Development of an endothelial-like cell line from the brain of the American eel and its use to study the effects of selenium

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

I hereby acknowledge the contributions of the co-authors listed in the pertinent chapters, and that my own contribution as the primary author conforms to the requirements stated by the University of Waterloo. The co-authors were involved in the data analysis, data collection and writing advice.

Abstract

A cell line, eelB, has been developed in this thesis from the brain of the American eel, *Anguilla rostrata* (Lesueur), an endangered species and belonging to a group of fish that for unknown reasons appear to be in worldwide decline. EelB was characterized and used to study the action of the selenium (Se), which is just one of many ecotoxicants that might be contributing to their decline.

EelB was developed from the outgrowth of adherent cells from brain explants of the American eel. EelB cells have been grown routinely in L-15 with 10 % fetal bovine serum (FBS), undergone over 100 passages, and cryopreserved successfully. The cells from late passage cultures (>45) were polygonal, formed capillary-like structures (CLS) on Matrigel, and stained immunocytochemically for von Willebrand Factor (vWF) and for three tight junction proteins, zonula occludens-1 (ZO-1), claudin 3, and claudin 5. These results suggest that eelB is an endothelial cell line, one of the few from fish and the first from brain. Despite this, eelB did not respond to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) with the induction of CYP1A protein. The cells from early passage cultures (<20) had more varied shapes and did not form CLS on Matrigel. Only cells from early passage cultures formed in suspension 3-dimensional aggregates that had some cells expressing alkaline phosphatase and nestin. These cells are thought to be neural stem cells and the aggregates, neurospheres. The emergence of endothelial-like cells upon the continued subcultivation of cells from early passage cultures that had neural stem cells has been described previously for mammals, but this is a first for

teleosts. Remarkably cells from all passage levels stained strongly for senescence-associated β-galactosidase (SA β-Gal) activity.

EelB was used to study the effects of Se deprivation and of Se addition. This was done with three exposure media: complete growth medium (L15/FBS), serum-free medium (L15), and minimal medium (L15/ex). L15/ex contains only galactose and pyruvate and no obvious source of Se, allowing Se deprivation to be studied. In L15/ex, eelB cells survived for at least 7 days, formed capillary-like structures (CLS) on Matrigel, and migrated to heal wounds. Three Se compounds were added to cultures: selenite, selenate, and selenomethionine (SeMet). Adding selenite or selenate to eelB cell cultures for 24 h caused dose-dependent declines in cell viability, regardless of the exposure media. Although varying with exposure media and viability endpoints, selenite was approximately 70-fold more cytotoxic than selenate. By contrast, 24 h exposures to either DL- or L-SeMet in the three media caused little or no cytotoxicity. However, for 7-day exposures in L15/ex, DL-, and L-SeMet were very cytotoxic, even at the lowest tested concentration of 31μM. By contrast in L15 and L15/FBS, cytotoxicity was only observed with 500 and 1,000 μM L-SeMet. In L15/FBS, eelB continued to migrate and form CLS in the presence of SeMet but at 500 μM cell migration appeared stimulated. As judged from a colony-forming assay over 14 days in L15/FBS, 500 and 1,000 μM DL- and L-SeMet inhibited cell proliferation. Overall, the responses of eel cells to selenium depended on the selenium form,

concentration, and exposure media, with responses to SeMet being most dependent on exposure media.

Cell lines are a resource for research into applied and basic scientific issues and eelB should be useful for a wide range of problems, especially exploring the actions of ecotoxicants and viruses of the eel, all of which might be contributing to their population decline.

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List of Abbreviations

AB	Alamar blue
AhR	Aryl hydrocarbon receptor
AhRR	Aryl hydrocarbon receptor repressor protein
ANOVA	Analysis of Variance
AP	Alkaline phosphatase
BAE	Bovine aortal endothelial cell line (*Bos taurus*)
BB	Blocking buffer
BBB	Blood-brain barrier
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
BLAST	Basic Local Alignment Search Tool
BLBP	Brain lipid binding protein
BOLD	Barcode of Life Data
BRCE	Bovine retinal capillary endothelial cells
BSO	Buthionine sulfoxine
C6	Rat glial cell line (*Rattus norvegicus*)
CF	5-carboxyfluorescein
CFDA-AM	5-carboxyfluorescein diacetate, acetoxymethyl ester
CLS	Capillary-like structures
COSEWIC	Committee on the Status of Endangered Wildlife in Canada
CYP1A	Cytochrome P450, family 1, subfamily A, polypeptide 1
D-PBS	Dulbecco's PBS
DAPI	4',6-diamidino-2-phenylindole
DCFH-DA	2,7-dichlorodihydrofluor-escein diacetate
dd H_2O	Double-distilled water

DICD	Detachment-induced cell death
DIO	Thyroid hormone deiodinases
DMSO	Dimethyl sulfoxide
DOI	Den Oever Index
EA.hy926	Human hybridized umbilical/carcinoma cell line
EC	Endothelial cells
EC50	Effective concentrations causing a 50% decline in cell viability
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EelB	Eel brain cell line (*Anguilla rostrata*)
EHS	Engelbreth–Holm–Swarm mouse sarcoma cells
EROD	7-ethoxyresorufin-0-deethylase
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FRC	Fibroblastic reticular cells
GBC4	Orange-spotted grouper cell line (*Epinephelus coioides*)
GFAP	Glial fibrillary acidic protein
GPx	Glutathione peroxidase
GSH	Gluthatione
H_2DCFDA	2,7-dichlorofluorescein diacetate
H_2Se	Selenide
HaCaT	Human keratinocyte cell line
HBMEC/ciß	Human brain cell line
HCT116	Human intestinal epithelial cell line
HeLa	Henrietta Lacks human cervical carcinoma cell line

Hepa	Mouse hepatoma cell line
HMEC-1	Human microvascular endothelial cell line
HTB123/DU4475	Human mammary tumour cell line
HTB140	Human melanoma cell line
JEECV	Japanese endothelial cells-infecting virus
K-562	Human erythroleukemia cell line
L15/ex	Exposure medium, i.e. basal medium salts with galactose and pyruvate
L15/FBS	Growth media, i.e. basal with FBS
L15	Leibovitz 15 basal medium
LLC-PK1	Porcine renal epithelial cell line
miRNA	Micro ribonucleic acid
MS-222	Tricaine methanesulfonate
MSC	Mesenchymal stem cell
MSeA	Methylselenic acid
MT-4	Human leukemia cell line
NAC	N-acetyl cysteine
NBT	Nitro-blue tetrazolium chloride
NCBI	National Center for Biotechnology Information
NF	Neurofilament
NPC	Neural progenitor stem cells
NR	Neutral red
NSC	Neural stem cells
NSERC	Natural Sciences and Engineering Research Council of Canada
NSPC	Neural stem/progenitor cells
P	Passage

PAD	Polyclonal Antibody Designation
PAH	Polycyclic aromatic hydrocarbon
PBLE	Eel peripheral blood leukocyte cell line (*Anguilla rostrata*)
PBS	Phosphate-buffered saline
PC	Pericytes
PC-2	Human prostate cancer cell line
PCB	Polychlorinated biphenyl
PFA	Paraformaldehyde
PLHC-1	Desert topminnow hepatoma cell line (*Poeciliopsis lucida*)
RFU	Relative fluorescence units
ROS	Reactive oxygen species
RTL-W1	Rainbow trout liver cell line (*Oncorhynchus mykiss*)
SA β-Gal	Senescence-associated β-galactosidase
SBB-W1	European seabass brain cell line (*Dicentrarchus labrax*)
SBP	Snubnose pompano brain cell line (*Trachinotus blochii*)
Se	Selenium
SeMet	Selenomethionine
SeNP	Selenium nanoparticle
SGIV	Singapore grouper iridivirus
SNU-1	Human gastric adenocarcinoma cell line
Src	Sarcoma
TB2	Tilapia brain cell line (*Oreochromis mossambicus*)
TCDD	2,3,7,8-tetrachlorodiobenzo-*p*-dioxin
TEM	Transmission electron microscopy
TJ	Tight junction

TrxR	Thioredoxin reductases
TY08	Human brain microvascular endothelial cell line
UV	Ultra violet
VECNE	Viral endothelial cell necrosis
VNNV	Viral nervous necrosis virus
vWF	von Willebrand Factor
WE-cfin11f	Walleye fin cell line (*Sander vitreus*)
WE-cfin11fe	Walleye fin cell line (*Sander vitreus*)
WE-spleen6	Walleye spleen cell line (*Sander vitreus*)
WEBA	Walleye bulbous arteriosus cell line (*Sander vitreus*)
WPB	Weibel-Palade bodies
ZO-1	Zona occludens-1

Chapter 1: General Introduction

The overarching scientific problem investigated in this thesis is the decline in American eel populations that has been observed over the last 30 years. This is a complex problem that is being studied by ecotoxicologists, environmental chemists, and fish biologists. The approach of this thesis is a cell biological one. There are two specific goals.

1.1 SPECIFIC GOALS

1. to develop and characterize a cell line from the American eel (Chapter 2).

2. to use the cell line to investigate the actions of selenium compounds on eel cells (Chapter 3)

1.2 AMERICAN EEL (*ANGUILLA ROSTRATA*)

1.2.1 Life Cycle

The American eel has a very complex life cycle (see Figure 1.1). It migrates up to 6000 km to spawn in the only known spawning place so far: the Sargasso Sea. It reaches its reproductive maturity at an age of 22-30 years. The American Eel goes through six different stages during its life cycle. In the first stage, the eggs hatch supposedly about one week after being laid (Tremblay, 2012). The hatched larvae are called leptocephali. During this stage they are in salt water and start to migrate mostly by drifting in the direction of coastal waters. The larva is willow leaf shaped and transparent, and develops in about 7-12 months. Their gut structure suggests that they

feed by absorbing dissolved organic carbon. However, observations of gut contents suggest that they occasionally feed also on zooplankton fecal pellets (Tremblay, 2012). In the third stage, the larvae develop into glass eels with shape closer to adult eels, but much smaller and still transparent. The glass eel will transition from salt water to freshwater during its migration. The metamorphosis into glass eels usually starts when they reach the continental shelf, and lasts about 55 days, while they are migrating to estuaries. Laboratory experiments on European eels suggest that glass eels are morphologically and physically not able to eat (Tremblay, 2012).

While the eels are heading towards the tributaries they start to become pigmented, entering the elver stage. As they start to pigment and enter that stage they have also been observed to feed on insect larvae. The elver stage lasts about 3-12 months and during this stage the eels are still sexually undifferentiated. Elvers spend much of their time swimming upstream, and the elver migration seems to be connected to environmental cues such as increases in water temperature and reduced flow streaming as well as to the tidal cycle. Once they enter coastal waters and transition into a new stage, they also transition from being in a pelagic state to being in a benthic state (Tremblay, 2012).

The next stage is the yellow eel stage. Yellow eels occupy a large range of habitats such as fresh water or salt water, rivers, creeks, lakes, tidal regions, estuaries, and marshes. In this stage, major growth and physical changes occur, as well as sexual differentiation. Their bellies become yellow, green, or brown, and their backs are

black. There is a large mucous production, which acts as protection. Not all factors are known that influence sexual differentiation. However, density was reported to play a major role. High densities favour male differentiation, while low densities favour female differentiation. Canadian waters seem to host mostly female eels except for the Scotia Fundy region where males seem to be prevalent (Tremblay, 2012).

Eel growth depends on different environmental factors such as temperature, salinity, food availability, and geography. For example, eels in salt water grow much faster than eels in fresh water. Yellow eels spend a large amount of time buried in sediment or substrates and usually come out at night for feeding during summer. They are benthic omnivores and feed mostly on molluscs, fish, insects and their larvae, worms, and plants. During winter they are reported to go into a state of torpor at temperatures below 5°C; however, some active eels have also been observed (Tremblay, 2012).

In the sixth stage, when eels start to prepare to migrate back to the Sargasso Sea to spawn, they metamorphosize into silver eels. The metamorphosis includes physical and physiological changes. Their colour changes to greyish, their digestive tracts degenerate, and their pectoral fins grow larger, their eyes change to adapt to the oceanic environment, and they store lipids for their journey. Their reproductive organs continue to develop during their migration (Tremblay, 2012).

Silver eels manifest considerable differences according to their geographic location. The farther from the spawning site, the larger the silver eels seem to be. Also, females

seem to be older and larger compared to males when they start to migrate (Tremblay, 2012).

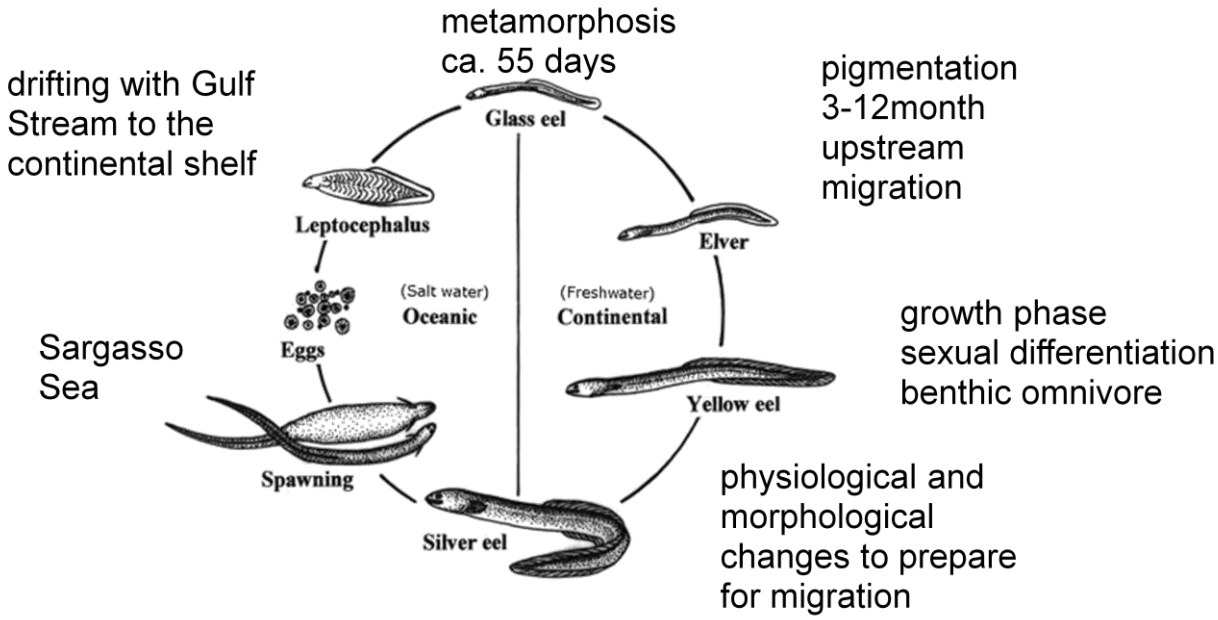

Figure 1.1 American eel life cycle. Adapted from Ministry of Natural Resources (2010).

1.2.2 Importance of American Eels

American eels are top predators and are therefore important to keep the species ratios in balance. If the American eel is missing, there is a higher chance of invasion by invasive species, such as the zebra mussel (Ministry of Natural Resources, 2010). As a top predator, the American eel plays an important ecological role, but also has a great potential for bioaccumulation/magnification of pollutants, for example selenium, through the food web. Furthermore, the American eel, with its complex and probably fragile life cycle, is also an important indicator of habitat integrity (MacGregor et al.,

2011). Moreover the American eel is a culturally and nutritionally important fish for native communities, as well as for the fisheries economy. The eel fisheries were valued at about $600,000 during the 1980's and the early 1990's (MacGregor et al., 2011; Ministry of Natural Resources, 2010).

1.2.3 Eel Population Decline

In the last three decades, a dramatic decline of the eel population in Canadian waters was observed. American eels made up about 50% of the fish biomasses and were therefore one of the most abundant species in Canadian waters (Tremblay, 2012). In the 1600's, fishers reported catches of up to a 1,000 eels per day. Up to the early 1980's, the commercial catches were 223,000 kg per year, but they declined to 11,000 kg in the year 2002 (Ministry of Natural Resources, 2010). The eastern ranges of the American eel still seem to be in decline but manifest a less important one. However, the density studies available are limited (Tremblay, 2012).

The most consistent, non-fishery-dependent population density data comes from the St. Lawrence River and Lake Ontario region (Tremblay, 2012). In 2012, the American eel status was changed from being of special concern to threaten.

1.2.4 Possible Causes of the Decline

The exact reason for the decline is not known; however, it is very likely that several factors play a major role in the decline. American eels living in fresh water apparently are more affected then American eels residing only in salt water. This difference implies that there might be a particular problem affecting fresh water (Tremblay, 2012).

According to Tremblay (2012), the main possible reasons for the decline are physical barriers disrupting migration, turbines of hydroelectric dams, human-produced chemical pollution, overfishing, climate change, change in oceanic conditions, and the occurrence of an exotic swim bladder nematode parasite.

Given the complex life cycle of the American eel it is very difficult to determine a definitive cause for the observed population decline. There might be multiple causes and some of them might occur during parts of the migration journey that are still unknown to us. It is possible that causes occur only in some of the geographical locations where the eel spends time during its life cycle and the symptoms arise at different geographical locations which are not related to the source of the symptoms. American eels lay their eggs in the Sargasso sea, and they might be affected by pollution or environmental changes due to natural or anthropological causes there; however, maternal transfer of pollutants, which might impair the proper development of the larvae is also possible (Byer et al., 2013a; Hodson et al., 1994). This is difficult to determine, because we do not have access to the larvae to take samples for the examination of these issues. The impaired development can manifest at different stages of their migration. For instance, deformed larvae might not even be able to start the migration in the proper way. On the other hand, development can also be impaired at a later stage of the migration, which makes the continuation of the migration difficult or impossible, or the impairment might manifest itself at the time of reproduction.

Eels will accumulate large amounts of lipid storage during their growth phase. Once eels start their migration, they will fast until they spawn; therefore, the lipid storage is essential for their migration, gamete production, and spawning. The accumulation of lipid storage as an energy source for their migration can bring a series of challenges in the present polluted environment. The lipid content has to exceed 20% of their body mass to be able to successfully migrate and spawn (Robinet & Feunteun, 2002). Pollutants today can either affect the ability of the eels to store lipids, or can accumulate in the lipid tissue. Unfortunately, they will be released gradually during the migration or at the time of the spawning. It can therefore harm the eel during its migration or affect the eggs (Robinet & Feunteun, 2002). The American eel today will encounter high levels of pollutants all along the way of its migratory journey and its different habitats. The composition of pollutants might change at each geographical location. Some might have greater or lesser effect, while some may have a cumulative or synergic effect. This makes it very difficult to pinpoint the problem. Pollutants might be just straightforwardly toxic and kill the eel, or they might have a slow impact on their lives by impairing their hormonal systems and other physiological processes. An impaired hormonal system might not allow for proper reproduction, and it can also impair the proper physiological signalling needed to start the different stages of migration. Chemical pollutants can be absorbed via different pathways, such as direct ingestion, absorption through the skin, or through bioaccumulation and biomagnifications through the food chain. The eel is a top predator and is therefore exposed to chemicals that are ingested by the organism the eel is feeding on. For

example, yellow eels feed on mussels. Mussels are known to be able to store and accumulate chemical pollutants and their level of tolerance is often higher than the tolerance of their predators (Linville, Luoma, Cutter, & Cutter, 2002). Furthermore, the food chain experiences changes, often also caused by human activities, such as the introduction of invasive species (Linville et al., 2002). An invasive species can radically change the food chain structure and have a deep impact on top predators, either by destroying the food source, or if the invasive species is able to accumulate and tolerate higher levels of chemical pollutants and becomes a new food source of the predator, it can intoxicate the predator. For instance, Hodson et al. (1994) reported that eels were contaminated with PCB and Mirex and other chemicals in the St. Lawrence River. However, they also observed a decline in contamination levels in the time span of their study. Also a later study conducted by Byer et al. (2013b) confirms that PCB levels remained low in less industrialized areas, while continuing to be high in industrialized areas. Albeit, given the long life cycle of the American eel, the effect of pollution peaks may be seen only at a later point in time than they occur. Especially with PCBs, which are known to be embryotoxic, it will only be possible to completely assess their effect when data obtained from larvae are available (Byer et al., 2013b). Castonguay et al. (1994) observed that the condition factor decreased over time, which affects the ability of the eels to complete their migration. The decrease of the condition factor can be caused by several stressors, like chemical pollution. Even if a decline in pollution is observed, sublethal chronic exposure can cause a decrease of the condition factor.

Eels use physiological cues, but also environmental cues, for their transition between life stages. As mentioned above, the physiological cues might be thrown off due to chemical pollution. Unfortunately, many environmental cues are also different then they used to be, because of anthropological changes in water systems (Verreault, Mingelbier, & Dumont, 2012). Many rivers have been diverted, their flows have become controlled, and many physical hindrances have been built. Water temperatures are changing due to climate changes or more directly because of landscape changes such as the removal of riparian vegetation or the use of river water for the cooling of industrial waters (Verreault et al., 2012). Females and males were observed to choose separate geographic locations to grow to sexual maturation before they meet in the Sargasso Sea to mate (Krüger & Oliveira, 1999). This geographical separation can bring a whole set of other issues, such as a potential female/male imbalance due to differing issues at each location for females or males. For example, they are exposed to a different range of chemical pollutants or more physical barriers that might impede or slow down the migration of one or the other mate, disturbing mating timing (Krüger & Oliveira, 1999). Verreault et al. (2004) reconstructed the extension of the original habitat range of the American eel in the St. Lawrence River watershed and reviewed the structural changes of the water systems. In the original habitat range, 151 dams with turbines and 8,260 without turbines were built. The dams without turbines are mostly used for drinking water, flood control, and for recreation purposes. In the same report, Verreault et al. (2004) document how these obstacles limit and obstruct access to a surface of 12,140 km² of fresh water suitable for eel growth. Carr et al. (2008)

provided 25 American eels with a sonic tag to observe their passage through a recently built hydroelectric dam in the Magaguadavic River. Of the 25 eels tagged, 19 eels ended up in the turbines and died. In 2004, Verreault et al. estimated a mortality rate of 40% for the eels that leave Lake Ontario for spawning and have to pass through the Moses-Saunders and the Beauharnois hydroelectric dams. Furthermore, turbine mortality was connected to eel length and turbine blade spacing, which puts the large female eels from the upper St. Lawrence River at a higher risk (Tremblay, 2012; Verreault et al., 2004). There has been an effort to provide eels with ladders at many hydroelectric dams; however, as the Carr and Whoriskey (2008) study confirms, not all the eels find the ladders. Verreault et al. (2012) also analysed migration data from a time period of 1843-1872 and the contemporary time period 1963-1990, and concluded that today, eels need, on average, 18 days more for their migration out of the freshwater system in the direction of the ocean. They speculated that the reasons for the delay might be obstacles or altered hydroclimatic conditions. Not only freshwater condition changed, but also the condition in the oceans. Ocean condition changes due to climate change; for example because of increased surface temperatures the mixing of the water column is less prominent, which has an influence on the currents, which are most probably used by the eels as cues to find their spawning places (Bonhommeau et al., 2008). Currents that the larvae most probably are using to drift to continental shelf are also altered because of climate change. Furthermore, the change in surface temperature also decreases the primary production and thus the food source for migrating larvae is compromised (Bonhommeau et al., 2008). Friedland et al. (2007) analysed the North

Atlantic oscillation (NAO), the surface temperatures, wind currents, ocean currents, and mixed layer depths, and compared them to the Den Oever Index (DOI), which is an index developed by a yearly sampling of eels since 1938 in Den Oever in the Netherlands. The comparison of the above-mentioned parameters and the DOI suggests a correlation with the recruitment decline (observed in European and American Eels) since the 1980s. However, the correlation can be only inferred, because we do not have access to sampling eels during their migration in the open ocean, at their spawning site, and at their larval stage.

1.3 FISH CELL LINES

The first reported fish cell line was described in 1962 by Wolf and Quimby and is derived from rainbow trout gonad cells. Initially, fish cell lines were used mostly in virology, but now fish cell lines are used in carcinogenesis, toxicology, genetics, and biomedical fields. Fish cell lines are easier to maintain then mammalian cell lines, because they thrive also when not frequently subcultivated, need less medium change and have a larger range of temperature tolerance (Fryer & Lannan, 1994). In 1994 Fryer and Lannan listed 125 reported fish cell lines from 52 species. In 2011 Lakra et al. listed 124 more published fish cell lines. Fish cell lines are useful for cell biology, but also for species-specific studies of diseases and toxicology at the cellular level. Furthermore, there are fish cell lines from all sorts of organs, such as the liver, heart, fins, skin, spleen, bladder, brain, and others. From the 125 cell lines listed in Fryer and Lannan (1994) only 14 were from tumorigenic tissue, all the rest were derived from normal tissue. Seemingly, the first investigators to use fish cell culture to study aquatic

toxicants were Rachlin and Perlmutter (1968). They compared zinc toxicity of whole fathead minnow to toxicity on a fathead minnow cell line. They used the mitotic index as an endpoint and concluded that the cell culture was more sensitive to the toxicant than the whole organism. Since then many more ecotoxicological studies have been performed with fish cell lines and several endpoints used and developed. Fish cell lines have been used, for instance, for chemical toxicity ranking, to study relative toxicity of a chemical, exploring synergistic and antagonistic reaction, and to study biotransformation (Babich & Borenfreund, 1991). Since the beginning, there is continuous research done to improve and establish the position of fish cell lines in ecotoxicology. For instance, an early study was carried out by Kocan et al. (1979), in which they explored the toxicity of 8 mutagens on 3 fish cell lines. Their endpoint was cell growth, i.e. they exposed the cells to the compounds and counted the cells after 72h. They concluded that fish cell lines are sensitive to the compounds tested and that the different cell lines can have different sensitivity levels to the same compound. In their study BF-2 responded more sensitively to most compounds tested compared to the other two cell lines (RTG-2 and STE) used. Marion and Denizeau (1983) studied the toxicity of lead and cadmium in two separate studies on the fish cell line RTG-2. They used the measurement of total protein, RNA, and DNA, as well as [3H]thymidine in DNA and [14C]uridine in RNA incorporation. In both studies the fish cell line was sensitive to the toxicants and the toxicity comparable to that of the whole organism. Bols et al. (1985) used RTG-2 to study the toxicity of phenols, benzenes, and anilines and compared the cytotoxicity to whole organism data. Their endpoint was the ability

of the cells to attach to the surface after chemical exposure. They concluded that the cytotoxicity strongly correlated to the whole organism but were about three orders of magnitude less sensitive. Babich et al. (1986) tested eighteen metal salts on BF-2 using neutral red as a viability test and concluded that there was a significant correlation between the *in vitro* and *in vivo* results. The above examples are the beginning of an important movement: since then many more studies to characterize fish cell lines for ecotoxicological studies were undertaken, and are still undertaken. The increase in pollution and awareness of its risk raises demand for time-efficient and economical ecotoxicological testing and tools. The fish cell lines have a large potential to satisfy this increasing demand. Therefore, the need of fish cell line characterization in regards to their maintained properties, sensitivities, and endpoints is crucial. In 2006, Schirmer reviewed and discussed the current status quo, and the different available fish cell lines and endpoints. Although the reviewed studies showed that the *in vivo*/*in vitro* relative toxicity levels correlate considerably, the absolute sensitivity does not. This is a major challenge for fish cell lines, because the minimal sensitivity makes it difficult to determine safe chemical levels. A limiting factor at this point is the limited target sites of a cell line with respect to a whole animal. However, one way to approach this challenge is the development and characterization of appropriate fish cell lines, which maintain the needed properties of the original tissues, as well as developing them from different species. In addition to obtaining the right fish cell lines, models made out of several fish cell lines can be developed, i.e. sets of successive fish cell lines, on which environmental toxicants and waste water can be screened or tested (Schirmer, 2006).

