Abstract: The present invention relates to methods of promoting the survival of cells by treating the cells with acid ceramidase. A kit for promoting ex vivo cell survival is also disclosed, as is a method of predicting in vitro fertilization outcome of a female subject.
ACID CERAMIDASE AND CELL SURVIVAL

This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/883,661, filed January 5, 2007, and U.S. Provisional Patent Application Serial No. 60/939,178, filed May 21, 2007, each of which is hereby incorporated by reference in its entirety.

This invention was made with government support under grant number ROI DK54830 awarded by The National Institutes of Health. The government has certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to methods of promoting cell survival using acid ceramidase.

BACKGROUND OF THE INVENTION

Due to its involvement in the human genetic disorder Farber Lipogranulomatosis ("FD"), acid ceramidase ("AC;" JV-acylsphingosine deacylase, I.U.B.M.B. Enzyme No. EC 3.5.1.23) is the most extensively studied member of the ceramidase enzyme family. The protein has been purified from several sources, and the human and mouse cDNAs and genes have been obtained (Bernardo et al., "Purification, Characterization, and Biosynthesis of Human Acid Ceramidase," J. Biol. Chem. 270:1 1098-102 (1995); Koch et al., "Molecular Cloning and Characterization of a Full-length Complementary DNA Encoding Human Acid Ceramidase. Identification of the First Molecular Lesion Causing Farber Disease," J. Biol. Chem. 271 1:331 10-5 (1996); Li et al., "Cloning and Characterization of the Full-length cDNA and Genomic Sequences Encoding Murine Acid Ceramidase," Genomics 50:267-74 (1998); Li et al., "The Human Acid Ceramidase Gene (ASAH): Chromosomal Location, Mutation Analysis, and Expression," Genomics 62:223-31 (1999)). Growing interest in the biology of this and other ceramidases stems from the fact that these enzymes play a central role in ceramide metabolism. Ceramide is a signaling lipid that is produced in response to various stimuli (Hannun, "Function of Ceramide in Coordinating

SUMMARY OF THE INVENTION

A first aspect of the present invention relates to a method of promoting the ex vivo survival of cells. This method involves providing one or more cells ex vivo and treating the one or more cells with acid ceramidase under conditions effective to promote survival of the one or more cells.

A second aspect of the present invention relates to a method of promoting in vivo survival of one or more cells in a female mammalian subject. This method involves administering to the female mammalian subject acid ceramidase under conditions effective to promote survival of one or more cells in the female mammalian subject.

A third aspect of the present invention relates to a kit for promoting ex vivo primary cell survival. The kit includes a cell culture medium and an acid ceramidase.

A fourth aspect of the present invention relates to a method of predicting in vitro fertilization outcome. This method involves providing a sample of serum or follicular fluid from a female subject and screening the sample for acid ceramidase activity level. The acid ceramidase activity level obtained through said screening is then correlated to a prediction of the outcome of in vitro fertilization for the female subject.

The present invention demonstrates that AC is one factor required for early embryo survival. Gene targeting has been used to inactivate the AC gene (Asahl) in mice (Li et al., "Insertional Mutagenesis of the Mouse Acid..."
Ceramidase Gene Leads to Early Embryonic Lethality in Homozygotes and Progressive Lipid Storage Disease in Heterozygotes,” Genomics 79:218-24 (2002)). Initial characterization of these animals revealed that heterozygous mice ("Asahl+/-") had a progressive lipid storage disease phenotype, and that a complete loss of AC activity led to the absence of mutant individuals. It remained unclear, however, whether the Asahl-I- embryos were not formed, or, alternatively, if they were formed, whether they died during early embryogenesis.

The present invention describes the use of a combination of molecular, biochemical, and morphological methods to follow the development of individual embryos obtained from Asahl+/− intercrosses. These analyses showed that Asahl-I- embryos could be formed, but underwent apoptotic death at the 2-cell stage. Importantly, these embryos could be rescued by adding SIP to the culture media, permitting their survival to at least the 4-8-cell stage. It was also demonstrated that Asahl is one of the earliest genes expressed in newly formed embryos. Further, AC is shown to be a predominant protein in unfertilized eggs, and expression of this protein and gene is decreased during egg aging unless fertilization occurs. Overall, these results demonstrate that AC is an essential component of newly formed embryos, and is required for their survival beyond the 2-cell stage.

