DEFINING THE CONDITIONS FOR *CAENORHABDITIS ELEGANS* GERM CELL

PRIMARY CULTURES

by

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(Under the Direction of Edward Kipreos)

ABSTRACT

This thesis discusses the creation of a cell culture system for germ cells from the nematode *Caenorhabditis elegans*. While a culture system exists for *C. elegans* embryonic cells, they have poor long term survival, and cannot be used for experiments after five days. As well, the media used for this embryonic culture leads to very rapid death of germ cells. Therefore, a better culture system is needed. Here, a method for harvesting germ cells is provided as well as a media, CeM1, that has been optimized so that these cultured germ cells can remain alive and at fairly constant numbers for at least 28 days. In addition, germ cell proliferation is shown for germ cells between days 1-4 in certain conditions, which is predicted to be a result of signals from bacteria.

INDEX WORDS: *C. elegans*, Germline, Cell culture, Growth media, CKI-2, GLP-1,

DAF-16

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DEDICATION

I would like to dedicate this thesis to my family and friends, who have supported me in my

endeavors to obtain my joint Bachelors/ Masters Degree.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTEER 1

INTRODUCTION

Purpose of the Study

There is currently no *Caenorhabditis elegans* germ cell line and no primary cell culture system

that can maintain germ cells in culture; the purpose of this study is to optimize the procedure for

culturing germ cells so that they can survive for an extended time. As well, effort will be put

into trying to provide conditions to allow these germ cells to proliferate. In order to create this

germ cell culture, mutated worms had to be used, which had mutations in the *cki-2* (lof), *glp-1*

(gof), and *daf-16* (lof) genes.

***Caenorhabditis elegans* use as a model organism**

Caenorhabditis elegans is a free-living (nonparasitic), pseudocoelomate roundworm from the

phylum Nematoda. There are 2 genders: hermaphrodites (XX) and males (XO). The males are

rare, and thus the hermaphrodites are capable of either self-fertilization or mating with males. In

the wild, *C. elegans* are soil living, residing in organic material that contains nutrients and

microbes (Kiontk and Sudhaus 2006). In the research lab, the nematodes are grown on agar

plates and are fed bacteria, usually *E. coli.*

C. elegans are a useful research tool

C. elegans are a very useful model organism and research tool. They are well studied so much is already known about them. The roundworm is a fairly simple organism, so it is easy to study and work with, and generally has less complex regulation than higher organisms. However, it is a multicellular animal so it has many conserved processes, which can be translated to higher animals, and research done with this animal has relevance for human disease. In fact, 43% of *C. elegans* genes have human homologs so there is a significant amount of overlap of conserved gene function, especially for essential genes, which can be important in understanding human diseases (Barr 2003). As well, *C. elegans* has a fast life cycle and grows rapidly, inexpensively, and they are easy to maintain.

      C. elegans development is well known. The complete invariant cell lineage is known, so the formation and differentiation of every cell in the body is understood (Schierenberg, Miwa et al. 1980; Kimble 1981; Sulston and Horvitz 1981; Sulston, Schierenberg et al 1983). The complete genome of *C. elegans* is also known, as it was the first multicellular organism to have its genome sequenced. Gene function is also well understood for many of the *C. elegans* genes due to being able to efficiently knockdown genes. There is a whole-genome RNA interference library and well-developed methods for gene knockouts (Fire, Xu et al. 1998). *C. elegans* has been useful for defining gene pathways, identifying new proteins and their functions, and modeling the molecular aspects of human diseases (Barr 2003). Also, *C. elegans* can be used for neuronal research. The morphology and connectivity of all *C. elegans* neurons is known (White, Southgate et al. 1986) and patch clamp techniques are well developed and optimized. Also,

primary cultures of *C. elegans* embryonic cells can be differentiated into neuronal cells (Christensen, Estevez et al. 2002).

There are many tools available to use for research with *C. elegans*. Mutant worms are readily available from the Caenorhabditis Genetics Center. Cosmid and yeast artificial chromosome clones spanning the genome are freely available (Christensen, Estevez et al. 2002). Fluorescent protein tags are available for use in order to easily visualize protein location in the worm (Chalfie, Tu et al. 1994; Yang, Moss et al. , 1996; Zhang, Ma et al. 2004). In addition, the worms' transparent body makes it easy to visualize *C. elegans* under a microscope while they are still alive.

The culture of C. elegans cells

Despite the many benefits of *C. elegans* as a useful research tool, there is one glaringly missing piece: there are no *C. elegans* cell lines. A cell line from *C. elegans* has remained very elusive. A cell line would be hugely beneficial, as it would substantially broaden the types of experiments capable to be carried out with *C. elegans*. A cell line would allow for analyses of specific cell types (Christensen, Estevez et al. 2002), and would be well suited for mutagenesis and forward genetic analysis (Strange, Christensen et al. 2007). The small size of the worm and its cells and the presence of a cuticle makes it difficult to access specific types of cells in vivo. Having a cell line would make these cells more accessible and easier to work with and manipulate (Strange, Christensen et al. 2007). As well, cell cycle progression could be observed in a population of cultured cells. Thus, the development of a *C. elegans* cell line would be hugely beneficial.

To date, the closest approximation of a *C. elegans* cell line has been the culture of embryonic cells from *C. elegans*. Bloom was one of the earliest researchers to describe his *C. elegans* culture although it had many problems (Bloom 1993). There was poor cell survival, it was difficult to attach the cells to the growth substrate, there were problems with cell differentiation, and difficulties with reproducibility of results (Strange, Christensen et al. 2007). Lois Edgar also described techniques to culture *C. elegans* cells, devising techniques to culture individual blastomere cells and watch their development, leading to the creation of an embryonic culture system (Edgar 1995). Edgar describes the removal of eggshells and vitelline membranes, and then the isolation of embryonic blastomeres. This procedure can be done for up to about 200 cells at a time, taking 15-40 minutes. When his procedure is done properly, it yields embryonic cells that continue to divide on schedule (Edgar 1995). These techniques allowed for the study of morphogenesis, but because it only provided the normal embryonic division and differentiation pattern, and not continuous cell division, its utility was rather limited.

Following Edgar, Strange described a technique for the large-scale culture of embryonic cells, perfecting methods to culture worms, isolate eggs, remove eggshells, dissociate embryos into individual cells, and maintain the embryos in culture for a limited period of time (Strange, Christensen et al. 2007). The cultured cells differentiate into the various cell types found in the newly hatched L1 larva (Christensen, Estevez et al. 2002). For instance, cultured embryonic cells will differentiate into nerve and muscle cells, which behave similarly to those in vivo, and which can then be used to perform many different types of experiments (Christensen, Estevez et al. 2002). The benefit of Strange's embryonic culture is that it can be used to study physiological and biological processes such as signal transduction, ion channel physiology, and

4

expression of genes (Strange, Christensen et al. 2007). However, it has limitations. Cells lose

some of their intercellular interactions and certain excreted factors and signals for cell-to-cell

communication, which may be important or necessary. However, this downfall is not unique to

Strange's culture, but rather is a characteristic of cell cultures. One limitation more unique to

Strange's culture is that the cultured embryonic cells can only be used for up to five days for

physiological experiments. Any older and they are no longer suitable for experiments. Strange's

cells have "excellent survival" for 2-3 weeks (about 70% survival after 22 days), but

dedifferentiate after that and do not proliferate beyond a few divisions immediately after

isolation (Strange, Christensen et al. 2007). So, while there is documentation of cultured *C.*

elegans cells, these cells are not primary cells that can proliferate in culture, or a defined cell line

that is capable of unlimited cell division.   There is much room for improvement in the

development of cell culture methods. One important consideration for culturing *C. elegans* cells

is considering that they are auxotrophs- there are certain nutrients that they require but cannot

produce themselves. Therefore, an effective media must provide these essential nutrients to

them.

C. elegans are auxotrophic organisms
C. elegans are auxotrophs, meaning that there are nutrients and metabolites that worms require

but cannot produce themselves. Therefore, they must obtain these compounds from their

environment and the bacteria they eat. *C. elegans* feed on bacteria, typically *E. coli,* in the lab,

and the particular diet of the worm can greatly impact the expression of genes, as well as the

physiology of the worms, as different bacteria contribute varying metabolites (Gracida and

Eckmann 2013).

One of the important metabolites that *C. elegans* require is heme. Hemin chloride is a

porphyrin complex that comes from erythrocytes that is used to provide heme to culture systems.

Heme is a ferrous ion surrounded by a porphyrin ring and is a component of hemoglobin.

Nematodes cannot synthesize heme, and they also lack the orthologs of genes for enzymes used

for heme biosynthesis, but they require hemoproteins for many important biological pathways

(Rao, Carta et al. 2005). Therefore, *C. elegans* must obtain heme from outside sources. Heme is

an important cofactor for many biological pathways and ingested heme is used as an iron source

and to make hemoproteins (Rao, Carta et al. 2005). Heme is also necessary for normal C.

elegans development (Hieb, Stokstad et al. 1970). Therefore, *C. elegans* are an auxotroph for

heme.

Cholesterol is another metabolite required by *C. elegans.* Cholesterol is a sterol required

by cells for proper cell membrane formation and as a precursor to other sterol compounds. Hieb

and Rothstein showed that nematodes have a nutritional requirement for cholesterol (Hieb and

Rothstein 1968). They compared the reproduction rate of *C. briggsae* grown with and without

sterols in *E. coli* containing medium. They found that those grown with the sterols in the

medium had rapid reproduction, while those grown in medium lacking sterols did not have any

reproduction. Therefore, *C. elegans* are an auxotroph for cholesterol, as well.

Vitamin B12 is another metabolite utilized by *C. elegans* that is provided by their diet. It

is an essential nutrient for many organisms but only synthesized by some bacteria species

(Bender 2003). Vitamin B12 regulates genes, speeds up development, and lowers fertility

(Watson, MacNeil et al. 2014). Vitamin B12 affects development and fertility by serving as a cofactor for two enzymes. First, vitamin B12 serves as a cofactor to methionine synthase, which works in the SAM cycle (methionine/S- Adenosylmethionine). As well, vitamin B12 serves as a cofactor to methylmalonyl-CoA mutase, which aides in the break down of propionic acid (a short chain fatty acid that can have toxic effects) (Watson, MacNeil et al. 2014).

By examining *C. elegans'* need for vitamin B12, Watson and MacNeil demonstrated both the requirement that worms have for certain metabolites and the variability in which these metabolites are provided by different strains of bacteria. For instance, worms that were feed a diet of *Comamonas aquatic* DA1877 had faster development, shorter lifespan, and lowered fertility, due to changes in *C. elegans* gene expression, than those fed *E. coli* OP50. It is noteworthy that this effect was seen even when DA1877 was mixed in small amounts with the *E. coli* OP50 (Watson, MacNeil et al. 2014). They found that vitamin B12 is produced by DA1877 and contributed to many of these observed gene changes. While vitamin B12 contributes to the different developmental rates between worms fed the two bacteria, it does not affect the observed change in lifespan. Vitamin B12 works in opposition to propionic acid: when there are high levels of vitamin B12, there are low levels of propionic acid and development speeds up. On the flip side, when vitamin B12 levels are low, propionic acids levels are high and development is slowed down. As well, vitamin B12 lowers fertility resulting in smaller brood sizes and altered egg-laying patterns. It is also interesting to note that vitamin B12 does not have to be modified by bacteria, as when it is added to either live or dead bacteria that lack vitamin B12, it has the same effect on *C. elegans*.

The *C. elegans* intestine is filled with microbiota and the bacteria that *C. elegans* eat provide the worm with metabolites that can greatly affect biological processes in the worm. Based on the bacteria species *C. elegans* consume, the bacteria can provide different metabolites and therefore elicit different metabolic and biological responses in the worm. Cellular sensory signals transmit information to the worms about the bacteria in the *C. elegans* gut, which leads to transcription of particular genes based on the signals (Gracida and Eckmann 2013). Because of the different metabolites that various bacteria species provide, worms with the same genotype can have different phenotypic appearances. Certain gene knockouts can have normal function restored when grown on new types of bacteria. For instance, mutation of *nhr* (nuclear hormone receptor)-*114*(-), which leads to germline mutations and sterility in worms grown on *E. coli* OP50, can have germline function restored when grown on *E. coli* HT115 bacteria (Gracida and Eckmann 2013). Interestingly, when *nhr-114* mutant worms were fed live *E. coli* OP50 supplemented with tryptophan, these worms were no longer sterile (Gracida and Eckmann 2013). However, when dead *E. coli* OP50 were supplemented with tryptophan, there was no longer a rescue in the germline function of the *nhr-114* mutants, indicating that the active agent is not tryptophan but a processed form of the metabolite that must be obtained from live bacteria (Gracida and Eckmann 2013). This shows that the HT115 bacteria can provide *C. elegans* with the particular tryptophan metabolite(s), or a substance that can bypass the need for this nutrient, and emphasizes the differences in metabolites provided by different bacterial species.

8

The Germline of *Caenorhabditis elegans*

The C. elegans gonad structure

There are two genders of *C. elegans* worms. Hermaphrodites, who are XX, produce oocytes and spermatocytes and can either self fertilize or mate with males. Males, who are XO, produce only spermatocytes and must mate with a hermaphrodite to propagate. Hermaphrodites have two gonadal arms while males have only one (Kimble and Crittenden 2005). The distal end of each gonad arm contains the stem cell niche, which is responsible for maintaining the germline stem cells. The germline cells are considered stem cells because they produce both more of themselves, the germline cells (self-renewal), as well as gametes (differentiation). The mitotic region is composed of about 225 to 250 germ cells, and is about 20 germ cell-diameters long along the distal-proximal axis (Kimble and Crittenden 2005). More proximally to the mitotic region is the transition zone, which is where the nuclei of these cells prepare for meiotic prophase and cells begin undergoing meiosis. The region more proximal to the transition zone contains cells undergoing gametogenesis (Kimble and Crittenden 2005; Crittenden, Bernstein et al. 2002).

The primordial germ cell is cellular through the L1 stage. However, the germline becomes syncytial by the L2 stage. This means that all of the cells in the germline nucleus and cytoplasm are partially enclosed by a single nuclear membrane (Kimble and Crittenden 2005). This phenomenon has implications when attempting to harvest germ cells from the *C. elegans* worm.

Development of the germline

The germline originates from one individual cell in the embryo, P_4, which is the first primordial

germ cell (PGC). Early during embryonic development, the lineage producing the PGCs

becomes distinct from the lineages producing somatic cells (Hubbard and Greenstein 2005).

During gastrulation, P_4 moves into the interior of the embryo and divides once to form the PGCs

Z_2 and Z_3. (Sulston, Schierenberg et al. 1983). The early PGCs Z_2 and Z_3 become incorporated

into the gonadal primordium during embryogenesis that also contains the somatic gonad

precursor cells Z_1 and Z_4, which flank the PGCs (Hubbard and Greenstein 2005). Z_2 and Z_3 do

not divide again until the worm reaches the L1 larval stage, at which time, under favorable

conditions, these cells will divide exponentially through the L3 larval stage. These cells, made

by the Z_2 and Z_3 divisions, form a somatic gonadal niche, where germline cells continue

undergoing mitosis (Kimble and Crittenden 2005). Once the worm enters the L3 stage, the cells

located in the most proximal region of the gonad arm begin undergoing meiosis. From this point

forward, germline mitosis is restricted to the most distal region of the gonad arm, while meiosis

occurs in the more proximal region (Kimble and Crittenden 2005).