The exposure environment has also an influence on the sensitivity. For example, serum can protect the cells and interact with the toxicant to be tested and in doing so, diminish the sensitivity. Therefore, in many cases, serum-free exposure media, for instance L15ex, might be favourable. But it depends also on the endpoint: for example, proliferation as an endpoint might need serum. Thus, when a cell line gets tested it is important to study also the aspects of how the cell reacts in different media (Schirmer, 2006). For instance, Schirmer et al. (1997) developed the exposure medium L15ex to measure photocytotoxicity. This was necessary because the UV radiation would react with the serum components and produce toxicity. Furthermore, it was useful to dissolve PAH, which had been a challenge in the past. This improved considerably the fish cell line as a model to study PAH. Dayeh et al. (2005) found that copper was only cytotoxic when it was dissolved in L15ex, but not when it was in L15, with or without serum.

The right choice of endpoints is also a very important criterion to enhance the sensitivity of fish cell lines. For acute toxicity, for instance, cell viability is a straightforward endpoint: there are different cell viability tests such as the Trypan blue exclusion test, or the combination of Alamar Blue (AB), 5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester (CFDA-AM) and Neutral Red (NR). AB measures metabolic activity, CFDA-AM measures cell membrane integrity, and NR measure lysosomal membrane integrity. These three dyes can be used together on the same set of cells, which makes a quick test that includes several cellular responses and can be easily used routinely (Bols, Dayeh, Lee, & Schirmer, 2005; Schirmer et al., 1997). Cell detachment and proliferation are also usable endpoints. More specific endpoints can be

the expression of CYP1A for dioxin-like compounds. Not all cell lines maintain the ability to express this enzyme, but several do, such as RTL-W1 and PLHC-1 (Bols et al., 2005). Another endpoint can be oxidative stress, and this has been measured successfully using the dye 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Selvaraj, Tomblin, Armistead, & Murray, 2013).

1.4 EEL CELL LINES

As discussed earlier, the American eel (*Anguilla rostrata*) is experiencing a considerable decline. To study the reasons for the decline is particularly challenging in the case of the eels, because of their complex and the wide geographic distribution of their life cycle. Therefore, models to study eel physiology and impact of pollution are sought. Cell lines derived from this species have a high potential to produce important information about the physiology and ecotoxicology of eels and give hints about their decline. It would be particularly useful to have cell lines from different organs of the eels. For instance, Garrick et al. (2005) used early American eel endothelial cell lines derived from different organs, such as rete mirabile, heart, and kidneys to study the induction of CYP1A and EROD activity and found a differentiated response by the different organs.

However, so far there are few established permanent cell lines derived from eel species in general. Japanese eel cell lines, which were derived from ovary, kidney, and viscera, have been reported (Fryer & Lannan, 1994; Lakra et al., 2011).

For the American eel, the early endothelial cell lines derived from the rete mirabile of adult eels were described by Garrick (2000). Dewitte-Orr et al. (2006) described and characterized a continuous American eel cell line derived from peripheral blood leukocytes of American eel PBLE. The cell line was susceptible to chum salmon reovirus. In this study another continuous eel cell line, derived from the eel brain, will be described, which expresses endothelial cell properties.

1.5 BRAIN CELL LINES

In this thesis the development of a continuous cell line of the brain of an eel is going to be described. The cell line arose from the explants of the brain of a female silver-stage American eel. The explants started to grow and over time developed into a continuous cell line with endothelial properties. In the last decades there have been several reports of brain fish cell lines from different fish species, which developed into a variety of cell types with different characteristics and with several potential uses. F. Zeng et al. (2014) describe a cell line from the brain of Japanese flounder which grew out of seeded brain fragments. The cell developed into epitheloid-like cells. They stained for glial fibrillary acidic protein (GFAP) which is considered a marker for astrocytes, therefore the authors concluded that the cells were astrocytes. Huang et al. (2011) described a brain cell line of a red spotted grouper EAGB. In the initial culture the cells were epithelial and fibroblast-like, but with time they became only fibroblast-like. The cells were tested for virus susceptibility and were positive for viruses such like Singapore grouper iridovirus (SGIV) and viral nervous necrosis virus (VNNV). Wen et al. (2008a) isolated a tilapia brain cell line TB2. The cell line expresses a

manifold combination of immunoreactivity, such as A2B5 for oligodendrocyte progenitor cells, GFAP for astroglia, brain lipid binding protein (BLBP) for radial glia, and others. The authors concluded that the cell line is an astroglial progenitor cell line with the potential to differentiate into neurons and oligodendrocytes. In another report Wen et al. (2010) describe another complex brain cell line derived from a snubnose pompano which was positive for oligodendrocyte markers and disposed tanycyte characteristics. These latter two cell lines are suggested to be used to elucidate further the differentiation of brain cells. Servili et al. (2009) developed a brain cell line from a sea bass. The cell line formed neurospheres, which were positive for nestin, a neural stem cell marker. The cell line included cell morphologies of glial and neural cells. It also was positive for other neural markers such as GFAP, neurofilament (NF), as well as Sox 2.

These few examples of reported brain cell lines give an idea of the potential for research in different fields, such as physiology of the nervous system, neurodegenerative diseases, neurotoxicity, and ecotoxicology. The potential lies in the general physiological and toxicological as well as in species-specific research. EelB with its endothelial characteristics will add to this pool of invaluable resources.

1.6 ENDOTHELIAL CELLS

Endothelial cells (EC) line the vascular system all over the organism and can therefore have a large variety of origins. Depending on the origin the EC can have different functions, but in general they are active carriers of molecules and hormones.

In the brain, for instance, the EC form the brain blood barrier (BBB). This protects the brain from unwanted and potential dangerous chemicals. Therefore, EC cell lines are a large resource for studying pollutants, which are able to surmount the BBB. EC are also involved in angiogenesis, the process of new vessel formation. Thus, EC cell lines are useful to study inhibitors or abnormal stimulators of angiogenesis, as well as pathologies which cause abnormal stimulation or inhibition of angiogenesis. There is a need to understand vascular pathologies due to disease and toxicants, therefore there is also a need for endothelial cell models to do research on (Bouïs, Hospers, Meijer, Molema, & Mulder, 2001). Several human endothelial cell lines were developed; some prominent ones are EA.hy926 and HMEC-1. Like many human cell lines they are derived from tumorigenic tissues, which has its own set of issues and challenges (Bouïs et al., 2001). EA.hy926 was generated by the hybridization of two cell lines: human umbilical vein endothelial cells, and the cell line A549, a human carcinoma cell line. EA.hy926 expresses factor VIII-related antigen, which is characteristic of endothelial cells (Edgell, McDonald, & Graham, 1983). HMEC-1 was developed from human microvascular ECs which were transfected. Among other EC characteristics it shows typical EC cobblestone morphology and expresses von Willebrand factor (vWF), and forms tubes when plated on Matrigel (Ades et al., 1992). Recently, specifically BBB human cell lines were established such as TY08 (Sano et al., 2010) and HBMEC/ciβ (Kamiichi et al., 2012), which both retain important BBB properties such as limited molecule penetration and efflux abilities. There are also a choice of animal BBB barrier models originating from rats, mice, and bovine, which have been reviewed by Terasaki

et al. (2003). The vast majority of them were mortalized by transfection or using transgenic animals of origin (Terasaki et al., 2003). One presumable advantage of fish cell lines is that they mostly become immortalized spontaneously (Bols et al., 2005). However, there are only a couple of endothelial fish cell lines described so far. One is the WEBA (Vo et al., 2015), which is derived from the bulbus arteriosus of the Walleye. WEBA expresses vWF, forms tubes on Matrigel, and takes up ac-LDL. Endothelial fish cells are also an important tool to explore physiological processes, but since fish are extensively used for ecotoxicology they are an invaluable tool in this field. As discussed above, the more fish cell lines are available, the more species-specific and target-specific research can be done, and routine monitoring with whole organism can be replaced by fish cell lines.

1.7 SELENIUM

Selenium is an essential trace element and is a component of important enzymes in vertebrates. It was originally known for its toxicity in mammals, until it was discovered to have nutritional importance as a trace element, and was recognized to be a fundamental component of the enzyme glutathione peroxidase (Spallholz, 1994). At the present, there are at least 25 known selenoproteins that contain the amino acid selenocysteine, which is decoded from the codon UGA (Papp, Lu, Holmgren, & Khanna, 2007). Several of the selenoprotein are not yet characterized and their function unknown. However, well known selenoproteins are thioredoxin reductase (TrxR), glutathione peroxidase (GPx), and thyroid hormone deiodinase (DIO); the first two are involved in redox regulation and the latter in thyroid hormone metabolism (Papp et al.,

2007). Selenium is gaining more attention in the health sector due to its redox regulation functions, which confer anti-oxidants properties (Rayman, 2012). Campbell et al. (2007) for instance explored the protective properties of Se against peroxidative damage on the endothelial cell line EA.hy926. They found that subtoxic selenium concentration in from of sodium selenite increased the concentration of thioredoxin reductase-1, and increased the GPx activity, which protected the cell from peroxidative damage. Selenium in the form of selenomethionine (SeMet) was also observed to stimulate cell migration and angiogenic response (McAuslan & Reilly, 1986).

In general there is an immense body of research done of the effect selenium has on human health. Deficiency of selenium has been connected to high mortality risk, poor immune function, and cognitive decline (Rayman, 2012). Selenium supplementation has been a very large research topic. To selenium have been attributed anti-cancerogenous and immune system enhancing properties (Rayman, 2012). There have been several large supplementation trials, which gave mixed results, but it is generally accepted that selenium has beneficial action for prevention and cure of lung, prostate, colorectal, and bladder cancer (Rayman, 2012).

The other big research field in which selenium is playing a major role is as an environmental concern. In the late 1970s there was the first reported case of major selenium pollution, which was in Belews Lake in North Carolina, with the selenium source coming from an efflux of the ash pond of a coal-fired power plant (Lemly, 2002c). Since then there has been a lot of discussion on how to handle selenium

pollution. The challenge of selenium pollution lays in its complex biogeochemical cycling and in the fact that it expresses its toxicity with bioaccumulation, and in addition to this, for as-yet-unknown reasons, selenium water concentrations do not predict the amount of bioaccumulation and thus toxicity (Chapman, 2009). Another case shortly after Belews Lake was the Hyco reservoir, which is a cooling reservoir for a coal-fired power plant (Baumann & Gillespie, 1986). A later case in the 1990s was the Kesterson reservoir in California, where the selenium source was agricultural drainage. The Kesterson reservoir has been closed, but the agricultural drainage is still a problem in the San Joaquin valley, where the reservoir was located (Hamilton, 2004; Ohlendorf, Hothem, Bunck, & Marois, 1990). These case studies gave a lot of valuable information on selenium pollution.

1.7.1 Selenium Sources in the Environment

Selenium is a metalloid and is present in the earth's crust and is distributed in the environment through natural processes and human activities. Natural processes include processes such as volcanic activity and rock weathering and may cause a regional threat. Human activities are much more effective in redistributing selenium and cause often more global hazards. Selenium is mobilized by human activities including oil refining, fossil fuel combustion, agriculture, and mining. These activities usually involve the "contact of a Se-containing matrix with water" and therefore the transportation of selenium into aquatic ecosystems (Chapman et al., 2010). The selenium is often accumulated in regions where the raw material is also present,

therefore increasing the potential of selenium-related harm to the environment (Chapman et al., 2010).

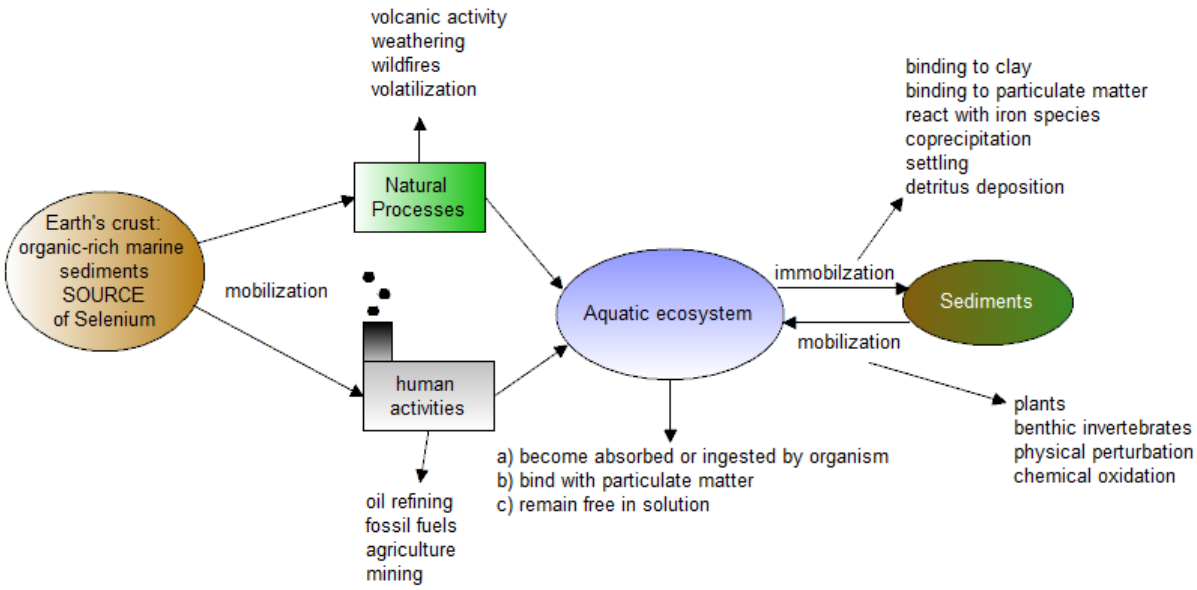

Figure 1.2 Selenium Cycling.

Selenium reaches aquatic systems mainly in the inorganic forms sodium selenate and sodium selenite (Chapman et al., 2010; Lemly, 2002b). In an aquatic system it is quickly taken up by microorganisms, plants, and invertebrates. Plants and invertebrates have a larger tolerance to selenium and also tend to accumulate selenium (Chapman et al., 2010).(Chapman et al., 2010) The inorganic forms get transformed to organic selenium forms by the microorganisms and by the plants and invertebrates that accumulate it (Chapman et al., 2010; Fan, Teh, Hinton, & Higashi, 2002). The most common organic product is selenomethionine, which is then more bioavailable to vertebrates (Chapman et al., 2010). Vertebrates are less tolerant to selenium accumulation, especially viviparous species such as fish and birds (Chapman et al.,

2010; Lemly, 2002b). Through the degradation of plants and other organisms selenium gets incorporated into the sediments of lakes, where it can accumulate to considerable concentrations, and become temporarily immobilized (Chapman et al., 2010; Lemly, 2002b). However, benthic life is able to mobilize the selenium from the sediments through physical and biological processes (Chapman et al., 2010; Lemly, 2002b). For example, a mussel invasion is able to loosen up the sediments and temporarily free the selenium from the sediment; furthermore, mussels are able to accumulate selenium and bring it into the food chain (Linville et al., 2002).

1.7.2 Selenium Toxicity

Epidemiological studies showed that the incidence of cancer in the population varies with selenium content in the soil in different geographical locations. Locations with medium and high content of selenium appear to have lower cancer incidence (Shamberger & Frost, 1969). In consequence, there has been an increased interest in the *in vitro* study of cytotoxicity of selenium to cancerous cells. In the environment excess selenium toxicity expresses itself mainly in deformities and embryo toxicity in fish and wildfowl (Hamilton, 2004). Selenium toxicity can be either acute, due to elevated selenium concentrations in waters, or chronic, usually via the food chain (Hamilton, 2004). The chronic exposure is of major concern, because of the complexity of the selenium cycle and the food chain, as well as the species-specificity of selenium toxicity (Hamilton, 2004). Selenium toxicity used to be attributed to the substitution of sulphur by selenium in proteins. The proteins are then not able to form a sulphur bond anymore and this would cause a teratogenic effect (Chapman et al., 2010). However,

this hypothesis is uncertain; selenium gets included in the amino acids selenomethionine and selenocysteine and none of the two amino acids seems to alter the structure of proteins (Chapman et al., 2010). The other and currently more accepted hypothesis, that oxidative stress plays a major role, is gaining importance (Chapman et al., 2010). Apparently, inorganic selenium is metabolized by GSH, and during this metabolism there are different points at which ROS can be produced (Letavayová, Vlčková, & Brozmanová, 2006).

1.7.3 Selenomethionine

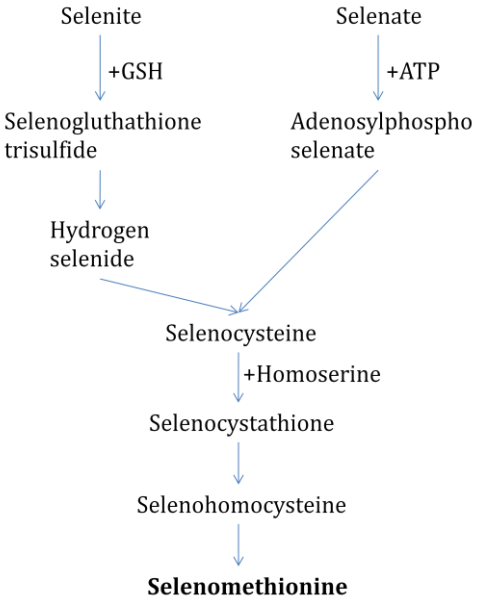

Figure 1.3 Biosynthesis of Selenomethionine in plants, algae, and brewer's yeast. Adapted from Schrauzer 2000.

Selenomethionine is a naturally occurring amino acid. In plants and microorganisms selenomethionine can be incorporated in place of methionine, because the tRNA does not differentiate between methionine and selenomethionine (Papp et al., 2007). The amount of selenomethionine incorporated depends on the selenium available in the environment (Schrauzer, 2000). Right now selenomethionine is considered the largest environmental concern of all selenium compounds (Hamilton, 2004). The reason for these concerns is that it is the most common form produced and accumulated by microorganisms and plants (See Figure 1.3). Selenomethionine especially harms

viviparous vertebrates and top predators, because they accumulate it in their egg yolks, where it causes embryotoxicity. Selenomethionine does not appear to be acutely toxic, but seems to have severe long term consequences for aquatic wildlife (Hamilton, 2004). Fan et al. (2002) studied the biotransformation of selenium in the selenium-laden drainage waters, some of the organisms they studied were carps and stilts. They found elevated selenomethionine levels in the carp liver, which were associated with ovarian lesions. Furthermore, they observed deformation in stilt embryos, which had also higher selenomethionine levels. In a laboratory study where adult mallards were given a SeMet diet, there was no mortality among the adult mallards fed SeMet, but at a concentration of 16ppm all the hatchling were dead and had deformation mostly of the eyes, bills, legs and feet (Heinz, Hoffman, & Gold, 1989). In the field, teratogenic deformities were also observed in fish, which might be the result of waterborne and also dietary selenium in the form of SeMet (Lemly, 1997). However, laboratory studies confirm the effect caused specifically by SeMet. For instance, rainbow trout larvae exposed to SeMet for 90days showed an impaired growth (Vidal, Bay, & Schlenk, 2005), and juvenile fathead minnows manifested impaired swim performance, metabolic capacity and energy homeostasis after 60 days of SeMet exposure (McPhee & Janz, 2014). Palace et al. (2004) studied SeMet metabolism in rainbow trout embryos, and found that the presence of glutathione, SeMet caused superoxide radicals. Interestingly, the superoxide formation did not occur at all embryo stages, and was the greatest during the eleuthro-embryo stage, which is just after early liver development

while SOD is not yet developed, and stopped after superoxide dismutase was developed (Palace et al., 2004).

SeMet is not directly able to produce superoxide, but its metabolite methylselenol is, as confirmed by a study by Spallholz et al. (2004), in which SeMet was used by methioninase as substrate to form methylselenol (See Figure 1.4 and Figure 1.5). Misra et al. (2012) observed oxidative stress caused by SeMet in primary hepatocytes of rainbow trout. They also found increased caspase levels which hints that cell death might be also caused by apoptosis.

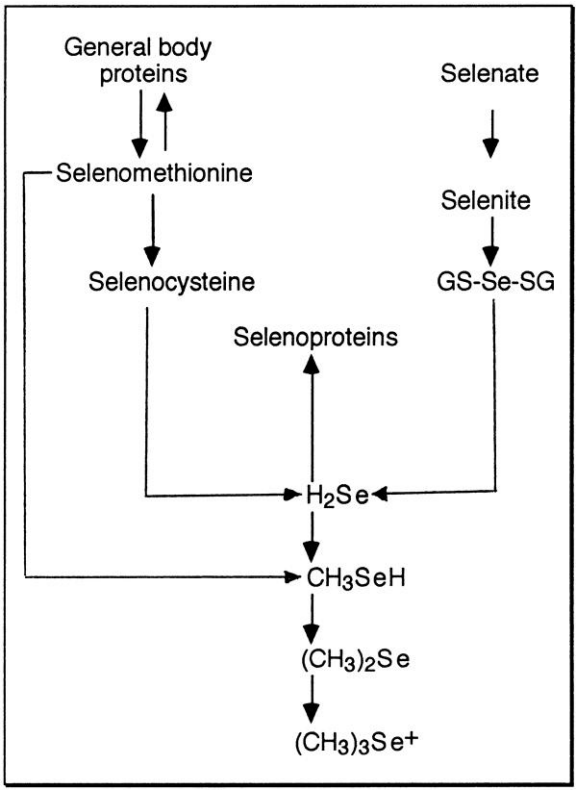

Figure 1.4 Metabolism of selenomethionine, selenite, and selenate. A metabolite is methylselenol CH_3SeH. Published in Schrauzer 2000.

1.7.4 Sodium Selenite

Sodium selenite is an inorganic selenium compound occurring at mildly oxidizing environments and neutral pH`s. Selenite can be absorbed by particles and chemically or biochemically reduced to elemental selenium (Uden, 2005). Plants have a higher tolerance for selenite than vertebrates have and are able to transform it and store it as

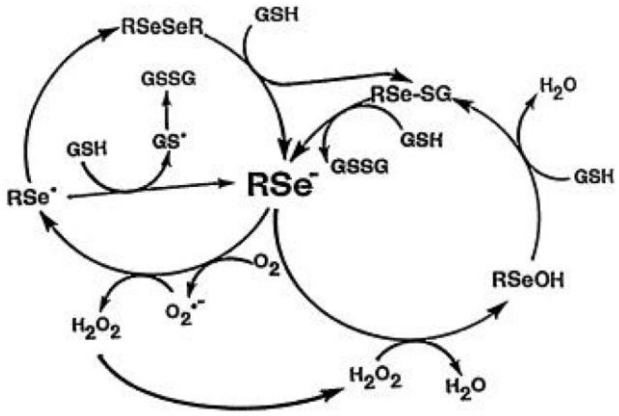

Figure 1.5 Metabolism of methylselenol, which produces superoxide. Published in Chaudière 1992.

selenomethionine (Fan et al., 2002; Spallholz, 1994). It can get accumulated in sediments and from there it can resuspend in the water column.

Selenite reacts with GSH and forms selenopersulfide anion which quickly reacts with O_2 to form superoxide (See Figure 1.6.) (Letavayová et al., 2006; Spallholz, 1994). Therefore toxicity might be caused at selenite concentrations at which GSH gets depleted and the ROS defending role of GSH is impaired (Misra & Niyogi, 2009).

In laboratory studies, sodium selenite has caused respiratory distress, myocardial necrosis, and pulmonary edema, as well as a decrease of vitamin E in the liver, which was attributed to oxidative stress in Lambs (Tiwary, Stegelmeier, Panter, James, & Hall, 2006). In an older study, sodium selenite caused lower egg weight and hatchability of laying chickens (Ort & Latshaw, 1978). Hodson et al. (1980) found the

Figure 1.6 Redox cycle of Selenite, generation of superoxide. Originally published by Seko 1989.

LC50 for rainbow trout to be 8.1mg/L for 96 hours of exposure. Furthermore, chronic selenite exposure caused an increased mortality of eyed eggs and a decreased volume of red blood cells. In a recent study the measured oxidative stress indicators where all elevated after 4 weeks of sodium selenite exposure of juvenile red sea breams. The indicators were superoxide dismutase activity, glutathione S-transferase activity, and glutathione levels in the gills and liver (J.-H. Kim & Kang, 2015).

1.7.5 Sodium Selenate

Sodium selenate and sodium selenite are the two most common selenium sources in water (Sappington, 2002). As with sodium selenite, sodium selenate can also get accumulated in the sediments and resuspended into the water column. It is most stable in alkaline and oxidizing environments (Uden, 2005). Selenate can easily leach from soil and end up in the water and is even more easily taken up and accumulated by plants as selenomethionine then sodium selenite (Spallholz, 1994; Uden, 2005). Sodium selenate generally has not been found to directly induce oxidative stress. It does not react with glutathione to produce superoxide (Spallholz, 1994). Currently it is generally accepted that selenate will only be toxic after being reduced to sodium selenite or selenol (Spallholz, 1994). In general there is less information on sodium selenate toxicity, except that is considerably less toxic than selenite. Brasher and Ogle (1993) compared the sodium selenite and sodium selenate toxicity on the amphipod *Hyalella azteca*, and reported that sodium selenite was 2-4 times more toxic than sodium selenite. Also Hamilton and Buhl (1990) reported that sodium selenate was significantly less toxic than sodium selenite to Coho and Chinook salmon.

1.7.6 *In vitro* Cell Biology of Selenium

Several early studies on human and rodent cancer cell lines showed the levels at which selenium was essential for cell growth. These levels varied with selenium compound and cell line. McKeehan et al. found an increased growth in WI-38 diploid human fibroblasts at 30 nM selenious acid supplementation, and in the Chinese hamster cell line at 0.1 nM selenious acid supplementation (McKeehan, Hamilton, & Ham, 1976). H. Zeng found an optimal growth stimulation of HL-60 cells with a medium supplemented with 0.25 µM selenomethionine and sodium selenite (H. Zeng, 2002).

The effect of selenium compounds on specific cell types has been examined *in vitro*, with endothelial cells being one example. Campbell et al. (2007) explored the protective properties of Se against peroxidative damage on the endothelial cell line EA.hy926. They found that subtoxic selenium concentration in from of sodium selenite increased the concentration of thioredoxin reductase-1, and increased the GPx activity, which protected the cell from peroxidative damage. Another property of selenium in the form of selenomethionine (SeMet) was observed by McAuslan and Reilly (1986). The authors investigated the effect of cell migration and angiogenesis of SeMet. The clonal capillary endothelial cell line BRCE that was exposed to SeMet, showed a considerable cell migration. They also performed a corneal pocket and chorioallantoic membrane assays with SeMet, and both resulted in an increased angiogenic response. The author suggested that this may be an indication that selenium plays a role in Se-deficiency vascular diseases.

Perhaps the most *in vitro* work on selenium compounds has been done on their cytotoxicity to human tumour cell lines. This is because selenium compounds are thought to be promising chemotherapeutic agents (Wallenberg, Misra, & Björnstedt, 2014a). The hope is to find selenium compounds that kill tumour cells at concentrations that do not affect normal cells. For this purpose, selenite, selenate and selenomethionine have been examined. Selenite was more cytotoxic to a human melanoma cell line (HTB140) than to normal human skin melanocytes and keratinocytes (Bandura, Drukala, Wolnicka-Glubisz, Björnstedt, & Korohoda, 2005). Selenite caused growth inhibition and cytotoxicity in human lymphocyte and brain cell lines (Spyrou, Björnstedt, Skog, & Holmgren, 1996; Sundaram et al., 2000).