The present invention also demonstrates that acid ceramidase increases the survival rate of cells in culture. The use of acid ceramidase has several advantages over other potential anti-apoptotic factors: low toxicity, easy delivery, and its unique and specific function.

Being a natural component of normal cells, acid ceramidase should have little or no toxic effects. In addition, providing cells with the precursor (inactive) form would allow the cell itself to control the rate of activation and the amount of the active protein required for survival. Furthermore, controlling ceramide metabolism and producing sphingosine/sphingosine-1-phosphate are the only known functions of acid ceramidase. Thus, increasing acid ceramidase activity in a cell should not affect other cellular signaling pathways.

Acid ceramidase has a natural ability to enter cells through mannose receptors and/or mannose-6-phosphate receptors located on various cell types, including oocytes, neurons, and synovial fibroblasts. Additionally, cells
that do not have these receptors contain "scavenging" receptors that can lead to internalization of AC. This implies that administering acid ceramidase into a culture medium can increase the level of the enzyme inside cells in the culture, leading to a reduction in ceramide levels within the cell. It also appears that acid ceramidase can cross the zona pellucida of oocytes, something most molecules cannot do.

[0016] Increasing ceramide levels in cells almost always leads to cell death, and ceramidases are the only enzymes that can hydrolyze ceramide. Expression of acid ceramidase in cells has at least two consequences: removal of ceramide, and the production of sphingosine and sphingosine-1-phosphate (two well-characterized, anti-apoptotic lipids). Therefore, without being bound by theory, it is expected that acid ceramidase promotes cell survival in at least two ways: by removing ceramide and by producing sphingosine and sphingosine-1-phosphate. Acid ceramidase is the only known molecule that does both of these.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0017] Figure 1 is an agarose gel. Long and nested amplification of the wild-type Asahl allele ("wt") produced ~9kb and 180bp DNA fragments, respectively. Amplification of the disrupted Asahl-/- allele ("mutant") produced ~7kb and 255bp DNA fragments, respectively. Genotype analysis was performed on individual 2-cell embryos obtained from Asahl+/- intercrosses using this method, and a representative gel of the nested PCR amplification is shown. Asahl-/- embryos are shown to exist at this stage (lane 3). The negative control ("T(reg. con.") did not have template DNA added to the reaction mixture. Lanes "Con. Asahl+/-" and "Con. Asahl+I-" contain template DNA from adult mice known to have the indicated genotype. The size of individual marker fragments in a DNA ladder are indicated on the left side of the panel.

[0018] Figures 2A-F are light micrographs (Figures 2A and 2D), Annexin V-stained images (Figures 2B and 2E), and merged images (Figures 2C and 2F), showing that Asahl-/- embryos undergo apoptotic death during the 2-cell stage. These figures show the cellular morphology (Figures 2A, 2C-D, and 2F) and Annexin V staining pattern (Figures 2B-C and 2E-F) of representative wild-type
"wt") and Asahl-I- ("mutant") 2-cell mouse embryos obtained from Asahl+I-
tercrosses (see Example 9). "PB" indicates polar bodies. Bar =10 μm.

Figure 3 is a graph of AC activity and a representative western blot
(inset) of cell extracts from unfertilized mouse eggs. Cell extracts from 400
pooled eggs were analyzed by western blot (see Example 10). A goat anti-human
AC IgG was used to detect the murine AC precursor protein (55kDa) and AC β-
subunit (40kDa). For AC activity assays, cell extracts were prepared from 65
pooled eggs, incubated for 22 hours at 37°C with BODIPY-conjugated C12-
ceramide, and then analyzed by HPLC. The AC activity in these extracts was
significantly higher in comparison to the blank control (t-test, p<0.005). These
data show that AC is expressed at high levels in unfertilized, healthy mouse eggs.
The data represent mean ±S.E.M; n=3 independent experiments.

Figures 4A-G are representative immunohistochemistry images of
fixed, unfertilized eggs. Goat IgG was used against human AC (Figures 4A and
4D), and rat IgG was used against the lysosomal marker Lampl (Figures 4B and
4E). Figures 4C and 4F show merged images. Localization of the primary
antibodies was visualized using a fluorescent secondary antibody (Cy-3/2) and
laser-scanning confocal microscopy (see Example 5). Eggs labeled only with
secondary antibodies were used as a control (Figure 4G). Bar =10 μm. The data
represent three independent experiments, and confirm that AC is expressed at high
levels in healthy mouse eggs.

