THE ROLE OF BEC-1 IN OOCYTE QUALITY

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ABSTRACT

In women, oocytes arrest development during prophase of meiosis I and remain inactive for years. Over time the quality and quantity of these oocytes decreases; therefore, fertility declines as human females age. *C. elegans* display a similar trend showing a decrease in the quality of oocytes as they grow older. Oocyte quality is assayed in *C. elegans* by determining the fraction of oocytes that produce viable eggs following fertilization. A decreased ability to eliminate genetically defective oocytes could account for this drop in oocyte quality. However, mutations that only prevent cell death in response to DNA damage have little effect on embryonic lethality. By contrast, mutations in *C. elegans* that block all cell deaths or germline specific cell deaths result in a drastic early decline in oocyte quality. Furthermore, mutants in which cell death could still function but the recycling of the corpse was prevented, lowered the quality of oocytes. This evidence indicates that developmental programmed cell deaths allow the proper allocation of resources to growing germ cells. This function of apoptotic cell death is the main contributor to oocyte quality rather than the removal of cells with damaged DNA. Therefore, oocytes compete for resources in the germline and apoptosis is vital for the efficient distribution of these resources required for proper oocyte development. This experiment sought to develop a method to study the influence a specific gene involved in the cell death pathway ultimately has on oocyte quality. *bec-1* is linked to apoptotic cell death, since inactivation of *bec-1* triggers *ced-3*/caspase-dependent programmed cell death. There is an interaction between *bec-1* and *ced-9* that is required for *ced-9* to function properly. Without *bec-1* excessive cell death occurs. *bec-1* is also a gene involved in the intracellular process of autophagy. Autophagy maintains the balance between synthesis, degradation and recycling of cellular products. As seen in the case of apoptosis, this reallocation of resources may be required for proper oocyte development and quality. These findings make *bec-1* an extreme gene of interest for its role in oocyte quality. Since *bec-1* mutants are not viable, to study its effect on oocyte quality its activity must be reduced only in the germline. To perform germline specific RNAi, the *rrf-1* strain was used. *rrf-1(pk1417)* mutants lack an RNA dependent RNA polymerase necessary for an RNAi to be successful in the somatic cells but not in the germline. It was found that *bec*-1 activity can be reduced specifically in the germline of *rrf-1(pk1417); bec-1(RNAi); fog-2 (oz40)* animals. This result allows the direct study of the role *bec-1* plays in oocyte quality in *C. elegans*.

INTRODUCTION

As women age, the quality and quantity of their oocytes decline. This results in an increased chance of having a child with birth defects or experiencing a miscarriage. This also makes it much more difficult for an older woman to become pregnant [1]. The National Survey of Family Growth found that the probability of a woman achieving pregnancy in one year or less was significantly higher in women who were less than thirty years of age compared to women older than thirty-five. A similar study found that the monthly probability of conception leading to a live birth remained optimal until the age of thirty-one. The monthly probability of conception after thirty-one progressively decreased thereafter. Once a woman has reached the age of thirty-

eight, probability of conception has dropped to one quarter of that in women less than thirty years old [2]. The oocytes are of lower quality in aging women resulting in eggs that are less likely to mature and eventually be fertilized. Additionally, among older women, there is a larger fraction of oocytes that have chromosomal abnormalities [2]. These abnormalities can be attributed to defects in recombination and cohesion during meiosis [3].

C. elegans can be used to study how oocyte quality is regulated during aging [4]. Oocyte quality can be assayed by determining the fraction of oocytes that produce viable eggs following fertilization. This characteristic of *C. elegans* is the most crucial and pertinent for the scope of this experiment. Furthermore, the time required for *C. elegans* fully to develop is minimal. XX animals are self-fertile hermaphrodites, making crosses very easy. XO animals are considered male. These males can be used in crosses where self-fertilization of the XX animal is not desired. Additionally, *C. elegans* are transparent, making for easy analysis of their body form under a microscope. At 15^{o}C, the first 60-80 germ cells in the hermaphrodite develop into spermatocytes. This results in approximately 300 sperm. Germ cells following the initial production of spermatocytes develop into oocytes. The female gonad consists of two symmetrical U-shaped tubes connected to a uterus at the center of the animal. As germ cells develop, they move from the distal tip towards the proximal portion of the gonad and meiosis ensues. The germ cells increase in size as they mature, but more than half of the developing oocytes undergo apoptosis. The reason for these germ cell deaths is debated. However, proper regulation of these cell deaths is imperative for the normal development of healthy ooctyes [5]. In other words, when cell death is improperly regulated, oocyte quality declines.