The mechanisms by which selenite kills cancer cells are still debated. Kitahara et al. (1993) exposed primary rat hepatocytes to sodium selenite. They observed increased oxygen consumption and a decrease in reduced GSH, followed by an increase in thiobarbituric acid and lactate leakage. Misra and Niyogi (2009) measured the induction of catalase, superoxide dismutase, and glutathione peroxidase in rainbow trout primary hepatocytes, which were all increased after the exposure of sodium selenite. As well, Selvaraj et al. (2013) measured an increased production of reactive oxygen species in the fish cell line PLHC-1. These results suggest oxidative stress as the toxicity mechanism of sodium selenite, but a very interesting one has recently been proposed. Bao et al. (2015) found that intracellular metabolism of selenite generated endogenous selenium nanoparticles (SeNPs). SeNPs sequestered critical proteins, such as glycolytic enzymes.

To date, selenate has shown less promise. Selenate failed to be cytotoxic to a human mammary tumour cell line (HTB123/DU4475), a human leukemic cell line (Jurkat E6-1), and a human prostate cancer cell line (PC-2) (Lunøe et al., 2011; Yan, Yee, Boylan, & Spallholz, 1991). Selenomethionine was cytotoxic to a variety of rodent and human tumour cell lines (Kajander et al., 1990) and possibly had unique effects on the transcript profiles of prostate cancer cells (H. Zhao & Brooks, 2007).

In mammalian cell lines the reports on SeMet cytotoxicity are ambiguous. All report that SeMet is the least toxic compared to selenate and selenite, where selenite is the most toxic (Lunøe et al., 2011; Maraldi et al., 2011; Misra et al., 2012; Stewart, Spallholz, Neldner, & Pence, 1999). Several studies report SeMet to be more toxic to cancerous cells than to normal cells (Menter, Sabichi, & Lippman, 2000; Redman et al., 1998). Zeng et al. (2012) found that when they incubated SeMet with methioninase with a cancerogenous cell line, methylselenol inhibited the cancer cells more than normal cells. On the other hand, Weiller et al. (2004) did not find any selectivity for SeMet for cancer cells. Stewart et al. (1999) report that SeMet does not generate any superoxide in mammalian cells, which is almost confirmed by Lin and Spallholz (1993), which found only little superoxide produced by SeMet. However, in another study with prostate cancer cells it is confirmed that SeMet in the presence of methioninase produces superoxides which mediate cancer cell death via apoptosis (R. Zhao, Domann, & Zhong, 2006).

Chapter 2: Development from the American Eel Brain of a Cell Line Expressing Endothelial Cell Properties

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2.1 INTRODUCTION

Fish cell lines are useful in ecotoxicological research for studying the cellular actions of environmental contaminants (Bols et al., 2005; Castaño et al., 2003). Cell lines can be cryopreserved indefinitely and used at any time as a source of experimental material. Just one example is the rainbow trout liver cell line, RTL-W1 (Lee et al., 1993). RTL-W1 has properties of teleost liver stem cells (Malhão et al., 2013), expresses the aryl hydrocarbon receptor (AhR) (Billiard et al., 2002), and responds to 2,3,7,8-tetrachlorodiobenzo-p-dioxin (TCDD) and several polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) with the induction of the cytochrome P450 superfamily member, CYP1A (Bols et al., 1999; Clemons, Dixon, & Bols, 1997). Additional cell lines from other organs and expressing structural markers and functions of different cell types in the organ would expand the range of toxic mechanisms that can be examined. Cell lines from different species allow them to be compared for their relative sensitivity to a toxicant. However, cell lines from endangered species are perhaps most valuable because once established they allow experiments to be done on the cellular systems of the species without the need to sacrifice individuals of the species.

The American eel (*Anguilla rostrata*) is a species that has been identified as threatened by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC), and environmental contaminants might be playing a role in the decline of their populations (Hodson et al., 1994). However, only one cell line, PBLE, which is from peripheral blood leukocytes, has been described from the American eel and this

cell line did not show CYP1A induction and was not characterized further (DeWitte-Orr et al., 2006). A possible source of additional cell lines is the brain because stem cells have been found in the brains of higher and lower vertebrates (Bergström & Forsberg-Nilsson, 2012; English, Sharma, Sharma, & Anand, 2013; Sîrbulescu et al., 2015).

Several stem cells have been studied in the adult vertebrate brain, although sometimes a consensus on what exactly constitutes a stem cell has been difficult to reach (Götz, Sirko, Beckers, & Irmler, 2015). Broadly speaking stem cells proliferate or self-renew but also can differentiate into specialized cells. Cells identified in the mammalian brain as having stem cell properties include neural stem cells (NSCs) (English et al., 2013), neural progenitor stem cells (NPCs) (Franco & Müller, 2013), radial glial cells (Malatesta & Götz, 2013), reactive astrocytes (Götz et al., 2015), and mesenchymal stem cells (MSCs)/pericytes (PCs) (Lange et al., 2013; Paul et al., 2012). Although much less studied, adult fish brains also have stem cells, which are sometimes designated as neural stem/progenitor cells (NSPCs) (Sîrbulescu et al., 2015). Mammalian brain stem cells have been intensively studied *in vivo* and *in vitro*, with stem cells *in vitro* showing a much greater potential to differentiate into different cell types than *in vivo* (Götz et al., 2015). In at least one case, NSCs differentiated into endothelial and smooth muscle cells as well as neurons and the glial cells, astrocytes and oligodendrocytes (Ii et al., 2009). A spontaneously immortalized cell line from the human fetal brain had NSC properties, retaining a tripotent differentiation capacity (Y. Sun et al., 2008).

Most fish cell lines have arisen through spontaneous immortalization (Bols et al., 2005) and several from the brain appear to have neural stem cell properties. These are TB2 from tilapia (Wen et al., 2008a), GBC4 from the orange spotted grouper (Wen, Huang, Ciou, Kao, & Cheng, 2009), SBB-W1 from the European sea bass (Servili et al., 2009) and SBP from snubnose pompano (Wen et al., 2010). These cell lines have been examined for several marker sets, suggesting that they are slightly different. TB2 appeared to be an astroglial cell line (Wen et al., 2008a). GBC4 was proposed to be an immature astroglial cell line or multipotent neural progenitor cells similar to tanycytes (Wen et al., 2009). SBP was thought to be a NSC line, expressing markers for both oligodendrocyte progenitor cells and tanycytes (Wen et al., 2010). SBB-W1 was possibly a NSC line as well (Servili et al., 2009).

In this chapter the development of a cell line from the American eel brain is described and designated eelB. During the course of long-term cultivation, eelB cultures acquired endothelial cell properties, including forming capillary-like structures (CLS) and von Willebrand factor (vWF). These cultures are only the second fish cell line to express multiple endothelial properties (Vo et al., 2015d) and the first endothelial-like cell line from the fish brain. Although they did not respond to TCDD with the induction of CYP1A, eelB will be useful for studying brain endothelial cell biology and for studying other classes of environmental toxicants.

2.2 MATERIAL AND METHODS

2.2.1 Cell Culture Media and Vessels

The basal medium Leibovitz's L-15 and fetal bovine serum were purchased from Hyclone (ThermoFisher Scientific, Burlington, ON, Canada). L-15 supplemented with 10 % FBS and 1 % penicillin-streptomycin solution (10,000 units/ml penicillin, 10 mg/ml streptomycin, P/S) was the routine growth medium and is referred to as L-15/FBS or L-15 with FBS. Sigma-Aldrich (Oakville, ON, Canada) was the source of P/S, Fungizone, and the cryoprotectant, dimethyl sulfoxide (DMSO). In some experiments the FBS concentration was varied. The highest concentration was 25 % and the lowest was 0 %, which is just referred to as L-15 or L-15 alone. For the initial primary cultures from eel brain, the L-15 had 25 % FBS and the antifungal agent Fungizone at 2.5 µg/ml. Other tissue culture supplies were purchased through VWR International (Mississauga, ON) and included Versene (0.2 % ethylenediaminetetra acetic acid, EDTA), trypsin (Lonza, Basel, Switzerland) for subcultivation or passaging, cryovials, 12.5 cm^2, 25 cm^2 and 75 cm^2 flasks (BD Falcon), 4-chamber slides (BD Falcon), slide flasks (Nunc), and 96 well ultra-low attachment plates (Corning, New York, NY).

2.2.2 Fish

The fish that ultimately gave rise to the cell line was a female silver-phase American eel, *Anguilla rostrata* Lesueur. The eel was from a group of eels obtained from commercial fishermen before the species had been considered threatened and held

at the University of Waterloo as part of a study to evaluate methods for attaching telemetry transmitters (Cottrill et al., 2006). An overdose of the anaesthetic, MS-222 (tricaine methanesulfonate), was used to euthanize the fish.

2.2.3 Primary Cultures

The cranial surfaces of the eel were cleaned with alcohol and a sterile scalpel was used to create an incision in the cranium and to remove the brain. The brain was placed into a petri dish with a small volume of L-15 with 25 % FBS and Fungizone and cut with sterile scissors into small fragments or explants. The explants were incubated in L15 with 25 % FBS in 12.5 cm^2 polystyrene flasks at 18 ºC and over a few days adhered to the polystyrene surfaces. Over a period of a month cells migrated out from the explants to cover most of the surface between mounds adherent explants. The cells were removed the surface of one of these flasks with trypsin and transferred to two new flasks where they attached and grew to form monolayers after approximately a month. These cells were trypsinized and placed into a 75 cm^2 flask in L-15 with 20 % FBS and hereafter referred to as the cell line, eelB.

2.2.4 EelB

EelB were grown routinely at room temperature in L-15 with 10 % FBS in 75 cm^2 flasks and subcultivated with trypsin into two new flasks (1 to split) upon reaching confluency. The cells grew very slowly at first with the first subcultivation been done after two months. The time between subcultivations shortened to several weeks by eight months and to approximately a week after a year. By eight months, the time

between subcultivations had shortened to several weeks and by fourteen months had stabilized at approximately one week. Cells were cryopreserved after different passage levels. The cryoprotectant was DMSO and the cells in cryovials were stored in liquid nitrogen as described previously for fish cell lines in general (Bols & Lee, 1994). Cryovials were prepared from cultures at different passage levels that have arbitrarily been defined as early passage (5 to 20), middle passage (21 to 45) and late passage (>45) passages.

After 63 passages, confirmation of eelB as originating from American eel was sought through DNA barcoding, based on the amplification of a short DNA fragment from the 5' end of mitochondrial cytochrome c oxidase subunit 1 (COX1) gene. The PCR primer cocktail was designed for teleosts (Cooper et al., 2007; Ivanova, Zemlak, Hanner, & Hebert, 2007) as previously described for fish cell lines in particular (Sansom et al., 2013). A 655 bp region of the eel COX1 gene was sequenced. DNA sequence was compared and matched to the species identification in both the Barcode of Life Data (BOLD) (Ratnasingham & Hebert, 2007) as well as the NCBI BLAST databases (http://www.ncbi.nlm.nih.gov/BLAST).

A few cryovials of cells were thawed within a few weeks of freezing to ensure that cryopreservation had been successful but the rest were stored in liquid nitrogen for three years before further characterization of the cell line was undertaken. As many more vials of cells had been cryopreserved from late passage cultures than early or middle passages cultures, the cell line characterization began with late passage cells.

Subsequently vials of early and middle passage cells were thawed in order to describe the evolution of some cellular properties in eelB.

2.2.5 Other Cell Lines

Several established fish cell lines were used as controls for different procedures that were used to characterize eelB. PBLE is the only other American eel cell line (DeWitte-Orr et al., 2006) and was used in the examination of eelB for senescence-associated β-galactosidase (SA β-Gal) activity. WEBA and WE-spleen6 are from respectively the walleye bulbus arteriosus and spleen, whereas WE-cfin11f and WE-cfin11fe are from the walleye fin. WEBA is an endothelial cell line (Vo et al., 2015d); WE-spleen6 has properties of fibroblastic reticular cells (FRC) (Vo et al., 2015a); and WE-cfin11f and WE-cfin11e represent fibroblasts and epithelial cells respectively (Vo et al., 2015b; Vo, Bender, Lumsden, Dixon, & Bols, 2015c). RTL-W1 from the rainbow trout liver responds to very low 2, 3, 7, 8 tetrachlorodibenzo-p-dioxin (TCDD) concentrations with strong induction of CYP1A (Clemons et al., 1997) and is widely used to study dioxin-like compounds (Heinrich, Diehl, Förster, & Braunbeck, 2014).

2.2.6 Common Immunofluorescent Staining Steps

EelB was characterized for intermediate and tight junction proteins as described later in specific detail but described here are the common steps for these procedures. Cell cultures were established in Nunc slide flasks. EelB cells were seeded into Nunc slide flasks at approximately 5 x 10^5 cells per flask and allowed to grow at 26 °C for 3 to 7 days at 26 °C. For the analysis of tight junction proteins, the cultures were

maintained for 7 days to allow the cells to grow into tightly packed monolayers. These were rinsed several times in phosphate buffered saline (PBS) prior to the fixation and staining. Fixation was done in 3% paraformaldehyde (PFA) for 20 min at 4 °C, except for two proteins, followed by cell permeabilization with 0.1% Triton X-100 in PBS for 10 minutes. For vimentin and zonula occludens-1 (ZO-1), fixation was done in ice-cold absolute methanol for 20 min at 4 °C, followed by a quick wash in PBS to rehydrate cells. Cultures were then rinsed in PBS and incubated for 1 h in a blocking buffer (BB): 10% goat serum, 3% bovine serum albumin and 0.1% Triton X-100 in PBS. The primary antibodies were then applied as documented later. After incubation with the primary antibody, cultures were washed with PBS three times with rocking, incubated with Alexa Flour 488®-conjugated goat anti-rabbit or anti-mouse IgG secondary antibodies (1:1,000 in PBS) for 1 h, and washed five times with PBS. The cells were allowed to dry and mounted in Fluoroshield medium containing DAPI. Fluorescence images were taken with a Zeiss LSM 510 laser-scanning microscope and confocal images were acquired and analyzed using a ZEN lite 2011 software.

2.2.7 General Properties of EelB

2.2.7.1 Cell Morphology

Throughout the development and maintenance of eelB, cultures were monitored by phase contrast microscopy. At some points phase contrast micrographs were taken.

2.2.7.2 Cell Proliferation

The proliferation of eelB was monitored at the population and single-cell levels. The influences of fetal bovine serum (FBS) concentration and incubation temperature on cell proliferation were examined at the population level for eelB cells from cultures at between 30-40 passages. The ells in L-15 with 10 % FBS were added to 12-well plates at 5×10^4 cells per well and allowed to attach overnight at 26 °C. At this point 3 wells were counted with a Guava EasyCyte™ flow cytometer (EMD Millipore, Billerica, MA). Three replicate wells were used for each culture condition. For FBS, the medium in the remaining plates was changed to L-15 either with 0%, 5%, 10%, or 20 % FBS. The plates were incubated at 26 °C and cells counted 3, 6, 9 and 12 days later. For the effect of temperature, the remaining plates were then incubated at 4°, 14°, 20°, 26° or 30 °C and cells counted on days 3, 6, 9, and 12. The ability of single cells to form colonies was evaluated for eelB cells from early, middle and late passage cultures under the optimal serum and temperature conditions for population growth. Approximately 50-100 eelB cells in L-15 with10% FBS were plated into each well of 6-well culture plate. These plates were incubated at 26 °C and observed for colonies by phase contrast microscopy for up to 2 weeks when the cultures were fixed and stained with crystal violet. A colony was defined as tight clusters of 6 or more cells, with an example presented in Figure 2.3.

2.2.7.3 Chromosome Number

Metaphase chromosome spreads were prepared and counted by standard procedures as described previously for other fish cell lines (DeWitte-Orr et al., 2006; Ganassin, Schirmer, & Bols, 2000). Briefly, subconfluent cultures that had dividing cells were exposed to 0.4 μg/ml demecolcine (Sigma) for 2.5 h. Cells were removed by trypsinization and collected by centrifugation at 400 X g for 10 min. The cell pellet was suspended in .075 mol/L KCl (Sigma) for 8–10 min after which the cells were collected by centrifugation. The cells were resuspended in fixative (3:1 methanol–glacial acetic acid) for 6 h at 4 °C, collected again by centrifugation, and resuspended again in the fixative. Two to three drops of cell suspension dropped onto a glass slide (VWR, West Chester, PA). Multiple slides were prepared, stained with Giemsa stain (Sigma), and observed under oil immersion with a Nikon Optiphot microscope (Japan). Chromosome numbers in 43 and 55 metaphase spreads were counted from cultures at respectively 19 and 45 passages.

2.2.7.4 Senescence-Associated β-Galactosidase Staining

Cultures of eelB and PBLE were stained for SA β-Gal activity with the Senescence Cells Histochemical Staining Kit (CS0030) from Sigma-Aldrich (St Louis, MO). Cultures were initiated in 12 well plates at near confluency and staining done the next day. The staining kit was used according to the manufacturer's instructions, with a minor modification for the fish cells as described previously (Vo et al., 2015b; Vo, Mikhaeil, Lee, Pham, & Bols, 2015e). Cultures were incubated in the staining solution

for 20 h at 26°C rather than at 37 °C. Cells positive for SA β-Gal activity appeared blue.

2.2.8 Endothelial Properties of EelB

As on occasion capillary-like structures appeared spontaneously 1 to 2 days after eelB cultures had been passaged, the influence of Matrigel on the behaviour of eelB was studied. Matrigel is a commercial, soluble extract of basement membrane proteins from a mouse tumour (EHS) and supports the development of capillary-like structures by endothelial cells *in vitro* (Kleinman & Martin, 2005). Other markers that have been used to identify mammalian endothelial cells were examined immunocytochemically. These were vimentin, which is the most abundant intermediate filament protein of endothelial cells (Schnittler, Schmandra, & Drenckhahn, 1998), tight junction proteins, which are needed the for endothelial barrier functions (Song & Pachter, 2003), and von Willebrand factor (vWF), which is a blood clotting factor synthesized by the endothelium (Craig, Spelman, Strandberg, & Zink, 1998).

2.2.8.1 Formation of Capillary-Like Structures (CLS) on Matrigel

Matrigel (BD 35423) was purchased from BD Biosciences (Mississauga, ON, Canada). Surfaces in 24-well tissue cultures plates were coated with Matrigel according to the manufacturer's instructions. Approximately 10^5 eelB cells from early and late passage cultures were plated per well of 24 well plates. In most cases the plates were incubated at room temperature or 26 ºC for up to a week and observed periodically and photographed under an inverted phase contrast microscope. Some

plates were stained for SA β-Gal activity by the procedure described earlier. In a few cases after the cells had attached to the Matrigel-coated surface, the medium was changed the next day to medium with Matrigel to create a Matrigel sandwich, which is thought to more closely mimic the *in vivo* situation and improve tubule formation (Staton, Reed, & Brown, 2009). In some cases 96 well plates were coated with Matrigel. EelB cells seeded on these were used to assay for EROD activity as described below in the TCDD section.

2.2.8.2 Vimentin

The anti-vimentin monoclonal antibody (Clone V9, V6389) that had been raised against pig vimentin and was purchased from Sigma-Aldrich. The antibody was used overnight at a 1:200 dilution and previously has been used successfully in immunohistochemical studies of the fish brain (Arochena, Anadón, & Díaz-Regueira, 2004; Sîrbulescu et al., 2015) and in immunocytochemical characterization of fish cell lines (Vo et al., 2015d, 2015a, 2015c).

2.2.8.3 Tight Junction Proteins

Commercial epitope-affinity-purified polyclonal antibodies from Invitrogen (Burlington, ON, Canada) were obtained against synthetic peptides of three tight junction proteins: aa 463-1109 of human ZO-1 (Polyclonal Antibody Designation (PAD): Z-R1), C-terminal peptide from mouse claudin 3 (PAD: Z23.JM), and C-terminal peptide from mouse claudin 5 (PAD: Z43.JK). For ZO-1, the antibody was

applied overnight at a 1:200 dilution; for claudins 3 and 5, the antibodies were applied overnight at a 1:100 dilution.

2.2.8.4 Von Willebrand Factor (vWF)

A rabbit polyclonal antibody that was raised against human vWF was obtained from Sigma-Aldrich in an affinity-purified form. Previously, this antibody detected vWF in zebrafish (Carrillo, Kim, Rajpurohit, Kulkarni, & Jagadeeswaran, 2010) and in WEBA (Vo et al., 2015d). The antibody was used at a 1:200 dilution for 1.5 h on confluent eelB cultures.

2.2.9 EelB Neurospheres

EelB cells from early and late passage cultures were examined for their capacity to form aggregates in suspension and for cells from these aggregates to express neural stem cell markers, which if they did, would indicate that the aggregates were neurospheres. In mammalian neurobiology, neurospheres are 3 dimensional aggregates of stem cells, progenitor cells and differentiated cells that form when the cells are kept in suspension, providing a culture system for expanding the population of stem cells (Pastrana, Silva-Vargas, & Doetsch, 2011).

2.2.9.1 Neurosphere Formation

Aggregates of eelB cells from either early or late passage cultures were formed in hanging drop cultures or in ultra-low attachment plates (Corning). The formation of aggregates in hanging drop cultures was done as described for a sea bass brain cell line (Servili et al., 2009) and in non-adherent plates as described for a zebrafish embryonic

stem cell line except that the plate manufacturer was Corning (Xing et al., 2008, 2009). EelB cells from early and late passage aggregates, as well as from early and late passage conventional cultures, were added to Nunc slide flasks and allowed to adhere and spread before being examined for alkaline phosphatase and nestin.

2.2.9.2 Alkaline Phosphatase (AP)

The cytochemical detection of AP activity was done with the Leukocyte Alkaline Phosphatase Kit (Sigma 85L3R) (Sigma-Aldrich). The kit was used according to the manufacturer's instructions. The cells were fixed using a citrate-buffered acetone solution for 30 seconds and washed for 45 seconds with deionized water. After this wash the cells were exposed to an AS-MX phosphate alkaline solution mixed with a diazonium salt solution for 0.5 h, followed by a wash in deionized water for 2 minutes. The slide was counterstained with Mayer's hematoxylin solution for 10 minutes and rinsed in tap water. AP was seen as pink granular coloring within cells.

2.2.9.3 Nestin

The immunocytochemical detection of nestin was sought with a rabbit antibody to the C-terminus of mouse nestin (SAB4200394, Sigma-Aldrich, St Louis, MO) that was used at a 1 to 500 dilution.

2.2.10 TCDD Exposure

EelB cells from late passage cultures were exposed to TCDD at concentrations of up to 1561 pM. The dosing procedure for fish cell cultures has been described previously (Ganassin et al., 2000). CYP1A protein levels were monitored as 7-

ethoxyresorufin-O-deethylase (EROD) activity and through western blotting. Cultures of RTL-W1 served as the positive controls.

2.2.10.1 EROD Activity

For EROD induction, the exposure and the enzyme assay were done in 96 well plates. The standard exposure was for 48 h at 26 °C. Exposure conditions were varied in several ways in order to see if more EROD activity could be induced. Exposures were extended for up to 96 h. Also exposed to TCDD were eelB cells that had formed capillary-like structures on Matrigel coated wells. After all exposures, the EROD assay was done at room temperature as described in step by step detail for fish cell lines in general (Ganassin et al., 2000) and for RTL-W1 specifically (Bols et al., 1999).

2.2.10.2 Western Blotting

Western blotting was done on eelB cultures in 75 cm^2 flasks as previously described in detail for RTL-W1 and walleye cell lines (Vo et al., 2015d, 2015a, 2015c). The primary antibody was a commercially available monoclonal antibody (clone C10-7) that had been raised against peptide 277–294 of rainbow trout CYP1A (*Oncorhynchus mykiss*). This antibody is advertised by the manufacturer as recognizing CYP1A orthologs from a wide range of fish species including carp, Atlantic salmon, Atlantic cod, turbot, Lemon sole and mudfish (Cedarlane, Burlington, ON). Goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma) was the secondary antibody and used at 1:20,000 for 1 h. NBT/BCIP substrates were used to develop the protein bands.

2.3 RESULTS

2.3.1 General Properties

2.3.1.1 Cell Morphology

The cellular morphology subtly changed during the course of eelB development but ultimately stabilized. Several cell morphologies were seen in early passage cultures (Figure 2.1A). These included cells that were star-shaped (neuronal-like), long and bipolar (fibroblast-like), and polygonal. With more subcultivations (>20), the cultures became more uniform. At approximately 40 passages, predominately polygonal cells predominated but a few long, bipolar cells were still present (Figure 2.1B). After more than 70 passages, nearly all the cells in eelB cultures were polygonal and this morphology has persisted in all subsequent passages.

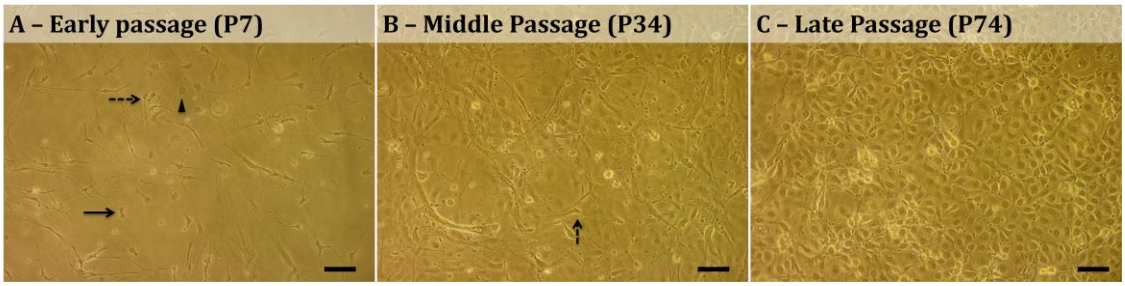

Figure 2.1 Morphology of eelB cells in cultures at different passage levels. Cultures after early (A), mid- (B), and late (C) passages (P) were viewed by phase contrast microscopy and photographed approximately 1 wk after the last passage or subcultivation. In (A), examples of single star-shaped (arrow), bipolar (dotted arrow), and epithelial-like (delta arrowhead) cells are pointed out. In (B), most cells had polygonal shapes but an example of a bipolar cell is marked (dotted arrow). In (C), polygonal cells predominated. Scale bar = 100 μm.

2.3.1.2 Cell Proliferation

FBS and temperature profoundly influenced the proliferation of eelB cells (Figure 2.2). FBS was required for proliferation. Cell number over 12 days was unchanged at 26 °C in cultures without FBS but increased in cultures with 5 to 20 % FBS (Figure 2.2A). In cultures with 10 % FBS, cell number over 12 days was unchanged in cultures held at 4 °C but increased in cultures incubated at 14, 20, 26 and 30 °C, with the highest increase being at 26 °C (Figure 2.2B).

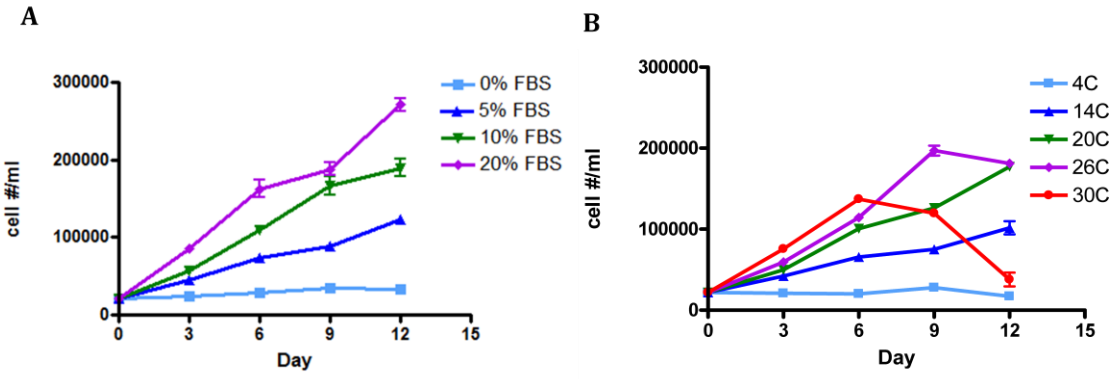

Figure 2.2 Effect of FBS concentration and of incubation temperature on eelB cell proliferation. The mean numbers of cells per well (±standard deviation, n = 3) are plotted against time of culture under conditions of different serum concentrations and incubation temperatures.