Maintenance of the germline

The somatic Distal Tip Cell (DTC) is located at the distal end of the gonad arms and maintains

the stem cell niche (Kimble and Crittenden 2005). The distal tip cell has been shown to be both

necessary and sufficient for maintenance of germline cells in mitosis (Sulston, Schierenberg et

al. 1983; Byrd and Kimble 2009). Kimble and White showed that the DTC was necessary by

removing the DTC and observing that all germline cells entered meiosis (Kimble and White

1981). Kimble and White, and Feng and Zhong showed that it was sufficient: when the distal tip cell was moved to a new location or duplicated, germline cells developed wherever the DTC was (Kimble and White 1981; Feng, Zhong et al. 1999).

The Distal Tip Cell regulates the germline by providing signals via the Notch Signaling Pathway that keeps these cells as germline cells that undergo mitosis. When cells leave the somatic gonadal niche, they begin differentiation. This means they stop mitotically dividing and begin meiosis and gametogenesis (Kimble and Crittenden 2005).   RNA binding proteins play an important role in deciding whether cells divide or differentiate (Kalchhauser, Farley et al. 2011).

The GLP-1/Notch signaling pathway

The notch-signaling pathway determines cell fate through certain RNA regulators; these regulators dictate to the cell to either remain in mitosis or enter meiosis (Byrd and Kimble 2009). It is the notch-signaling pathway that keeps germline cells undergoing mitosis. There are many components of this pathway: LAG-2 is the signaling ligand, GLP-1 is the ligand receptor, LAG-1 and LAG-3/SEL-8 are transcription factors, and FBF-1 and FBF-2 are RNA binding proteins, which suppress genes that promote entry of cells into meiosis. FBF-1 and FBF-2 are nearly identical, and thus are collectively called FBF. FBF is proposed to work, at least in part, by suppressing Gld-1 (Crittenden, Bernstein et al. 2002). LAG-2, GLP-1, LAG-1, and LAG-3/SEl-8 are all required for cells to stay in mitosis, as depletion of any of these components cause the germline cells to enter meiosis. In the opposite extreme, unregulated LAG-2 or GLP-1 levels lead to increased germline proliferation and a germline tumor (Kimble and Crittenden 2005). FBF-1/FBF-2 double mutants cause all cells to enter meiosis after the L4 stage and there is no

switch of cells from spermatogenesis to oogenesis (Kimble and Crittenden 2005). Interestingly, in the FBF double mutant, early germline development appears normal and thus, it could be seen that FBF is required for maintenance of the germline stem cells but is not required for its early development (Crittenden, Bernstein et al. 2002). Depletion of FBF-1 causes smaller mitotic regions. Depletion of FBF-2 causes larger mitotic regions (Kimble and Crittenden 2005).

LAG-2 is expressed in the DTC and its activity is tethered by the LAG-2 transmembrane domain (Kimble and Crittenden 2005). GLP-1 is expressed in germ cells and receives signals from the ligand LAG-2 that promotes mitosis. The GLP-1 protein is located only in the mitotic region, while GLP-1 mRNA protein is located throughout the germline (Kimble and Crittenden 2005). This variability in distribution of GLP-1 and the restriction in expression of LAG-2 to the region around the distal tip cell allows for maintenance of mitosis in cells in only the most distal regions.

FBF plays a role in maintaining mitosis in the distal region. FBF binds the 3' UTR of target mRNA, suppressing their expression (Kimble and Crittenden 2005). Two of its most important targets are GLD-1 and GLD-3, which both promote meiosis (Kimble and Crittenden 2005). Therefore, FBF inhibits transcription of meiosis inducing genes. FBF also targets and regulate themselves. FBF-1 inhibits FBF-2, and FBF-2 inhibits FBF-1, which is done to keep their levels relatively low so the worm isn't entirely undergoing meiosis (Kimble and Crittenden 2005).

Germ cell transition from mitosis to meiosis

There are four main RNA regulators of entry of germline cells from mitosis into meiosis: GLD-1, which is a repressor of GLP-1 and which works by binding the GLP-1 3'UTR to repress it (Kimble and Crittenden 2005); NOS-3, which does not have its methods for promoting meiosis completely understood but is believed to somehow aid GLD-1 (Kimble and Crittenden 2005); and GLD-2 and GLD-3, which work together, and which are proposed to activate mRNAs to promote meiosis, as they are found to bind together in vitro, and co-immunoprecipitate (Wang, Eckmann et al. 2002). Thus GLD-1 and NOS-3 work together in one pathway and GLD-2 and GLD-3 work together in another. Removal of one of the components in both pathways causes significantly reduced entry into meiosis with germ cells remaining mitotic throughout the gonad. In contrast, removal of both of the components of only one of the pathways still shows normal entry to meiosis, thereby showing that the two pathways work redundantly to promote meiotic entry.

CKI (cyclin dependent kinase inhibitor)-2 and Cell Cycle Inhibition

Cyclin dependent kinase inhibitors (CKIs) play a role in deciding whether cells remain in or exit the cell cycle. There are two CKIs in *C. elegans*: CKI-1 and CKI-2. The inactivation of either CKI promotes extra rounds of ectopic cell divisions during larval stages (Buck, Chiu et al. 2009). CKI-1 and CKI-2 appear to act in independent but redundant pathways, as double mutants have more rounds of cell division and a more extreme phenotype than knockdowns of either CKI alone (Buck, Chiu et al. 2009).

The decision of whether to divide or not is made in the G1 phase of animal cells, and once the cell passes the restriction point, it commits to the completion of the cell cycle. Thus, there must be ample regulation to ensure the cell only enters the cell cycle at appropriate times. The two CKIs work with pRB (retinoblastoma tumor suppressor gene) to inhibit the cell cycle. pRB inhibits genes that are needed for S phase (for example, cyclin E), while CKIs inhibit the kinase activity of the CDK-2-Cyclin E complex (Buck, Chiu et al. 2009). Buck and Chiu found that CKI-1 affects many tissues while CKI-2 predominantly affects vulval precursor cells (VPCs) (Buck, Chiu et al. 2009), and that CKI-1, CKI-2, and pRb all function redundantly in VPC to suppress cell cycle (Ambros 2009).

Kalchhauser and Farley found that repression of CKI-2 inhibition is important to maintain germline stem cells (Kalchhauser, Farley et al. 2011). CKI-2 is inhibited by FBF in order to keep germline stem cells dividing and remaining germline stem cells. In the absence of FBF, CKI-2 levels increase and germ cell division is blocked (Kalchhauser, Farley et al. 2011). Kalchhauser demonstrated that the FBF inhibition of CKI-2 is important for the germ cell arrest by showing that the loss of all mitotic germ cells in FBF-1/FBF-2 double mutants is rescued by also inactivating CKI-2. (Kalchhauser, Farley et al. 2011). CKI-2 is the predominant CKI in the adult germline. It is absent in germline stem cells undergoing mitosis in the germline, but becomes present when the cells begin to undergo meiosis (Kalchhauser, Farley et al. 2011).

The DAF-16/FOXO Transcription Factor and Longevity Determination

There are three main pathways through which the DAF-16/ FOXO transcription factor regulates

C. elegans lifespan longevity. They are the Insulin/ Insulin-like Growth Factor Pathway, the

Target of Rapamycin Pathway, and the Germline Signaling Pathway.

The decrease of worm longevity through the insulin/ insulin-like growth factor pathway

The insulin/insulin-like growth factor (IGF) pathway decreases *C. elegans* worm longevity

largely through the inhibition of the FOXO transcription factor DAF-16 (Lapierre and Hansen

2012). DAF-16 works in opposition to the insulin/IGF-1 pathway to promote *C. elegans* worm

longevity and stress resistance. AKT and SGK-1, which work downstream of insulin signaling,

inactivate DAf-16 by phosphorylating it, thereby preventing DAF-16 from moving into the

nucleus and regulating transcription (Lapierre and Hansen 2012). Many other mechanisms also

control DAF-16 nuclear localization in response to cues from hormones and the environment.

Once in the nucleus, DAf-16 interacts with other proteins to mediate transcription in response to

reduced insulin/IGF-1 signaling. For instance, SMK-1/SMEK and HCF-1, which are

transcription factors that are coregulators with DAF-16, allow for DAF-16/FOXO protection

against microbes, DNA damage, and oxidative stress. HSF-1 works with DAF-16 to transcribe

heat shock protection genes. DAF-16 also transcribes genes involved in metabolism,

detoxification, proteostasis, and immunity.

Increased glucose in *C. elegans* worms reduces lifespan because increased glucose levels

also increases insulin signaling, which inhibits the DAF-16/FOXO transcription of worm

longevity genes (Lapierre and Hansen 2012). On the flip side, decreased glucose extends lifespan.

Worm longevity is decreased by the target of rapamycin pathway

The target of rapamycin (TOR) pathway regulates growth and reproduction based on the availability of amino acids and growth factors. Inhibition of TOR extends lifespan, while increased expression of TOR lowers lifespan (Lapierre and Hansen 2012). There are two types of TOR: TORC1 and TORC2. TORC1 controls cell proliferation and size based on mitogen and nutrients. Its coactivator is DAF15/Raptor, and it affects longevity with a number of GTPases: RAGA-1/RAGC-1, RHEB-1/RHEB, and DAF-15/Raptor. TORC1 inhibition increases cell longevity, due to DAF-16/FOXO and SKN-1/NRF, which are necessary to increase lifespan in the TORC1 knockdown. TORC2 regulates the shape of the cells, at least in mammalian cells, and its coactivator is RICT1/Rictor. TOR and Insulin/ IGF-1 have converging effects when it comes to some of their transcription factors, target genes, and effector mechanisms (Lapierre and Hansen 2012).

DAF-16 controls worm longevity through the germline signaling pathway

The germline controls aging by integrating nutrient signals, which it then communicates to other parts of the worm. Removal of the germline extends lifespan significantly. Removal of the somatic gonad in addition to the germline cancels out this lifespan extension. It was found that DAF-16/FOXO is required for the lifespan extension in animals lacking germlines, mainly by affecting genes in the intestinal cells. There are factors used in the insulin/IGF-1 pathway that

also control aging along the germline pathway, such as DAF-18/PTEN, SMK-1/SMEK, and HSF-1, as well as factors that only work along the germline signaling pathway to extend the lifespan of germline free worms (Lapierre and Hansen 2012). There is also a lipophilic hormone/steroid signaling pathway, involving DAF-9, DAF-36, and DAf-12, that is needed for proper function of DAF-16/FOXO localization to the nucleus in the intestine and the lifespan extension seen in animals lacking germlines (Lapierre and Hansen 2012). Worms that have a *glp-1* (lof) mutation, and do not have mitotic germ cell divisions, have extended lifespans. Thus, there seems to be an inverse relationship between *C. elegans* longevity and germline cell function and proliferation.

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CHAPTER 2

CEM1 MEDIA IS DEVELOPED TO ALLOW FOR PROLONGED SURVIVAL OF

CULTURED *CAENORHABDITIS ELEGANS* GERM CELLS

Purpose

The aim of the research described in this chapter was to develop a media, CeM1, that is capable

of maintaining *Caenorhabditis elegans* germ cells in tissue culture conditions. This media that

was developed is distinct from the media developed by Strange for culturing embryonic cells in

multiple aspects that are required for germ cell viability. The use of CeM1 can keep *C. elegans*

germ cells alive in culture for at least one month.

Materials and Methods

CeM1 media preparation

CeM1 Media is prepared with the ingredients listed in Table 2.1: Schneider's Insect Medium

(Life Technologies, 21720-024), Leibovitz's L-15 Medium without phenol red (Life

Technologies, 21083-027), fetal bovine serum (Atlanta Biologicals), penicillin/ streptomycin

(Sigma-Aldrich, P4333), hemin chloride (MP Biomedicals, 0219402501), RPMI vitamins

(Sigma-Aldrich, R7256), L-glutathione, reduced (Sigma-Aldrich, G4251), normocin (Sigma-

Aldrich, P4333), cholesterol (J.T. Baker, 1580-01), and trehalose (Sigma-Aldrich, T0167).

Hemin chloride is prepared as a stock solution immediately before use. To make the stock

solution, 0.013 g of the hemin chloride is added to 9.8 ml water. Then 0.2 ml of 5N NaOH is added so that the hemin chloride will dissolve. Cholesterol is added from a 10 mg/ml stock solution in ethanol. Once prepared, the media is adjusted to a pH of 6.5, using NaOH to raise the pH and HCl to lower the pH. The media is then checked for osmolarity and adjusted to 390 mOsm. A freezing point osmometer is used for the measurements (Advanced Digimatic Osmometer 3DII from Advanced Instruments Inc.). Additional trehalose is added to the media to raise the osmolarity and media lacking trehalose is added to lower the osmolarity.

FBS treatment

CeM1 media contains fetal bovine serum from the lot G11012. Several treatments are performed on the FBS to prepare it for use in the media. First, the FBS is heat inactivated. This is done by heating the FBS at a specified temperature (usually at 65°C, although FBS heat inactivation was also tested at 56°C and 74°C) in a water bath for 30 minutes. Following the heat inactivation, the FBS is treated with Amberlite IRA 400-CL (Sigma Aldrich, 247669), which is a strongly-basic gel-type resin. To do this, the amberlite beads are first prewashed with water. Then the media is incubated with the beads (at a concentration of 50 mg/ml) on a rotator for 4-6 hours and kept at 4°C. After the incubation period, the media is transferred to a new tube and incubated with new Amberlite IRA 400-CLbeads at the same concentration overnight at 4°C. Following the amberlite treatment, the FBS is treated with charcoal dextran (Sigma Aldrich, C6241). The charcoal dextran is added to the media at a concentration of 100 mg/ml and the media is rotated with in the charcoal overnight at 4°C. The next day, the charcoal dextran is separated from the

FBS by two sequential centrifugations, each for 30 min at 3500 rpm. Following these treatments, the FBS is ready to be added to the media.

Worm preparation

Triple mutant worms were created by combining loss of function alleles for the *cki-2* gene and *daf-16* gene, and a gain of function allele of the *glp-1* gene. This strain is ET507: *daf-16(*mu86) I; *cki-2*(ok2105) II; *glp-1*(ar202) III. A *glp-1* gain-of-function mutation causes the worm to form tumors in their germline, meaning these worms are unable to produce offspring as all germline cells self renew (none of the germline cells differentiate into oocytes or spermatocytes). Therefore, to maintain this strain, the *glp-1* mutation must be temperature sensitive, so that at lower temperatures (16°C), the worms are normal and can produce offspring, but at high temperatures (25°C), the worms have germline tumors.