Fourteen genes have been identified that function in the cell death pathway (Figure 1). This pathway may be broken down into four main steps. Step number one is the decision of the cell to undergo programmed cell death. Step two is characterized by the actual killing of the cell. Step three is the engulfment of that cell and step four its degradation [6]. The same genetic pathway is used to carry out all cell deaths in *C. elegans*. However, hermaphroditic development in *C. elegans* has very different developmental origins. For this reason, there may be several ways to induce cell death that is cell-type-specific. Ultimately, different signals that induce cell death may converge to activate the same pathway [7]. The genes *ced-3* and *ced-4* are required for all cell deaths to occur. They are the key genes in step two where the actual killing of the cell happens. Without these genes, no cell death may take place [7]. *ced-9* protects *C. elegans* from undergoing programmed cell death [6]. A gain of function mutation in *ced-9* results in the survival of cells that would die normally. Inactivation of *ced-9* results in the death of many cells that would normally survive. There is an interaction between the *bec-1* gene and *ced-9* that is required for *ced-9* to function properly. Without *bec-1* excessive cell death occurs [6]. Engulfment of the dying cell takes place next. Mutations in the seven genes (*ced-1,2,4,6,7,8,10*) block this engulfment process. The gene *nuc-1* is required to degrade the DNA of the dead cell. In mutants that lack *nuc-1* activity, the cells die and are engulfed, but the DNA of the dead cells is not degraded (Figure 1) [6].

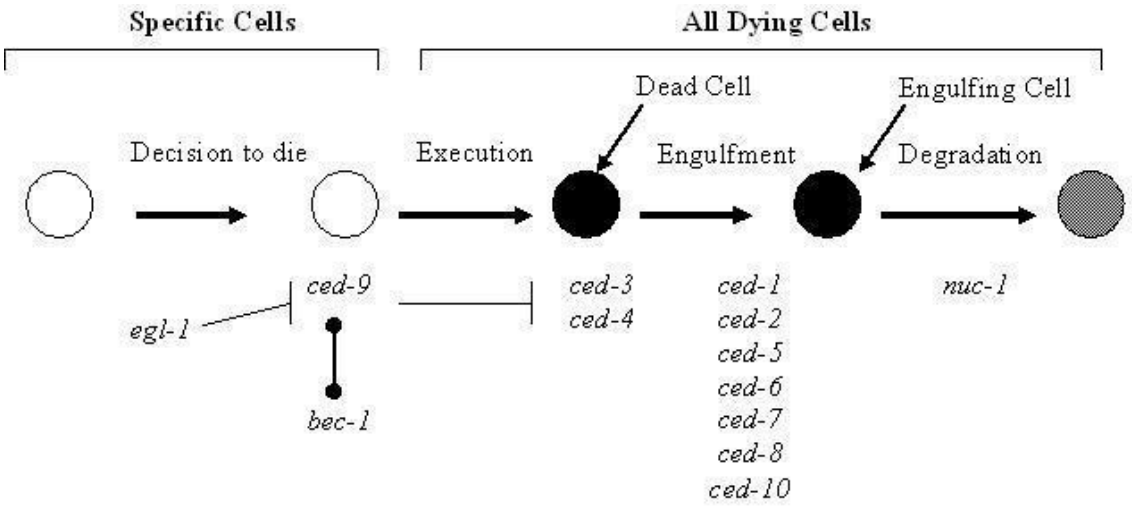


Figure 1. The genetic pathway for programmed cell death in *C. elegans* is shown. Mutations in any of the 14 genes affect programmed cell deaths. There are four steps involved: decision to die, execution, engulfment, and degradation. The genes that act in the last three steps are common to all programmed cell deaths. The genes in the first step affect specific cell types.