At 26 °C with 10 % FBS, eelB from early passage cultures did not form colonies but approximately 25 and 50 % of eelB cells from respectively middle and late passage cultures did. The colonies from middle passage cultures were scattered whereas from late passage cultures the colonies were made of tightly packed polygonal-shaped cells (Figure 2.3).

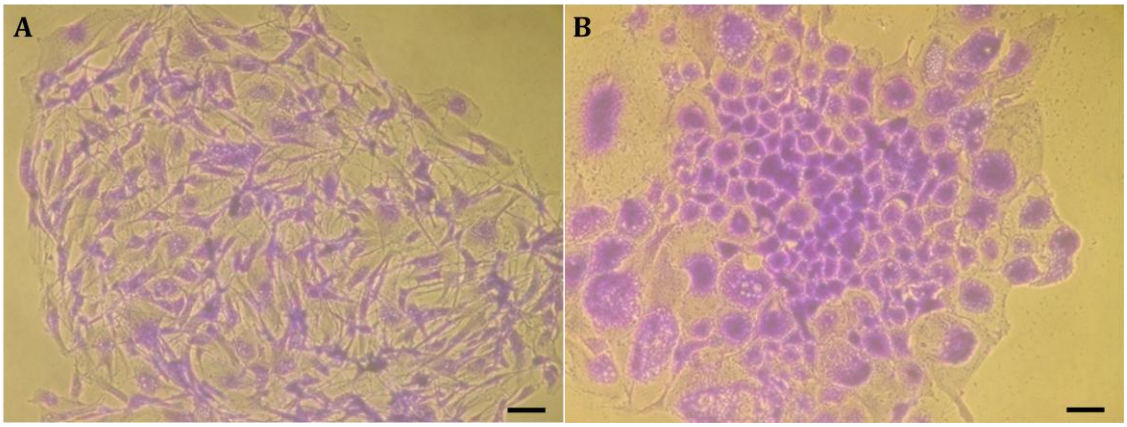

Figure 2.3 Appearance of eelB colonies. Cells were plated at approximately 100 cells per well of 6-well plates, which was incubated for 2 wk before the colonies were stained with crystal violet. Colonies formed by eelB cells from mid- (A) and late-passage (B) cultures are shown. Scale bar=100 μm.

2.3.1.3 Chromosome Number

As eelB cells were continuously passaged, the cell line appeared to become heteroploid, which is not having the exact multiple of the haploid chromosome number. The diploid chromosome complement for the American eel is 38 (Sola, Gentili, & Cataudella, 1980). In eelB cultures after 19 passages the modal chromosome number was 38. However, after 45 passages, no clear modal number was seen. Chromosome number ranged from 28 to 114, with possible modal peaks occurring at 57, 76, and 95.

2.3.1.4 Senescence-Associated β-Galactosidase Staining

Throughout the development of eelB, cultures stained for SA β-Gal. This was shown for eelB cells from early (7) and late (76) passage cultures (Figure 2.4) and also for cells that had formed capillary-like structures on Matrigel (Figure 2.4). The

staining was an intense blue and appeared cytoplasmic. By contrast, no cells stained in cultures of the only other American eel cell line, PBLE (Figure 2.4).

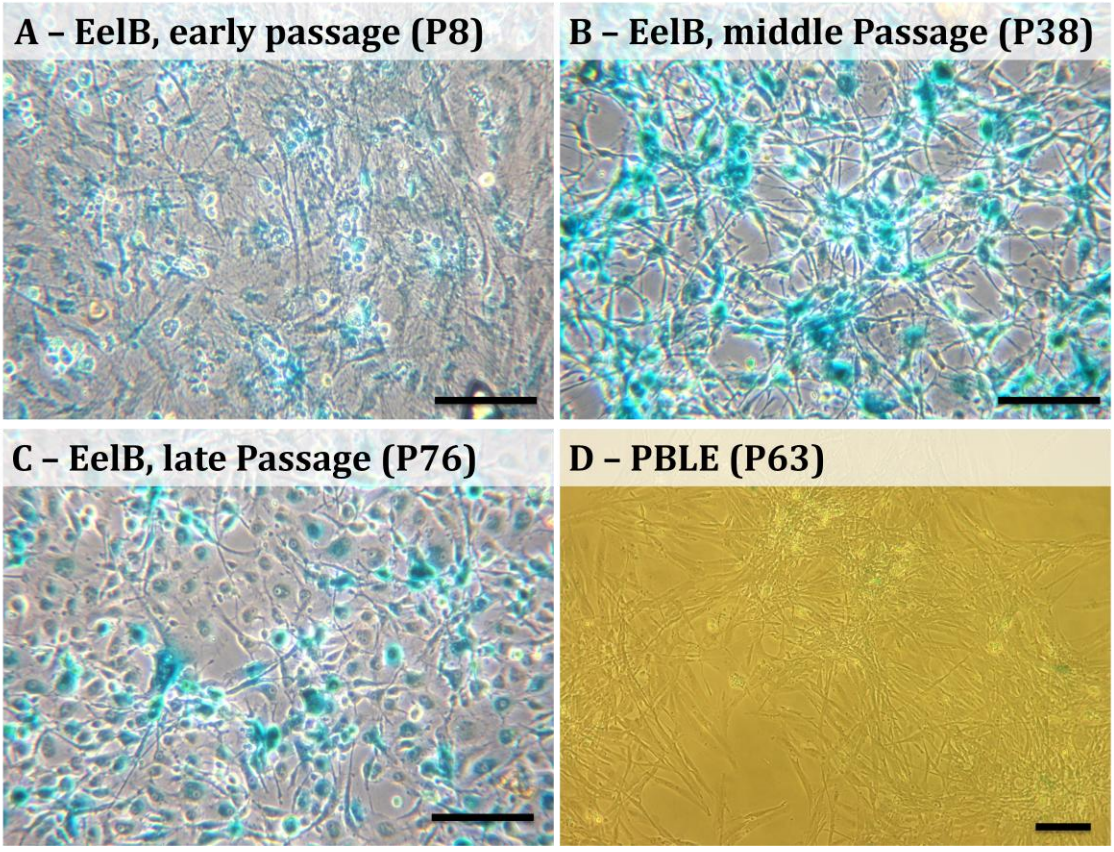

Figure 2.4 Cytochemical staining for the senescence-associated β-galactosidase activity (SA β-Gal) in cultures of eelB and PBLE. Staining was done with the Senescence Cells Staining Kit from Sigma-Aldrich (CS0030), with dark blue indicating activity. EelB cultures were stained at early (A), mid- (B), and late (C) passages (P). PBLE cultures were examined after 63 passages (D). Scale bar=100 μm.

2.3.2 Endothelial Properties of EelB

2.3.2.1 Formation of Capillary-Like Structures (CLS)

EelB cells from late passage cultures consistently formed CLS in the presence of Matrigel (Figure 2.5A). By contrast cells from low passage cultures did not. The CLS

were stable and could persist for up to 7 days. EelB cells in CLS continued to stain for SA β-Gal activity (Figure 2.5B).

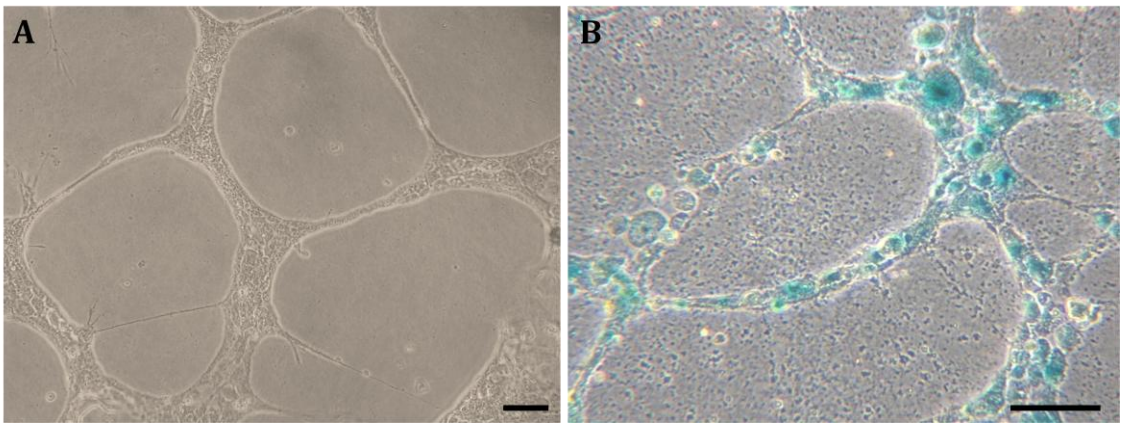

Figure 2.5 Formation of capillary-like structures (CLS). Organization of eelB cells from late-passage cultures on Matrigel. CLS are shown as they appear under the phase contrast microscope (A) and after staining for SA β-Gal activity (B). Scale bar=100 μm.

2.3.2.2 Vimentin

Vimentin was detected immunocytochemically in eelB cells. In early passage cultures, vimentin was in stellate shaped cells with a radiating pattern of long fibres, whereas in late passage cultures vimentin was in polygonal shaped cells with a complex network of short fibres (Figure 2.6).

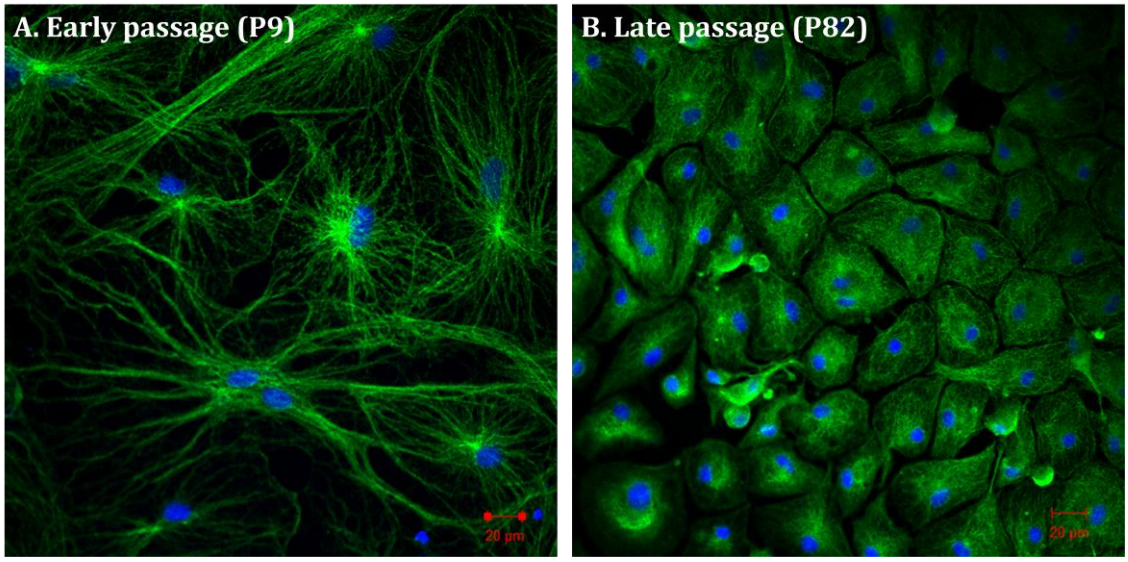

Figure 2.6 Immunofluorescence staining for vimentin in eelB cells. The primary antibody was mouse monoclonal anti-vimentin and was applied to eelB cells from early- (P9) and late- (82) passage cultures. The secondary antibody was goat anti-mouse with Alexa Fluor 488 (green). The nuclei were counterstained with DAPI (blue). Scale bar=20 μm.

2.3.2.3 Expression of Tight Junction (TJ) Proteins

EelB cells from late passage cultures stained for three tight junction proteins: zona occludens-1 (ZO-1), claudin 3, and claudin 5. No staining was observed for a fourth tight junction protein, occludin. ZO-1 staining was strong at the cell periphery where cell-to-cell contacts were established, whereas staining in the cytoplasm was diffuse (Figure 2.7). Anti-claudins 3 and 5 stained eelB around the cell periphery where cell-to-cell contacts were made but for anti-claudin 3 the staining appeared more as dots than continuous peripheral staining (Figure 2.7).

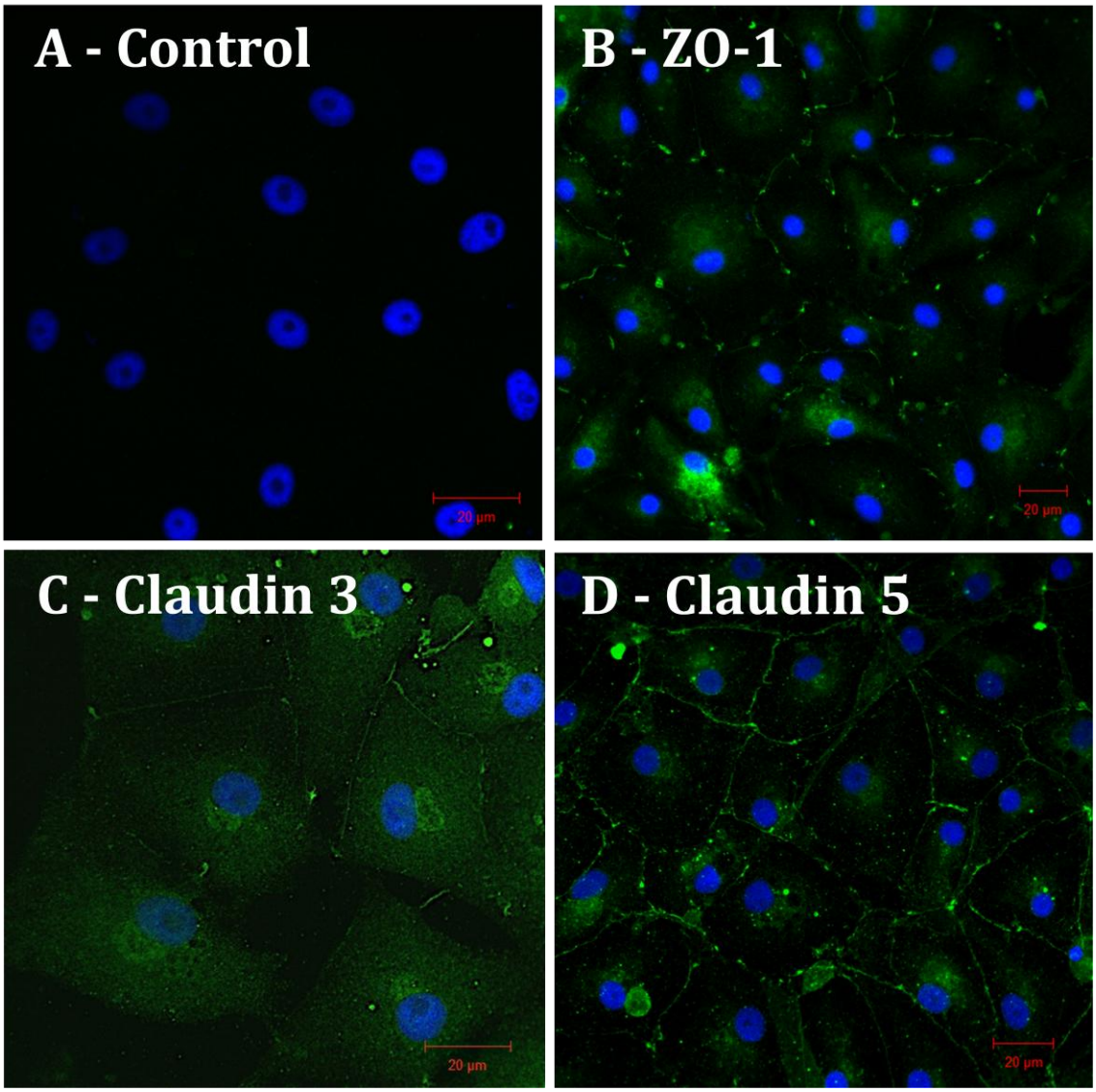

Figure 2.7 Immunocytochemistry of tight junction proteins in eelB cells from late-passage cultures. The negative controls were cultures without primary antibodies (A). The primary antibodies were rabbit polyclonal affinity-purified anti-ZO-1 (B), anti-claudin 5 (C), and claudin 3 (D). The secondary antibody was Alexa Flour 488 (green)-conjugated goat anti-rabbit IgG. DAPI (blue) was used as a counterstain for the cell nuclei. Scale bar=20 µm.

2.3.2.4 Von Willebrand Factor (vWF)

The cytoplasm of eelB cells from late passage cultures stained for vWF in a granular pattern (Figure 2.8). By contrast, a walleye spleen cell line that had the properties of fibroblastic reticular cells and like eelB formed capillary-like structures on Matrigel (Vo et al., 2015a) did not stain for vWF (Figure 2.8). The staining of eelB cells from early passage cultures was weak or equivocal (data not shown).

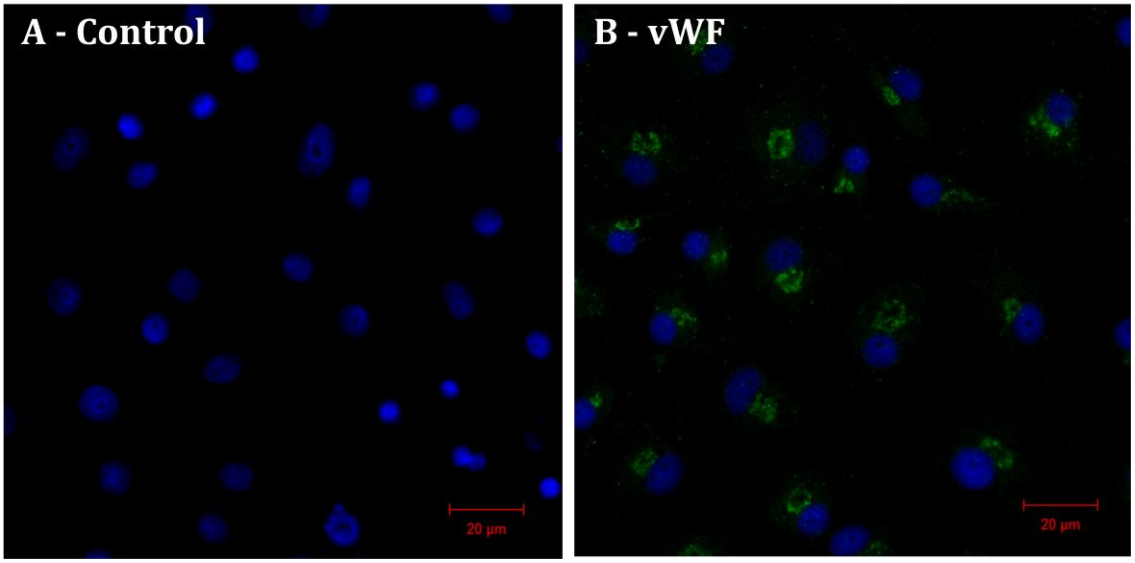

Figure 2.8 Immunocytochemistry of vWF in eelB cells from late passage cultures. (A) Cultures without primary antibodies acted as negative controls. (B) The primary antibody was a rabbit polyclonal affinity-purified anti-vWF added to the cultures at 1:200 for 1.5 h. The secondary antibody was Alexa Flour 488 (green)- conjugated goat anti-rabbit IgG. DAPI (blue) was used as a counterstain for the cell nuclei. Scale bar=20 µm.

2.3.3 EelB Neurospheres

When kept in suspension either in hanging drop cultures or in wells with an ultra-low attachment surface, eelB cells from either early or late passage cultures organized

into 3-dimensional aggregates (Figure 2.9 A & B). After persisting for up to 7 days at room temperatures, the aggregates could upon addition to conventional plastic culture surfaces adhere. From the adherent aggregates, cells migrated out and spread over the culture surface (Figure 2.9 C & D). These cells were monitored for alkaline phosphatase activity and the intermediate filament protein, nestin, in order to determine whether stem cells were present and the aggregates were neurospheres.

2.3.3.1 Alkaline Phosphatase (AP)

EelB cells migrating out from early passage aggregates stained strongly for AP, whereas cells migrating out from late passage aggregates did not (Figure 2.9 E & F). When cultured in a conventional manner, a few eelB cells from early passage cultures stained weakly for AP whereas cells from late passage cultures did not (data not shown).

2.3.3.2 Nestin

EelB cells from early passage neurospheres stained for nestin but no cells from late passage neurospheres did (Figure 2.9 G & H).

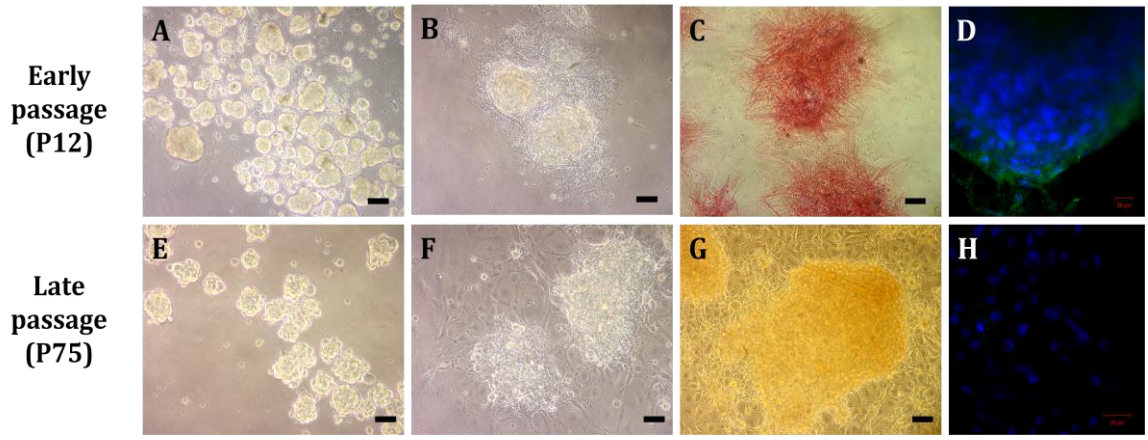

Figure 2.9 Stem cell marker expression in eelB cells after being maintained in suspension as cell aggregates. Phase contrast microscopy appearance of cell aggregates formed by culturing cells from early- (A–D) and late- (E–H) passage cultures for 3 d in ultra-low attachment plates (A,E) and subsequently allowed to attach for 3 d to conventional plates (B, F). Cytochemical and immunocytochemical staining, respectively, for alkaline phosphatase activity (C, G) and nestin (D, H) in eelB cells migrating out of aggregates from early- (C, D) and late- (G, H) passage cultures. Scale bars for A–C and E–G are equal to 100 µm. Scale bars for D and H are equal to 20 µm.

2.3.4 TCDD Exposure

EelB cultures exposed to TCDD at concentrations as high as 1561 pM and for up to 48 h showed no morphological change and no increase in CYP1A levels, which were monitored as EROD activity or in western blots.

2.3.4.1 EROD Activity

EROD activity was absent without TCDD and barely became detectable with high TCDD exposure (Figure 2.10A). When the cells had been allowed to attach and form CLS on Matrigel before being exposed to TCDD, the cultures continued to show little or no EROD activity.

2.3.4.2 Western Blotting

CYP1A was undetectable in eelB cultures, even upon exposure to TCDD at up to 1561 pM (Figure 2.10B).

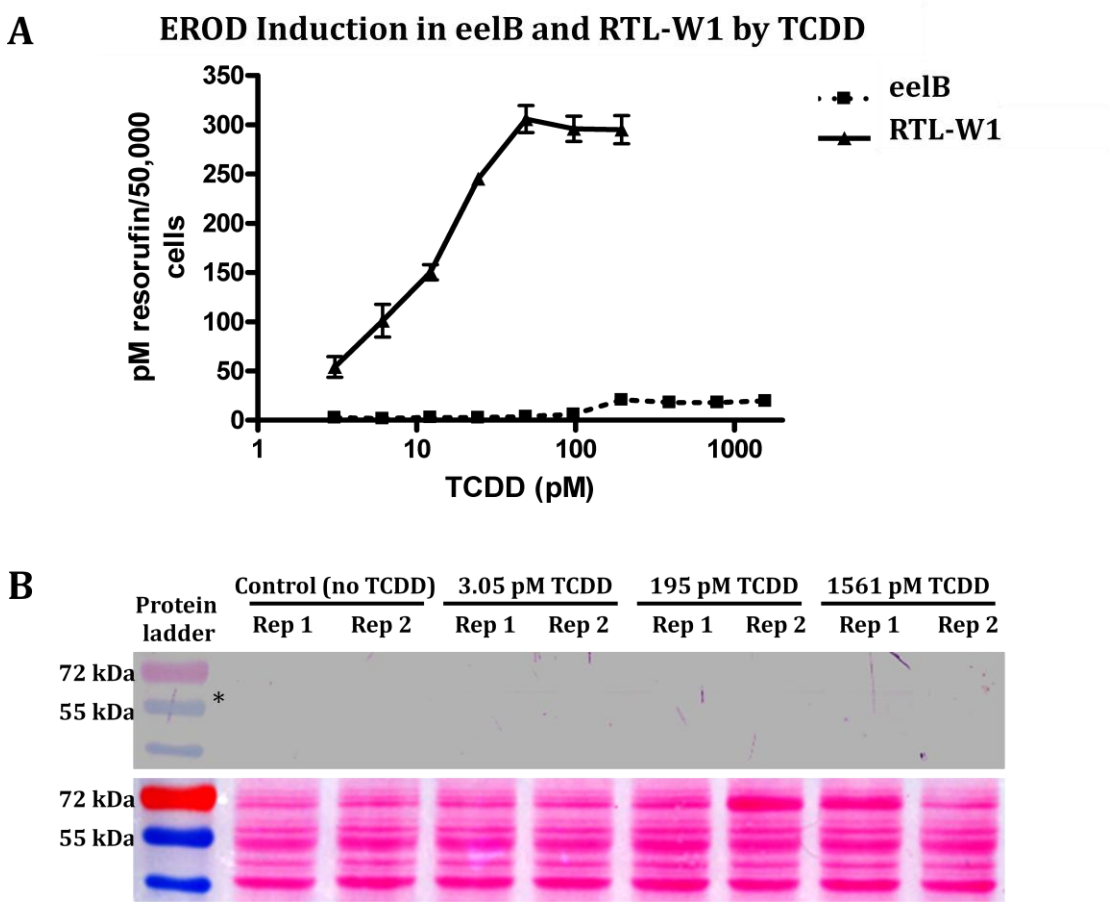

Figure 2.10 Effect of TCDD on CYP1A levels in eelB cells. After eelB cells from late-passage cultures had been exposed to different TCDD concentrations for 48 h, CYP1A was monitored as EROD activity (A) or as an antigen in Western blots (B). RTL-W1 is a cell line that consistently responds to TCDD with EROD induction (Bols et al. 1999) and was exposed at the same time. (A) shows strong induction of EROD activity for RTL-W1 (solid line) and little activity for eelB (dotted line). In (B), extracts of eelB were run but no band appeared at the position expected for CYP1A, which is identified as an asterisk. A band did appear in RTL-W1 extracts (data not shown).