The process of harvesting worms begins by sodium hypochlorite treatment of the cki-2; glp-1; daf-16 worms grown at 16°C, so that only eggs from these worms remain. These eggs are then transferred to a 3xNGM plate (Wood 1988) covered with a lawn of bacteria and grown at 25°C for four days to ensure that all worms have become young adults with germline tumors. When germline cells are harvested from these worms, the worms must be completely free of live bacteria, including the *E. coli* that they grew up on. Therefore, the worms must be transferred to heat-killed bacteria plates. To make heat-killed bacteria plates, 3xNGM plates are placed in a 67°C incubator for 24 hours. When worms are transferred to heat-killed plates, they are washed off of their plates with M9 solution (Wood 1988) and spun at 1000 RPM for 1 min, so that all worms collect at the bottom of the tube, and the M9 is then aspirated. The worms are washed

this way four times and then put onto fresh heat-killed plates. The worms are sequentially

transferred to three heat-killed plates for a minimum of two hours for each transfer. After each

transfer, dead worms and debris are picked off the plate. After completion of the three transfers

to heat-killed plates, worms are incubated in an antibiotic solution overnight. The antibiotic

solution is prepared by mixing 2.5 ml of M9 with heat-killed bacteria (so that it is suspended in

the M9), penicillin/streptomycin (100 units Penicillin and 0.1mg Streptomycin per ml),

tetracycline (0.125 mg/ml) (Sigma-Aldrich, 87128), and normocin (0.7 mg/ml). Worms are

transferred from the last heat-killed bacteria plate (after being washed as described previously) to

this solution in a 25 ml flask and incubated overnight at 25°C.

Harvesting of C. elegans germ cells

Following their incubation in the antibiotic solution overnight, worms are ready to be harvested.

Worms are washed with M9 four times, and then suspended in 1.5 ml of CeM1 media in a small

dish. Adult worms with well-developed germline tumors are then individually picked from this

dish into another dish that contains 80 μl of media in its center, making sure to leave behind dead

worms, young worms, and any debris. Once all of the worms have been transferred to this new

dish, they are cut into quarters using needles, which allows the germ cells to be released.

Twenty-five worms are picked for each 1 ml well used.

Germ cell culture

Following the dissection of the adult *cki-2; glp-1; daf-16* mutant worms, the worm pieces and

cells are pipetted repeatedly against the dish and then collected into a 15 ml tube, where they are

25

spun at 1000 RPM for 1 minute. The majority of the media is aspirated off so that only 100μL of media remains at the bottom of the tube, which contains the worm cells and pieces. This is then distributed evenly between the wells of media in a 12 well plate, with each well containing 1 ml of media. These plates are stored at 20°C.

Counting the number of germ cells

Two stains are used to assess the harvested germ cell culture. The first is Calcein AM dye (Calcein acetoxymethyl ester, Sigma-Aldrich, C1359) and it stains live cells green. Calcein AM, which is not florescent, gets taken up by living cells, where it gets hydrolyzed by the living cell into Calcein, which fluoresces green. This dye is used at a concentration of 1 μM. The other dye used is ethidium homodimer (Sigma-Aldrich, E1903), which stains dead cells red. Ethidium homodimer has a weak red fluorescence until bound to DNA, which allows it to fluoresce strongly red. Ethidium homodimer is unable to enter living cells, but dead cells cannot keep this dye out, and therefore show red fluorescence. This dye is used at a concentration of 0.1 μM.

In order to stain the cells and count them, cells are dispersed evenly into the media in their well by pipetting, and then a 20μl sample is removed for counting. The sample is then mixed with 0.2μl of dye (100x stock solution in DMSO) and the mixture is kept covered from the light, and the dye is given enough time to stain the cells, 30 minutes. The sample is then loaded onto a hemacytometer to be counted (Nexcellom Cellometer Counting Chambers, CHT4-PD100). All of the cells located on the hemacytometer grid are counted. This is first done on the green filter, looking for living cells (the total number of individual cells is counted and the total number of clumps of cells and the number of cells in each clump is counted). Then, the

hemacytometer is counted on the red filter, looking for the total number of dead cells (the number of individual cells and cells in clumps are combined). Three samples for each well are taken for separate counts. These counts and pictures were made with a Microscope Axio Observer.A1 (Zeiss, 491911-0002-000). Cells are counted on days 1, 4, 7, 14, 21, and 28 after harvesting.

Rationale and Results

Description of primary culture system of C. elegans germ cells

A *C. elegans* germ cell line has ben a long sought but elusive research tool for the model organism *C. elegans*. Even the culture of *C. elegans* somatic cells has been far from ideal. The best somatic cell culture system techniques, which were developed by the Strange lab for embryonic cells are not very good (Strange, Christensen et al. 2007). Cells do not proliferate beyond completing cell divisions that they were already undertaking, they have poor cell survival, cell numbers drop significantly even after a week, and the conditions do not permit cells to be experimentally used after they are five days old. In this chapter, a worm genotype and media is described that allows for prolonged maintenance of cultured cells at constant numbers, creating a potentially experimentally-amenable cell culture system for *C. elegans* germ cells. The media is called *C. elegans* Media 1 (CeM1) and is described in Table 2.1. Unless otherwise stated, CeM1 has: a pH of 6.5; osmolarity of 390 mOsm; uses FBS from Atlanta Biologicals, lot G11012; and the FBS is heat inactivated, amberlite treated, and charcoal treated. Pictures of these cultured cells are shown in Figure 2.1.1 and 2.1.2, which shows that there are high numbers of living cells in cultures 7 days and 28 days old.

27

Normal germline cells are syntical, meaning that the germ cells are all connected through an opening in their respective plasma membranes to a central common cytoplasm termed the rachis. Thus, wild-type, N2, worms could not yield a germ cell line, as there are no individual fully cellularized germ cells, and attempting to harvest them by cutting the worm would simply cause media to enter the germ cells to kill them. Therefore, in order to create a germ cell culture system, the use of a mutated *C. elegans* worm with cellularized germ cells is necessary. Many different mutations were tried, and in addition to looking for mutations that cause cellularization of the germline, mutations were tried that increase proliferation of the germline or that inhibit genes that either cause the germline to differentiate and enter meiosis or that directly inhibit the cell cycle. This work was carried out by myself and others and will not be covered in this Thesis. Ultimately, the most long-lived, healthy worm genotype tested was the strain ET507: *daf-16*(mu86) I; *cki-2*(ok2105) II; *glp-1*(ar202) III. These cells are cellularized, tumorous germ cells that under the appropriate conditions, will remain viable in culture for least a month (28 days) without a significant decrease in their cell numbers. These worms will be referred to as *cki-2*; *glp-1*; *daf-16* worms.

The *cki-2*(ok2105) mutation is a loss of function mutation in a cyclin-dependent kinase inhibitor, and as described in the first chapter, CKI-2 acts to prevent progression of cells through the cell cycle. Therefore, by inactivating *cki-2*, the cell is more likely to make the decision to progress through the cell cycle and undergo mitosis (Buck, Chiu et al. 2009). The *glp-1*(ar202) mutation is a gain of function mutation. GLP-1 is a part of the notch-signaling pathway, serving as the ligand receptor. It causes germline stem cells to remain mitotic stem cells and continue undergoing mitosis. This mutation is primarily responsible for the creation of the germline

tumor and therefore is maintained as a temperature sensitive mutation, so that the germline

tumors only form at higher temperatures (Kimble and Crittenden 2005). *daf-16*(mu86) is a loss

of function mutation in a FOXO transcription factor that normally promotes animal longevity.

One of its downstream effects is to reduce the rate of germ cell division and therefore, by

knocking it out, germline proliferation should increase (Lapierre and Hansen 2012). The triple

mutant of *cki-2*(ok2105); *glp-1*(ar202); *daf-16*(mu86) is the strain of worm used throughout this

paper.

Development of CeM1 media

In his 2002 journal article describing somatic cell culture, Strange describes his method for

obtaining *C. elegans* embryonic cells and maintaining them in tissue culture (Strange,

Christensen et al. 2007). The media that the Strange lab proposed is currently considered the

best media to use for *C. elegans* cell culture. Strange used L15 media without phenol red, heat

inactivated fetal bovine serum at a concentration of 10%, penicillin & streptomycin (at 50 U/m

and 50 mg/ml, respectively), and sucrose to bring the osmolarity to 340 mOsm. However,

because this media was designed for use in embryonic cells (not germ cells) and because it had

poor long-term cell survival even for embryonic cells, a better media needed to be developed.

Strange's media was used as a starting point, being slowly modified to create this better media,

CeM1.

Strange's media had used only Leibovitz L-15 media as a base media, but it was thought

that perhaps combining a mammalian and insect medium would be better for cell survival, as

each might provide a different combinations of ingredients that are beneficial or necessary to the

cultured cells. The importance of the two types of base medias was tested by trying different ratios of the two: 100% Schneider's Insect Medium, 75% Schneider's: 25% L-15, 50% Schneider's: 50% L-15, 25% Schneider's: 75% L-15, and 100% Leibovitz L-15 Medium. Leibovitz L-15 Medium is a medium used for culturing mammalian cells, while Schneider's Insect Medium is used for culturing insect cells. Therefore, the two have significantly different ingredients because of the different requirements of the two target organisms. I observed that 75% Schneider's: 25% L15 was the best ratio for cell survival, while high amounts of L-15 Medium, which is similar to what Strange used (75% L15 and 100% L15) were shown to be quite bad for the overall survival of the cells (Figure 2.2).

Cells require the correct pH to function properly and can be very susceptible to even minor changes in pH. Many physiological processes require a narrow pH range to occur and many proteins require the correct pH in order to be active and functional. If proteins are at an inappropriate pH, they can lose important intramolecular bonds that hold the protein in the appropriate functional shape and cause the protein to become inactive. Strange never specifies that they adjust the pH of the media, making the most likely situation that the overall pH is the pH of L-15 media, which is 7.4. Here it is shown that using a specific, correct pH is very important for germ cell culture. The results show quite clearly that a pH of 6.5 is ideal for cultured germ cell survival (Figure 2.3). There is a sharp decrease in cell survival even when the pH is just adjusted up or down half a pH unit (pH 6 or pH 7). There is much worse survival when the pH of the media is shifted up by an entire pH unit (pH 7.5), and cells die almost immediately when pH is adjusted down an entire pH (pH 5.5).

The correct osmolarity is also very important for the survival of cells, and cells can be quite affected by small changes in osmolarity. If the osmolarity is too high, cells can shrivel and die, as the osmotic pressure forces water out of the cell into the environment. On the reverse extreme, if the osmolarity is too low, cells can swell and burst as osmotic pressure forces water into the cell from the environment. Strange used an osmolarity of 340 mOsm, but it was uncertain if this was the ideal osmolarity for cultured *C. elegans* germ cells, so the optimal osmolarity needed to be determined. CeM1 media uses trehalose to adjust the osmolarity of the media, while Strange uses sucrose to adjust the osmolarity. It was found that an osmolarity of 390 and 400 mOsm produced the best germ cell survival (Figure 2.4). After 390 and 400 mOsm, it is found that a lower osmolarity is better than a higher osmolarity. Osmolarities of 370 and 380 mOsm allow moderate cell survival over time, while cells die quickly in 410 mOsm. Cells in the osmolarity of Strange's media, 340 mOsm, died fairly rapidly.

Fetal Bovine Serum is one of the few ingredients added to L-15 in Strange's media and is used at a concentration of 10%. It needed to be determined if FBS was a necessary ingredient and, if so, what the ideal concentration is. FBS is the non-cellular portion of blood from slaughtered fetal cows that also lacks the anti-coagulants found in plasma. In addition to the major protein bovine serum albumin (BSA), serum also contains growth factors, hormones, amino acids, glucose, trypsin inhibitors, and lipids. FBS relative to other animal serums is especially beneficial because it is very rich in growth factors and low in factors that inhibit cell growth. FBS is found to be a key ingredient in CeM1 media, as shown by the rapid cell death seen in cells grown with 0% FBS (Figure 2.5). Adding the correct concentration of FBS is also

important, as it has peak benefit at an 8% concentration, while percentages higher or lower produces suboptimal results (Figure 2.5).

FBS can be manipulated to modify its properties. Strange's media uses only heat inactivated FBS (no other treatments are done to it) (Strange, Christensen et al. 2007). Lois Edgar also describes heat inactivating FBS at 56°C for 30 minutes prior to adding it to her media for use with *C. elegans* embryonic cells (Edgar and Goldstein 2012). The Edgar study did not provide experimental evidence for the necessity of heat-inactivating FBS or tests for the ideal temperature for heat inactivation. Heat inactivation at 56°C is known to destroy compliment (Nimura, Muneta et al. 2010). However, Heating above 56°C can also cause loss of growth factors and other components. I tested the effect of heat inactivation of FBS for 30 min at 56°C, 65°C, and 74°C. I found that heat inactivation is necessary, as cells rapidly begin dying when not heat inactivated (Figure 2.6). Heat inactivation at 65°C is best for prolonged cell survival, with heat inactivation at 56°C producing results similar to 65°C treatment initially, but then being less capable of maintaining cell viability over an extended period (Figure 2.6). Heat inactivation at 74°C is very detrimental, as these cells die very quickly, similar to cells that lack FBS altogether (Figures 2.5, 2.6). These results suggest that incubation at 65°C inactivates deleterious component(s) of FBS, while incubation at 74°C inactivates beneficial component(s) of FBS.

Christine Nguyen and Edward Kipreos in our laboratory discovered that treatment of heat-inactivated FBS with Amberlite IRA 400-CL and charcoal dextran improved the ability of the FBS to maintain *C. elegans* germ cell viability. Amberlite is an ion-exchange resin that binds and removes negatively-charged molecules from FBS. Activated charcoal is used to remove

non-polar, lipophilic substances, such as hormones, steroids, and growth factors. The effect of these treatments had not been quantitated with cell counts. I found that heat-inactivated FBS treated with only one method (amberlite or charcoal) does not support long-term cell viability, while the failure to treat heat-inactivated FBS with either method led to a fairly rapid decline in cell numbers (Figure 2.7). Treatment with both amberlite and charcoal allows *C. elegans* germ cells to stay at constant numbers throughout the 28 day counting period.

Individual tests of various aspects of Strange's media showed that alterations of several components led to noticeable improvements in germ cell cultures: base media components, pH, osmolarity, FBS concentration, and FBS treatment. However, it is necessary to show that these various conditions all combine to make CeM1 media better than Strange's media at maintaining cultured *C. elegans* germ cells. As expected, CeM1 media was found to be much better than Strange's media for germ cell maintenance (Figure 2.8). Strange's media lead to rapid death of most of the germ cells. CeM1 media, on the other hand, allows for prolonged survival of the germ cells, with the number of viable cells remaining steady over the 28 day period examined.