Oocyte quality declines in *C. elegans* as they age as in human women [8],[5]. A decreased ability to eliminate genetically defective oocytes could account for this drop in oocyte quality. If this were the case, then specifically preventing all cell death of oocytes with damaged chromosomes would result in a high rate of embryonic lethality. These cell deaths require CEP-1 which acts through *egl-1* to regulate *ced-9* activity. Any loss-of-function mutation in these genes prevents cell death in response to DNA damage but does not affect physiological germ cell deaths. However, only 12% of eggs in these mutant mothers die before hatching. Physiological germ cell deaths require *ced-3* and *ced-4* but are not affected by mutations in *egl-1* or *ced-9(gf)* [5]. A more likely scenario is that these numerous cell deaths seen in the germ line are required to provide nutrients to other oocytes so that they may develop properly. Mutations in *C. elegans* that block all cell deaths or germline specific cell deaths result in a drastic early decline in oocyte quality. Mutations that only prevent somatic cell deaths have no effect on oocyte quality. Furthermore, mutants in which cell death could still function but the recycling of the corpse was prevented lowered the quality of oocytes [5]. Therefore, developmental programmed cell deaths in the germline permit the proper allocation of resources to growing germ cells which can explain the drop in oocyte quality. This function of apoptotic cell death is the main contributor to oocyte quality rather than the removal of cells with damaged DNA. The evidence shows, as *C. elegans* age, the competition for resources increases and apoptosis is vital for the efficient distribution of these resources required for proper oocyte development [5].

The study of specific genes involved in the cell death pathway could further elucidate the precise factors and mechanisms that are required for proper oocyte development. *bec-1* is a gene that is directly involved in the cell death pathway [9]. *bec-1* is a highly conserved gene from *C. elegans* to mammals making it a gene of great interest in the study of infertility. It has been linked to apoptotic cell death in *C. elegans,* when *bec-1* binds the antiapoptotic protein *ced-9*. As previously mentioned, the improper regulation of cell death causes a drop in oocyte quality. However, *bec-1* also plays a crucial role in the intracellular process of autophagy. Autophagy is the degradation of the cell's own components in which *bec-1* is required for the formation of the autophagosome [10]. Since autophagy influences both apoptosis and the reallocation of resources in the cell, it might help regulate oocyte development and quality.

The two roles *bec-1* plays in autophagy and apoptotic cell death make it a gene worth investigating for its possible role in oocyte quality. However, in mutants where *bec-1* was deleted, approximately 90% of the animals underwent embryonic arrest [11]. Therefore, to study the role of *bec-1* in oocyte quality, an animal is required whose gene activity is reduced only in the germ line. This can be accomplished by performing a modified RNAi experiment. In this study, double stranded RNA for *bec-1* was injected into the distal portion of the gonad of *rrf-1(pk1417); fog-2(oz40)* mutants. *f*og-2 is a sex determination gene. XX *C. elegans* are normally hermaphrodites; therefore, they produce both sperm and eggs. A balance between the levels of *fem-3* and *tra-2* determines if spermatogenesis or oogenesis takes place. A high ratio of *fem-3*/*tra-2* promotes spermatogenesis. Meanwhile, a low ratio of *fem-3*/*tra-2* promotes oogenesis. In XO animals, *her-1* inhibits *tra-2* so that sperm is produced continuously (Figure 2). In XX animals, *her-1* activity is absent. *tra-2* is negatively regulated post-transcriptionally by *gld-1* and *fog-2* to permit the onset of hermaphrodite spermatogenesis. Therefore, *fog-2* mutants lack *fog-2* activity necessary to inhibit *tra-2* post-transcriptionally. With this uninhibited *tra-2* activity, *fem-3* levels are low and oogenesis persists (Figure 2). This makes these XX animals essentially female [12]. This permits control of the exact time the eggs of these animals are fertilized.