2.4 DISCUSSION

2.4.1 General Properties of EelB

2.4.1.1 Cell Morphology

The ultimate cell morphology of eelB appears to be unique for cell lines from the fish brain. As judged by phase contrast microscopy, the morphology of eelB changed from being varied in early-passage to becoming predominantly polygonal in late-passage cultures. Others have found that cell shape changes during on the on-going proliferation of cell lines from teleost brains (Cheng et al., 2010; Huang et al., 2011; Wen et al., 2008a). The most complex changes were seen in a brain cell line from cobia that went from neuron-like to fibroblast-like to epithelial-like (Cheng et al., 2010) In early passage eelB cultures, some neuron-like cells were seen among the mainly epithelial-like cells, but after about 40 passages, polygonal-shaped cells dominated and have continued to do so with subsequent passaging. DNA bar-coding of cultures after 65 passages confirmed that eelB was from the American eel. EelB appears to be the only fish brain cell line with primarily polygonal-shaped cells.

2.4.1.2 Cell Proliferation

The conditions under which eelB cells proliferated were as anticipated for a fish cell line generally and for the American eel more specifically. Like most cell fish cell lines (Bols et al., 2005), eelB needed FBS in order to be grown continuously *in vitro*. EelB cell proliferation was best at between 18 and 26 °C, slowed at higher temperatures, with cells ultimately dying at 37 °C, and stopped at 4 °C, although the cells survived for at

least several weeks at this low temperature. The responses to these temperatures were very similar to what were observed with PBLE, the only other American eel cell line (DeWitte-Orr et al., 2006) and generally mirror the thermal biology of the American eel (Kleckner, McCleave, & Wippelhauser, 1983) and the closely related European eel (Sadler, 1979). EelB cells from late but not early passage cultures were capable of forming colonies. Colony formation is often poor or nonexistent with teleost cell lines (Bols et al., 2005) but for mammalian cell lines, this capability is often a characteristic of immortal cell lines (Freshney, 2010).

2.4.1.3 Chromosome Number

As eelB cells were continuously passaged, cultures appeared to develop aneuploidy, which for mouse cells in culture often accompanies or precedes spontaneous immortalization (Padilla-Nash et al., 2012; Rasnick, 2000). Yet, aneuploidy has also been seen paradoxically to have inhibitory effects on mammalian cell proliferation (Sheltzer & Amon, 2011) and the impact on fish cell proliferation appears largely unexplored. With fish cell lines the relationship between aneuploidy and immortalization is uncertain because the cells appear to undergo immortalization spontaneously and early in culture before ploidy levels have been determined (Bols et al., 2005). However, apparently immortal fish cell lines have been observed to be diploid, with the American eel cell line, PBLE being one example (DeWitte-Orr et al., 2006). Previously both diploid (Zheng et al., 2015) and heteroploid (Wen, Lee, Wang, Cheng, & Huang, 2008b) cell lines have been described from the brains of other fish species. Yet, the development of aneuploidy in eelB might reflect their brain origin.

This is suggested by recent research on the mammalian brain. In the human brain, neural progenitor cells are frequently found to be aneuploid (Devalle et al., 2012; Rehen et al., 2005), and during the long-term cultivation of mouse neural precursor cells, aneuploidy and an increased differentiation potential developed (Nguyen et al., 2013).

2.4.1.4 Senescence-Associated β-Galactosidase (SA β-Gal)

Throughout the development of the cell line, eelB cells stained for senescence-associated β-galactosidase (SA β-Gal) activity, which is exceptional among animal cell lines that appear to be immortal. Usually, finite or mortal rodent and human cell lines acquire SA β-Gal activity with prolonged passaging, whereas immortal cell lines never stain for SA β-Gal activity (Dimri et al., 1995; Gendron, Liu, Paradis, Adams, & Kao, 2001). Recently most immortal piscine cell lines were also found to lack SA β-Gal activity (Vo et al., 2015e). SA β-Gal staining in apparently immortal animal cell lines has been seen in only one other case. The other exception was a fish cell line from the bulbous arteriosus of walleye, which is termed WEBA and has several endothelial characteristics (Vo et al., 2015e). EelB also appears to be endothelial because as discussed in the next section the cells formed capillary-like structures (CLS). In these CLS, eelB cells continued to stain for SA β-Gal activity. Possibly the level of SA β-Gal activity is high in fish endothelial cells and eelB cells retain this property. This will be interesting to explore *in vitro* and *in vivo* in the future.

2.4.2 Endothelial Properties of EelB

2.4.2.1 Formation of Capillary-Like Structures

When plated onto Matrigel, eelB cells consistently formed capillary-like structures (CLS). The only other fish cell line that to date has been shown to develop CLS in response to Matrigel is a walleye spleen stromal cell (Vo et al., 2015a). Matrigel and other gels such as collagen and fibrin cause many mammalian endothelial cell lines to form CLS (Bouïs et al., 2001; Morin & Tranquillo, 2013; Olyslaegers, Desmarets, Dedeurwaerder, Dewerchin, & Nauwynck, 2013; Rahmanian et al., 1997; Tsuda, Ohtsuru, Yamashita, Kanetake, & Kanda, 2002). Yet, Matrigel also supports the formation of CLS by a variety of tumour cell types (Harrell et al., 2014). This is termed vascular mimicry. In vascular mimicry tumour cells mimic endothelial cells and form tubular channels (Cao et al., 2013). Thus one possibility was that eelB cells had acquired one or more tumorigenic properties, including vascular mimicry, and this was the cause of CLS. The nature of the lumen in the CLS as revealed transmission electron microscopy (TEM) might help delineate this in the future. However, as cells in eelB cultures expressed other endothelial properties, the simplest explanation is that the endothelial character of eel cells was responsible for the CLS.

2.4.2.2 Vimentin

EelB cells from early and late passage cultures stained strongly for vimentin, which could indicate their identity as neural stem/progenitor cells (NSPCs) or endothelial cells. In teleosts, strong staining for vimentin has been seen in cerebellar NSPCs of knifefish brain (Sîrbulescu et al., 2015) and in immature astroglial cells of the grey

mullet brain (Arochena et al., 2004). The cells staining in early passage eelB cultures had long processes like fish astroglial/radial glial cells (Mack & Tiedemann, 2013) and could represent NSPCs. However, the cells staining from late passage cultures had the polygonal morphology of endothelial cells. Vimentin is abundant in mammalian endothelial cells and thought to protect the endothelium from mechanical force (Schnittler et al., 1998) and to promote sprouting angiogenesis (Kwak et al., 2012). Vimentin stained strongly in the walleye endothelial cell line, WEBA (Vo et al., 2015d). Thus vimentin in eelB cells from late passage cultures is consistent with their other endothelial-like properties.

2.4.2.3 Expression of Tight Junction (TJ) Proteins

EelB cells in mid and late passage cultures expressed several of the tight junctions (TJs) proteins that are characteristically found in the microvascular endothelial cells that make up the vertebrate blood brain barrier (BBB). TJs between capillary endothelial cells are the structural basis of the BBB and are composed of several protein families, including claudins, occludin, and zonula occludin-1 (ZO-1). Some endothelial cell lines from rodent and human brains express this spectrum of TJ proteins (Song & Pachter, 2003; Watanabe et al., 2013; Weksler et al., 2005). EelB cells expressed claudins and ZO-1, but not occluding. Interestingly, occluding could not be demonstrated in the zebrafish BBB (Jeong et al., 2008). In the TJs of the BBB, expression of claudin-5 is uniquely strong in mammals (Nitta et al., 2003) and possibly in teleosts as well (J. Zhang, Liss, Wolburg, Blasig, & Abdelilah-Seyfried, 2012). Although several brain endothelial cell lines from mammals express TJ

proteins, few express claudin-5 (Watanabe et al., 2013). By contrast eelB did have claudin-5 and, and like some rodent cell lines (Roux & Couraud, 2005), should be useful for studying the blood-brain barrier.

2.4.2.4 Von Willebrand Factor (vWF)

EelB cells stained immunocytochemically for vWF, which is generally considered to be an excellent marker for endothelial cells in culture (Craig et al., 1998). A commercial polyclonal antibody to human vWF (Factor VIII-related antigen) was used and previously has detected vWF in endothelial cells of zebrafish arteries and veins (Carrillo et al., 2010) and in the walleye endothelial cell line, WEBA (Vo et al., 2015d). Several mammalian brain endothelial cell lines express vWF (Camalxaman et al., 2013; Weksler et al., 2005), but for some endothelial cell lines from other mammalian tissues vWF expression declined with the continued maintenance of the cells in culture (Hormia, 1982; Lou & Hu, 1987; Maciag, Hoover, Stemerman, & Weinstein, 1981; van Leeuwen et al., 2000; Yosef & Ubogu, 2013). However, eelB cultures at all passage levels had at least some cells staining for vWF and staining seemed more noticeable with increased passaging. For the endothelial cells of many mammalian tissues, the vWF is localized to cytoplasmic Weibel-Palade Bodies (WPB) (Wagner, Olmsted, & Marder, 1982) but the brain might be an exception. High levels of vWF were found in cerebral endothelial cells (Suidan et al., 2013; Yamamoto, de Waard, Fearns, & Loskutoff, 1998), but relative to other tissues, the brain had few WPB (Herrlinger, Anzil, Blinzinger, & Kronski, 1974; Plendl, Neumüller, Vollmar, Auerbach, & Sinowatz, 1996). Instead the vWF in brain endothelial cells was found in

cytoplasmic granules associated with the rough endoplasmic reticulum and Golgi (Craig et al., 1998; Dorovini-Zis & Huynh, 1992). The vWF appeared in cytoplasmic granules but whether these were WPB will require further study, including TEM examination.

2.4.3 Neurospheres of EelB

The possible presence of neural stem cells in eelB cultures was investigated by determining whether cells from early and late passage cultures could form neurospheres. Previously neurospheres have been demonstrated in primary cultures from the knifefish brain (Hinsch & Zupanc, 2006) and with a sea bass brain cell line (Servili et al., 2009). EelB cells from both early and late passage levels formed 3-dimensional cell aggregates when prevented from attaching to a culture surface. However, only the cell aggregates from early passage cultures might be considered neurospheres, because only cells from these aggregates expressed two neural stem cell markers, alkaline phosphatase and nestin.

2.4.3.1 Alkaline Phosphatase (AP)

Commonly AP has been used as a marker for embryonic stem cells of mammals (Štefková, Procházková, & Pacherník, 2015) and teleosts (L. Sun, Bradford, Ghosh, Collodi, & Barnes, 1995) and of neural stem cells from mammals at least (Langer, Ikehara, Takebayashi, Hawkes, & Zimmermann, 2007). Some eelB cells from early passage monolayer cultures stained weakly for AP and cells from early passage neurospheres stained strongly for AP. By contrast, AP staining was absent in eelB cells

from late passage cultures whether they were in adhering neurospheres or in monolayer. Under the staining conditions of this study most fish cell lines did not stain for AP (Kawano et al., 2011; Vo et al., 2015a; Xing et al., 2009), although a zebrafish embryo cell line did (Xing et al., 2008).

2.4.3.2 Nestin

Nestin is an intermediate filament protein that has commonly been used to identify mammalian neural stem cells in neurospheres (Pastrana et al., 2011), although recently nestin has been noted in a few other cell types (Tampaki et al., 2014). For fish cell lines, only a few cells in a sea bass brain cultures stained weakly for nestin (Servili et al., 2009). Yet, eelB cells from early passage neurospheres stained for nestin but no cells from late passage neurospheres did. Together the AP and nestin results suggest that neural stem cells are present in early passage eelB cultures but are absent or at a very low level in late passage cultures.

2.4.4 TCDD Exposure

EelB cells did not respond to TCDD with CYP1A induction, although the American eel appears to contain the AhR signalling cascade. Previously CYP1A induction by dioxin and dioxin-like compounds has been demonstrated *in vivo* with the European and American eel (Bonacci, Corsi, Chiea, Regoli, & Focardi, 2003; Schlezinger & Stegeman, 2000) and *in vitro* in primary cultures of endothelial cells from the heart of the American eel (Garrick et al., 2005). However, TCDD did not induce CYP1A in primary cultures of capillary endothelial cells from the rete mirabile of the American

eel (Garrick et al., 2005). This suggests that the expression and/or regulation of CYP1A might differ in endothelial cells from different organs of the eel. Possibly the failure of eelB to respond to TCDD with CYP1A induction might reflect their origin from the brain.

Alternatively AhR signalling system might have become dysfunctional during the course of the cell line development. Previously, TCDD was found not to induce EROD activity in the only other cell line from the American eel, PBLE (DeWitte-Orr et al., 2006), suggesting that when cells of this species become cell lines some components of the AhR signalling pathway might be diminished whereas suppressors of the pathway might be maintained. For example, AhR levels might be too low to support induction. This has been suggested as part of the reason for the poor CYP1A induction in human fibroblasts (Tigges et al., 2013). Additionally, the regulation of AhR signalling cascade might be different in unresponsive cells like eelB or human fibroblasts. In the case of human fibroblasts, poor CYP1A1 induction had been attributed to high levels of AhR repressor protein (AhRR) (Haarmann-Stemmann et al., 2007). However, this idea was rejected recently (Tigges et al., 2013), leaving open the possibility that repression might occur at the level of CYP1A mRNA translation (Tigges et al., 2013). Interestingly, a microRNA (miRNA), miR-892a, recently was shown to inhibit CYP1A1 expression in a human epithelial cell line (Choi et al., 2012). Thus the levels of AhR and AhRR in the signalling cascade and the involvement of miRNA in CYP1A expression could be parameters to explore in the future with eelB cells.

2.4.5 Conclusions

A cell line, eelB, has been established from an endangered species, the American eel, and should be useful in several disciplines because its development and properties are unique. Late-passage eelB cells have endothelial characteristics and are one of the few vertebrate brain cell lines to express vWF and claudin-5, which might allow *in vitro* models for the blood–brain barrier to be developed and used to study the effects of environmental toxicants. The endothelial nature of eelB emerged from prolonged passaging of the cells, which is an unprecedented phenomenon for teleost cell cultures but does have precedence in mammalian neurobiology. Over the last decade, rodent and human NSCs/NPCs have been found to differentiate beyond neural lineage cells, such as neurons and astrocytes, and into other lineages, including endothelial cells (Ii et al., 2009; Sekiguchi et al., 2013; Wurmser et al., 2004). During long-term culture, murine NPCs showed a dramatic reduction in their neuronal and glial differentiation capacity and an increase in their capacity for mesodermal differentiation (Nguyen et al., 2013). Possibly, early-passage eelB cells can be used to explore the signals that cause neural stem cells to differentiate into specific cell types.

Chapter 3: Responses of an American Eel Brain Endothelial-Like Cell Line to Selenium Deprivation or to Selenite, Selenate and Selenomethionine Additions

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3.1 INTRODUCTION

The American eel (*Anguilla rostrata*) is a species that has been identified as threatened by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC), and environmental contaminants could be one contributing factor to their population decline (Hodson et al., 1994) and to the general world-wide decline in eel populations (Geeraerts & Belpaire, 2010). Selenium (Se) contaminates aquatic environments (Muscatello & Janz, 2009; Simmons & Wallschläger, 2005) and recently has been linked to subtle changes in European and American eel populations (Laporte et al., 2016). How Se acts in eels has rarely been examined and has been made more difficult to study by their endangered status. One way around this problem is to use cell lines.

Few cell lines are available from the American eel (DeWitte-Orr et al., 2006), but one is an endothelial-like cell line developed from the brain and termed eelB (Bloch et al., 2016). In the context of Se, endothelial cells are interesting because Se modulates endothelial cell functions (Alturkmani et al., 2012; McAuslan & Reilly, 1986; Ren et al., 2016) and Se deficiency is associated with cardiovascular disease (Oster & Prellwitz, 1990). With respect to Se supply and retention, the vertebrate brain appears to be a privileged organ (Whanger, 2000) and mammalian brain cell lines express some specific selenium-containing proteins (Hoppe et al., 2008). The little information on Se cytotoxicity to brain cells has been obtained with human glioblastoma and glioma cell lines (Hazane-Puch et al., 2016; Sundaram et al., 2000; Zhu et al., 1996).

There are two aspects to Se and animal cell cultures. On one hand, Se is an essential trace element for the nutrition of mammals and fish (Combs Jr & Combs, 1986) and is central to antioxidant defences (Papp et al., 2007; Tapiero, Townsend, & Tew, 2003). As a result, a little Se is added to cell cultures either as part of the basal media or in the serum supplement (Karlenius et al., 2011) and is necessary to support cell survival (Irmak, Ince, Ozturk, & Cetin-Atalay, 2003; Saito, Yoshida, Akazawa, Takahashi, & Niki, 2003), proliferation (McKeehan et al., 1976) and differentiation (Gu, Royland, Wiggins, & Konat, 1997; Speier, Baker, & Newburger, 1985; Stewart, Davis, Walsh, & Pence, 1997), although the Se requirement for fish cells has rarely been investigated. On the other hand, too much Se is cytotoxic. This has been intensively studied in mammalian cell cultures in order to develop Se compounds as chemotherapeutic agents and to understand and manipulate differences in their cytotoxicity to normal and tumour cells (Bandura et al., 2005; Lunøe et al., 2011; Wallenberg et al., 2014a; Yan et al., 1991; H. Zeng et al., 2012). The actions of Se compounds on fish cells in culture have been examined much less frequently (Babich, Martin-Alguacil, & Borenfreund, 1989; Misra et al., 2012; Misra, Peak, & Niyogi, 2010; Misra & Niyogi, 2009; Selvaraj et al., 2013). Generally animal cell responses depend on the Se form (organic, inorganic or elemental). For mammalian cells, a wide range of Se compounds have been evaluated, but perhaps selenite and selenate have been most thoroughly investigated. Although fish cell studies have been few, SeMet has been studied most commonly because SeMet is the major form found in the food for fish at Se contaminated sites (Phibbs et al., 2011).

In this report we have investigated the responses of eelB to the absence of Se and to the addition of selenite, selenate or SeMet. Unlike previous reports on the actions of Se compounds in cell cultures, this was done in three different media: L15/FBS, L15 and L15/ex. L15/FBS consists of a basal medium, Leibovitz's L15 (Leibovitz, 1963), with a supplement of fetal bovine serum (FBS), and was chosen because this is commonly used for fish cell cultures (Bols et al., 2005). L15 and L15/ex were used so eelB survival in the absence Se could be studied. L15/ex has only the galactose, pyruvate, and the salts of L15 (Schirmer et al., 1997) and was used to exclude possible complicating interactions between Se compounds with L15 amino acids, such as cysteine and methionine, that *in vitro* can modulate toxic actions of metals and selenium compounds (Dayeh et al., 2005; Frisk, Yaqob, Nilsson, Carlsson, & Lindh, 2000; Tobe, Ueda, Ando, Okamoto, & Kojima, 2015). In the absence of Se, eelB survived for at least 7 days. Upon the addition either of selenite or selenate, eelB showed a dose-dependent decline in cell viability, and regardless of exposure media, selenite was much more cytotoxic than selenate. SeMet caused significant cytotoxicity only in L15/ex, and in L15/FBS, inhibited cell proliferation only at high concentrations.

3.2 MATERIAL AND METHODS

3.2.1 Cells and Routine Cell Culturing

The eelB cells originated from the American eel brain and were routinely grown in 75 cm^2 flasks with non-vented caps. Most commonly Falcon flasks manufactured by BD Bioscience (San Jose, CA, USA) and distributed by VWR (Mississauga, ON,

Canada) were used. The cells could also be routinely maintained in BioLite 75 cm^2 flasks from Thermo Fisher Scientific (Burlington, ON, Canada). The cells were grown in basal medium Leibovitz's L15 medium with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Burlington, ON, Canada), and 1% penicillin/streptomycin solution (Sigma-Aldrich, Oakville, ON, Canada). When the cells became confluent in flasks, they were subcultivated so that the contents of one flask were split into 2 flasks. Subcultivation or passaging was done with TrypLE™ Express (manufactured by Gibco, Grand Island, NY, USA and distributed through Life Science Technologies, Burlington, ON, Canada). TrypLE™ was used as recommended by the manufacturer except all solutions and manipulations were done at room temperature. In some cases rather than TrypLE™, trypsin was used to subcultivate cultures as described previously (Bols & Lee, 1994). Cells from cultures at between passages 70-100 were used in the following experiments. Routine growth and all subsequent experiments were done at room temperature.

3.2.2. Studying the Effects of Se Deprivation

Cultures were deprived of selenium as described below and monitored generally by observing and photographing cultures with a Nikon Coolpix Camera attached to a Nikon Eclipse TS100 inverted phase contrast microscope or a Leica EC4 Camera attached to an Olympus CKX41 microscope and specifically for cell viability, cell migration, and angiogenesis.

3.2.2.1 Depriving EelB Cultures of Se for Subsequently Measuring an Effect on Cell Viability

EelB were seeded in 24 well plates at a density of 150,000 cells per well in L15/FBS. After 24h the media was removed and the cells washed with L15/ex and the media replaced with either L15/ex or L15. At this point photographs and AB, CFDA-AM, and NR measurements as described below were taken for 12 wells. The other 12 wells were photographed and assayed the same way 7 days later.

3.2.2.2 Alamar Blue (AB)

Alamar Blue (AB) was used to assess the metabolic activity of cell cultures. AB is a commercial preparation of the dye resazurin by Immunocorp (O'Brien, Wilson, Orton, & Pognan, 2000) and was obtained from Medicorp (Montreal, PQ, Canada). Resazurin has the IUPAC name of 7-hydroxy-10-oxidophenoxazin-10-ium-3-one and is also known as diazo-resorcinol and rezoresorcin (Rampersad, 2011). Resazurin easily crosses cellular membranes, is non-toxic, and monitors the reducing environment of living cells. Resazurin is a non-fluorescent but when reduced by metabolically active cells becomes the fluorescent product resorufin. The reduction of AB has been attributed to cellular oxidoreductases in the cytoplasm and mitochondria and to the oxidation-reduction activity of the mitochondrial electron transport chain (O'Brien et al., 2000; Petrenko, Gorokhova, Tkachova, & Petrenko, 2005; Rampersad, 2011). Thus AB reduction provides a general measure of cellular metabolism.

AB was used to monitor metabolism of cells in 96 well plates by a protocol that has been described previously in a step-by-step fashion (Dayeh et al., 2005) and will only briefly be presented here. AB comes in pre-mixed solutions of 25 mL and 100 mL volumes ready to be prepared as a working solution. A 5% (v/v) working solution of Alamar Blue was prepared in L15/ex. AB solutions could be used as outlined previously (Ganassin et al., 2000). However, most commonly AB was combined with CFDA-AM so that the two different viability endpoints could be measured at the same time on the same culture wells (Schirmer et al., 1997). How this was done is described after the section below on CFDA-AM.

3.2.2.3 5-Carboxyfluorescein Diacetate (CFDA-AM)

CFDA-AM is 5-carboxyfluorescein diacetate acetoxymethyl ester and is an esterase substrate that is converted by the non-specific esterases of living cells from a non-polar, non-fluorescent dye into a polar, fluorescent dye (Dayeh et al., 2005). The substrate diffuses into cells rapidly whereas the product, 5-carboxyfluorescein (CF), diffuses out of cells slowly. Measuring CF production most directly measures esterase activity. When being used to monitor esterase activity in cell cultures that began with the cells attached to the surface of a culture well and subsequently had the medium removed prior to the addition of CFDA-AM, CF formation provides a measure of plasma membrane integrity. The intactness of the plasma membranes supports esterase activity in three ways. Functioning plasma membranes help keep cells on the plastic surface, retain esterases inside the cells, and maintain the cytoplasmic milieus to support esterase activity. Different experimental treatments might impact differently these

three ways that plasma membranes contribute to esterase activity. This could account for those rare situations where the CFDA-AM assay appears at odds with other viability endpoints (Dayeh, Chow, Schirmer, Lynn, & Bols, 2004). However, overall a decline in CF formation represents a loss in plasma membrane integrity (Dayeh et al., 2005; Ganassin et al., 2000).

CFDA-AM was used to monitor plasma membrane integrity of cells in 96 well plates by a protocol that has been described previously in a step-by-step fashion (Dayeh et al., 2005). The procedure will only briefly be presented here. CFDA-AM in crystalline form was produced by Molecular Probes (Eugene, OR, USA) and obtained from Life Science Technologies (Burlington, ON, Canada). Anhydrous DMSO (# 27,685-5, Aldrich, Milwaukee, WI, USA) was added directly to the CFDA-AM vial to give a 4 mM CFDA-AM stock solution. The CFDA-AM stock solution was diluted 1:1,000 in L-15/ex to make a working solution of 4 μM CFDA-AM. CFDA-AM solutions could be used as outlined previously (Ganassin et al., 2000). However, most commonly CFDA-AM was combined with AB so that the two different viability endpoints could be measured at the same time on the same culture wells (Schirmer et al., 1997) as described in the next section.

Plasma membrane integrity and cellular energy metabolism were monitored concurrently in individual culture wells of 96 well plates as described previously (Dayeh et al., 2005; Ganassin et al., 2000; Schirmer et al., 1997). The working solution for this was prepared by mixing 8μl of the CFDA-AM stock solution with 0.4

mL of Alamar Blue in 7.6 mL of either L-15/ex or L-15/salts to yield 4 μM CFDA-AM and 5 % (v/v) Alamar Blue. To each culture well 100 μL of this working solution was added after the wells had been emptied of medium either by dumping or aspiration. The plates were incubated in the dark for 30 or 60 minutes at room temperature and then read on a CytoFluor series 4000 microplate reader. For AB, the excitation and emission wavelengths were respectively 530 nm and 595 nm. For CFDA-AM, the excitation and emission wavelengths were respectively 485 nm and 530 nm. The results were recorded as relative fluorescent units (RFUs), and for each treatment or condition 8 replicate wells were used. The mean RFUs for the experimental wells were expressed as a percentage of the mean RFUs for control wells.

3.2.2.4 Neutral Red (NR)

Neutral red (NR) (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) was used to measure plasma membrane integrity and lysosomal activity (Dayeh et al., 2005). The principle behind the use of this fluorescent dye is that like other lipophilic weak bases, NR accumulates in acidic cytoplasmic compartments, such as endosomes, Golgi apparatus, and most prominently, lysosomes (Aki, Nara, & Uemura, 2012; Borenfreund & Puerner, 1985; Goldman, Funk, Rajewski, & Krise, 2009). The mechanism is ion trapping (Goldman et al., 2009). NR diffuses through the plasma membrane into the cytoplasm and then into the lysosome. The low lysosomal pH causes the dye to become charged, trapping and accumulating NR inside lysosomes (Goldman et al., 2009; Morissette, Moreau, René, & Marceau, 2004). For this mechanism to work, the lysosomal pH must be maintained low (pH ~5.5). A vacuolar

(V)-ATPase does this by pumping protons into the lumen of lysosomes (Goldman et al., 2009). This requires that the cells maintain an intact plasma membrane and continue to produce ATP to support the V-ATPase. Therefore, a decline in NR accumulation or retention indicates cell injury or a loss of cell viability. Yet sometimes an increase in NR accumulation is seen. This has been attributed to cytoplasmic vacuolization (Dayeh, Schirmer, & Bols, 2009; Michalik, Pierzchalska, Pabiańczyk-Kulka, & Korohoda, 2003) and/or increase in autophagic vacuole formation (Martins, Severino, Souza, Stolf, & Baptista, 2013).