Tables and Figures
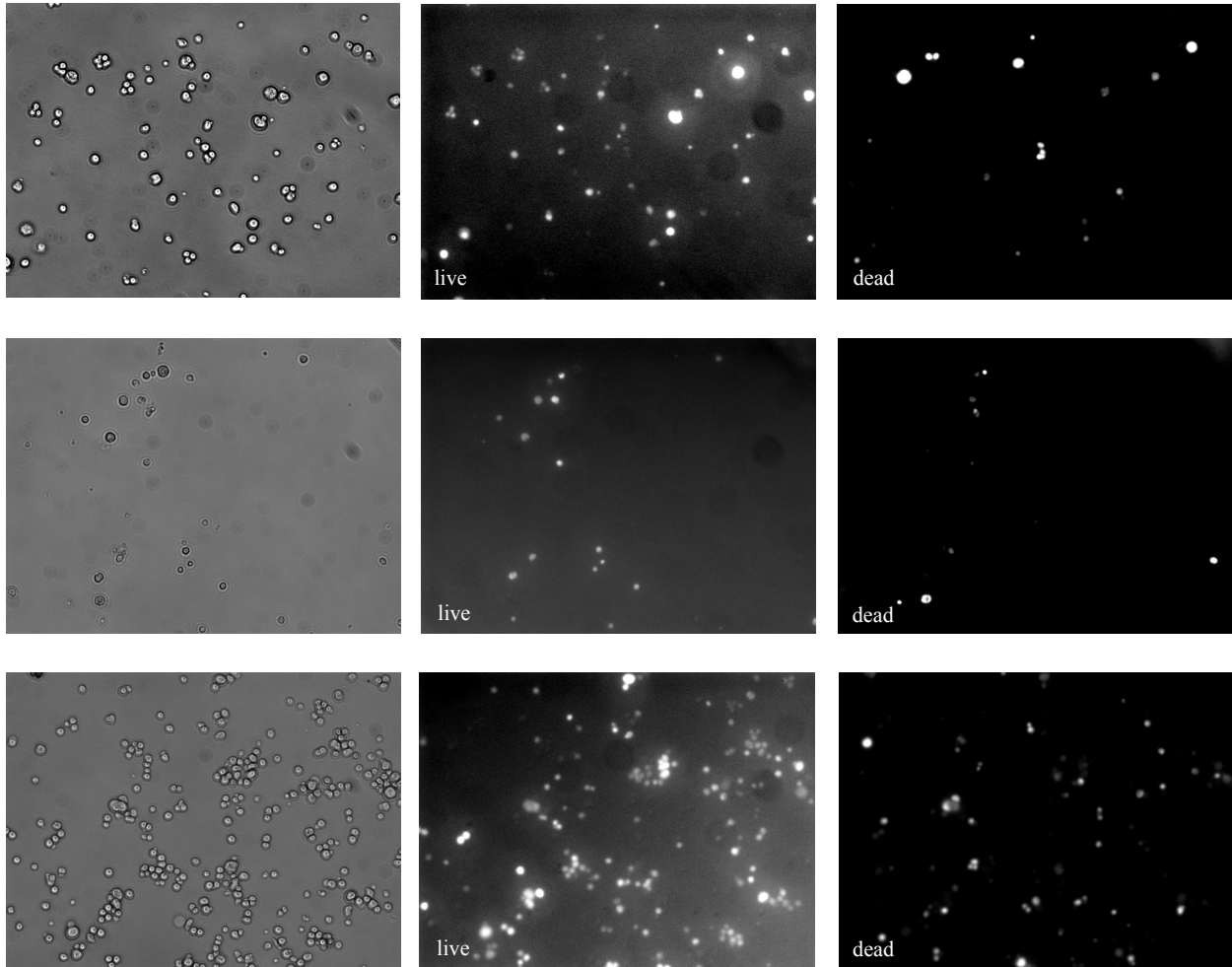
Figure 2.1.1: Pictures of 7 day old isolated *C. elegans* germ cells grown in CeM1 media. From left to right: differential interference contrast (DIC) picture of all cells (left), live cells fluorescing with Calcein AM (center), and dead cells fluorescing with Ethidium homodimer (right). Each row is a different sample of cells.
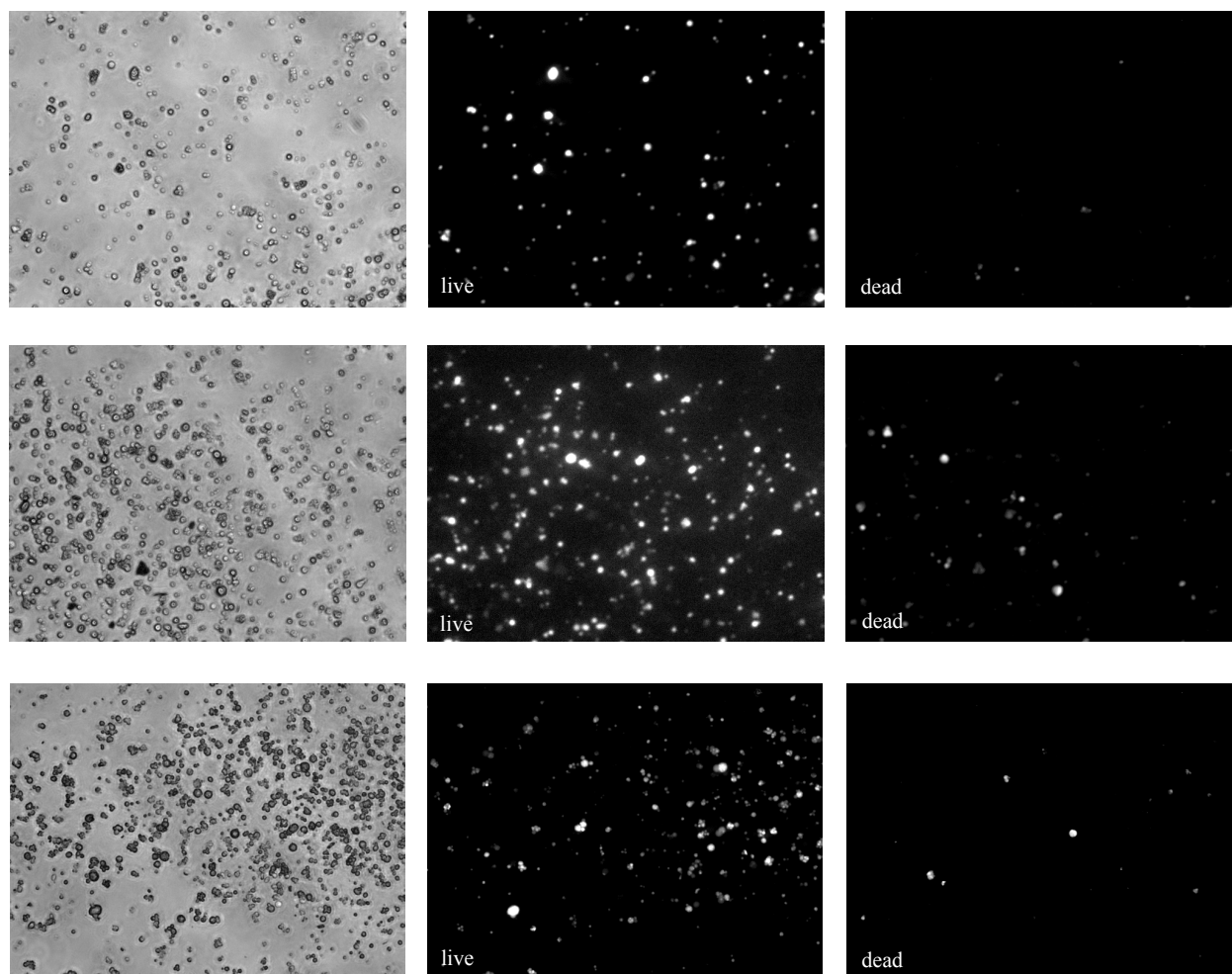
Figure 2.1.2: Pictures of 28 day old isolated *C. elegans* germ cells grown in CeM1 media. From left to right: differential interference contrast (DIC) picture of all cells (left), live cells fluorescing with Calcein AM (center), and dead cells fluorescing with Ethidium homodimer (right). Each row is a different sample of cells.

Table 2.1: List of ingredients found in CeM1 media.

| Ingredients	Concentration
Schneider's Insect Medium w/ glutamine	67.2%
Leibovitz's L-15 Medium w/ glutamine w/o phenol red	22.4%
Fetal Bovine Serum - heat inactivated, amberlite treated, charcoal treated, lot G11012	8.0%
Penicillin-Streptomycin	1.0%
Hemin Chloride	0.2%
RPMI Vitamins	1.0%
Glutathione, reduced (302 MW)	0.6 mg/ml
Normocin	0.1%
Cholesterol in EtOH	0.1%
Trehalose (to 390 mOsm)	19 mg/ml
pH	6.5
Osmolarity	390 mOsm

Effect of Base Media Ratio
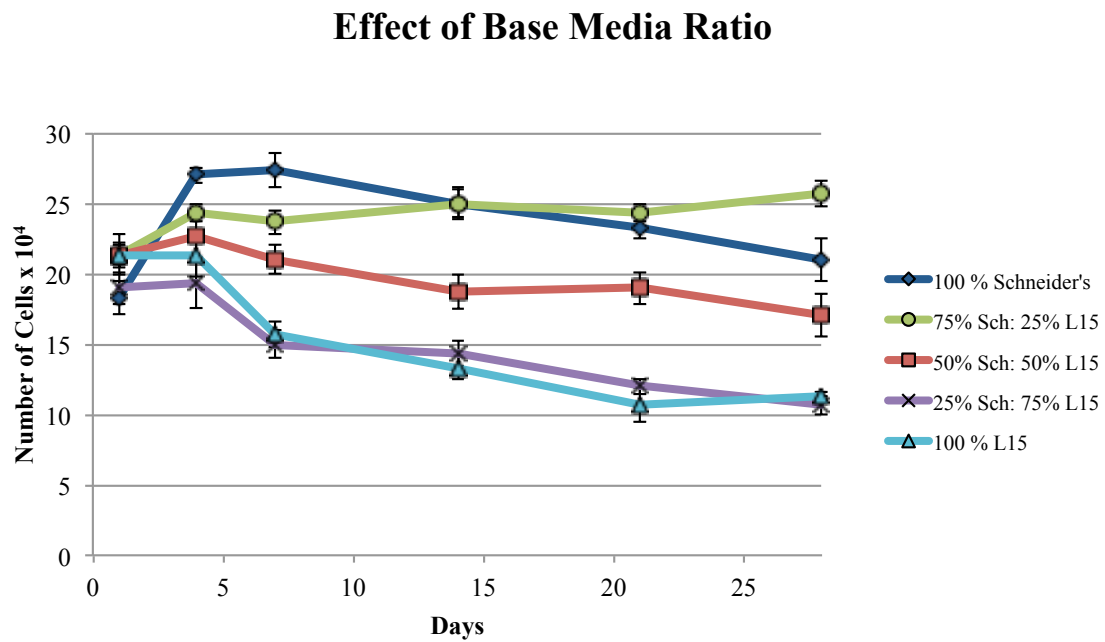
Figure 2.2: A graph of viable cell numbers that compares the ratio of Leibovitz's L-15 Medium and Schneider's Insect Medium in CeM1 media.
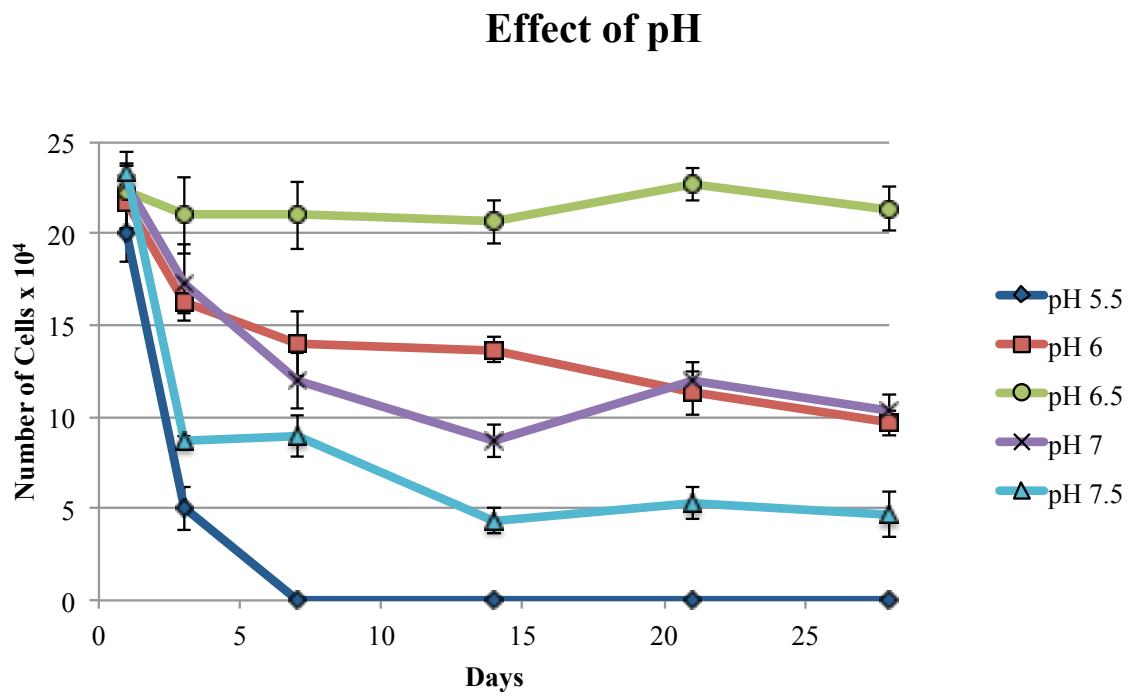
Figure 2.3: A graph of viable cell numbers in CeM1 media with differing pH levels.

Effect of Osmolarity
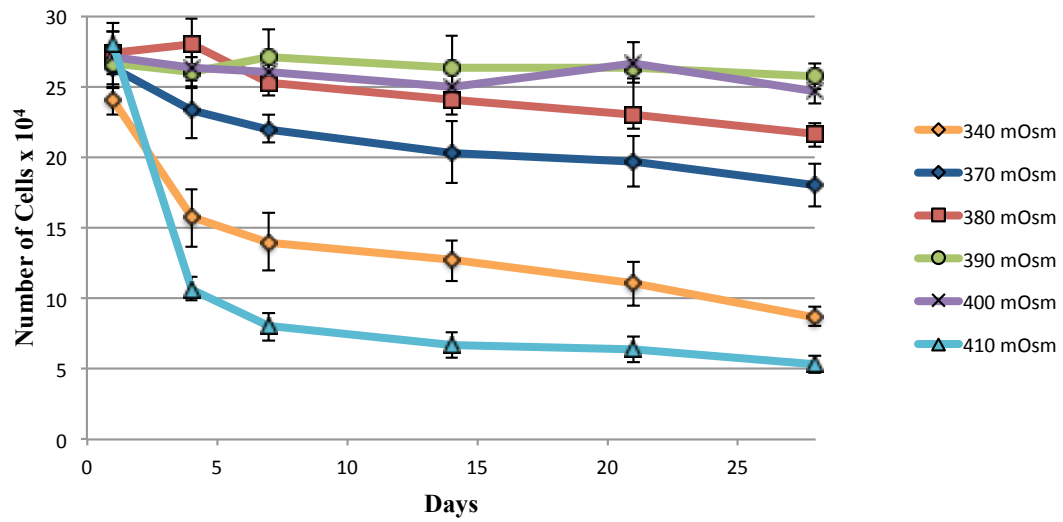
Figure 2.4: A graph of viable cell numbers in CeM1 media with various osmolarities.