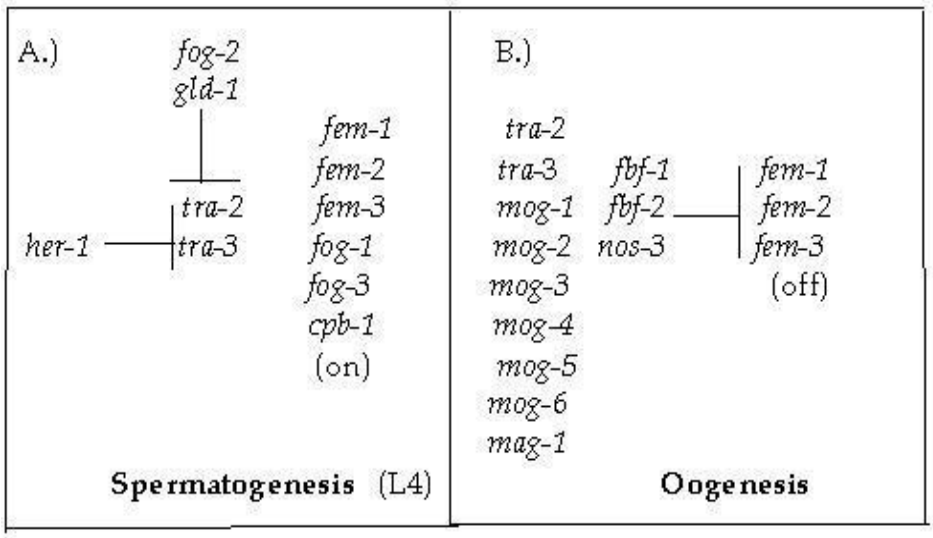


Figure 2. The pathway of *C. elegans* germline sex determination is shown. A balance between the levels of *fem*-3 and *tra*-2 determines whether spermatogenesis or oogenesis takes place. **A:** Spermatogenesis occurs during the L4 (fourth larval) stage. Low levels of *tra*-2 are required to achieve high levels of *fem*-3 activity. **B:** In adults, post-transcriptional regulation of *fem*-3 is required to permit the switch to oogenesis.

The *rrf-1* gene codes for an RNA dependent RNA polymerase. RNA dependent RNA polymerases copy messenger RNAs and spread the amplified sequences throughout the cell. This amplification and spread of the injected dsRNA is required for a successful RNA interference experiment [13]. The double stranded *bec-1* mRNA that is injected into the gonad of the worm requires the *rrf-1* gene in the somatic cells for its amplification and dispersal. However, *rrf-1* is one member of a family of RNA dependent RNA polymerases. Other RNA dependent RNA polymerases are present in the germline allowing *rrf-1* mutants to be *bec-1* deficient in the germline only. Therefore, *rrf-1* mutants lack the RNA-dependent RNA polymerase necessary for an RNAi to be successful in the somatic cells, but not in the germline [14]. Thus, these animals

should be deficient in *bec-1* in the germline, but normal in other tissues. If so, this technique would allow direct study of the effect *bec-1* has on oocyte quality.

MATERIALS AND METHODS

C. elegans were handled using standard methods [15]. The animals were maintained on NG plates at 15°C (NG Agar: 6 g NaCl, 9 g KH_2PO_4, 1.5 g K_2HPO_4, 12 g tryptone, 60 g Agar, and 1 ml cholesterol in ethanol (15 mg/ml) were added to 3 L dH_2O and autoclaved). Female animals were aged to the early adult stage before being used for microinjection.