Figure 5 is a graph of the relative mRNA levels in young
unfertilized eggs ("Eggs"), old unfertilized Mil eggs ("Old eggs"), and 2-cell
embryos, of mice. "mAC": AC mRNA; "mActb": actin beta mRNA; "mG3":
glyceraldehyde-3-phosphate dehydrogenase mRNA, "RPSH": ribosomal protein
S11 mRNA. The data represent mean ±S.E.M.; n=3 independent experiments.
These results demonstrate that AC expression decreases in unfertilized mouse
eggs as they age. However, if fertilization occurs, AC increases in healthy 2-cell
embryos.

Figures 6A-B are stained images of mouse oocytes incubated in
the absence ("-AC") (Figure 6A) or presence ("+AC") (Figure 6B) of acid
ceramidase. Bar =10 mm.
Figure 7 is a graph illustrating the rate of apoptosis of oocytes incubated in: M2 media without AC ("M2 -AC"), media collected from a parental CHO cell line that does not express AC ("CHO -AC"), M2 media supplemented with pure AC (10ug/ml) ("M2 +AC"), or media collected from a CHO cell line that stably expresses and secretes AC ("CHO6 +AC"). Data represent three independent experiments.

Figures 8A-P are stained images of denuded and fixed human oocytes at the germinal vesicle stage ("GV"), germinal vesicle breakdown stage ("GVBD"), MI stage, or MI stage. The oocytes were incubated with polyclonal anti-AC antibody (red; Figures 8C, 8G, 8K, and 8O), polyclonal anti-LAMP antibody (green; Figures 8B, 8F, 8J, and 8N), or the Hoechst DNA-specific fluorochrome 33342 (blue; Figures 8A, 8E, 8I, and 8M). Figures 8D, 8H, 8L, and 8P show the preceding three images superimposed upon each other ("Merge"), to identify the co-localization of AC with LAMP and/or the cellular DNA. Bar =10µm. These data are the first to demonstrate expression of AC in human oocytes.

Figures 9A-D are stained images of denuded and fixed low grade human embryos. The embryos were incubated with the Hoechst DNA-specific fluorochrome 33342 (blue; Figure 9A), anti-acid sphingomyelinase antibody ("ASM") (green; Figure 9B), or polyclonal anti-AC antibody (red; Figure 9C). Figure 9D shows Figures 9A-C superimposed upon each other ("Merge"), to identify the co-localization of AC with DNA and/or ASM. Localization of the primary antibodies was imaged using secondary antibodies Cy-3 or Cy-2 and Laser-scanning confocal microscopy. Embryos were graded according to the morphology of the inner and outer cell masses. The data represent three independent experiments. These data are the first to demonstrate expression of AC in human embryos.

Figures 10A-D are stained images of denuded and fixed high grade human embryos. The embryos were incubated with the Hoechst DNA-specific fluorochrome 33342 (blue; Figure 10A), anti-ASM antibody (green; Figure 10B), or polyclonal anti-AC antibody (red; Figure 10C). Figure 10D shows Figures 10A-C superimposed upon each other ("Merge"), to identify the co-localization of AC with DNA and/or ASM. Localization of the primary antibodies was
imaged using secondary antibodies Cy-3 or Cy-2 and Laser-scanning confocal microscopy. Embryos were graded according to the morphology of the inner and outer cell masses. The data represent three independent experiments.

Figure 11 is a western blot of human follicular fluid samples using antibodies against the AC precursor protein or the AC α-subunit. A 1 μl sample was loaded onto lane 1 ("FF 1λ") and a 10 μl sample was loaded onto lane 2 ("FF 10λ"). Pure AC was loaded onto lane 3 as a control ("Con AC").

Figure 12 is a plot of AC activity in human follicular fluid as a function of maternal age (in years).

Figures 13A-B are slides of immature human oocytes cultured in vitro with (Figure 13B) or without (Figure 13A) AC.

Figures 14A-D are stained images (Figures 14A-B) and slides (Figures 14C-D) of human oocytes cultured with ("+AC") or without ("-AC") AC. Oocyte DNA was stained with the Hoechst DNA-specific fluorochrome 33342, and the fluorescent signal visualized by Laser-scanning confocal microscopy. Condensed chromatin ("Condensed"), the metaphase plate, and polar bodies ("PB") are indicated. The data represent two independent experiments.