Effect of FBS Percent
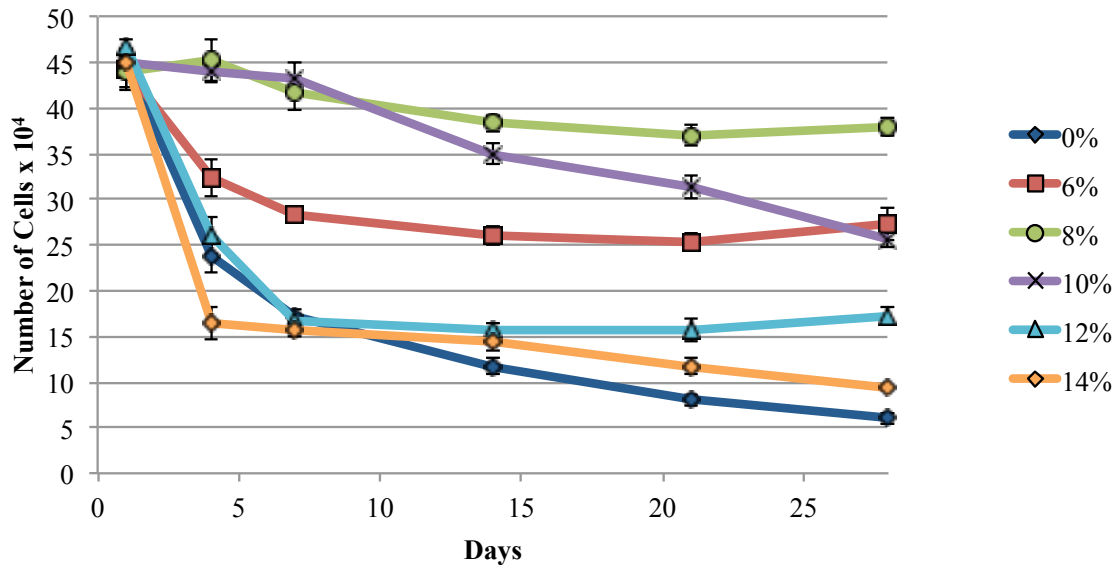
Figure 2.5: A graph of viable cell numbers in CeM1 media that have varying fetal bovine serum concentrations. FBS is shown to be an important ingredient in CeM1 media, and is most effective at a concentration of 8%. FBS is heat inactivated at 65°C, treated with amberlite and charcoal, and comes from lot G11012.

Effect of FBS Heat Inactivation
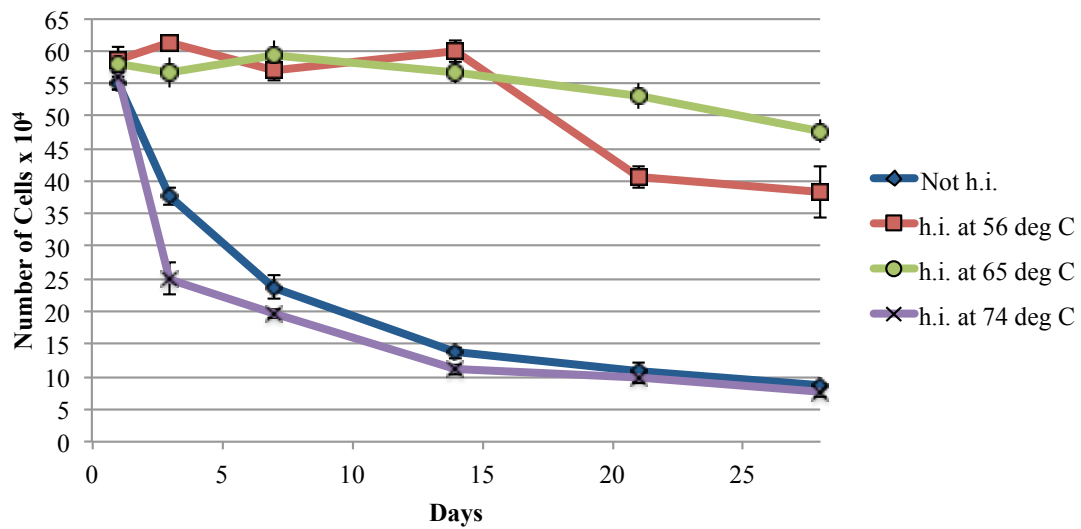
Figure 2.6: A graph of viable cell numbers in CeM1 media that have varying temperatures at which FBS heat inactivation occurred. FBS is used at a concentration of 8%, treated with amberlite and charcoal, and comes from lot G11012.

Effect of FBS Treatment
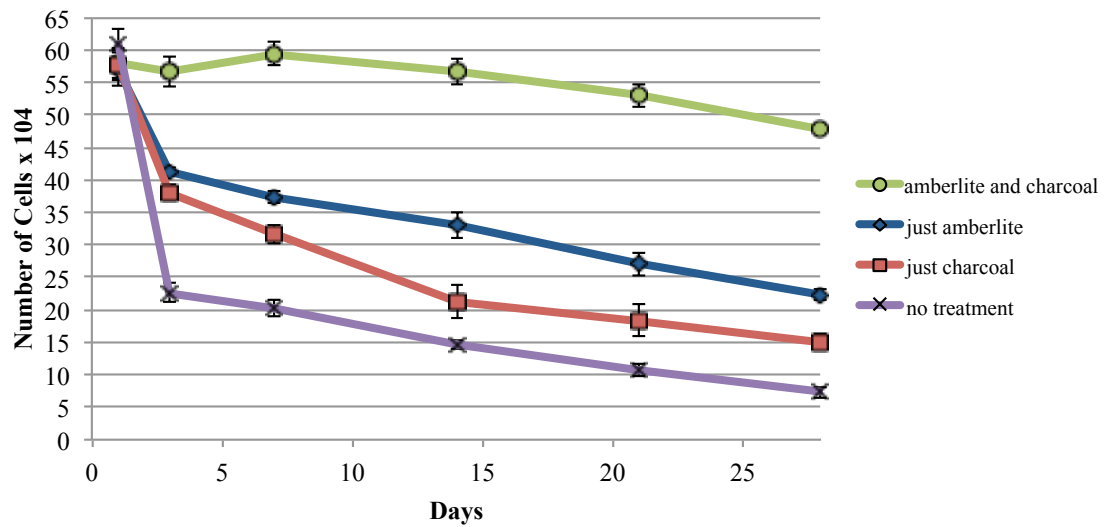
Figure 2.7: A graph of viable cell numbers in CeM1 media that compares the affects of FBS treatments: no treatment, just amberlite, just charcoal, and amberlite and charcoal together. FBS is used at a concentration of 8%, heat inactivated at 65°C, and comes from lot G11012.

Comparison of CeM1 and Strange's Media
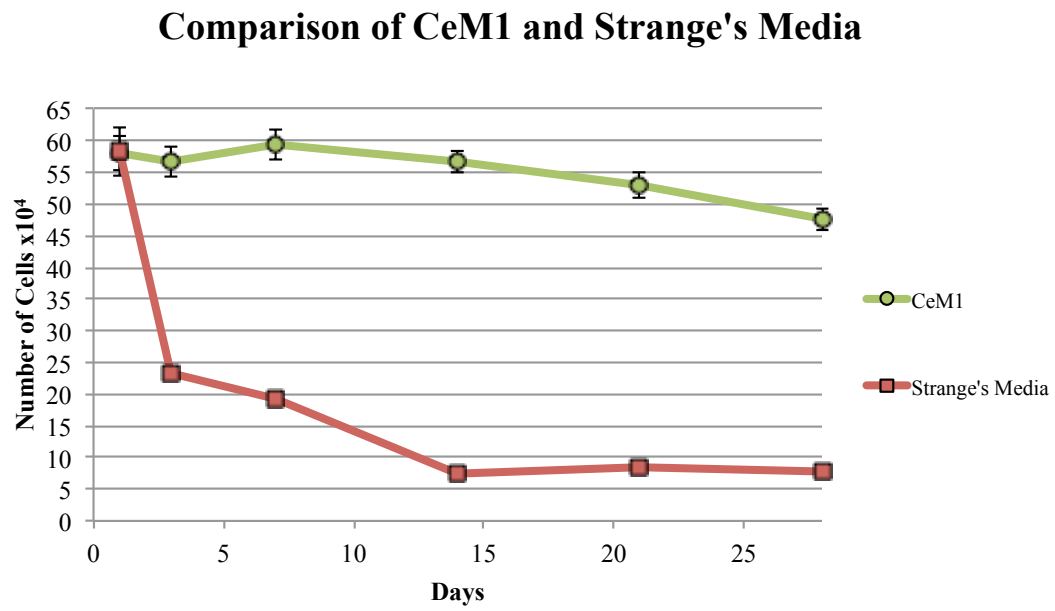
Figure 2.8: Comparison of CeM1 media to the established media for *C. elegans* culture: Strange media.

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CHAPTER 3

THE COMPONENTS OF CEM1 MEDIA ALLOW FOR SURVIVAL OF CULTURED

CAENORHABDITIS ELEGANS CELLS FOR AN EXTENDED PERIOD OF TIME

Purpose

The purpose of this chapter is to show that CeM1 media is optimal for the prolonged survival of

cultured *C. elegans* germ cells and that all ingredients contribute to its beneficial effect. As well,

several media additives are tested to see if they are beneficial to the cell culture.

Materials and Methods

Media preparation

CeM1 Media is prepared using the ratios and ingredients described in Table 2.1. The media is

used at a pH of 6.5 and an osmolarity of 390 mOsm. The standard Fetal Bovine Serum (FBS)

used is from Atlanta Biologicals, lot G11012. The FBS is heat inactivated, amberlite treated, and

charcoal treated. Further description on the preparation of media is described in the Materials

and Methods of Chapter 2.   The worm strain used is ET507: *daf-16*(mu86) I; *cki-2*(ok2105) II;

glp-1(ar202) III. Germ cells are harvested from worms, set up in 12 well plates, and counted

with calcein AM and ethidium homodimer as described in the Materials and Methods in Chapter

2.

45

Dropout media is prepared by individually removing a single ingredient found in Table 2.1: hemin chloride, RPMI vitamins, glutathione, cholesterol, and trehalose. Schneider's Insect Media and Leibovitz's L-15 Media are added in a 3:1 ratio to compensate for the amount of total media volume missing due to the removed ingredient. When testing the effect of trehalose on cell survival in the dropout experiment, sucrose is used in the place of trehalose as it is another sugar that can be used to adjust the osmolarity of the media to the proper concentration.

Base media components

The base medias used in standard CeM1 media are Schneider's Insect Medium and Leibovitz L-15 Medium, and they are used in a ratio of 75% Schneider's Medium and 25% L-15 Medium. However, other insect medias were also tested (at the 3:1 ratio): Grace's Insect Medium (HiMedia, IM001), Mitsuhashi & Maramorosch Insect Medium (HiMedia, IM002), Shield's & Sang's Insect Medium (HiMedia, IM004), TNM-FH Insect Medium (HiMedia, IM008), IPL-4 Insect Medium (HiMedia, IM006), TC-100 Insect Medium (HiMedia, IM007), and D-22 Insect Medium (HiMedia, IM005). These base medias were purchased in powder form and made into liquid medias as specified by Himedia Inc. Grace's Insect Medium, Mitsuhashi & Maramorosch Insect Medium, TNM-FH Insect Medium, and TC-100 Insect Medium were recommended to be used with $NaHCO_3$, so these were tested with both the recommended amount of $NaHCO_3$ and without any $NaHCO_3$. Shield's & Sang's Insect Medium was recommended to be used with $KHCO_3$, so it was tested with both the recommended amount and without any $KHCO_3$. IPL-4 Insect Medium was recommended to be used with $CaCl_2$ and $KHCO_3$, so it was tested with both

the recommended amount and without any $CaCl_2$ or $KHCO_3$. D-22 Insect Medium was not recommended to be used with any additives, so was tested as a single formulation.

Media additives

Vitamin B12 was added at a concentration of 3.2×10^{-6} mM. MEM amino acids were added at 2x concentration (at 2% of the media) (Sigma-Aldrich, M5550). Schneider's Insect Media and Leibovitz's L-15 Media were removed in a 3:1 ratio to compensate for any volume changes resulting from media component additions. BME Vitamins were used to replace RPMI vitamins and were added at a concentration of 1% (Sigma-Aldrich, B6891).

Dafachronic acid (Cayman Chemical, 14101) was prepared and stored in a 1 mM stock in DMSO. Dafachronic Acid was tested at a concentration of 0.0125 µM, 0.025 µM, 0.125 µM, and 0.25 µM.

Sphingosine-1-Phosphate (S-1-P) (Sigma-Aldrich, S9666) was added to the media at concentrations of 0.5 µM, 1 µM, and 2 µM.

Rationale and Results

All ingredients in CeM1 media are found to be important

A media dropout experiment was performed to determine the importance of each ingredient in CeM1 media. Individual ingredients were removed from the media and it was observed how much faster cells died when the media lacked a particular ingredient. Through this method, it was found that each ingredient of the media is important for prolonged cultured germ cell survival.

Hemin chloride was added to the media because it was shown that nematodes, such as *C. elegans*, cannot synthesize heme, yet *C. elegans* require hemoproteins for many important biological pathways (Rao, Carta et al. 2005). The RPMI vitamins in CeM1 refers to the vitamins found in RPMI 1640 Media, which is used for culturing mammalian cells: choline chloride, D-biotin, D-Ca-pantothenate hemicalcium, folic acid, niacinamide pyridoxine hydrochloride, riboflavin, thiamine hydrochloride, vitamin B12, i-Inositol, and p-Aminobenzoic acid (PABA). Trehalose is added, rather than other sugars such as sucrose, because it is the major sugar found in the nematode haemolyph (Behm 1997). Therefore, it makes sense that this would be the preferred sugar of cultured *C. elegans* cells. In *C. elegans* worms, trehalose is both a major source of energy, plus serves a role in protecting the worm from freezing and dehydration, egg hatching, and helps the worm to take-up glucose (Behm 1997). Glutathione is added to the media because it is often added to primary cultures as an antioxidant ingredient (Franco and Cidlowski 2009). Cholesterol is added to the media because it was shown that nematodes have a nutritional requirement for cholesterol (Hieb and Rothstein 1968). Penicillin/ streptomycin and normocin were not tested in the media dropout as they are added as antibiotics, not as ingredients to improve the media. Penicillin is used to protect against gram positive bacteria, streptomycin is used to protect against both gram positive and gram negative bacteria, and normocin is used to protect against mycoplasma, bacteria, and fungi.

The dropout experiment showed that all ingredients are important. This was concluded because there were worse cell survival curves for each ingredient that was removed from the media (Figure 3.1).

Determining the effect of various insect medias

Because of the benefits seen by adding the insect media Schneider's Insect Medium in combination with L-15 medium, it is possible that there is another insect media that is even more beneficial for cell survival, and that could potentially even allow for cell division. Seven different insect mediums were compared to Schneider's Insect Medium: Grace's Insect Medium, Mitsuhashi & Maramorosch Insect Medium, Shield's & Sang's Insect Medium, TNM-FH Insect Medium, IPL-4 Insect Medium, TC-100 Insect Medium, and D-22 Insect Medium. However, none of the other insect medias, either with added $NaHCO_3$, $KHCO_3$, or $CaCl_2$ (as recommended by Himedia) or without it, were as beneficial to germ cell survival over time as the Schneider's Insect Medium (Figure 3.2.1 and 3.2.2).