A PCR was run to amplify a DNA template for *bec-1*. Primers were designed for *bec-1* that have T7 promoter sequences. The forward primer sequence was 5'- TAA TAC GAC TCA CTA TAG GGA GAG TTT GTA ATG ATT GCT CTG ACG CT-3'. The reverse primer sequence was 5'- TAA TAC GAC TCA CTA TAG GGA GAT CCA TGT CGA TGC CAT TAC GAC GA -3'. The DNA was purified using a QIAquick column. The concentration of the template was measured using a spectrophotometer. The DNA concentration obtained was 75 nanograms/microliter. To confirm that the correct fragment was obtained, an agarose gel (0.8%) was run. The predicted size of the DNA strand was 644 nucleotides. The gel showed a band of approximately that length, when compared to a 100 base pair ladder (Figure 3).

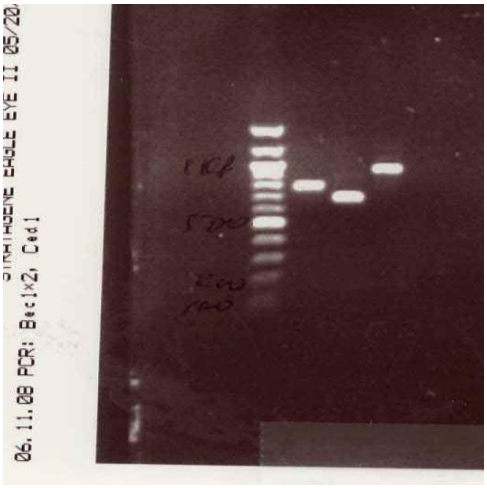


Figure 3. Agarose gel (.8%) showing the fragment size of the *bec-1* DNA (sample 2) compared to a 100 base pair ladder. The DNA strand measured 644 nucleotides. The DNA concentration obtained was 75 nanograms/microliter.

A Megascript reaction (Ambion), was then conducted to transcribe the DNA into its corresponding RNA using this DNA template. The reaction was allowed to run overnight. The RNA was purified using a Megaclear kit. The single-stranded RNA was then annealed by being heated to 70 °C and returning to room temperature in approximately twenty-five minutes. The concentration, which was once again measured using a spectrophotometer, was approximately 800 nanograms/microliter.

The microinjection technique was used to inject the double-stranded RNA into the distal portion of the gonad of young adult female *C. elegans*. First, *fog-2(oz40)* mutants were injected. They were then transferred to new plates to lay their eggs. The progeny were screened for *bec-1(RNAi)* phenotypes, were many of which observed. Some of these defects were seen in the ventral nerve cord, the intestine, the developing vulva, the pharynx, and the gonad. However, the most distinct and easily observed was the pharynx defect. The pharynx in these mutants did not develop properly. Defective worms were photographed at 1000v DIC optics. Forty of these

animals were scored. Next, the *bec-1* double-stranded RNA was injected into *rrf-1(pk1417);fog-2(oz40)* mutants. Once again, the injected worms were transferred to new plates to await egg production. Forty of the progeny of the injected *rrf-1(pk1417);fog-2(oz40)* worms were screened for defects. Additionally, forty *fog-2(oz40)* animals that were not injected were screened for defects as a control.

RESULTS

bec-1 is expressed in the pharynx as well as the intestine, the developing vulva, and the ventral nerve cord. The worms shown were photographed at 1000v DIC optics. The first specimen is a wild type nematode displaying a normal pharynx (Figure 4). The second animal, a *bec-1(RNAi); fog-2(oz40)* mutant, shows a pharynx defect (Figure 4). Improper buccal cavity attachment can be seen. The metacarpus and terminal bulb also failed to develop properly resulting in severe structural defects. The pharynx defect was used as an indicator of a lack of *bec-1* activity because it is the most easily observed defect. Double stranded RNA of *bec-1* was injected into the distal portion of the gonad of the parents of these worms. Control *fog-2(oz40)* worms that were not injected with *bec-1* dsRNA showed no defects in the pharynx. A significant portion of the *bec-1(RNAi);fog-2(oz40)* mutants screened, displayed the expected phenotype exhibiting obvious pharynx defects (Figure 4). The RNAi worked in these worms and prevented the expression of *bec-1*. The next question was if *bec-1* activity could be reduced specifically in the germline.