Figures 15A-D are TUNEL-stained images (Figures 15A-B) and slides (Figures 15C-D) of human oocytes cultured with ("+AC") or without ("-AC") AC. The metaphase plate and polar bodies ("PB") are indicated. The data represent two independent experiments.

Figure 16 is a graph of the percentage of apoptotic cells in primary rat hippocampal neuron cultures grown in normal culture media ("control"), or with amyloid-β peptide ("Abeta"), amyloid-β peptide and AC ("Abeta+AC"), hydrogen peroxide ("H2O2"), or hydrogen peroxide and AC ("H2O2+AC") added to the culture media. *p<0.01 based on three independent experiments.

Figure 17 is a graph of sphingosine-1-phosphate ("SIP") levels in primary rat synovial fibroblasts grown with ("+AC") or without ("-AC") human AC. *p<.001.

Figure 18 is a graph of cell proliferation in primary cat synovial fibroblasts cultured with (closed circles) or without (open circles) human AC, as determined using the MTS reagent (quantified by the absorbance at 490 nanometers). *p<0.001.
Figure 19 is a western blot of mouse embryonic stem cells, showing the presence of the AC precursor protein (55kDa) and the AC β-subunit (40kDa). This shows that AC is expressed at high levels in undifferentiated mouse ES cells.

Figures 20A-C are western blots of mouse embryonic stem cells, showing the relative amount of poly(ADP-ribose) polymerase ("PARP") (Figure 20A), Bax (Figure 20B), and actin (Figure 20C) (control) in cells incubated with ("+AC") or without ("-AC") AC. These data demonstrate that when mouse ES cells are grown in the presence of AC, the expression levels of several apoptotic markers (PARP and Bax) are reduced. Proteins were detected using polyclonal antibodies against the respective proteins.

Figure 21 is a graph of the ceramide levels in rat neuronal cell cultures with ("+") or without ("-" ) amyloid-β peptide ("Aβ") and/or recombinant human AC ("rhAC"). *p<0.05, compared to normal brains. Values are expressed as the mean ± S.D (N=3).

Figure 22 is a graph of the caspase 3 activity in untreated ("control") rat neuronal cell cultures, and cultures exposed to amyloid-β peptide with ("+Aβ +AC") or without ("+Aβ") recombinant human AC pre-treatment. **p<0.01, compared to normal brains. Values are expressed as the mean ± S.D. (N=3).

**DETAILED DESCRIPTION OF THE INVENTION**

A first aspect of the present invention relates to a method of promoting the ex vivo survival of cells. This method involves providing one or more cells ex vivo and treating the one or more cells with acid ceramidase under conditions effective to promote survival of the one or more cells.

Acid ceramidase ("AC") is an enzyme that catalyzes the hydrolysis of ceramide to sphingosine and free fatty acid (Bernardo et al, "Purification, Characterization, and Biosynthesis of Human Acid Ceramidase," J. Biol. Chem. 270(19): 11098-102 (1995), which is hereby incorporated by reference in its entirety). Mature AC is a ~50kDa protein composed of an α-subunit (~13kDa) and a β-subunit (~40kDa) (Bernardo et al., "Purification, Characterization, and
Biosynthesis of Human Acid Ceramidase," *J. Biol. Chem.* 270(19): 11098-102 (1995), which is hereby incorporated by reference in its entirety. It is produced through cleavage of the AC precursor protein (Ferlinz et al., "Human Acid Ceramidase: Processing, Glycosylation, and Lysosomal Targeting," *J. Biol. Chem.* 276(38):35352-60 (2001), which is hereby incorporated by reference in its entirety), which is the product of the *Asahl* gene (NCBI UniGene GeneID No. 427, which is hereby incorporated by reference in its entirety). The present invention demonstrates that AC promotes cell survival.