Determining the effect of various media additives

Vitamin B12 has been shown to be an important component of a lifespan extension pathway (working as an inhibitor of the pathway), and it regulates genes, speeds up development, and decreases fertility (Watson, MacNeil et al. 2014). Because it is not well understood if and how this pathway affects the proliferation of germ cells, the effect of vitamin B12 was examined. However, because there is an interaction between DAF-16 (a transcription factor that extends worm lifespan) and germ cell proliferation (Lapierre and Hansen 2012), it was thought that vitamin B12 may be important and provide a signal to the cultured germ cells to signal them to divide. Therefore, additional vitamin B12 was added to CeM1 to determine if it would have an affect (RPMI vitamins already provide a small amount of vitamin B12). The amount added,

3.2×10^{-6} mM, was the optimal amount indicated by Watson and MacNeil to see vitamin B12's affects (Watson, MacNeil et al. 2014). However, because this pathway it is not completely understood in relation to germ cell proliferation, and it is not known whether vitamin B12 would be beneficial or deleterious, an altered CeM1 media was also prepared that lacked vitamin B12: BME vitamins were substituted for RPMI vitamins, which have most of the same vitamins as RPMI, but which lack vitamin B12 (choline chloride, D-biotin D-Ca-pantothenate hemicalcium, folic acid, niacinamide pyridoxine hydrochloride, riboflavin, thiamine hydrochloride, and i-Inositol). Watson and MacNeil also described a number of amino acids that are important in the vitamin B12 lifespan extension pathway (glycine, threonine, isoleucine, valine, cystine, methionine), which work for the most part antagonistically against vitamin B12, so an altered CeM1 was created which had MEM amino acids (which contains arginine, cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine) added.

It was found that adding additional vitamin B12 did not allow for an increase in cultured *C. elegans* germ cell numbers. However, it was also not deleterious as the cells grown with extra vitamin B12 had cell numbers remain fairly constant, similar to the cell numbers seen in cells grown in normal CeM1 (Figure 3.3). Vitamin B12 was found to be a necessary ingredient, as cells grown with BME vitamins, lacking vitamin B12, had a fairly rapid decrease in cell numbers. As well, MEM amino acids were found to be harmful, as cells grown with the MEM amino acids (which did not lack vitamin B12 as they had RPMI vitamins) also had a fairly rapid decrease in cell numbers.

50

Dafachronic acid is a bile acid-like steroid and is proposed to function as an endogenous ligand of DAF-12 (Mahanti, Bose et al. 2014). It works along the DAF-16/ Insulin pathway and can expand the lifespan in germline free animals that lack *daf*-9/*cyp*-27 or *daf*-36/oxygenase (Lapierre and Hansen 2012). Dafachronic acid has been shown to interact with DAF-12 to prevent entry of worms into the Dauer stage (Sommer and Ogawa 2011). Because this pathway involving dafachronic acid is not fully understood, it was tested as an additive to CeM1 to see its effect on the cultured germ cells. It was believed that the dafachronic acid might provide a signal to the cultured germ cells that would signal them to proliferate. However, when tested, it was found that the addition of dafachronic acid hurt the cells and the higher the concentration, the more deleterious it was (Figure 3.4).

Sphingosine-1-phosphate has been shown to be involved in regulating many aspects of cell physiology: growth, differentiation, senescence, and apoptosis (Mosbech, Kruse et al. 2013). Overall, S-1-P has been shown to play a role in extending lifespan, especially when the worm is experiencing a restricted diet (Mosbech, Kruse et al. 2013). The relationship between lifespan extension and germline proliferation is not well understood. Therefore, it was considered to be worthwhile to test the effect of S-1-P on germ cell proliferation in culture, with the hopes that this molecule would provide a signal to the germ cells to stimulate them to divide. However, when S-1-P is added to CeM1, it is found that the S-1-P was in fact deleterious to the health of the germ cells (Figure 3.5). Therefore, S-1-P is a deleterious substance to the cultured germ cells.
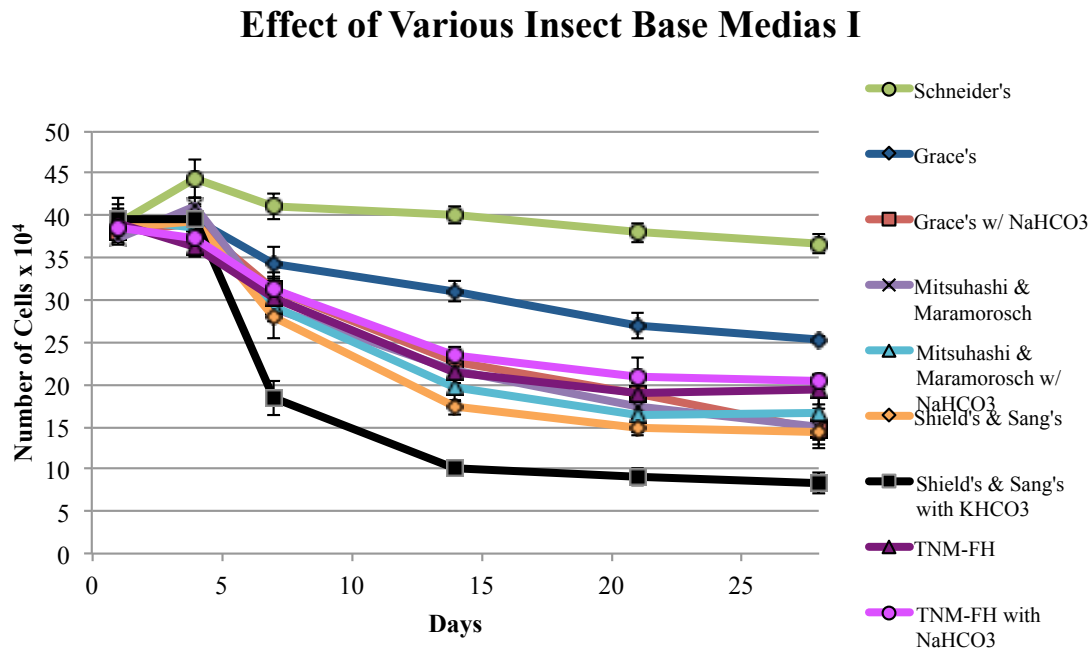
Effect of Media Components
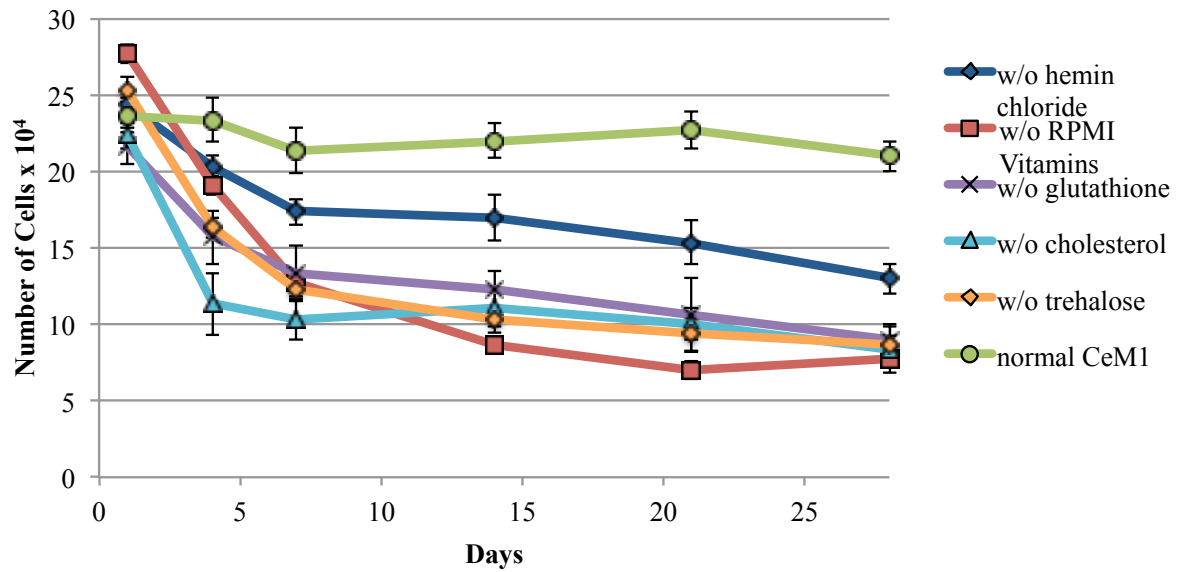
Figure 3.1: A graph of viable cell numbers in CeM1 media lacking specific ingredients. This

shows that all CeM1 media components are necessary for increased cell survival.

Effect of Various Insect Base Medias I

Legend:
- Schneider's
- Grace's
- Grace's w/ NaHCO3
- Mitsuhashi & Maramorosch
- Mitsuhashi & Maramorosch w/ NaHCO3
- Shield's & Sang's
- Shield's & Sang's with KHCO3
- TNM-FH
- TNM-FH with NaHCO3

Figure 3.2.1: A graph of viable cell numbers that compares five insect medias, with and without recommended additives, in CeM1 media. The best insect media to be used is determined to be Schneider's Insect Medium.

Effect of Various Insect Base Medias II
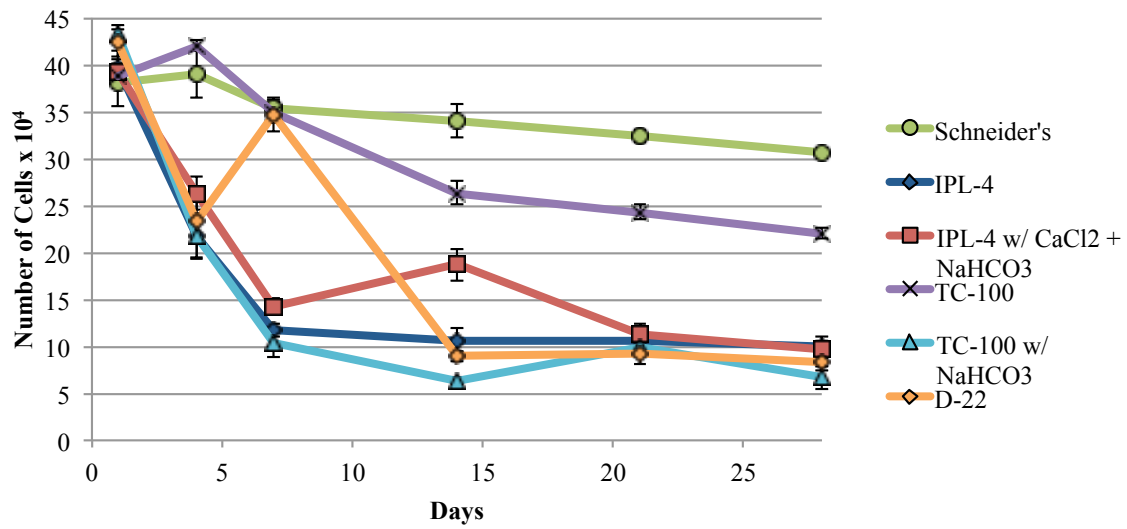
Figure 3.2.2: A graph of viable cell numbers that compares four insect medias, with and without recommended additives, in CeM1 media. The best insect media to be used is determined to be Schneider's Insect Medium.
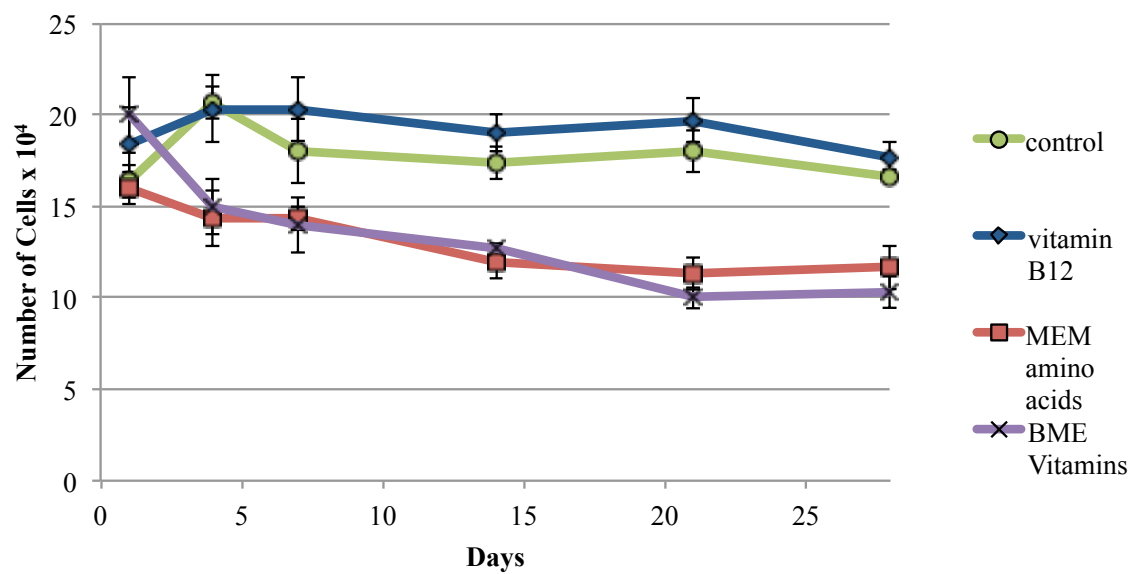
Effect of Vitamin B12 and Amino Acis

Figure 3.3: A graph of viable cell numbers that examines the effect of vitamin B12 in CeM1 media.

Effect of Dafachronic Acid
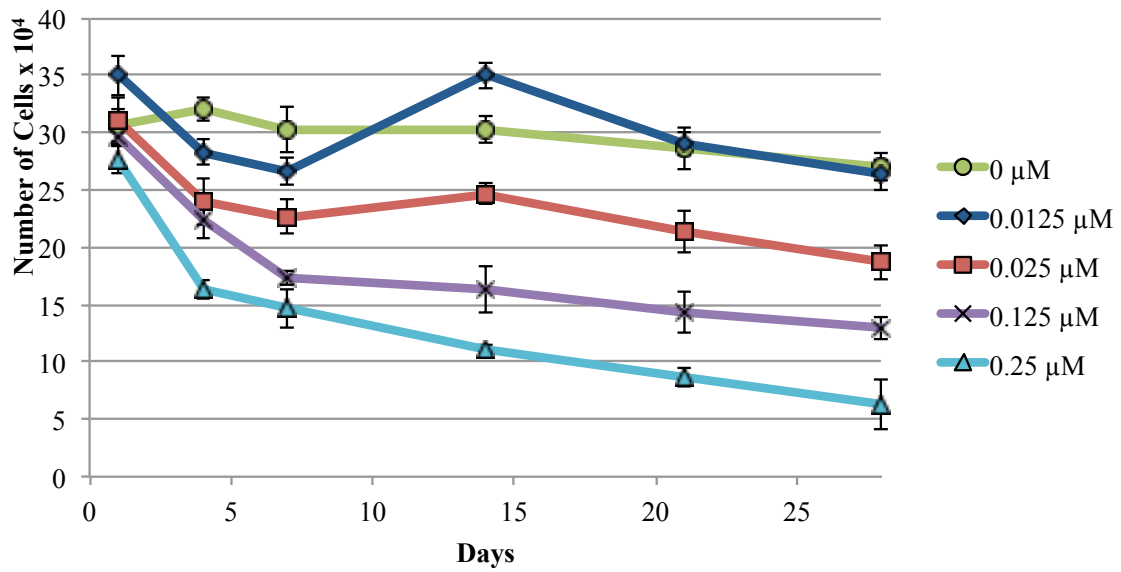
Figure 3.4: A graph of viable cell numbers that examines the effect of dafachronic acid in CeM1 media.