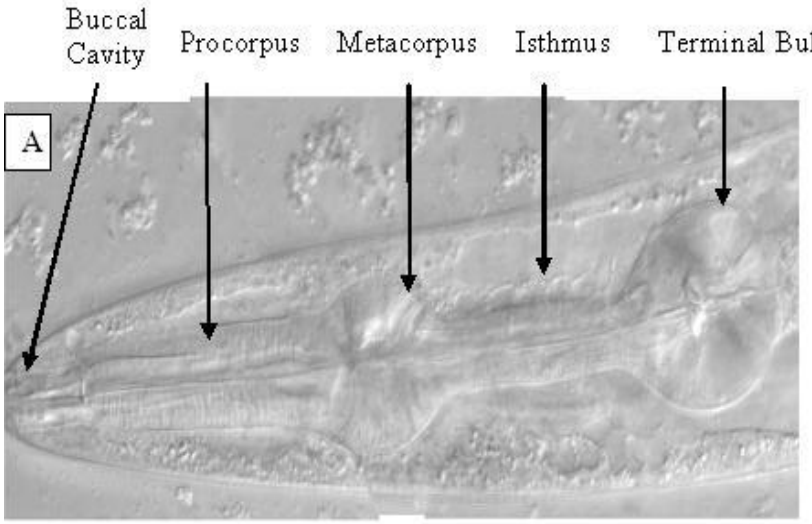
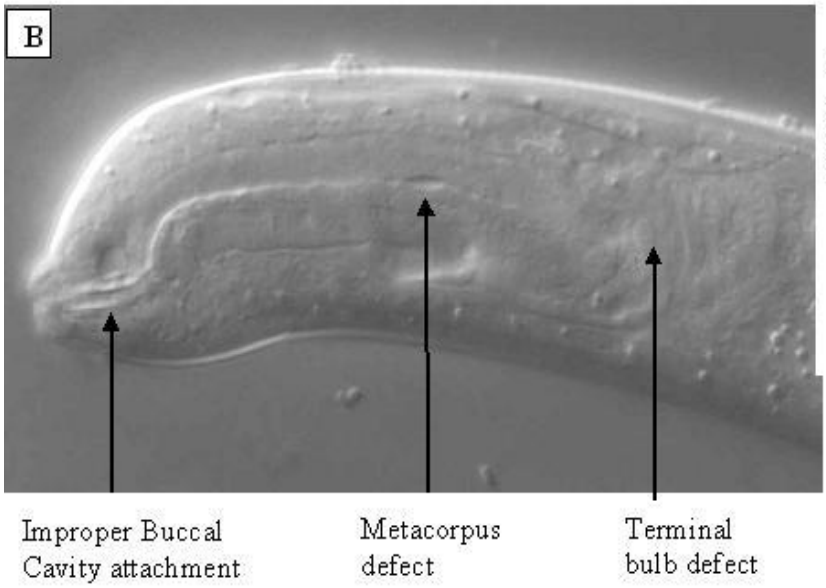


Figure 4. (A) Normal pharynx of wildtype *C. elegans*. Properly developed terminal bulb, isthmus, metacarpus, procorpus, and buccal cavity displayed.



(B) Pharynx defect in *bec-1(RNAi); fog-2(oz40)* mutant. Defects seen in the terminal bulb, metacorpus, and buccal cavity.

***bec-1* Activity Can Be Prevented Specifically in the Germline of *rrf-1(pk1417); bec-1(RNAi); fog-2(oz40)* Animals**

Forty worms of three different strains were scored using 1000v DIC optics. A control *fog-2 (oz40)* strain, a *bec-1(RNAi); fog-2(oz40)* strain, and *rrf-1(pk1417); bec-1(RNAi); fog-2(oz40)* strain were examined. The presence of a pharynx defect was scored indicating the successful prevention of ubiquitous *bec-1* expression.