In this thesis NR usually was used to evaluate the viability of cell cultures in 96 well plates. A stock solution of NR (3.3 mg/mL) in Dulbecco's PBS (D-PBS) was purchased from Sigma (St Louis, MO). This was diluted in L-15/ex just before use to give a working solution of 3.3 μg/mL. The step-by-step procedures for NR assay have been presented previously (Dayeh et al., 2005) and will only be briefly presented here. 100 μL of the NR working solution was added to each culture well after the wells had been emptied of medium either by dumping or aspiration. The plates were incubated in the dark for 60 minutes at room temperature. After 60 minutes the wells were emptied of NR solution by inverting the plate over a catch basin and blotting on a stack of paper towels. The wells were rinsed with 100 μL of L-15/ex, followed by the addition of 100 μL of fixative. The fixative was 0.5% [v/v] formaldehyde and 1% [w/v] $CaCl_2$ in deionized, distilled (dd) H_2O. Fixation lasted only 1 min and was terminated by again inverting the plate over a catch basin. Then 100 μL of extraction solution was added to

each well. The extraction solution was 1% (v/v) acetic acid and 50% (v/v) ethanol in dd H_2O. Extraction occurred for 10 minutes, and during this time the plate was agitated gently on an orbital shaker. The plates were read on a CytoFluor series 4000 microplate reader, at excitation and emission wavelengths of 530nm and 645nm, respectively. The results were recorded as relative fluorescent units (RFUs), and for each treatment or condition 8 replicate wells were used. The mean RFUs for the experimental wells were expressed as a percentage of the mean RFUs for control wells.

3.2.2.5 Studying the Effects of Se Deprivation on Cell Migration and Angiogenesis

A cell migration or wound healing assay was done in Culture-Insert 2 wells from Ibidi (Madison, WI) that were inserted into 12 well plates from Falcon. Each insert consists of 2 chambers open at the top and the bottom. The edges at the bottom have a sticky surface so that when placed into a well of a 12 well plate the insert forms a seal with the tissue culture plastic, making within the well two small rectangular chambers into which cells can be added from the top. Approximately 50,000 eelB cells in L15/FBS were seeded into each chamber, which has a growth area of 0.22 cm^2. Once the cells within these chambers had become confluent, usually 24 h, the cells were washed in PBS then L15/ex or L15/FBS were added into each chamber. After 24 h, the culture inserts were removed as described in Ibidi Application Note 21, leaving within the well of a 12 well plate two small rectangular monolayers separated by a 500 μm gap. To a well from which an insert had been removed, 1 ml of either L15/ex or L15/FBS was immediately added. The well was then observed under an inverted phase contrast microscope and the gap between the two monolayers photographed. ImageJ

1.50i was used to calculate the area of the initial (time 0) gap or wound. The movement of cells into this area was monitored by the same methods for up to 10 days afterwards. The area of the wound that became covered with cells was calculated and expressed as a % of the initial wound area.

As a measure of their capacity to undergo angiogenesis in the absence of Se, eelB cells were assayed for the formation of capillary-like structures (CLS) on Matrigel (BD 35423, BD Biosciences, Mississauga, ON) as described previously (Bloch et al., 2016) but in L15/ex. Cultures were initiated on Matrigel without Se in two ways and monitored on Matrigel by phase contrast microscopy for up to three days. One way was to have grown the cells in L15/FBS as usual and then collect and plate the cells in L15/ex onto Matrigel. The second way was to have maintained the cells in L15/ex for several days and then collect and plate the cells in L15/ex onto Matrigel.

3.2.3 Studying Effects of 24 h Selenite Exposures in 3 Different Media on Cell Viability

The effect of selenite on eelB cell viability was evaluated by procedures that have been documented in detail for investigating the toxicity of environmental contaminants with fish cell lines (Dayeh, Bols, Tanneberger, Schirmer, & Lee, 2013). Briefly, eelB cells were plated in 96 well plates at a density of 40,000 cells per well in L15/FBS. After 24h the cells had formed monolayers and the media was removed and the monolayers rinsed with L15/ex or phosphate buffered saline (PBS). At this point culture wells received different concentrations of sodium selenite either in L15/ex, L15 or L15/FBS. For each concentration and exposure media, generally six identical wells

were set up. Six wells received L15/ex, L15, or L15/FBS and were the control cultures. After 24 h, all wells were evaluated for cell viability with AB, CFDA-AM, and NR and the results recorded as RFUs. The RFUs for the selenite cultures were expressed as percentages of the control cultures. Dose-response curves were analyzed using the graphing software of GraphPad Prism (GraphPad Software, San Diego, CA USA) and the effective concentrations causing a 50% decline in cell viability (EC50s) were determined. Statistical analyses were done using GraphPad InStat (version 3.00). EC50s from the different cell viability endpoints or from exposures in different media were compared by one-way analysis of variances (ANOVA). If the ANOVA was significant, a Tukey-Kramer multiple comparisons test was performed to find the pairs of means that were significantly different. In all cases a p-level <0.05 was considered as significantly different.

3.2.4 Studying Effects of 24 h Selenate Exposures in 3 Different Media on Cell Viability

The effect of selenate on eelB cell viability was evaluated and analyzed as described above for selenite. For each cell viability endpoint, the selenite and selenate EC50s were compared by unpaired t-test ($p < 0.05$). In one experiment eelB cells were exposed to selenate in L15/FBS in 12 well plates in order to better photograph the cultures.

3.2.5 Studying Effects of 24 h SeMet Exposures in 3 Different Media on Cell Viability

The effects of L-SeMet on eelB cell viability were investigated with 24 h exposures as described above for selenite but also in 24 h exposures that occurred after the cells had been in the three exposure media, L15/ex, L15, and L15/FBS, for 7 days before the L-SeMet was added.

3.2.6 Studying Effects of 7-Day SeMet Exposures in 3 Different Media on Cell Viability

The effects of DL and L-SeMet on eelB cell viability were investigated for exposures of 7 days either in L15/ex, L15, or L15/FBS. As described for selenite, cultures were set up in 96 well plates and evaluated for cell viability with AB and CFDA-AM.

3.2.7 Studying the Effects of SeMet on Cell Proliferation

Approximately 2,000 eelB cells in L15/FBS were seeded per 25 cm^2 flask and allowed to attach for 24 h. At this time, the medium was removed and replaced with L15/FBS or L15/FBS with DL- or L-SeMet up to 1 mM. Ten small squares, 1.6 cm^2 in total, were marked on each flask, and the cells inside the squares were observed periodically over 14 day. On day 14 the cells were fixed with methanol and stained with 0.5% Crystal Violet. A cluster of 50 or more cells arising from a single cell was defined as a colony. The number of colonies inside the squares were counted and multiplied up to give the estimated number of colonies in the whole flask. The mean

colony numbers for the SeMet flasks were compared with the mean colony number for the control flasks through a one-way ANOVA. If $p < 0.05$, this was followed by Dunnett's test to determine which treatment flasks were different from the control flasks ($p < 0.05$).

3.2.8 Studying the Effects of SeMet on Cell Migration and Angiogenesis

The effect of DL-SeMet on cell migration was assayed with Ibidi Culture-Insert 2 wells as described above in section 2.2.5 for Se deprivation. Approximately 50,000 eelB cells in L15/FBS were seeded into each chamber. After 24h the cells had become confluent within these chambers and the culture inserts were removed to establish gaps. To wells from which inserts had been removed, 1 ml of media was immediately added to each well. The control medium was L15/FBS and the treatment media were L15/FBS with 31.25, 125 or 500μM DL-SeMet. The gaps between monolayers were photographed immediately to give time 0 values and at times afterwards for up to 3 days. The areas in the gap with and without cells were calculated with ImageJ 1.50i. A one-way ANOVA was used to compare in the different media areas with cells. When the ANOVA had a $p > 0.05$, Dunnett's test was used to compare areas from treated cultures with the control cultures ($p > 0.05$). For graphical presentation, the areas of the wound that became covered with cells was expressed as a % of the initial wound area that began free of cells.

As a measure of their capacity to undergo angiogenesis in the presence of SeMet, eelB cells were assayed for the formation of CLS on Matrigel (BD 35423, BD Biosciences, Mississauga, ON) as described previously (Bloch et al., 2016). Cells from

cultures routinely grown in L15/FBS were plated onto Matrigel in L15/FBS (control) and in L15/FBS with 31.25, 125 or 500 μM DL-SeMet. Alternatively cells from cultures routinely grown in L15/FBS without any further addition or in L15/FBS with 31.25, 125 or 500 μM DL-SeMet were plated onto Matrigel in their respective media. Cultures were monitored by phase contrast microscopy for up to four days.

3.3 RESULTS

The ability of eelB cells to withstand selenium (Se) deprivation was studied first and then the effects of adding sodium selenite (Na_2SeO_3, selenite), sodium selenate (Na_2SeO_4; selenate), and DL- or L-selenomethionine (DL- or L-SeMet) to cultures were examined.

3.3.1 Effects of Se Deprivation

When cultures were switched to either L15 or L15/ex, which should have no Se, eelB cells remained viable for at least a week. During 7 days in L15 or L15/ex, eelB cell monolayers remained adherent to the culture surface and maintained a similar appearance (Figure 3.1).

These monolayers showed little decline in viability as assessed with the indicator dyes, Alamar Blue (AB) for energy metabolism, CFDA-AM for plasma membrane integrity, and neutral red (NR) for lysosome activity, although lysosomal activity appeared to be better maintained in L15. These results are shown in Figure 3.1 and illustrate that selenium is not needed to maintain the general viability of eelB cells. The eelB cells were also able to migrate and form capillary-like structures (CLS) in medium

without Se (data not shown). The cells migrated to close a wound in L15/ex but the migration was much slower in L15/ex than in L15/FBS (Figure 3.2). After having been in L15/ex or L15 for up to seven days and then replated in L15/ex or L15 but onto Matrigel, the cells could still formed CLS (data not shown).

3.3.2 Effects of 24 h Selenite Exposures in Three Different Media on Cell Viability

Selenite at high concentrations was cytotoxic to eelB cells. For up to 50 µM little change was observed in the appearance and viability of cultures exposed for 24 h to selenite either in L15/ex, L15 or L15/FBS. In cultures with higher concentrations cells lost their regular shape and began to round in the higher concentrations. Exposure to selenite concentrations above 100 µM in all three media caused a dose-dependent decline in cell viability as evaluated either with AB, CFDA-AM, and NR (Figure 3.3 A, B, & C). The concentrations causing a 50 % decline in cell viability (EC50) are summarized in Table 3.1. The EC50s were similar, despite different exposure media and cell viability different measures of cytotoxicity gave similar EC50s. However, as indicated by the standard deviations, the EC50s were more variable for L15/FBS.

Figure 3.1 Viability of eelB cells after 7 days of selenium (Se) deprivation in either L15/ex (top panels) or L15 (bottom panels). (Next page.) The eelB cells in L15/FBS were added to wells of 24 well culture plates. After 24 h the cells had attached and spread to form monolayers and the medium was removed. The monolayer cultures were well rinsed in L15/ex and then received either L15/ex or L15, both of which would have no obvious source of Se. Immediately some cultures were photographed (A & D, scale bar = 100 μm) and 12 cultures were evaluated for cell viability. Cell viability was evaluated with Alamar Blue (AB) for energy metabolism, 5-Carboxyfluoroscein Diacetate Acetoxymethyl ester (CFDA AM) for plasma membrane integrity, and neutral red (NR) for lysosomal activity and the results recorded as relative fluorescent units (RFUs). The RFUs from these assays established the base line viability at day 0. After 7 days at room temperature, some wells were photographed (B & E, scale bar = 100 μm) and 6 wells for each medium were evaluated with AB, CFDA AM, and NR. For each assay the RFUs were expressed as a percentage (Y axis) of the RFUs at day 0, which was set at 100%. The bar graphs show the mean % ± standard deviation (n=6) (C & F).

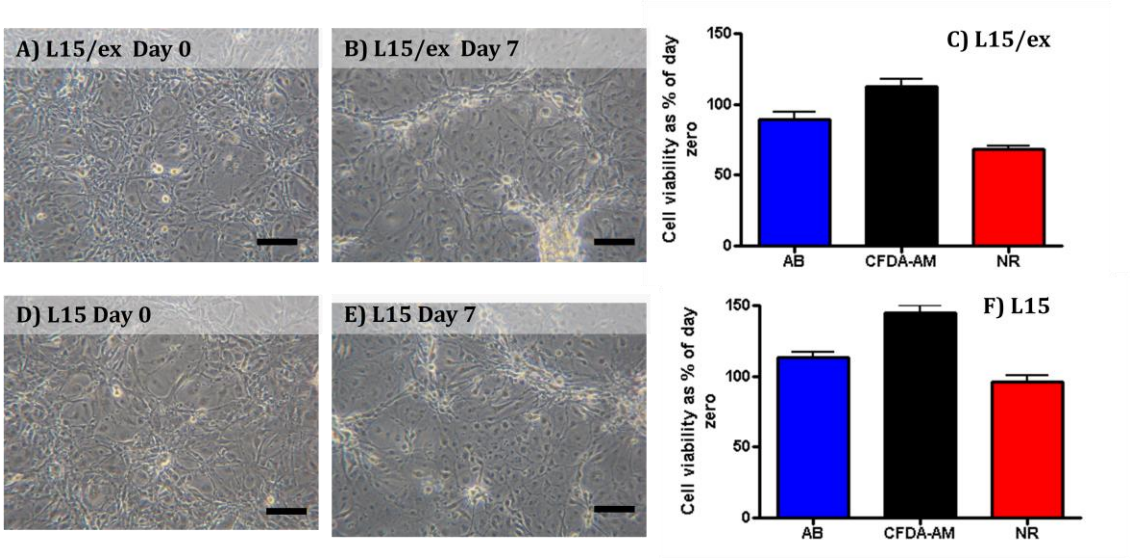
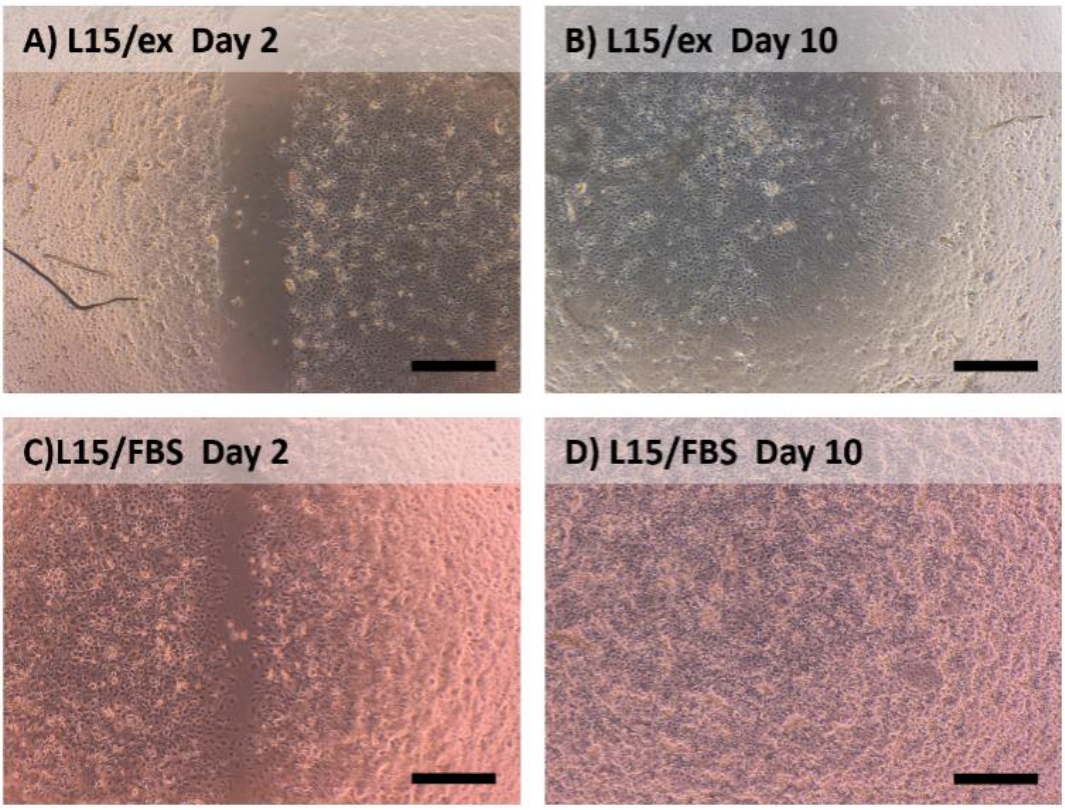

A) L15/ex Day 0
B) L15/ex Day 7
C) L15/ex
Cell viability as % of day zero
150
100
50
0
AB
CFDA-AM
NR
D) L15 Day 0
E) L15 Day 7
F) L15
Cell viability as % of day zero
150
100
50
0
AB
CFDA-AM
NR

Figure 3.2 Migration of eelB cells during Se deprivation in L15/ex. (Next page.) After the eelB cells had attached and formed monolayers in the chambers of 2-Well Culture Inserts from Ibidi, the medium (L15/FBS) was removed, the chambers rinsed, and for one 2-Well Culture Insert L15/ex was added to both chambers and for another L15/FBS was added to both chambers. After 24 h, the Inserts were lifted to create a 500 μm gap or "wound" at which point the cells could begin migrating in either L15/ex, which would have no obvious source of Se, or L-15/FBS, which would have Se due to the FBS. Photographs of the gap were taken immediately (day 0) and for up to 10 days afterwards and are shown in A) for day 2 and B) for day 10 in L15/ex and in C) for day 2 and D) for day 10 in L15/FBS. The photographs were used to calculate areas of the gap covered with cells. In E) the bar graph shows for days 2 and 10 the percentage of the gap or "wound" covered with cells relative to the area of the gap immediately upon being made (day 0). Scale bar = 500 μm.


A) L15/ex Day 2
B) L15/ex Day 10
C)L15/FBS Day 2
D) L15/FBS Day 10

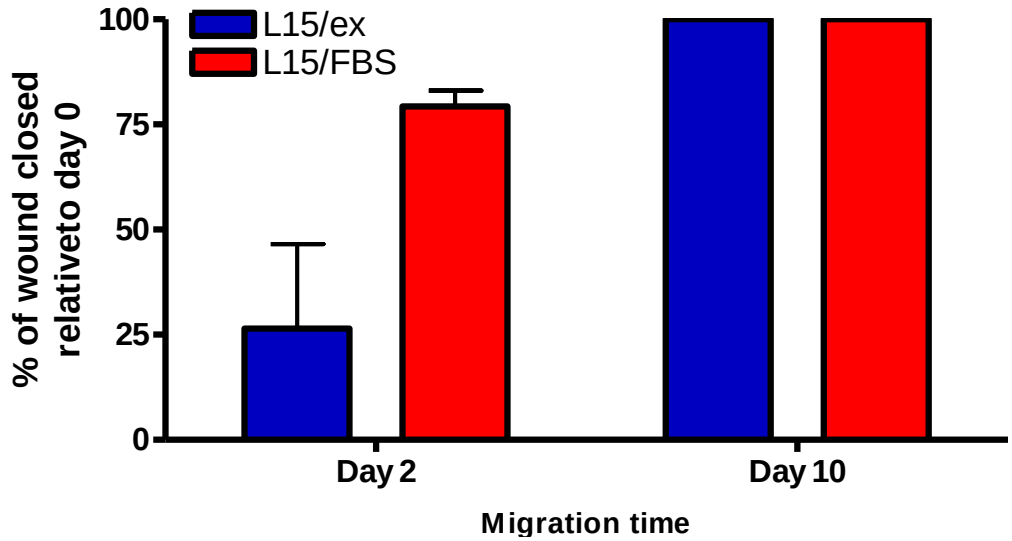

100
75
50
25
0
L15/ex
L15/FBS
% of wound closed relativeto day 0
Day 2
Day 10
Migration time

Figure 3.3 Effect on cell viability of 24 h exposures to either sodium selenite or sodium selenate in either L15/ex, L15 or L15/FBS. (Next page.) Confluent cultures of eelB cells in 96 well plates were exposed to sodium selenite (A, B, & C) or sodium selenate (D, E & F). As described in Figure 1, cell viability was monitored with AB (A & D), CFDA AM (B & E) and NR (C & F), with 6 wells used for each assay and culture condition. The results were recorded as RFUs and used to calculate EC50s (Table 3.1 & Table 3.2). For each assay the RFUs were expressed as a percentage (Y axis) of the RFUs in control cultures (100%) and the plots show for one experiment the mean % ± standard deviation (n=6).

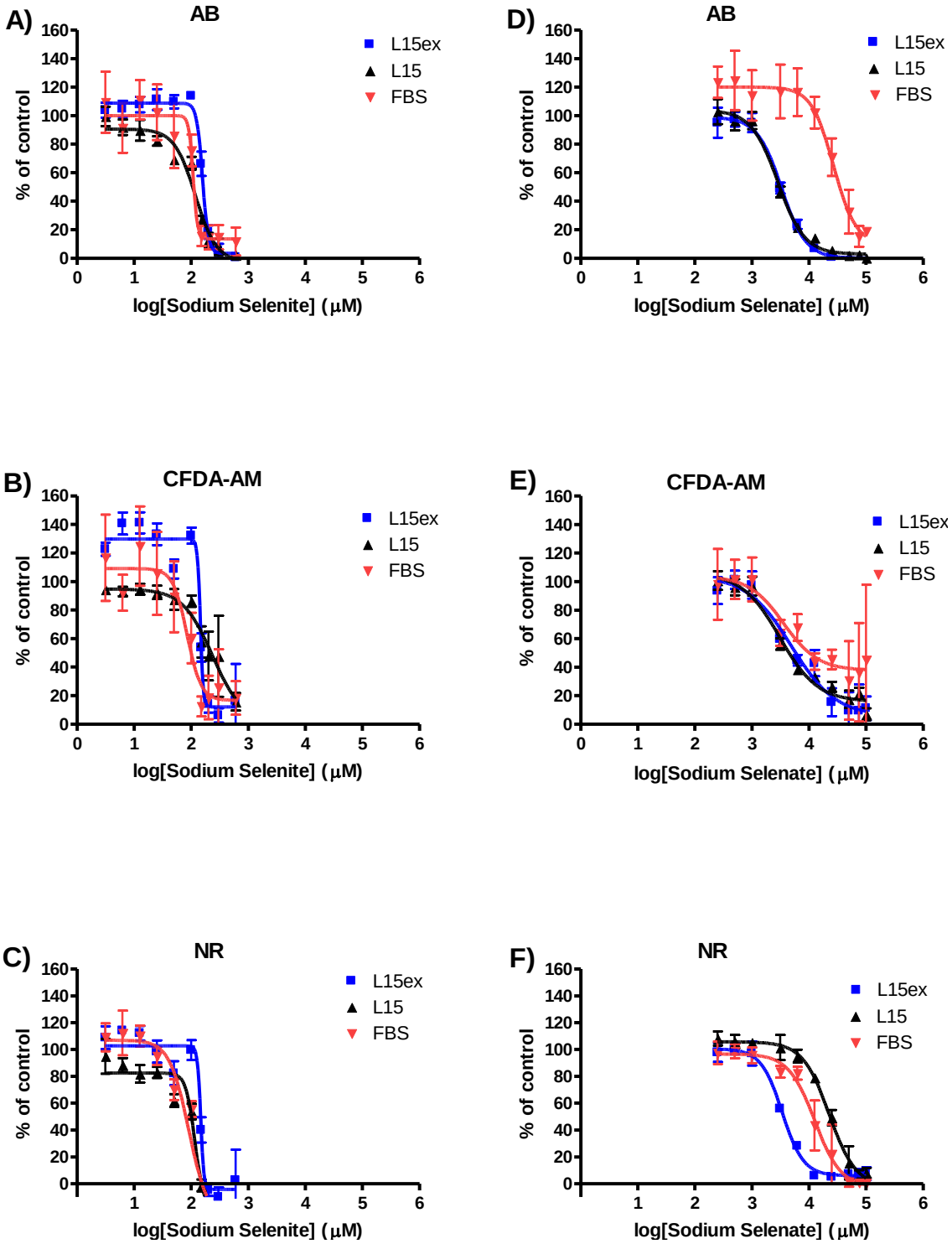

A)
AB
L15ex
L15
FBS
% of control
log[Sodium Selenite] (µM)
D)
AB
L15ex
L15
FBS
% of control
log[Sodium Selenate] (µM)
B)
CFDA-AM
L15ex
L15
FBS
% of control
log[Sodium Selenite] (µM)
E)
CFDA-AM
L15ex
L15
FBS
% of control
log[Sodium Selenate] (µM)
C)
NR
L15ex
L15
FBS
% of control
log[Sodium Selenite] (µM)
F)
NR
L15ex
L15
FBS
% of control
log[Sodium Selenate] (µM)

Table 3.1 Cytotoxicity of selenite to eelB cell after 24 h exposures in different media

Media	Cell viability assays for evaluating cytotoxicity			comparison[2]
	EC50[1] means in µM with standard deviation			
	Alamar Blue (AB)	CFDA-AM (CF)	Neutral Red (NR)	
I) L15/ex	**146.4 ±23.7**(n=5)	**128.8 ±29.6** (n=5)	**113.5 ±38.3** (n=5)	**AB=CF=NR**
II) L15	**148.9 ±25.4** (n=4)	**164.6 ±39.1** (n=4)	**121.6 ±27.2**(n=4)	**AB=CF=NR**
III) L15/FBS	**118.0 ±73.5** (n=5)	**117.0 ±128.0** (n=5)	**61.4 ±50.8** (n=5)	**AB=CF=NR**
comparison[3]	**I=II=III**	**I=II=III**	**I=II=III**	

[1]Sodium selenite concentration that resulted in 50 % cell viability in eelB cultures.

[2]ANOVAs were done across rows and $p>0.05$.

[3]ANOVAs were done down columns and $p>0.05$.

3.3.3 Effects of 24 h Selenate Exposures in Three Different Media on Cell Viability

Selenate at high concentrations was cytotoxic to eelB cells. For up to 1,000 µM, little change was observed in the appearance and viability of cultures exposed for 24 h to selenate either in L15/ex, L15 or L15/FBS. In cultures with higher concentrations cells lost their regular shape and began to round and detach in the higher concentrations. Exposure to selenate concentrations above 25000 µM in all three media

caused a dose-dependent decline in cell viability as evaluated either with AB, CFDA-AM, and NR (Figure 3.3 D, E&F). Despite the different viability endpoints, the EC50s were similar (Table 3.2). However, for the exposure media, EC50s in L15/FBS were higher than in the other media but they also had higher standard deviations so only the EC50 for AB was statistically different ($p < 0.05$).

Table 3.2 Cytotoxicity of selenate to eelB cell after 24 h exposures in different media

Media	Cell viability assays for evaluating cytotoxicity			comparison[2]
	EC50[1] means in µM with standard deviation			
	Alamar Blue (AB)	**CFDA-AM (CF)**	**Neutral Red (NR)**	
I) L15/ex	**3,349.2 ±439.9** (n=5)	**4,053.6±1,690.8** (n=5)	**4,059.8±769.8** (n=5)	**AB=CF=NR**
II) L15	**3,820.2 ±1461.9** (n=5)	**4,243.75±1,384.19** (n=4)	**5,060.4±2,181.8** (n=5)	**AB=CF=NR**
III) L15/FBS	**17,119.8 ±6939.1**(n=5)	**11,047.4 ±19,212.0** (n=5)	**12,617.2±6,483** (n=5)	**AB=CF=NR**
comparison[3]	**I=II<III**	**I=II=III**	**I=II=III**	

[1]Sodium selenate concentration that resulted in 50 % cell viability in eelB cultures.

[2]ANOVAs were done across rows and $p > 0.05$.

[3]ANOVAs were done down columns, and when $p < 0.05$, the Tukey-Kramer Multiple Comparisons Test was done to find significant comparisons ($p < 0.05$).

The EC50s for selenate are summarized in Table 3.2 and are higher than the EC50s for selenite (Table 3.1). The magnitude of the differences varied with the exposure media and to a lesser extent the cell viability endpoints. In L15/ex or L15, the EC50s for selenate were 23 to 42 fold higher. In L15/FBS, the EC50s for selenate were 94 to 206 fold higher but again the standard deviations were very high in this exposure medium.

