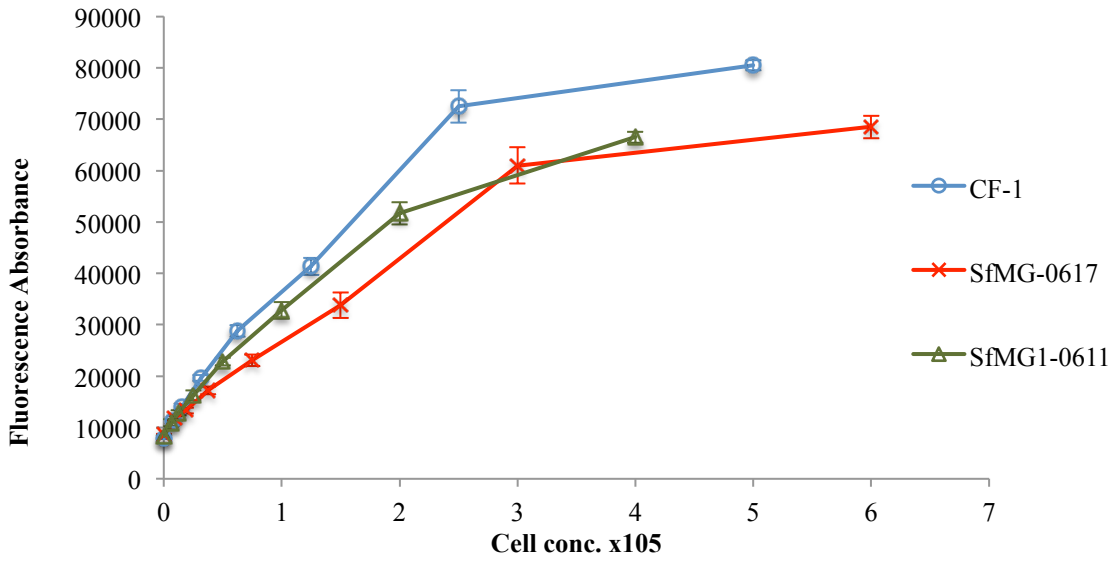

Figure 2-18. Cell sensitivity and linear range of *S. frugiperda* midgut cell lines and CF-1 cells using the CellTiter Blue assay.

These response curves were created after 3 h incubation and the linear range is determined for each cell line. For CF-1 cells, the linear range is 0-2.5 x10^5 cells/ml; for SfMG-0617 cells, the linear range is 0-3 x 10^5 cells/ml; for SfMG1-0611 cells, the linear range is 0-2 x 10^5 cells/ml.

2.4. Discussion

Overall, insect midgut cell lines generated from *D. virgifera, C. hilaris, N. viridula*, and *S. frugiperda* will become powerful tools for pesticide discovery programs, including both mode of action and screening assays. We obtained valuable experience in midgut cell cultures initiation, maintenance and medium optimization. This new knowledge is a

major contribution to the field of insect cell lines because it sets a baseline for establishing midgut cell lines from select orders of insects and possibly other invertebrates of commercial interest.

Despite the presence of symbiotic bacteria in the midgut, I was able to obtained viable, fungus-free egg cultures and midgut cell cultures from *D. virgifera* larvae reared on semi-sterile artificial diets. The artificial diet itself contains low concentrations of antibiotics, which moderately suppress symbiotic bacteria in the gut. Fungal contamination was noted for some cultures, which may possibly due to the errors during the sterilization process. Minced tissues were often attached to the flasks, which showed a high possibility to stretch and replicate. However, the primary cell cultures remain attached for two or three weeks, which indicates that the cell culture medium is not the optimal for cell metabolism. The most related study was done by Lynn and Stoppleworth (1984), who established two embryonic cell lines from southern corn rootworm, *Diabrotica undecimpunctata*, growing in cell culture media IPL-52B and IPL-76 in a 3:1 ratio with 9% fetal bovine serum.

Regarding the existence of midgut symbiotic bacteria in western corn rootworm and southern green stinkbug and green stinkbug, many studies have been conducted that show bacterial existence. The reliance on gut symbionts for successful development seems to vary depending on the species of stinkbug. Taylor et al. (2014) investigated the importance of gut symbionts to the brown marmorated stinkbug *Halyomorpha halys*. The developmental time and survivorship of *H. halys* was negatively impacted by the prevention of gut symbionts and the impact is significant from the first generation throughout the subsequent generations. However, Hirose et al. (2006) reported that the

symbiotic bacteria species *Klebsiella pneumonia* (Schroeter) and *Enterococcus faecalis* (Andrewes and Horder) exist in the crop/stomach (ventriculus 1-3) of the *Nezara viridula* gut, and possibly *Pantoea sp.* in the gastric caeca (ventriculus 4). Elimination of the symbiotic bacteria does not affect the insect development or cause mortality, but does reduce adult weight. They also suggested eliminating the bacteria by using the antibiotic kanamycin, which I also tested in this study. I found that kanamycin has a limited efficacy in inhibiting bacteria growth in stinkbug midgut culture. This may be due to the difference of in how the antibiotic is applied. Instead of adding kanamycin to the diets, I applied the antibiotics directly into the cell cultures, which minimized the slow-release effect of the kanamycin. To further confirm this, midgut cultures will need to be initiated from artificial diet-fed stinkbugs. Diet-reared nymphs may also be a good source for cell line initiations. Application of antibiotics effectively suppressed microbial growth in the midgut cultures from *D. virgifera,* but not for *C. hilaris* or *N. viridula*. I infer that the application of antibiotics is required in both rearing and cell culture stages to effectively suppress the presence of symbiotic bacteria.