Genotype	Pharynx Defect

fog-2 (oz40)	0.0%
bec-1 (RNAi); fog-2 (oz40)	25.0%
rrf-1 (pk1417); bec-1 (RNAi); fog-2 (oz40)	2.5%

Table 1. Number of worms of *bec-1(RNAi); fog-2(oz40)*, *rrf-1(pk1417); bec-1(RNAi); fog-2(oz40)* and *fog-2(oz40)* displaying a pharynx defect is shown. Forty worms of each strain were scored.

Ten of the forty *bec-1(RNAi);fog-2(oz40)* mutants screened displayed the expected phenotype (Table 1). These worms had severely deformed pharynxes (Figure 4). However, among forty *rrf-1(pk1417); bec-1(RNAi); fog-2(oz40)* worms, only 2.5% of animals scored had a defect (Table 1). The *rrf-1(pk1417); bec-1(RNAi);fog-2(oz40)* worms also had a normal germline. This indicates that the *rrf-1(pk1417); bec-1(RNAi);fog-2(oz40)* worms have no *bec-1* activity in the germline but are otherwise healthy. This means that the role of *bec-1* in oocyte quality can be directly studied using these worms.

DISCUSSION

The quality and the number of oocytes decrease dramatically in human females over their lifespan. At embryogenesis, a woman has millions of oocytes. By the onset of puberty, approximately 300,000 oocytes remain. This number decreases by the hundreds each month after puberty. In their late thirties, this rate of loss increases until menopause. At menopause, approximately 1,000 oocytes are left [16]. As the number of oocytes decreases so does the quality of those oocytes. Therefore, older females are less likely to become pregnant and are at a higher risk for complication. The decline in the quality of oocytes seen in human females increases the chances of a miscarriage or birth defects in any children. Oocyte quality declines in *C. elegans* as in mammals [5]. This makes *C. elegans* a very appropriate model for the study of factors that influence oocyte quality.

Most germ cells in mammals and nematodes undergo programmed cell death. In humans, for example, a woman is born with close to seven million oocytes. Nearly half of these die. Only 400 follicles ovulate in the lifetime of a woman [17]. A similar pattern is seen with the nematode. Approximately 1700 oocytes are produced during the lifetime of the nematode. Only half of those reach maturity. On average, the regular female hermaphrodite lays 300 eggs [18]. Logically, programmed cell death is a major contributor to the quality of oocytes [5]. Several schools of thought have hypothesized on the exact influence cell death has on oocyte quality. One theory suggests that the removal of defective oocytes helps for the maturation of healthy oocytes. Another claims that some oocytes are selected to nourish healthy, developing oocytes and later undergo apoptosis. Finally, one theory states some oocytes die because there are only enough nutrients for a select few to reach maturity [19]. It is possible that apoptosis eliminates oocytes with genetic defects. Since chromosomal abnormalities increase with age, programmed cell death might maintain oocyte quality through the elimination of oocytes with DNA-damage. However, if germ cell deaths removed only oocytes with genetic defects, then preventing these deaths should result in a high rate of embryonic lethality. The *ced-9 (gf)* and *egl-1 (lf)* mutations,

which only influence germ cell deaths induced by DNA damage, do not lower oocyte quality [5]. The most logical reason for these cell deaths is the proper allocation of resources among oocytes in the germline. In *ced-3* or *ced-4* mutants that lack all cell deaths, oocyte quality dropped dramatically [5]. In addition, oocyte quality declined in *ced-1* and *ced-6* animals, when cell corpses could not be engulfed and recycled [5]. In both the cases of *ced-3* or *ced-4* mutants and *ced-1* or *ced-6* mutants, the quality of oocytes worsened as the females aged. These observations substantiate the hypothesis that as *C. elegans* age, the competition for resources increases, and apoptosis is vital for the efficient distribution of these resources required for proper oocyte development.