Examining cultures closely by phase contrast microscopy during the 24 h exposures to selenite or selenate in different media revealed a possible cause for the variability in

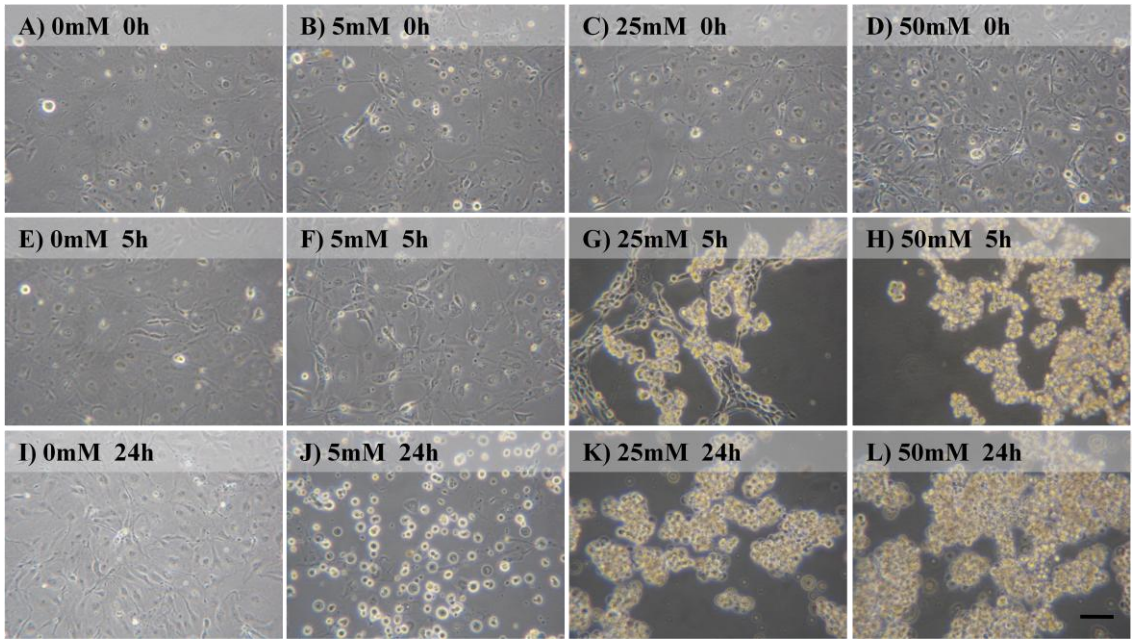


Figure 3.4 Sodium Selenate detaches eelB cells in L15/FBS. Confluent EelB in 12 well plates were exposed up to 24h to 5mM, 25mM and 50mM sodium selenate in L15/FBS and pictures were taken just after dosing (A-D), after 5h (E-H), and after 24h (I-L). After 5h cells detached in the 25mM and the 50mM concentration, and after 24h the cells are detached in all 3 media. Scale bar=100µm.

EC50s in L15/FBS. In cultures with L15/ex or L15 the cells generally remained attached to the culture surface over the course of the selenite or selenate exposures. However, in L15/FBS, the cells in some but not all experiments started to detach in sheets or large clumps. This was most evident with selenate in L15/FBS (Figure 3.4). Whether dead or alive, detached cells would be recorded as dead in the viability assays because they would be removed prior to the indicator dyes. When the clumps or sheets in L15/FBS were added to new cultures wells, they did not reattach, suggesting that all the cells were dead. However, live cells might have been present in the clumps/sheets but they might be unable to reattach because they are caught up in the network of dead cells.

3.3.4 Effects of 24 h SeMet Exposures in Three Different Media on Cell Viability

In contrast to selenite and selenate, SeMet for 24 h, even at concentrations as high as 10 mM, caused no cytotoxicity to eelB cultures in L15 and L15/FBS (data not shown). Yet in L15/ex, 10 mM L-SeMet was significantly cytotoxic, with the three cell viability assays showing a 60 to 70 % drop (Figure 3.5 A, B, & C). At lower concentrations declines in cell viability were minor, less than 20 % (Figure 3.5 A, B, & C). As nutrient deprivation appeared to sensitize eelB cells to SeMet, cultures were maintained in L15/ex for 7 days prior to 24 h L-SeMet exposures. Surprisingly, 10 mM L-SeMet was now less cytotoxic, with the declines in cell viability being

approximately 30% in AB and CFDA- AM assays and not being observed with NR (Figure 3.5 D, E, & F).

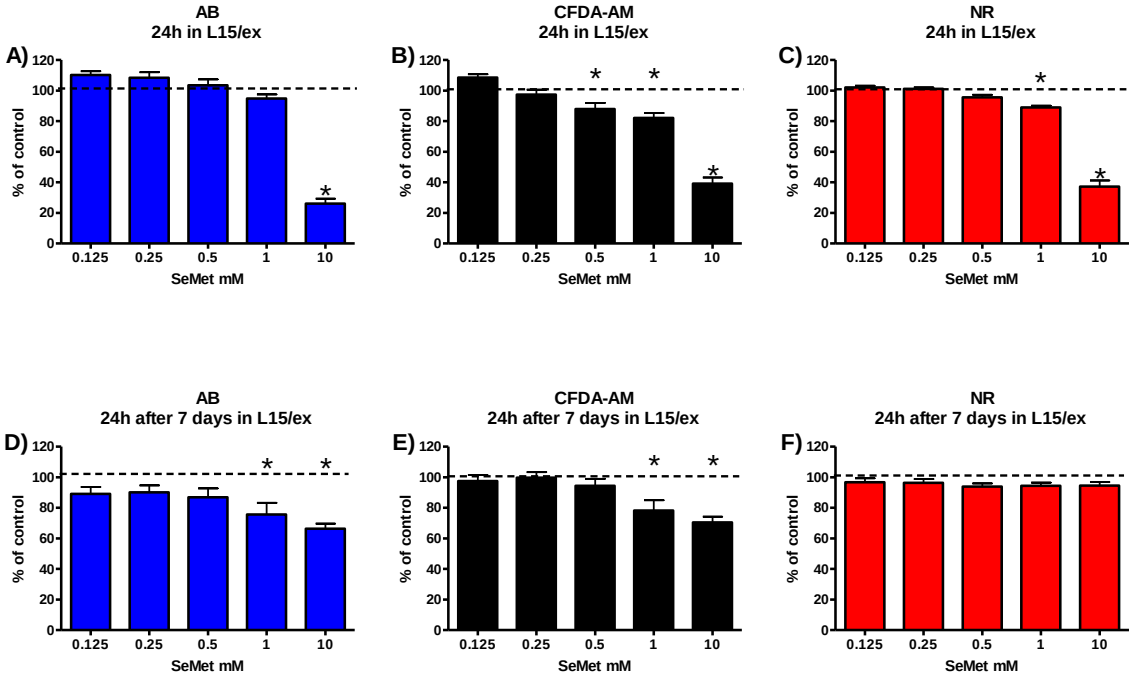


Figure 3.5 Effect on cell viability of 24 h exposures to SeMet in L15/ex. Confluent cultures of eelB cells in 96 well plates were exposed for 24 h to L-SeMet concurrently with the change to L15/ex (A, B, & C) or 7 days after the change to L15/ex (D, E & F). Cell viability was monitored with AB (A & D), CFDA AM (B & E) and NR (C & F). As described in Figure 3, viability assays were done in 8 wells for each assay and culture condition and recorded as RFUs. RFUs were subjected to an ANOVA, and if significant ($p < 0.05$), the results compared to the control (no SeMet) with Dunnett's test, $p < 0.05$). Values statistically different from the control are marked with an asterisk ($p < 0.05$). For each assay the RFUs were expressed as a percentage (Y axis) of the RFUs in control cultures, which was set at 100 % (dotted line), and the percentage means ± standard deviations (n=8) are plotted.

3.3.5 Effect of 7-Day SeMet Exposures in Three Different Media on Cell Viability

In contrast to 24 h exposures, 7-day SeMet exposures in all three media were cytotoxic. However the eelB cells were most susceptible in L15/ex and least in

L15/FBS, and L-SeMet was more cytotoxic than DL-SeMet. In L15/ex, both DL- and L-SeMet were cytotoxic at all concentrations tested (31.25-1,000 μM) (Figure 3.6), with the declines in cell viability being greater than 90 % at 125 μM. As judged with both AB and CFDA-AM, DL-SeMet was cytotoxic in L15 only at 1,000 μM and the declines in cell viability were less than 30 %, whereas L-SeMet in L15 was cytotoxic at 125 μM and at 1,000 μM the declines cells viability were greater than 90 %. In L15/FBS, DL-SeMet was cytotoxic only at 1,000 μM and the declines in cell viability were less than 25 %. L-SeMet in L15/FBS was cytotoxic at 500 μM and at 1,000 μM the declines in cell viability were in the 50 to 75% range.

3.3.6 Effects of SeMet on Cell Proliferation

SeMet at high concentrations inhibited eelB cell proliferation, as judged by the ability of cells to form colonies. At a low cell density, eelB cells formed colonies in L15/FBS with or without SeMet but at 500 and 1,000 μM SeMet, fewer colonies formed (Figure 3.7 A & B) and the colonies were smaller but with larger cells, some being binucleated (Figure 3.7 C & D). When colonies of 50 or more cells were counted, no colonies were recorded at 1,000 μM of either DL- or L- SeMet (Figure 3.7). At 500 μM no colonies were seen in L-SeMet and in DL- SeMet colony formation was reduced by approximately 70 % (Figure 3.7). Colony formation was unaltered by lower SeMet concentrations (Figure 3.7).

Figure 3.6 Effect on cell viability of 7 day exposures to SeMet in either L15/ex, L15 or L15/FBS. (Next page.) Confluent cultures of eelB cells in 96 well plates were exposed for 7 days to either DL- SeMet (open bar) or L-SeMet (filled bar) in either L15/ex (A & B), L15 (C & D), or L15/FBS (E & F). As described in Figures 1 and 4, cell viability was monitored with AB (A, C & E) and CFDA AM (B, D & F), recorded as RFUs, and plotted as a percentage of the control. RFUs were subjected to an ANOVA, and if significant ($p < 0.05$), the results compared to the control (no SeMet) with Dunnett's test, $p < 0.05$). All values were significantly different from the controls in panels A and B. Values statistically lower than the controls are marked with an asterisk ($p < 0.05$) in panels C, D, E & F.

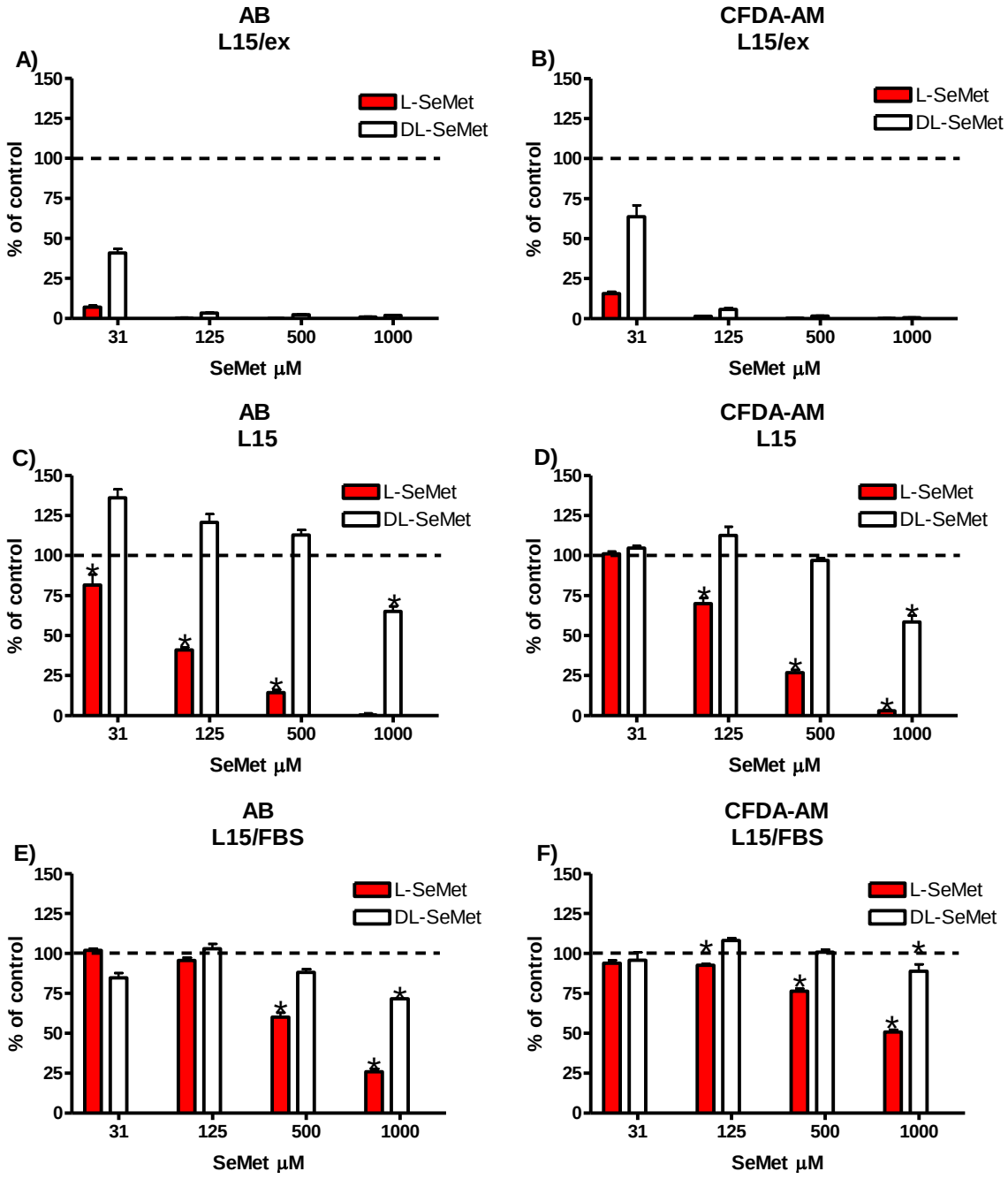
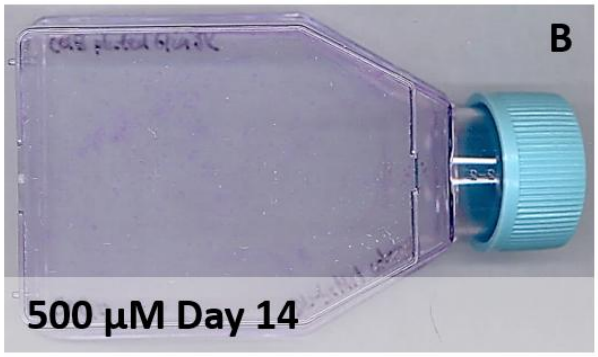
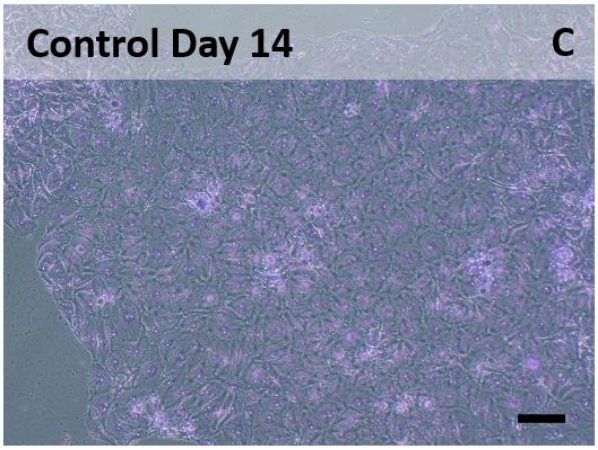
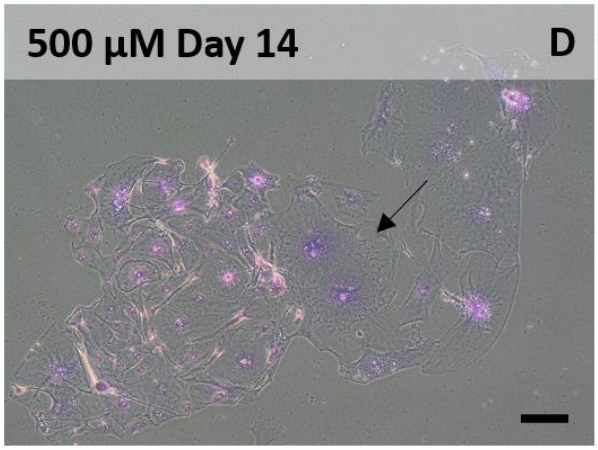

AB
L15/ex
A)
CFDA-AM
L15/ex
B)
AB
L15
C)
CFDA-AM
L15
D)
AB
L15/FBS
E)
CFDA-AM
L15/FBS
F)
L-SeMet
DL-SeMet
% of control
SeMet µM
0
25
50
75
100
125
150
31
125
500
1000

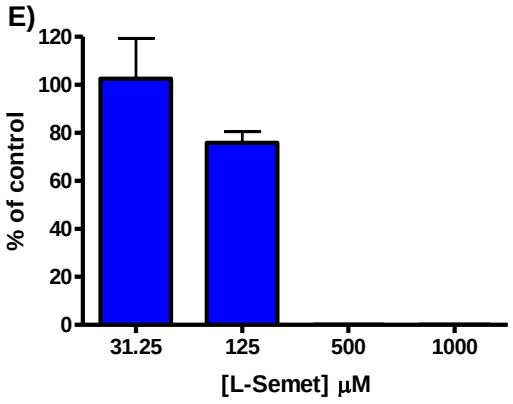
Figure 3.7 Appearance and Ability of eelB to form colonies in different concentrations of L-SeMet or DL-SeMet. (Next page.) Approximately 2,000 eelB cells in L15/FBS were seeded per T-25 flask. After the cells had been allowed to attach for 24 h, the medium was removed and replaced with L15/FBS (control, A & C) or L15/FBS with 500 μM DL-SeMet (B & D). The flasks were incubated at room temperature for 14 days at which time the cells were stained with 0.5% Crystal Violet. In A & B the flasks were photographed at approximately 1X to illustrate overall formation of colonies in the two culture conditions. In C & D, the flasks were photographed at 100X to show the appearance of individual colonies (the scale bar indicates 100μm). The arrow points to a binucleated cell (D). Scale bar = 100μm. For E & F, cultures were initiated in T-25 flasks and after 24 h the L15/FBS was changed to L15/FBS (control) and to L15/FBS with increasing concentrations of either L-SeMet (E) or DL-SeMet (F). After 2 weeks the cultures were stained and the numbers of colonies with ≥50 cells were counted. In control flasks (L15/FBS) the mean number of colonies with standard deviations was 120 ± 9 (n=3). The mean colony numbers for the SeMet cultures were compared with the means for control cultures through a one-way ANOVA followed by Dunnett's test, $p < 0.05$. The asterisk marks cultures that were significantly different from the control. For graphic presentation the results for the SeMet flasks were expressed as a percentage of the colonies in the control flasks. The bars indicate mean percentages with standard deviations (n=3).


A
Control Day 14


B
500 µM Day 14


Control Day 14
C


500 µM Day 14
D


E)
% of control
120
100
80
60
40
20
0
31.25
125
500
1000
[L-Semet] µM

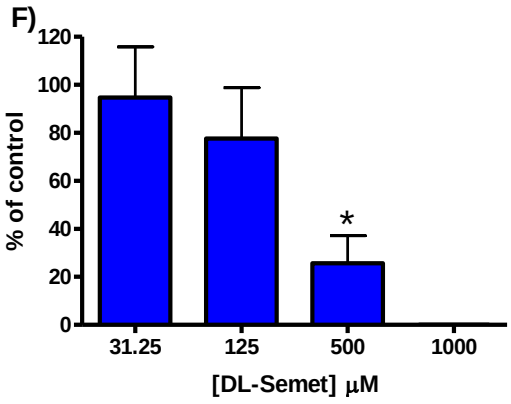
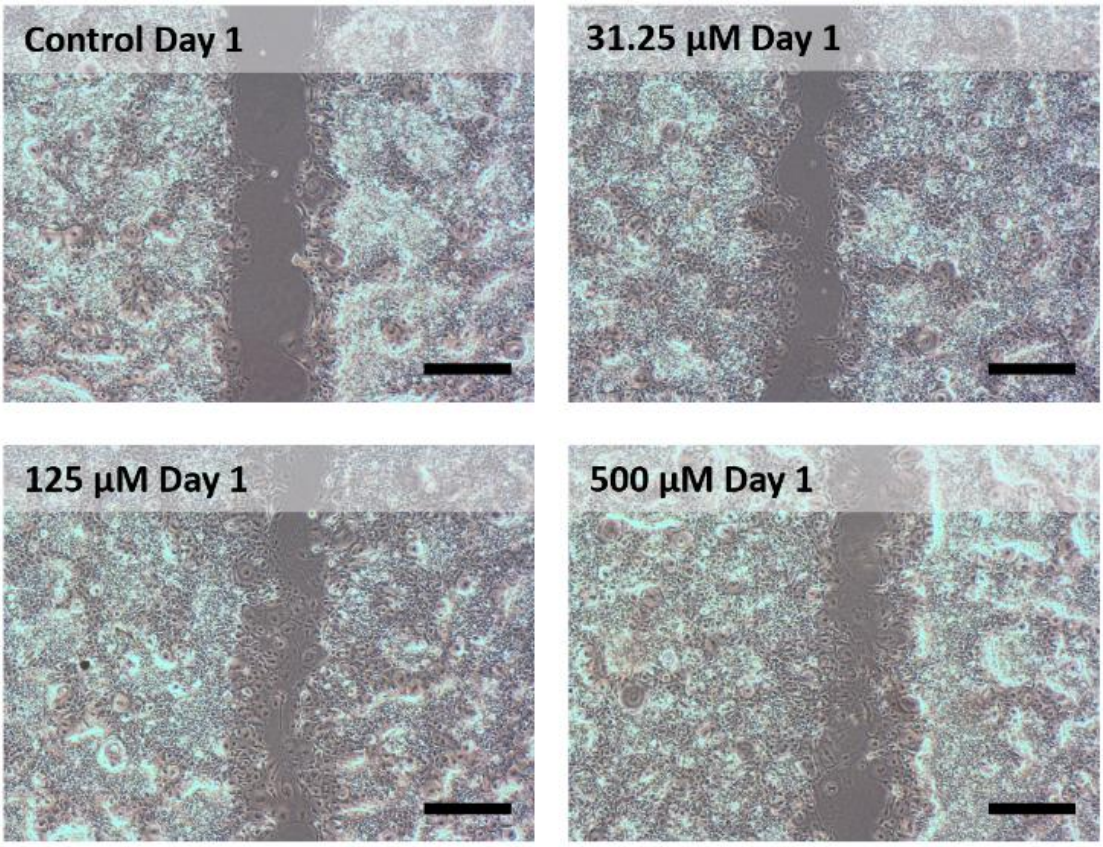
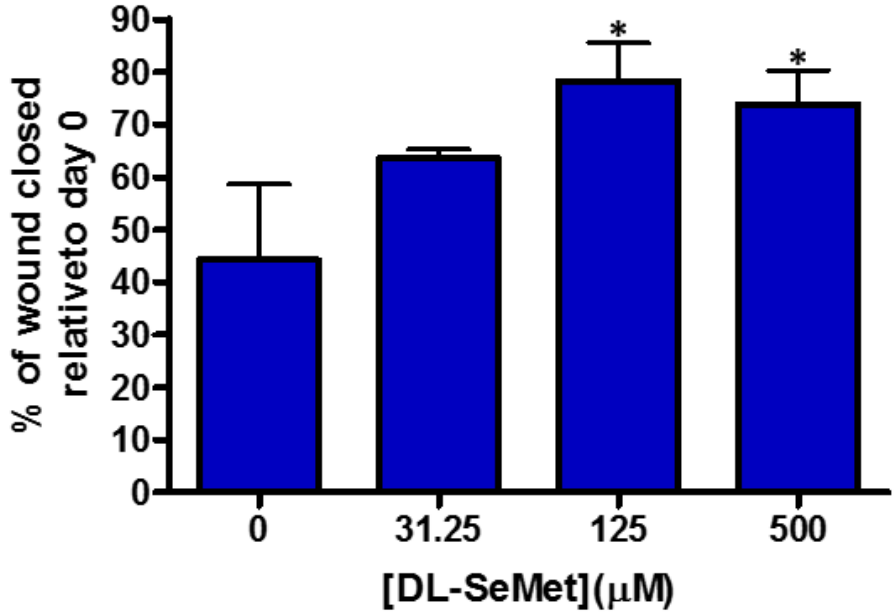

F)
% of control
120
100
80
60
40
20
0
*
31.25
125
500
1000
[DL-Semet] µM

Figure 3.8 Effect of DL-SeMet on eelB cell migration in L15/FBS. (Next page.) After the eelB cells had attached and formed monolayers in the chambers of 2-Well Culture Inserts from Ibidi, the medium (L15/FBS) was removed, the chambers rinsed, and the control or test media were added to the chambers. After 24 h, the Inserts were lifted to create a 500nm gap or "wound" at which point the cells could begin migrating in either L15/FBS (control) or L15/FBS with 31.25, 125 or 500 μM DL-SeMet (test media). Photographs of the gap were taken immediately and 1 and 2 days afterwards and used to calculate areas of the gap covered with cells. Photographs in the panel on the left hand side of the figure show the gaps at day 1 for the 4 different culture conditions. In the bar graph on the right hand side of the figure, the percentage of the gap or "wound" covered with cells relative to the area of the gap immediately upon being made (day 0) is plotted. The asterisk marks areas with cells in DL-SeMet cultures that were statistically different from the areas with cells in the control culture and in the 31.25 μM DL-SeMet culture (one-way ANOVA followed Tukey's multiple comparison test, $p < 0.05$). Scale bar = 500μm.


Control Day 1
31.25 µM Day 1
125 µM Day 1
500 µM Day 1


% of wound closed relativeto day 0
90
80
70
60
50
40
30
20
10
0
*
*
0
31.25
125
500
[DL-SeMet](µM)

3.3.7 Effects of SeMet on Cell Migration and Angiogenesis

The eelB cells were also able to migrate and form CLS in L15/FBS that had 31.25, 125 or 500μM DL-SeMet. Indeed 125 or 500μM DL-SeMet stimulated cell migration (Figure 3.8). In all DL-SeMet concentrations eelB cells were able to form CLS (data not shown) but the rate of CLS formation was not followed.

3.4 DISCUSSION

Cells of the brain endothelial cell line, eelB, from the American eel, survived for at least seven days in the absence of selenium (Se), and responded to addition of Se in different ways, depending on the Se species, which were selenite, selenate or selenomethionine (SeMet), and the exposure media, which were L15/ex, L15 or L15/FBS. After a consideration of Se deprivation first, the responses of eelB to the Se compounds are discussed below in the context of the *in vitro* literature on Se and animal cells, largely cancer cells (Wallenberg et al., 2014a).

3.4.1 Effects of Se Deprivation

In the absence of selenium (Se), eelB cultures might have been expected to lose viability because this has been found to be the case for some mammalian cells (Irmak et al., 2003; Saito et al., 2003). For example, after being placed in Se-free basal medium, a human Jurkat cell line showed massive cell death 24 to 60 h afterwards (Saito et al., 2003) and a human hepatocellular cell line underwent apoptosis 3 to 7 days later (Irmak et al., 2003). Death in the absence of Se was attributed to the cells being unable to maintain antioxidant defences that depended on selenoenzyme activities, such as

glutathione peroxidases and thioredoxin reductase. By contrast, eelB cells in cultures without Se (L15 and L15/ex) survived for at least 7 days and formed capillary-like structures (CLS) on Matrigel and migrated to close wounds.