Effect of Sphingosine-1-Phosphate

Figure 3.5: A graph of viable cell numbers that examines the effect of sphingosine-1-phosphate in CeM1 media.

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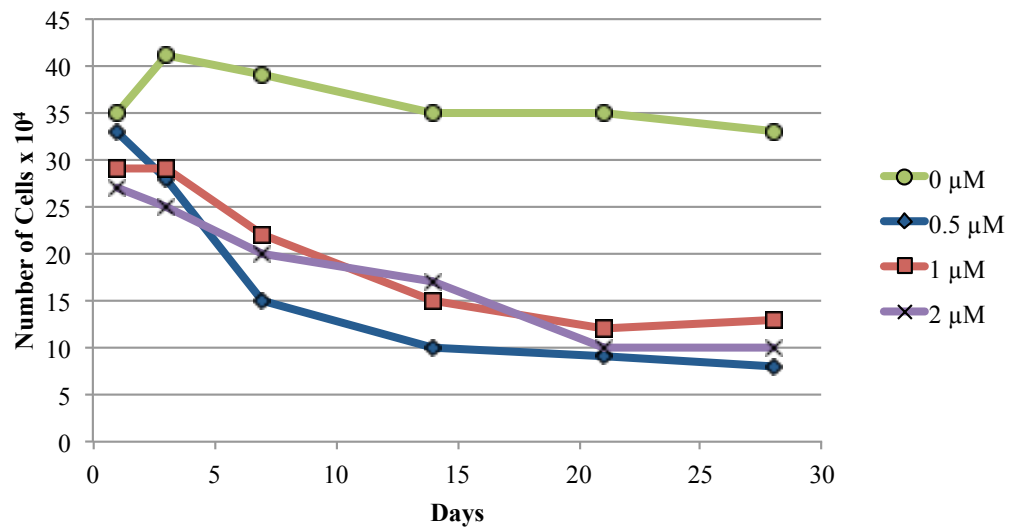Franco, R. and J. A. Cidlowski (2009). "Apoptosis and glutathione: beyond an antioxidant." <u>Cell Death and Differentiation</u> **16**(10): 1303-1314.

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CHAPTER 4

SIGNALS LINKED TO THE *CAENORHABDITIS ELEGANS*' BACTERIAL DIET ALLOWED

FOR AN INITIAL INCREASE IN *CAENORHABDITIS ELEGANS* GERM CELL NUMBERS

Purpose

C. elegans eat bacteria, and germ cells proliferate when C. elegans are well fed.   It is possible

that the ingested bacteria provide a signal for germ cells to proliferate.   In this Chapter,

experimental evidence will be provided that there are bacteria-derived signals that are capable of

inducing germ cell division.

Materials and Methods

CeM1 Media is prepared using the ratios and ingredients described in Table 2.1. The media is

used at a pH of 6.5 and an osmolarity of 390 mOsm. The standard fetal bovine serum (FBS)

used is from Atlanta Biologicals, lot G11012. The FBS is heat inactivated, amberlite treated, and

charcoal treated. Further description on the preparation of media is described in the Materials

and Methods of Chapter 2.   The worm strain used is ET507: *daf-16*(mu86) I; *cki-2*(ok2105) II;

glp-1(ar202) III. Germ cells are harvested from worms, set up in 12 well plates, and counted

with calcein AM and ethidium homodimer as described in the Materials and Methods in Chapter

2.

In addition to using FBS from lot G11012, FBS from nine other lots were tested: F12087, L11066, E1201, J12134, G12104, E12069, F1064, K13145, and B13029. All FBS samples were heat inactivated at 65°C, amberlite treated, and charcoal treated, as described in the Materials and Methods section of Chapter 2.

Preparation of bacterial extract

Bacterial Extract was prepared from *E. coli* HT115 bacteria that were grown in 2 liters of 2xYT incubated overnight in a shaker at 37°C. The bacteria was centrifuged and washed twice with PBS. The bacteria was frozen at -80°C, lyophilized, and split into 2 batches.

One of the batches was used for the creation of bacterial extracts E. This first batch of bacteria was mixed with 6 ml water and 6 ml phenol chloroform (heated at 65°C). The water/ phenol chloroform mixture was mixed with the bacteria and then vortexed until all of the bacteria was in solution, then spun at 3500 rpm for 10 minutes. The aqueous phase was removed and a chloroform extraction was performed by adding 6 ml chloroform, vortexing, spinning the solution at 3500 RPM for one minute, removing the aqueous phase, and repeating until all phenol odor was removed. When this is finished, 1.5 ml from the aqueous phase was set aside. This was labeled E-1 extract. The rest of solution was then heated at 60°C for 30 minutes, put on ice for 15 minutes, and spun at 3500 RPM for 10 minutes. 1.5 ml of this solution was saved. This was labeled E-2. The remaining 1.5 ml was treated with an endotoxin removal column. This was labeled E-3.

The other batch of lyophilized HT115 bacteria was used for the creation of bacterial extracts WA and WB. This batch was resuspended in 6 ml water and then the bacteria was

sonicated. The bacteria was then split into a 4 ml and a 2 ml group. The 4 ml group was spun at 3500 RPM for 10 minutes to remove debris and 0.8 ml was saved as WA-1 extract. The rest of the 4 ml sample was heated at 60°C for 30 minutes, put on ice for 15 minutes, and spun at 3500 RPM for 15 minutes to remove insolubles. The supernatant was removed and from this, 0.8 ml was saved as WA-2. The rest of the supernatant was treated with an endotoxin removal column, and saved as WA-3. The 2 ml group from above was not spun down (which left bacteria debris in the solution). This solution was heated at 60°C for 30 minutes, put on ice for 15 minutes, and spun at 3500 RPM for 15 minutes to remove insolubles. The supernatant was removed and from it, 0.8 ml is saved as WB-2. The rest of the supernatant was treated with the endotoxin removal column and saved as WB-3.

Endotoxin was obtained from the endotoxin that was removed by the endotoxin removal columns described above. Endotoxin was eluted from the endotoxin removal column using 2M NaCl that was washed several times through the column to release the endotoxins. This eluate was then heated at 65°C for 15 minutes, as endotoxin is resistant to heat treatment.

All of the bacterial extracts as well as the endotoxin were added individually to the media. First, all of the bacterial extracts were mixed 1:1 with CeM1 media and filtered (which produces a 1:2 dilution). The extracts were then tested by dilution series in a 200 μL/well, 48 well plate. The dilutions tested were 1:20, 1:200, 1:2,000, 1:20,000, 1:200,000, and 1:2,000,000.

Changing the media

To determine the effect of providing fresh media, media was changed either daily or every three days. When media was changed, 250 μl of media was removed from the side of the well (of a 12

well plate, containing 1ml of media) where there is a low concentration of cells. Care was taken

not to agitate the plate, as cells should remain in the center of the plate, away from the sides

where the media is removed. The removed media was kept and checked to make sure only a

minimal amount of cells were removed. 250 µl of fresh media was then added back to the well.

Rationale and Results

Determining the effect of various FBS lots

There is great variability in the components and their concentrations in different lots of fetal

bovine serum. Endotoxin, mycoplasma, total protein, and hemoglobin all occur at varying levels

in FBS. To determine the importance of FBS lots, seven different FBS lots were compared to

G11012 FBS lot: F12087, L11066, E1201, J12134, G12104, E12069, and F1064. It was found

that the previously believed best FBS lot, G11012, did not appear to be as good as the FBS lot

F12087. F12087 showed a 40% increase in cell numbers between day 1 and day 4, which then

plateaued. This was the first time an increase in cell number had ever been observed. All other

FBS lots were not as good as lot G11012 (Figure 4.1.1).

Among the tested lots, F12087 had the highest endotoxin levels (1.0 EU/ml), as described

in the data sheets provided by Atlanta Biologicals Inc. This result suggested that perhaps the

higher endotoxin levels were contributing to the beneficial effects of F12087 (Table 4.1).

Endotoxin, also known as lipopolysaccharide (LPS) is derived from the outer membrane of

Gram-negative bacteria and can cause an inflammatory response (Rietschel, Kirikae et al. 1994).

Because *C. elegans* are feed a diet of bacteria, mainly *E. coli*, a gram-negative bacteria, it would

be plausible for *C. elegans* to use signals or metabolites from the bacteria as signals for germ

62

cells to divide. In this hypothesis, the high endotoxin levels in the beneficial FBS lots may be a marker for a higher percentage of bacterial contamination, which would be associated with increased endotoxin and increased bacterial metabolites.

Because of the high endotoxin levels found in F12087, the next step was to compare this FBS, as well as G11012, to other FBS lots with very high endotoxin levels: K13145 (12.0 EU/ml) and B13029 (12.0 EU/ml). Although these new FBS lots had better cell survival than G11012, with slight increases between Day 0-4, neither of them had as large of an increase as seen with F12087 (Figure 4.1.2) And in the long run, K13145 was worse than G11012 and B13029 has similar cell survival to G11012. Therefore, endotoxin alone is not a definitive determinant of the utility of FBS lots.

Determining the effects of bacterial extracts

The positive correlation between initial germ cell division and endotoxin level led to the conclusion that bacteria might provide some essential signal that germ cells need to divide. Therefore, different bacterial extracts were created that were treated in various ways to inactivate and remove components such as proteins and lipids. Eight different extracts were made, as well processed endotoxin, which were tested using a dilution series: E1-3, WA1-3, WB 2-3, and purified endotoxin. The E endotoxin levels are all treated with phenol/chloroform, and the E2 is heated in a water bath, and E3 is heated in a water bath and passed through an endotoxin removal column. The W extracts all have their bacteria sonicated, and then WA2-3 and WB2-3 are heated in a water bath and WA3 and WB3 are passed through an endotoxin removal column. WA2-3 have these treatments done without bacterial debris and WB2-3 have these treatments done with

bacterial debris in solution. The effects of these additives were then analyzed by visual inspection. It was observed that the bacterial extracts in the E series appeared the best, especially E-1 at a ratio of 1:200 (with the second best ratio of extract: media being 1:20). From here, a new experiment was set up with bacterial extract E 1-3 at a ratio of 1:200 and cells were counted to quantitate the effects. All three of the extracts were beneficial, but E1 was the best and allowed for an ~40% increase in cell numbers between days 1-4 (Figure 4.2).

Changing the media of harvested C. elegans cells

Because there was the increase in cell numbers between days 1-4, which then plateaued, it was speculated that perhaps the plateau in cell numbers resulted either from a beneficial component of the CeM1 media being used up or a harmful substance that the cells release into the media that poisons the cells. By replacing 25% of the old media with fresh media either daily (for media made with either G11012 and F12087) or every three days (only for media made with G11012), it could be seen if providing fresh media would allow cell proliferation to continue for a more extended period beyond the first four days.

However, changing the media provided no benefit, for both media made with G11012 or F12087 (Figure 4.3.1). For the media made with G11012, there was only a slight decrease is cell numbers seen, with a slightly larger decrease for the media changed daily compared to media changed every three days (Figure 4.3.1). This decrease probably arose from removing small numbers of cells when changing the media. For the media made with F12087, between days 1 and 4, there was a greater increase in cell number in cells when the media was changed daily compared to not changing the media. However, after day 4, cell numbers decreased until they

64

returned to the same levels as seen in the sample where the media was not changed, and then

dropped below it, again probably due to removing cells during the media change (Figure 4.3.2).

Tables and Figures
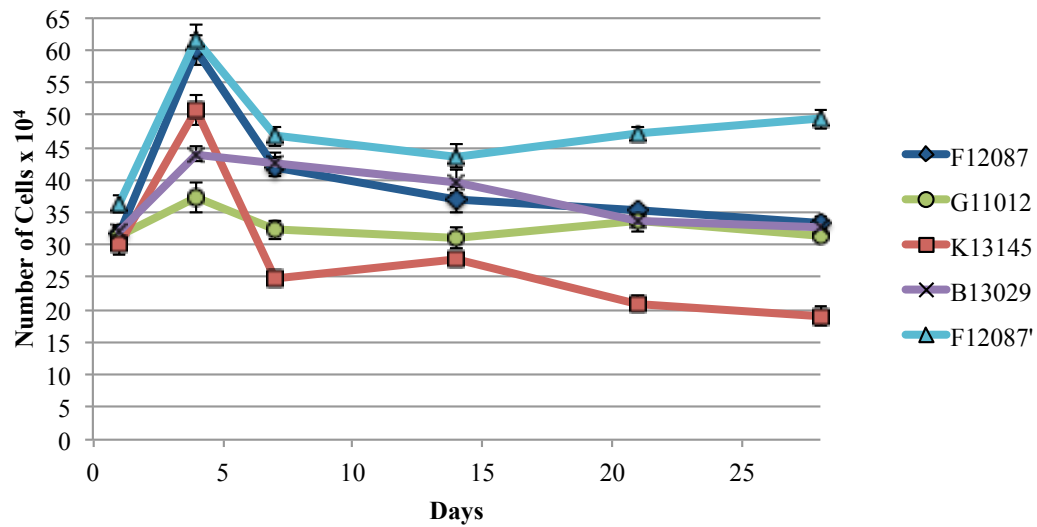
Effect of FBS Lot I
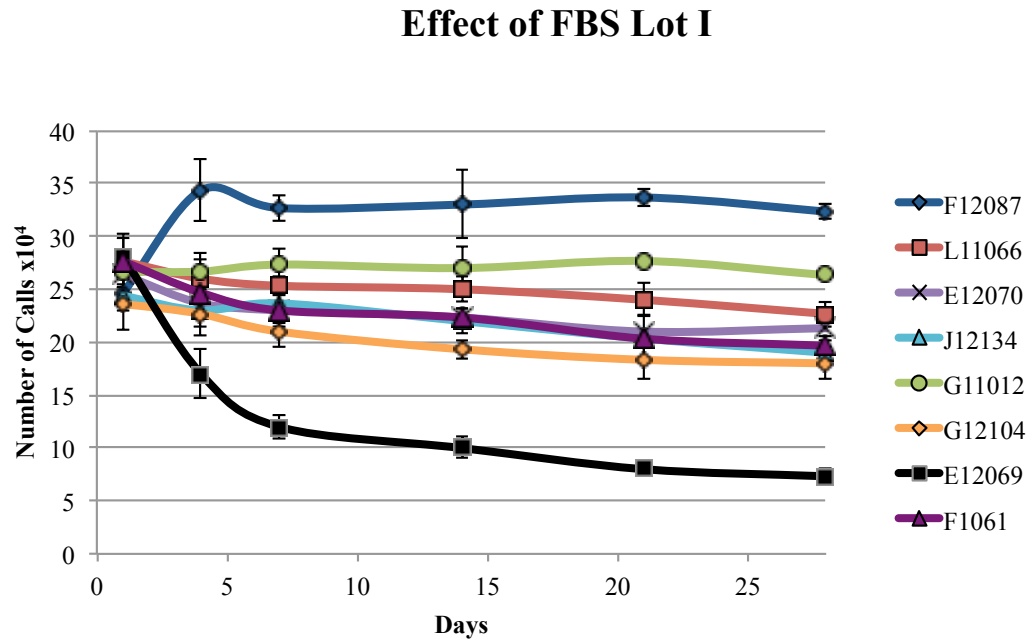
Figure 4.1.1: A graph of viable cell numbers that compares the variability in cell survival of 8 different lots of FBS in CeM1 media. FBS is used at a concentration of 8%, heat inactivated at 65°C, and treated with amberlite and charcoal.