In order better to understand the role apoptosis plays in oocyte quality, specific genes must be examined. *bec-1* is a gene directly involved in the cell death pathway [9]. It is also highly conserved from nematodes to humans, maintaining a homolog in mammals called *beclin*. *bec-1* has been linked to apoptotic cell death in *C. elegans*, where *bec-1* binds the antiapoptotic protein *ced-9*. When there is no *bec-1* activity, excessive cell death occurs [11]. The intracellular process of autophagy may also influence cell death. Autophagy influences the reallocation of resources in the cell. *bec-1* is required for the formation of the autophagosome [10]. For this reason, its activity might help regulate oocyte development and quality. Therefore, because of the two roles *bec-1* plays in autophagy and apoptosis, its function may provide valuable insight into exact mechanisms that determine oocyte quality.

bec-1 is an essential gene in *C. elegans*. In mutants where *bec-1* was deleted, approximately 90% of the animals underwent embryonic arrest [11]. Therefore, to study the role of *bec-1* in oocyte quality, a mutant is required in which the gene is knocked out only in the germline. This experiment found that obtaining such a mutant is indeed possible by performing an RNAi experiment. Double stranded RNA for *bec-1* was injected into the gonad of *rrf-1(pk1417);fog-2(oz40)* mutants. *rrf-1(pk1417)* mutants lack the RNA-dependent RNA polymerase necessary for a RNAi to be successful in the somatic cells, but not in the germline [11]. Hence, the offspring of the injected individuals should be *bec-1* deficient only in the germline. *bec-1* mutants that are otherwise healthy permit the direct study of the role *bec-1* plays in oocyte quality.

bec-1 is expressed in the pharynx as well as the intestine, the developing vulva, and the ventral nerve cord [3]. A gradient of phenotypes may be seen in *bec-1* RNAi treated animals. The severity and number of defects observed depends on the penetration of the RNAi performed. An RNAi experiment is not always one hundred percent penetrant. Approximately 25% of *bec-1(RNAi);fog-2(oz40)* worms displayed the phenotype expected when *bec-1* is disrupted. These worms had severely misshaped pharynxes. The pharynx mutation was scored because it is one of the easiest defects to observe. This result is consistent with the observation that *bec-1* is expressed in the pharynx, intestine, the developing vulva, and the ventral nerve cord [3]. The *rrf-1(pk1417); bec-1(RNAi); fog-2(oz40)* animals had normal pharynxes. This result confirms that *bec-1* can be specifically knocked down in the germ line of *C. elegans*. These animals will be useful because they permit the study of the role *bec-1* plays in oocyte quality in otherwise healthy *C. elegans*.

In the future, the eggs of these mutants can be scored to determine if there is a significant drop in oocyte quality after the inactivation of *bec-1*. Oocyte quality will be determined by the fraction of oocytes that produce viable eggs following fertilization. If there is a drop in the quality of oocytes, that could be the effect of the influence of *bec-1* on either apoptotic cell death or autophagy. To determine if defects in oocyte quality involve apoptosis, one can study *rrf-1(pk1417);ced-3(n2425);bec-1(RNAi);fog-2(oz40)* animals. If apoptosis is the contributing factor to a drop in oocyte quality, then knocking down *bec-1* activity in a mutant where cell death cannot occur should not change oocyte quality. To determine if defects in oocyte quality involve autophagy, one can study *rrf-1(pk1417);bec-1(RNAi);unc-51[APG1];fog-2(oz40)* animals. If autophagy is the cause of a drop in oocyte quality, then knocking down *bec-1* in a mutant where autophagy is already inactivated should have no further negative effect on oocyte quality.

As a result of the present work, the level of influence apoptotic cell death and autophagy have on oocyte quality in the future may be elucidated. The precise factors that contribute significantly to oocyte quality may then be pinpointed and examined. With these factors identified, more research may be performed to gain a better understanding of how to prevent the decline in oocyte quality in females. Knowledge of gene pathways involved in apoptosis and autophagy in the germ line is necessary for potential therapies to be developed. The results of these future experiments may provide information that can lead to techniques that can improve oocyte quality. These recent findings show a possibility of improving oocyte quality by manipulating cell death in the germ line. In mice, for example, mutations in *Bax*, which prevents many germ cell deaths, extend the reproductive lifespan of females [20]. It appears feasible that genetic manipulation could improve oocyte quality in older human females.

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