We report the establishment of two midgut cell lines from *S. frugiperda*, SfMG1-0611 and SfMG-0617, which are the first two midgut cell lines from this species. Many of the established fall armyworm cell lines now available were derived from embryonic or ovarian tissues, such as Sf21 cells that come from ovarian tissues (Vaughn et al., 1977). Our cell line was initiated from tissues of the digestive system, which is the main entry for pathogens and pest control agents. Insect midgut epithelial tissues largely consist of stem cells, columnar cells, and goblet cells (Hakim et al., 2010). The stem cells are the adult midgut precursor cells that remain undifferentiated. Columnar cells (a.k.a.

enterocytes) and goblet cells are differentiated from stem cells and then enlarge the midgut while molting. Using Ficoll-Paque^{TM} to centrifuge and extract midgut epithelial cells is quite effective (Castagnola et al, 2011), which can be used for the purification of midgut cells from other insect species. Compared to established ovarian cell lines Sf9, the doubling times of our midgut cell lines are relatively higher, but the lines are still consistently proliferating and at a reasonable rate of replication. Combined with the optimized CellTiter Blue assay, the two midgut cell lines are ready to be used in in the evaluation of cytotoxins, such as *Bt* cry toxins.

CHAPTER 3. THE SIGNIFICANCE OF THE CELL LINES

Insects act as herbivores that destroy plants and vectors of animal and plant diseases. Agricultural pest insects cause tremendous economic loss and health-related challenges. The insect midgut is an important site of entry of pathogens and pest control agents. Hakim et al. (2009) reported that primary midgut cell cultures from Lepidoptera have been used to identify factors that control midgut growth and differentiation, the effects of toxins on midgut growth, as well as the regulation of cell physiology. In comparison to the primary cell cultures, corresponding midgut cell lines are more convenient, consistent tools for performing similar studies.

Our midgut cell lines established from fall armyworm are the first two midgut cell lines in this species. Compared to other midgut cell lines, SfMG1-0611 and SfMG-0617 are fast-replicating with doubling time of 2-3 days. These two midgut cell lines tend to attach firmly to the flask bottom, for which a traditional subculture method of using trypsin is not able to detach cells.

3.1. Novel Target Sites

One of the important criteria that determine the value of cell lines is whether or not they retain target sites for potential insecticides. Hormone receptors are retained in many cell lines. Lynn et al. (1991) described a cell line that responds to ecdysone. Linser et al. (2014) pointed out that the posterior midgut of larval mosquitoes possess a massive apical brush border, and therefore increases the cell surface exposure to ingested materials. To confirm the existence of possible target sites, Zhang et al. (2008) purified

toxin receptor proteins from the apical plasma membrane. The proteomic analyses uncovered numerous novel targets for insect control, such as nutrient amino acid transporters, cadherins, alkaline phosphatase, sodium-proton antiporters and V-ATPase. Although midgut cell lines are not sufficient tools to determine insect gut structure-function relationships, they offer investigators effective tools to identify novel and vital insecticide target sites for pest control, as well as exploiting transporters for delivering toxins.

3.2. Enhance Screening Assays

For screening purposes, industries have demonstrated an increasing interest in the development of *in vitro* methods over the past decades. Along with the development of modern screening technologies, conventional animal toxicity tests are less efficient and more costly. Correspondingly, the available large inventory of natural and synthetic chemicals that is potentially useful in pest management requires more efficient, less expensive and faster screening methods.

Combined with cell lines from specific insect tissues, high-throughput screening (HTS) systems are becoming increasingly important in chemical discovery programs in the pharmaceutical and agricultural chemical industries. A high-throughput, *in vitro* assay for *Bacillus thuringiensis* insecticidal proteins was developed and evaluated for screening Cry protein variants produced by DNA shuffling (Willcoxon et al, 2016). There are a certain number of key requirements (Smagghe et al, 2007) that need thorough consideration before developing a cell-based testing procedure, such as dependable intra-

and inter-laboratory reproducibility, high predictivity, relevance to the type of compounds, procedure simplicity, low cost/benefit ratio and possibility for HTS with automation. Because midgut cell lines are a main entry for disease pathogens and pest control reagents, HTS will greatly enhance screening efficiency for potential compunds.

3.3. Mode of Action Studies

The feasibility of using insect cell lines to study the mode of action has been discussed and carried out for the past decades. Ward et al. (1988) found that chitin-like material was stimulated by 20-OH-ecdysone and inhibited by diflubenzuron in the cockroach (*Blatella germanica*) cell line UMBGE-4. More recently, Abo-Elghar et al. (2004) used cockroach integument cell cultures and cellular vesicles of *D. melanogaster* to study the mode of action of diflubenzuron. The newest results showed that the site of action of diflubenzuron is an ATP binding cassette transporter. Using insect midgut cell lines in mode of action studies possess advantages that are rarely found in *in vivo* assays. Studies on mode of action of pathogens or biopesticides like *Bt* have been carried out in midgut insect stem cells from Lepidoptera *in vitro* (Sadrud-Din et al., 1996). Using insect cell lines derived from certain species allows for the determination of the mode of action of insecticides at a cellular level. Cheng et al. (2011) used a Sf9 insect cell line to study the effect of Rhodojaponin-III (R-III), a natural botanic insecticide. R-III was found to induce a significant dose-dependent increase in the intracellular calcium and intracellular pH. Combined a the HTS system, engineered insect cell lines can be easily detected upon biological stimulus.

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