Several possible explanations can be advanced to explain the apparent Se independence of eelB for some activities. Firstly, eelB were not proliferating in these media so that selenoenzyme activities were not being reduced through cell division, as they were in the example of Jurkat cells. Secondly, the selenoproteins, which in the absence of Se decline in mammals by complex and incompletely understood mechanisms (Bermano et al., 1995; Legrain, Touat-Hamici, & Chavatte, 2014), might be better maintained in the absence of Se in eels. Thirdly, the vertebrate brain appears to be a privileged organ with respect to Se retention, efficiently retaining the element during conditions of deficiency (Chen & Berry, 2003), and perhaps eelB expresses brain specific retention mechanisms. Finally, Se-independent mechanism(s) might counter the reactive oxygen species (ROS) arising from the routine mitochondrial respiration of eel cells. Such an explanation has been given to account for the observation that many human cancer cell lines resist selenium-deficiency induced cell death (Irmak et al., 2003).

3.4.2 Effects of 24 h Selenite Exposures in Three Different Media on Cell Viability

When exposed to increasing concentrations of selenite over a period of 1 to several days in routine growth media, cell cultures from all animals that have been examined to

date show a dose-dependent decline in cell viability and this now includes the American eel. For eelB in the routine growth medium (L15/FBS), the selenite 24 h EC50s in ranged from 61 to 118μM, with three different cell viability endpoints. For other fish species, the 24 h IC50 value for selenite has been reported as 237 μM for the top minnow hepatoma cell line PLHC-1 (Selvaraj et al., 2013), 587 μM for primary rainbow trout hepatocytes (Misra & Niyogi, 2009), and 2.2 mM for bluegill cell line (Babich et al., 1989). In the later report, the EC50 for mammalian cell lines was 0.35 mM and approximately 7 fold lower than the fish cell line EC50s, but these values are much higher than all subsequent reports. Since then, selenite cytotoxicity on human tumour cell lines has been intensively studied and two generalizations have emerged. Cancer cells are more sensitive than their normal counterparts, with the EC50s commonly being below 100 μM (Wallenberg et al., 2014a). Secondly, even among human cancer cell lines and primary cell cultures, susceptibility to selenite killing has been found to vary (Lunøe et al., 2011; Sundaram et al., 2000). The EC50s or IC50s have been found as low 5 μM for HeLa and other human tumour cell lines (Wallenberg et al., 2014b, 2014a). On the other hand, little cytotoxicity has been seen in some lung tumour cell lines (Olm et al., 2009) and in cultures of normal human astrocytes (E. H. Kim et al., 2007). Too few species have been examined to say yet whether eel cells are more or less sensitive than the cells of other fish or of mammals but generally eelB is less sensitive than most human tumour cell lines but a little more sensitive to selenite than the few studies with fish cell cultures.

The type of exposure media appeared to have little influence on the dose-dependent decline in eelB viability by selenite. The expectation was that higher doses of selenite would be needed to kill eelB in L15/ex, because unlike L15 and L15/FBS, this medium lacks an extracellular thiol to stimulate selenite uptake. In a wide range of cellular systems, selenite uptake is stimulated by the addition to the growth media of extracellular thiols, such dithiothreitol, glutathione, or cysteine (Misra et al., 2012; Olm et al., 2009; Tarze et al., 2007). Among different human cancer cell lines, the cytotoxicity of selenite correlated with uptake (Olm et al., 2009). The authors speculated that the extracellular reduction of selenite led to the better uptake of a reduced form of selenite, possibly selenide, which then exerted intracellular cytotoxic actions, such as the generation of ROS (Olm et al., 2009). By contrast, selenite appeared as cytotoxic in L15/ex, with no extracellular thiol, as in L15 and L15/FBS with high levels of cysteine. Possibly, eelB cells in L15/ex had retained enough cysteine from their prior growth in L15/FBS to release cysteine over the 24 h selenite exposure period to provide a reductive extracellular microenvironment that supported selenite uptake and cytotoxicity. Alternatively, the extracellular cysteine values might indeed be low and selenite uptake less in L15/ex but selenite remained just as cytotoxic because the intracellular protective mechanisms, such as glutathione, were diminished in L15/ex.

The loss of viability by animal cells upon to selenite has been attributed to multiple interacting mechanisms and to several modes of cell death, but the ones acting on eelB are open to conjecture. Most commonly the loss of animal cell viability during selenite

exposures of one or two days has been attributed to the generation of reactive oxygen species (ROS) and oxidative stress (Lin & Spallholz, 1993; Misra & Niyogi, 2009). Selenite is reduced in cells to selenide (H_2Se) and in the process oxidative stress ensues (Kumar, Björnstedt, & Holmgren, 1992). Selenide has various fates, with one being metabolism to methylselenol, which can be cytotoxic (H. Zeng et al., 2012). Another fate for selenide is to give rise to elemental selenium (Se), also leading to ROS (Misra et al., 2010). Recently, the intracellular elemental Se has been found to form selenium nanoparticles (SeNPs) (Bao et al., 2015). The SeNPs caused cytotoxicity in several ways through the sequestering of proteins, such as glycolytic enzymes and tubulin (Bao et al., 2015). Whether all these mechanisms operate in eelB cultures and whether they do so to the same degree in the three media is unknown but the three cell viability endpoints gave little hint of differences.

3.4.3 Effects of 24 h Selenate Exposures in Three Different Media on Cell Viability

For cell cultures from a range of animals, selenite has been found to be more cytotoxic than selenate (Babich et al., 1989; Misra et al., 2010; Siwek et al., 1994) and this now includes cells from the American eel. If the results from the AB and NR assays for selenate exposure in L-15/FBS are excluded, selenite was between 23 and 41 fold more cytotoxic to eelB than selenate. The differences reported in the literature are hard to generalize because for some cell types no cytotoxicity is observed with selenate (Lunøe et al., 2011; Yan et al., 1991) and of course exposure conditions and viability endpoints vary from study to study. However, 30- and 50-fold differences have been

reported, respectively, for human leukemia MT-4 cells (Philchenkov, Zavelevich, Khranovskaya, & Surai, 2007) and melanoma cell line (Bandura et al., 2005). Generally, the requirement for higher selenate concentrations is attributed to the slower uptake and reduction of selenate in both fish and mammalian cells (Bandura et al., 2005; Misra et al., 2010).

Noticeable with selenate more than with selenite was the occasional detachment of cells as clumps or sheets, especially in L15/FBS, and suggests possible forms of cell death in these experiments. Cell detachment has been seen in some experiments with mammalian cells and Se compounds: detachment occurred after exposure to selenite (Řezáčová, Čáňová, Bezrouk, & Rudolf, 2016) or to selenium metabolites, methylselenic acid (MSeA) (Conley, McKay, Gandolfi, & Stamer, 2006) and methylselenol (H. Zeng et al., 2012). For a human prostate cancer cell line, the mode of cell death after exposure to MSeA was anoikis (Jiang, Wang, Ganther, & Lu, 2001). Anoikis is a type of apoptosis arising from cells having an inadequate or inappropriate cell-extracellular matrix (ECM) interactions but recently other forms of cell detachment-induced cell death (DICD) have been identified, such as autophagy and necrosis (Ishikawa, Ushida, Mori, & Shibanuma, 2015). For mammalian cells, modes of cell death have been investigated intensively with selenite but not with selenate, and several modes of cell death have been described, including apoptosis (Shi et al., 2013; Stewart et al., 1999), autophagy (E. H. Kim et al., 2007), necroptosis (Wallenberg et al., 2014b), necrosis (Shilo & Tirosh, 2003), and a new, non-specified modality (Řezáčová et al., 2016). These will be interesting to investigate in the future with eelB.

The selenium metabolite, methylselenol, offers one possible explanation for the occasional detachment of eelB cells as clumps/sheets, especially with selenate in L15/FBS. In several studies on mammalian cells and selenium compounds, cells were noted to detach as cell groups or sheets (Conley et al., 2006; Řezáčová et al., 2016; H. Zeng et al., 2012). For the human intestinal epithelial cell line, HCT116, cell detachment occurred in response to methylselenol (H. Zeng et al., 2012). Methylselenol also modulated the phosphorylation of Src and focal adhesion kinase (FAK) (H. Zeng et al., 2012), which together can regulate cell adhesion (Beierle et al., 2010). In eelB cultures with L15/FBS, more methylselenol might develop and/or cell adhesion might be more dependent on the FAK/Src complex so detachment occurs most often in this exposure medium. Methylselenol is a volatile compound and can diffuse from cultures (H. Zeng et al., 2012). In eelB cultures, the accumulation of methylselenol might vary between experiments. One way that this might arise is in how tightly 96 well plates were wrapped with Parafilm. The tightness of the wrapping might have been variable and caused methylselenol accumulation to vary and in turn cell detachment.

3.4.4 Effects of 24 h SeMet Exposures in Three Different Media on Cell Viability

SeMet for 24 h caused little or no cytotoxicity in eelB cultures, except when cultures were switched from the routine growth medium (L15/FBS) to nutrient-deprived medium (L15/ex) and concurrently received a very high SeMet concentration (10 mM). For rainbow trout hepatocytes, SeMet was cytotoxic at the highest tested

concentration (1 mM) (Misra et al., 2012), but was cytotoxic to murine hepatocytes at much lower concentrations (Hoefig, Renko, Köhrle, Birringer, & Schomburg, 2011). These results suggest that fish cells might be less susceptible to SeMet, at least over the short term. In other cell culture systems and in rainbow trout embryos SeMet generated ROS (Misra et al., 2012; Palace et al., 2004; Verma, Atten, Attar, & Holian, 2004). Perhaps ROS is produced in eelB cultures with 10 mM SeMet but oxidative stress and cytotoxicity develop only in L15/ex because L15/ex lacks the antioxidants of L15 and L15/FBS. L15 antioxidants would be the sulphur-containing amino acids (Atmaca, 2004), antioxidant B vitamins (Asensi-Fabado & Munné-Bosch, 2010), such as folic acid (Joshi, Adhikari, Patro, Chattopadhyay, & Mukherjee, 2001), inositol (Phillippy & Graf, 1997), and choline (Mehta, Arora, Gaur, & Singh, 2009). FBS contains antioxidant enzymes, such as catalase (Halliwell, 2003). Surprisingly, when the SeMet was added to eelB cultures after they had been in L15/ex for 7 days, cell viability as measured either with AB, CFDA-AM, or NR showed no significant diminishment. In this case, the 7 days in L15/ex might have caused eelB to have less capacity to generate ROS from SeMet and/or to have enhanced antioxidant defences. Support for the later suggestion is seen in the observation that starving fish can activate antioxidant defence mechanisms (Morales, Perez-Jimenez, Hidalgo, Abellán, & Cardenete, 2004).

3.4.5 Effects of 7-Day SeMet Exposures in Three Different Media on Cell Viability

In contrast to the 24 h treatments, 7-day SeMet exposures caused cytotoxicity, but the magnitude of the cytotoxicity depended on the exposure media and on whether

SeMet was the L or DL forms. Metabolism of SeMet is likely responsible for these effects because long exposures were needed for the loss of cell viability to develop and because in other cell systems cytotoxicity was caused by SeMet metabolism. Among the cytotoxic metabolites of SeMet were methylselenol for mammalian cells (H. Zeng et al., 2012) and selenohomocysteine and selenocysteine for yeast (Lazard, Dauplais, Blanquet, & Plateau, 2015). The addition of methionine protected mammalian cells and yeast against SeMet cytotoxicity by competing with SeMet for metabolism (Kajander et al., 1990; Lazard et al., 2015). For eelB, cytotoxicity was profound in L15/ex, which has no methionine, but much less so in L15 and L15/FBS, which would have at least 75 mg/L of L-methionine (0.5 mM). In mammalian cells L-SeMet was metabolized much more effectively than DL-SeMet (Kajander et al., 1990). As this is likely the case for other vertebrates as well, this would explain why L-SeMet was clearly more cytotoxic than DL-SeMet to eelB in L15. SeMet metabolism generates ROS that also contribute to cytotoxicity (Lazard et al., 2015; Misra et al., 2012; H. Zeng et al., 2012). Therefore, SeMet was likely more cytotoxic to eelB in L15 than in L15/FBS because the antioxidant systems of serum would be missing in L15. In the future, these different media should be useful for studying the cytotoxic interactions between SeMet and ecotoxicants and the modes of cell death in eelB but also in other cell types. Interestingly, rainbow trout hepatocytes appeared to die by apoptosis upon exposure to 1,000 μM L-SeMet in L15 (Misra et al., 2012).

3.4.6 Effects of SeMet on EelB Proliferation in L-15/FBS

The proliferation of eelB cells was inhibited by DL- and L-SeMet only at high concentrations (500-1,000 μM). These were the DL-SeMet concentrations that inhibited the proliferation of normal human fibroblasts (Redman et al., 1998). However for many mammalian tumour cell lines, proliferation has been found to be inhibited at much lower SeMet concentrations, in the 30 to 160 μM range (Kajander et al., 1990; Redman et al., 1998). Possibly, the higher SeMet concentration needed to inhibit eelB proliferation is due to their derivation from apparently normal tissue rather to their fish origin.

How the high SeMet concentrations inhibited eelB proliferation is open to several lines of speculation. Cytotoxicity is unlikely to be the cause because individual cells were observed over 14 days and most cells remained attached and spread on the growth surface. Additionally, as pointed out above for cell viability assays after 7-day exposures, DL-SeMet at 500 and 1,000 μM caused little impairment of cell viability. Yet 500 and 1,000 μM L-SeMet did cause cell death but this was not apparent in microscope examination of cells in the colony assay. However, in the colony assay for 500 and 1,000 μM L-SeMet, the cells did look abnormal. The cells were very large in some cases binucleated or multinucleated. Possibly the low cell density in the colony assay fails to produce sufficient SeMet metabolites to cause cytotoxicity and SeMet acts on cells in other ways. One way is to interfere with cell cycle progression. Several reports on mammalian cells hint at SeMet potentially doing this. For example, Redman

et al. (1998) noted aberrant mitoses in human tumour cell lines exposed to SeMet. For cultures of the human keratinocyte cell line, HaCaT, and the rat glial cell line, C6, SeMet at 1 and 10 μM arrested cells in respectively the S/G2 phase and G2/M phase of the cell cycle (Hazane-Puch et al., 2013; Yeh et al., 2006).

3.4.7 Effects of SeMet on Cell Migration and Angiogenesis

SeMet might have a slight potential to modulate angiogenesis in the eel. Cell migration is essential for the formation of capillary-like structures (CLS) and angiogenesis (Lamalice, Le Boeuf, & Huot, 2007), and high SeMet concentrations stimulated eelB migration. As well, eelB formed CLS after being grown in the presence of SeMet and plated on Matrigel and after being plated on Matrigel and incubated in the presence of SeMet. However, more detailed analysis will be needed in order to determine whether SeMet influences the timing of CLS formation and/or quantifying features of CLS, such as counting branching points (Khoo, Micklem, & Watt, 2011). This might be worth doing in the future because a report from 30 years ago also found evidence of SeMet stimulating not only bovine endothelial cell migration but also angiogenesis (McAuslan & Reilly, 1986). A very low SeMet concentration stimulated the migration of one bovine endothelial cell line but not another. At 0.1 μM SeMet, migration of BRCE, which was from capillary endothelial cells of the bovine retina, was stimulated but migration of BAE, which was from bovine aortal endothelial cells, was not. In two assays of angiogenesis *in vivo*, SeMet was found to be stimulatory: these were in the rabbit corneal pocket assay and chick chorioallantoic assay (McAuslan & Reilly, 1986).

3.4.8 Conclusions

Three selenium species caused different responses in the American eel brain endothelial cell line, eelB. Selenite was much more cytotoxic than selenate, and SeMet was only significantly cytotoxic in a nutrient deprived medium, L15/ex. In the normal growth medium, L15/FBS, SeMet modulated cell migration and proliferation. Comparing the concentrations at which Se compounds elicit responses *in vitro* and *in vivo* are extremely difficult to make (Selvaraj et al., 2013), especially for a species like the American eel for which there is little or no available Se toxicology information. However, the nature of the responses and how they might be modified are the strengths of the *in vitro* approach and can provide ecotoxicologists with information to direct and interpret future studies. Two examples emerge from the current work. The general similarity in how eelB and mammalian cells respond to the three Se compounds means that ecotoxicologists can look with some confidence to the much more comprehensive information on Se and mammalian cells for possible insights into how Se might be impacting eels. Secondly, as the blood brain barrier (BBB) might be critical for maintaining a proper Se levels in the central nervous system (Burk et al., 2014), the modulation of eelB by SeMet would suggest that in the future the structure of the BBB and brain Se levels should be examined in fish.

Chapter 4: Future Research Directions

A cell line, eelB, has been developed in this thesis from the brain of the American eel, *Anguilla rostrata*, an endangered species and belonging to a group of fish that for unknown reasons appear to be in worldwide decline. Cell lines are a resource for research into applied and basic scientific issues and eelB should be useful for a wide range of problems, especially exploring the actions of ecotoxicants and of viruses on the eel, as they might be contributing to the declines in eel populations. EelB was characterized as being endothelial-like and used to study the action of the ecotoxicant, selenium, in the forms of selenite, selenate and selenomethionine (SeMet). Some specific examples of how eelB and the results from the selenium study might be developed further in the future are outlined below.

4.1 EXPLORE THE FISH BLOOD BRAIN BARRIER (BBB) AND THE ACTIONS OF ECOTOXICANTS

EelB might be useful for studying the BBB and examining whether ecotoxicants act at this level. A key to this end would be to determine whether the tight junction (TJ) proteins in eelB cells form functioning TJs and a regulated permeable barrier. Some mammalian brain endothelial cell lines form tight junctions (TJs) and have been used as *in vitro* models to study BBB permeability and transport (Roux & Couraud, 2005). Recently, von Willebrand factor (vWF) and claudin-5 have been found to modulate the permeability of the BBB in mice (Suidan et al., 2013). EelB expressed both of these, suggesting that cells might be promising as tools for studying BBB permeability. Such

in vitro models would be useful in fish physiology but especially in toxicology. In mammals the BBB acts to protect the brain from neurotoxicants and is itself a target of some ecotoxicants. For example, in rats 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) impeded the development of the BBB (Miyazaki, Fujiwara, & Katoh, 2016) and polychlorinated biphenyls (PCBs) impaired BBB integrity (Selvakumar et al., 2012). Another class of ecotoxicant to investigate with an *in vitro* BBB system would be selenium. An *in vitro* system with a human enterocyte-like cell lines has been used to investigated intestinal uptake of selenium species (Thiry, Ruttens, Pussemier, & Schneider, 2013). Brain uptake would be interesting because of a possible link between selenium levels and neurodegenerative diseases (Burk et al., 2014; Cardoso, Roberts, Bush, & Hare, 2015).

4.2 EXPLORE THE ROLE OF OXIDATIVE STRESS IN THE CYTOTOXICITY OF SELENITE AND SELENATE TO EELB DURING 24 H EXPOSURE IN DIFFERENT MEDIA

Oxidative stress has been postulated to be the underlying mechanism for the cytotoxicity arising in eelB cultures from the 24 h exposures to selenite and selenate. Several experiments can be done to test whether the cytotoxicity is indeed dependent on oxidative stress. Intracellular reactive oxygen species (ROS) would be measured with 2,7-dichlorofluorescein diacetate (H_2DCFDA) (F. Zeng et al., 2014). ROS would be evaluated in control cultures and in cultures after 24 h exposure to selenite and selenate. The prediction would be that relative to the control cultures, ROS would be elevated in the selenite and selenate cultures. To test whether oxidative stress was the

cause of cell death, cultures would be exposed to the selenium compounds together with different concentrations of N-acetyl cysteine (NAC) to see if NAC protected. NAC could protect in two ways (F. Zhang, Lau, & Monks, 2010). NAC could serve as a precursor to glutathione (GSH), which is an important cellular antioxidant. NAC could also directly scavenge ROS. In L15 or L15/FBS, NAC would not be expected to be an important precursor because these media have an abundant supply of cysteine, also a precursor for GSH. Therefore, if NAC protected eelB from selenite and selenate in L15/FBS, NAC would likely be acting as a direct scavenger of ROS. If NAC protected eelB from selenite and selenate in L15/ex, the protection against oxidative stress would likely be by NAC scavenging ROS and supporting GSH synthesis.

If the ROS were not to be elevated by selenite or selenate, a different hypothesis would have to be advanced and tested, but an alternative cytotoxic mechanism is difficult to conceive. One line of possible research is to investigate a more direct role of GSH by modulating intracellular GSH levels because an increase in selenite cytotoxicity has been associated with an increase in GSH as well as a decrease in GSH (Shen, Yang, Liu, & Ong, 2000). Intracellular GSH levels has been lowered in fish cells in culture by treatment with buthionine sulfoxime (BSO) (Maracine & Segner, 1998). GSH levels might have been elevated by adding GSH to the L15/ex. In the case of HeLa cells, BSO pre-treatment protected against selenite and this was attributed to less selenodiglutathione being formed (Caffrey & Frenkel, 1991). If buthionine sulfoxime pre-treatment were to protect eelB cells to selenite or selenate and GSH increased their susceptibility to selenite or selenate, the interpretation would be that

selenite or selenate elicited cytotoxicity by reacting with glutathione to form selenodiglutathione.

4.3 EXPLORE THE ROLE OF METABOLISM AND ROS IN THE CYTOTOXICITY OF SELENOMETHIONINE TO EELB OVER 7 DAYS

As the cytotoxicity of long-term SeMet exposures to eelB in L15/ex has been attributed in this thesis to SeMet metabolism, the effect of interfering with metabolism on cytotoxicity should be examined. For rainbow trout hepatocytes, SeMet appears to be metabolized to methylselenol through two possible routes (Misra et al., 2010). The major pathway involves L-methionine γ-lyase. The second route involves the transulfuration pathway. Normally sulphur analogues, such as L-methionine and L-cysteine, rather than selenol- analogues would be used in these pathways. Adding high levels of L-methionine or L-cysteine singly or together to L15/ex might be expected to ameliorate SeMet cytotoxicity. They would do this by out competing SeMet as a substrate in these cellular metabolic pathways and slow the production of methylselenol, which could generate ROS inside cells (Misra et al., 2012). Finally the addition of NAC as a ROS scavenger might by itself provide protection and together with L-methionine and L-cysteine might completely protect against long-term exposure of eelB to SeMet in L15/ex.

4.4 USE EELB TO EXPLORE THE INTERACTIONS BETWEEN SELENIUM AND METAL CONTAMINANTS

Selenium has long been known to modulate the toxicity of metals to vertebrates (Diplock, Watkins, & Hewison, 1985) and interactions between the two have been explored *in vitro* with primary cultures and with cell lines. The cytotoxicity of cadmium to primary rainbow trout hepatocytes was reduced by the presence of either selenite or SeMet (Jamwal, Naderi, & Niyogi, 2016). For cell line examples, selenite protected against cadmium-induced apoptosis of a porcine renal epithelial cell line, LLC-PK1 (Liu, Zhang, & Cai, 2007) but SeMet did not protect the human erythroleukemia K-562 cells from cadmium (Frisk, Yaqob, & Lindh, 2002). Knowing that eelB cells survive in L15/ex alone and in L15/ex with low concentrations of selenite, selenate and SeMet should allow the design of experiments for evaluating the interaction between selenium and metals. In a previous study on the cytotoxicity of metals to the rainbow trout gill cell line, cytotoxicity was only expressed in L15/ex (Dayeh et al., 2005). Therefore, cell viability should be measured in eelB cultures in L15/ex after exposure to different concentrations of metals alone, which should generate dose-viability curves, and to metals together with non cytotoxic concentrations of selenite, selenate and SeMet, which might either protect, enhance or have no effect on the metal dose-viability curves. In this simple system, selenite, selenate and SeMet at non cytotoxic concentrations can be compared quickly for their capacity to interact with different metals. A particularly interesting combination will be selenite and zinc

because recently selenite uptake was found to be tightly associated with the presence of zinc (McDermott et al., 2016).

4.5 POTENTIAL OF LOW CONCENTRATIONS OF SEMET TO ELICIT CELLULAR CHANGES

Cellular changes elicited by SeMet at low concentrations might have the most importance in ecotoxicology because these concentrations are more likely to be found in the environment and so should be explored more thoroughly. One example of this is the potential of low SeMet concentrations to stimulate cell proliferation. At least four examples of this have been seen in the literature for mammalian cell cultures. The first is for two bovine endothelial cell lines: cell number was increased in cultures with 30 nM SeMet (McAuslan & Reilly, 1986). Secondly, SeMet at approximately 10 μM enhanced the proliferation of the mouse hepatoma cell line, Hepa (Kajander et al., 1990). Thirdly, 30 μM SeMet stimulated ^{3}H-thymidine incorporation into DNA in cultures of the human gastric adenocarcinoma SNU-1 cells (Verma et al., 2004). Finally the growth of primary bovine mammary epithelial cells was better in cultures with SeMet at 10 to 50 nM (Miranda, Purdie, Osborne, Coomber, & Cant, 2011). As FBS would contain a low level of selenium, an effect of adding very low doses of SeMet to eelB cultures might be best seen when L15 is supplemented with FBS after dialysis. This medium might also be useful for determining whether SeMet causes subtle changes in angiogenesis. Although eelB formed CLS in presence of SeMet, the timing of CLS formation and/or features of CLS might be subject to modulation. Some features of CLS that might be enumerated after they have formed in the absence or

presence of SeMet are branching points, tubule area, tubule numbers or combinations of these (Khoo et al., 2011).

4.6 POTENTIAL OF EELB TO WITHSTAND EXTREME STARVATION

As eelB survived 7 days only in L15/ex and because eels have been found to withstand rather extreme starvation, eelB might be useful for the exploring the cellular basis of this ability of eels to tolerate nutrient deprivation. Anecdotally and experimentally eels have been observed to live without feeding for up to 5 years (Boëtius & Boétius, 1985; Olivereau & Olivereau, 1997). Some preliminary experiments have been done with eelB in which the cells were completely deprived of nutrients by being placed in the basal salt solution, L15 salts. As a monolayer, eelB remained attached to the plastic growth surface for 28 days in L15 salts. However, during this time their capacity to reduce Alamar Blue declined but when trypsinized, the cells could still reattach to plastic, indicating that they were still alive. The cells might be responding to starvation by shutting down energy metabolism and surviving by hypometabolism. Whether cellular ATP levels were maintained over this time course would be interesting to determine and could be a first step in exploring the cellular basis of starvation resistance.

4.7 USE EELB TO DETECT, ISOLATE, AND STUDY EEL VIRUSES

As the decline in eel populations appears to be happening worldwide, researchers have searched for a possible role of pathogens, such as viruses, in their demise (Stone, 2003). One viral disease of concern is viral endothelial cell necrosis (VECNE). The

causative agent for this disease is thought to be a polyoma virus that has been termed Japanese endothelial cells-infecting virus (JEECV) (Mizutani et al., 2011). In VECNE, endothelial cells of blood vessels become infected with JEECV and undergo necrosis. The virus was not isolated until the development of a method for repetitively preparing primary endothelial cell cultures from the dorsal aorta of *A. japonica* (Ono, Wakabayashi, & Nagai, 2007). As permanent resource, cell lines might be much a more convenient source of cells for detecting and isolating viruses and studying viral pathobiology. Therefore it would be very interesting to determine whether eelB cells are susceptible to JEECV and to other eel viruses, such as a novel polyomavirus (AmPyV) (Wen, Chen, Wang, Liu, & Nan, 2015).

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