Table 4.1: A chart of the endotoxin levels of the FBS lots whose cell viability was tested.

FBS lot	Endotoxin (E.U./ml)
F12087	1.0
G11012	0.5
E12070	0.1
F1061	0.8
J12134	0.1
G12104	0.1
E12069	0.1
B13029	12.0
K13145	12.0

Effect of FBS Lot II

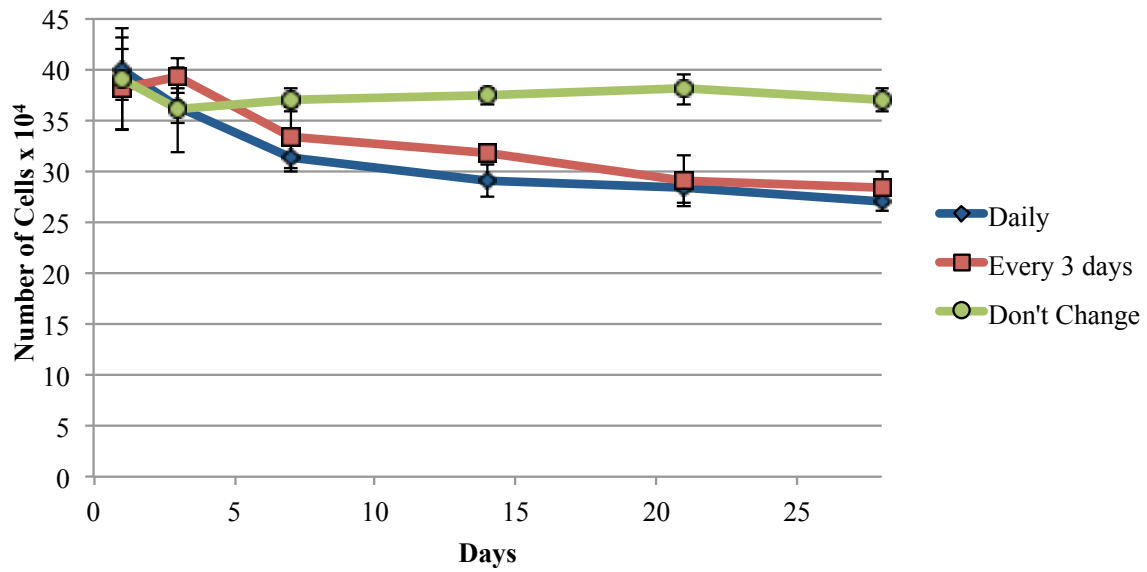**Figure 4.1.2:** A graph of viable cell numbers that compares the variability in cell survival in CeM1 media of 2 different lots of FBS to FBS lots G11012 and F12087. FBS is used at a concentration of 8%, heat inactivated at 65°C, and treated with amberlite and charcoal.

Effect of Bacterial Extract E
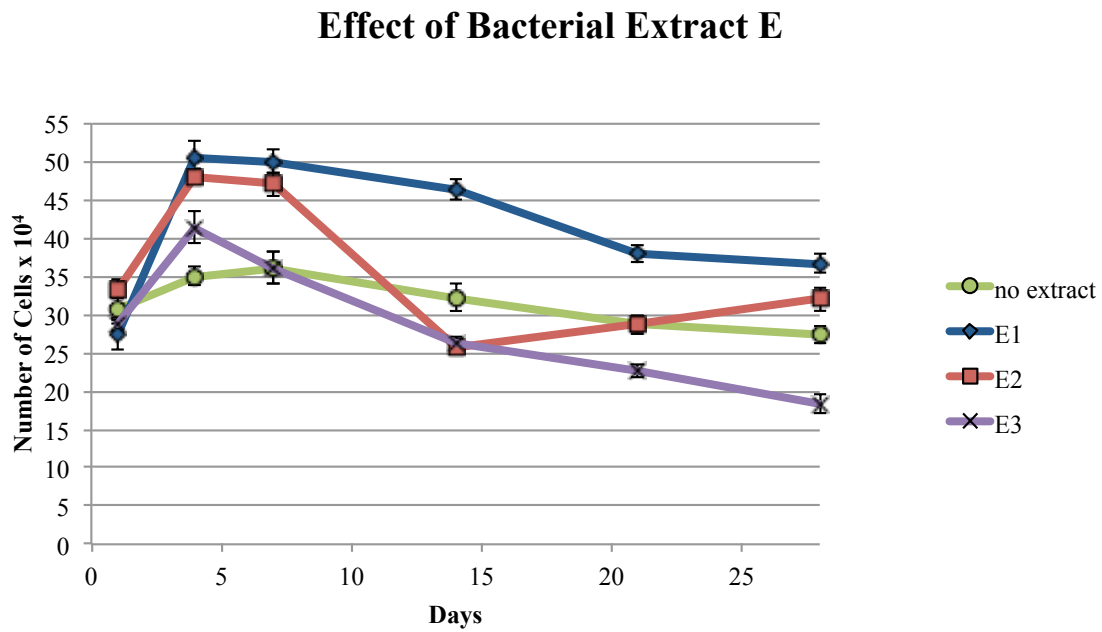
Figure 4.2: A graph of viable cell numbers that examines the effect of bacterial extract E in CeM1 media.

Effect of Changing Media: G11012

Figure 4.3.1: A graph of viable cell numbers that examines the effect of changing CeM1 media that contains FBS lot G11012.

Effect of Changing Media: F12087
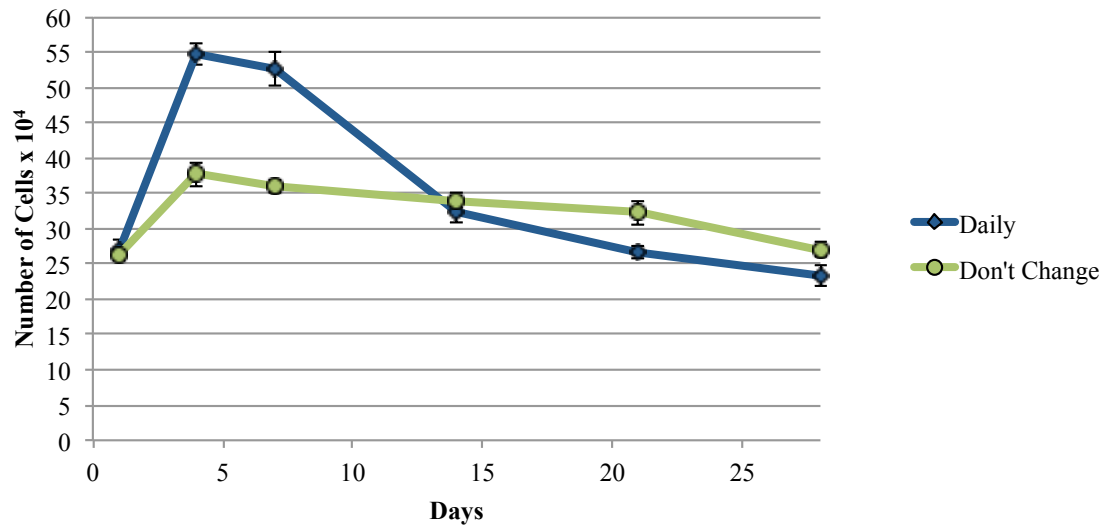
Figure 4.3.2: A graph of viable cell numbers that examines the effect of changing CeM1 media

that has FBS lot F12087 substituted for lot G11012.

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    activity and function." <u>FASEB Journal</u> **8**(2): 217-225.

CHAPTER 5

DISCUSSION

Conclusion

In this thesis, I have documented the ingredients and conditions of *C. elegans* Media 1 (CeM1) that allow for prolonged survival of cultured *C. elegans* germ cells. CeM1 was created by making adaptions to the previously accepted media for *C. elegans*, developed by Kevin Strange (Strange, Christensen et al. 2007). This CeM1 media can support germ cell survival for at least 28 days without significant drops in cell numbers. In addition several additives were tested to see if they would allow for increased cell proliferation, and while most were harmful, vitamin B12 was found to be an essential ingredient for *C. elegans* germ cells. Finally, cell proliferation was observed between days 1-4 post-harvesting with FBS lot F12087 and with certain bacterial extracts. These substances should be further explored to see if they can be modified to allow for prolonged cell proliferation.

Designing Media and Considering Species Auxotrophy

Using Strange's media as a starting point, a better media, CeM1, was designed to allow for prolonged survival of cultured *C. elegans* germ cells. However, simply optimizing Strange's conditions (base media component, pH, osmolarity, FBS concentration, and FBS treatment) was not sufficient to create a successful media. In order to make this media optimized for prolonged germ cell survival, it required substances that *C. elegans* are auxotrophic for. The knowledge

that *C. elegans* require hemin chloride and cholesterol but cannot produce it themselves, and that the main sugar they use is trehalose allowed for significant improvement of the media. However, it also appears that simply customizing the media so that it contains these required compounds is not sufficient for creating a proliferating cell line. While these components keep the worms alive, the cells presumably require additional signals to tell them to divide, which must be elucidated in order to achieve cultured germ cell proliferation.

It is therefore quite interesting that germ cell proliferation is observed between days 1-4 in cells grown with FBS from lot F12087 (which has a high bacterial endotoxin level) and when grown with certain bacterial extracts. It therefore appears that germ cells require a signal to proliferate that is provided from their diet - the bacteria they eat. Further research should be carried out to identify what these signals are.

The Relationship between *Caenorhabditis elegans* Longevity, Starvation, and Germ Cell Proliferation

There appears to be an interesting, inverse relationship between germ cell proliferation and animal longevity. This relationship first began to be discerned from the observation that *daf-16* loss of function mutant worms have increased germ cell proliferation (Lapierre and Hansen 2012). DAf-16 is a FOXO transcription factor that activates genes to increase worm longevity. As well, removal of the germ line allows *C. elegans* worms to live for an extended period time (Lapierre and Hansen 2012). While this relationship initially seems perplexing, I will propose a quite probable, but speculative, explanation for why this relationship occurs.

DAF-16 is thought of as a protein that promotes animal longevity, working within a longevity pathway. However, I do not feel that thinking of DAF-16 and other proteins that promote prolonged worm survival should be thought of as "longevity genes. " Rather, they should be thought of as "starvation response" genes. In a laboratory environment, when genes that promote the longevity pathway are upregulated or genes that inhibit the longevity pathway are knocked down, the *C. elegans* worm live for longer than normal- hence, they are known as longevity genes. But this is a very artificial environment and the worms are grown up on *E. coli* seeded agar plates providing them with ample food to feed on and the genes are forced on at a time they would not be activated in the natural environment. I believe that the worm lifespan longevity is a side effect, not a direct result, of these genes.

To understand the actual purpose of these genes, one must look at what signals cause these longevity genes to become activated naturally. One of the key longevity pathways is the insulin/insulin-like growth factor (IGF-1) pathway, with insulin inhibiting the longevity pathway and DAf-16 promoting the longevity pathway. When glucose levels are high (meaning that the worm has ample food supply), insulin signaling is elevated, and the DAF-16/FOXO transcription factors are inhibited, meaning the longevity genes are not transcribed. On the other hand, when glucose levels are low (meaning that the worm is starving), insulin signaling is reduced, and the DAf-16/ FOXO transcription factors are activated, and the longevity genes are transcribed (Lapierre and Hansen 2012). And in wild, starving worms, the activation of these longevity genes does not necessary lead to an increase in the worm's lifespan. Rather the worm is minimizing energy expenditure so that it can survive until it eventually finds food sources. These longevity genes work by inhibiting non-essential functions, such as worm growth and

75

reproduction. In the wild, starving *C. elegans*, this means that the worm is not wasting resources that it cannot spare on processes that are not essential for its immediate survival. However, in these mutated, non-starving worms grown in the lab, this means that non-essential processes are still being inhibited, so resources aren't being spent on fueling them, but the worms also have ample food supply, and therefore, the worms are functioning in a sort of "power saver mode" allowing them to live longer than normal. Therefore, it seems logical that DAF-16 and the longevity pathway would inhibit germ cell proliferation, as this is part of the non-essential task of reproduction. Insulin is an important regulator to use in this pathway as insulin levels are a good indicator of how much food/energy the worm is receiving, and therefore indicates whether the worm has enough resources to invest in nonessential tasks or if it needs to conserve energy and shut down non-essential processes.

It is worth noting that, while these processes are non-essential in the short term, or possibly for the individual worm, in the long term, and for the species as a whole, it is very important that these tasks are not permanently inhibited for the worm in order to have prolonged lifespan, as this would lead to the production of too few progeny.

In Chapter 3, four different additives were tested to see their effect on germ cell proliferation, and while it was unknown how they would impact the germ cells when the experiments were set up, in retrospect, looking at them from the perspective of the inverse relationship between longevity promoting genes and germ cell proliferation, the obtained results make logical sense. Vitamin B12, which works against the longevity pathway, was found to be essential for germ cell survival (although it did not promote cell proliferation). On the other hand, dafachronic acid, amino acids (that regulate the vitamin B12-dependent dietary senor/

worm longevity pathway) (Watson, MacNeil et al. 2014), and sphingosine-1-phosphate, which all promote cell longevity, were found to be detrimental to the germ cells.

In Chapter 4, the results indicate that *C. elegans* germ cells require a signal from bacteria in order to proliferate. While it is only speculation, one possible explanation to explain this necessity is that the bacteria works against this same longevity pathway, akin to the insulin. Just as the insulin provides signals to the worm, telling it that it is receiving enough food to invest in non-essential tasks, such as reproduction, the bacteria might provide a similar signal. Therefore, it makes logical sense that the germ cells might require a signal from the bacteria, telling them that they are receiving enough food to undergo reproduction and don't need to activate the longevity/starvation pathway. In the mutant *cki-2; glp-1; daf-16* worms, they cannot form gametes, and therefore the bacteria signal tells the germ cells to divide but because they cannot differentiate, this explains the initial increase in germ cell numbers.

However, it is troubling that this increase is only seen in the first few days and then the cell number plateaus. An important next step would be identifying how to keep the germ cells dividing. Perhaps the worms are running out of signals from the added bacterial extract and therefore, more bacterial extract must be supplemented to the media every few days. Perhaps there are other signals in addition to this bacterial signal, which must be identified to keep the proliferation going. It would also be beneficial to try other additives that inhibit the longevity pathway. And it is also possible that the bacterial extract gives a necessary signal unrelated to the longevity pathway.

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