in its entirety), eggs, embryos, neurons, sperm, synovial fibroblasts, and embryonic stem cells. Preferred cell types are eggs (fertilized or unfertilized), embryos, primary cells (e.g., neurons), sperm, synovial fibroblasts, and embryonic stem cells. Moreover, the ceramide apoptosis pathway appears to be conserved across mammalian species (Lee & Amoscato, "TRAIL and Ceramide," Vitam. Horm. 67:229-55 (2004); see also, Samadi, "Ceramide-induced Cell Death in Lens Epithelial Cells," MoI. Vis. 13:1618-26 (2007) (humans); Parra et al, "Changes in Mitochondrial Dynamics During Ceramide-induced Cardiomyocyte Early Apoptosis," Cardiovasc. Res. (2007) (rat); de Castro E Paula & Hansen, "Ceramide Inhibits Development and Cytokinesis and Induces Apoptosis in Preimplantation Bovine Embryos," Mol. Reprod. Devel, DOI No. 10.1002/mrd.20841 (2007) (cows), each which is hereby incorporated by reference in its entirety). Therefore, it is expected that, for each of the cell types recited above, suitable cells include those of humans, monkeys, mice, rats, guinea pigs, cows, horses, sheep, pigs, dogs, and cats. In a preferred embodiment, this method is used to prolong the survival of eggs and/or embryos during in vitro fertilization procedures, facilitating the identification and selection of healthy embryos for reimplantation, especially for older human women and for veterinary breeding procedures.

[0042] Cells according to this aspect of the present invention can be provided by methods that will be apparent to the skilled artisan. By way of example, the cells can be obtained from an animal or from an existing ex vivo source (e.g., a tissue sample, a cell culture, etc.) using standard techniques. Treating cells ex vivo includes treating cells present in a homogeneous culture, as well as cells present in a heterogeneous culture (e.g., a tissue sample).

[0043] Acid ceramidases that can be used in this and all aspects of the present invention include, without limitation, those set forth in Table 1. In this and all aspects of the present invention (including the in vivo methods discussed below), the acid ceramidase can be homologous (i.e., derived from the same species) or heterologous (i.e., derived from a different species) to the one or more cells being treated.
Table 1. Exemplary Acid Ceramidase Family Members

<table>
<thead>
<tr>
<th>Homo sapiens</th>
<th>Caenorhabditis elegans</th>
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<tbody>
<tr>
<td>UniProt</td>
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<tr>
<td>NCBI Accession</td>
<td>Q13510, AAC73009</td>
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<table>
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<th>Mus musculus</th>
<th>Danio rerio</th>
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<tbody>
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<tr>
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<tr>
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<table>
<thead>
<tr>
<th>Gallus gallus</th>
<th>Rattus norvegicus</th>
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<tbody>
<tr>
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<table>
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<th>Pan troglodytes</th>
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<tbody>
<tr>
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<tr>
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</tr>
<tr>
<td>NCBI RefSeq</td>
</tr>
<tr>
<td>NCBI UniGene</td>
</tr>
</tbody>
</table>

[0044] Treating according to this aspect of the present invention is carried out by contacting the cell(s) with the acid ceramidase, using methods that will be apparent to the skilled artisan.

[0045] In some embodiments, treating is carried out by introducing into the cell an acid ceramidase precursor protein, which is then converted into an active acid ceramidase protein by the cell. In particular, the AC precursor protein undergoes autoproteolytic cleavage into the active form (composed of α- and β-subunits). This is promoted by the intracellular environment, and based on highly conserved sequences at the cleavage site of AC precursor proteins across species, is expected to occur in most, if not all, cell types. Suitable acid ceramidase precursor proteins include those set forth in Table 1, supra. As will be apparent to the skilled artisan, the precursor protein could optionally be contained in a culture medium to which the cell is exposed. Embodiments in which the precursor protein is taken up by the cell of interest and converted into active acid ceramidase thereby, as well as embodiments in which the precursor protein is converted into acid ceramidase by a different cell or agent present in the culture medium, are both contemplated.
[0046] An approach for delivery of proteins or polypeptide agents (e.g., acid ceramidase, acid ceramidase precursor protein) involves the conjugation of the desired protein or polypeptide to a polymer that is stabilized to avoid enzymatic degradation of the conjugated protein or polypeptide. Conjugated proteins or polypeptides of this type are described in U.S. Patent No. 5,681,811 to Ekwuribe, which is hereby incorporated by reference in its entirety.

[0047] Yet another approach for delivery of proteins or polypeptide agents involves preparation of chimeric proteins according to U.S. Patent No. 5,817,789 to Heartlein et al, which is hereby incorporated by reference in its entirety. The chimeric protein can include a ligand domain and the polypeptide agent (e.g., acid ceramidase, acid ceramidase precursor protein). The ligand domain is specific for receptors located on a target cell. Thus, when the chimeric protein is delivered to the cell or culture medium, the chimeric protein will adsorb to the targeted cell, and the targeted cell will internalize the chimeric protein.

[0048] In some embodiments, the acid ceramidase may be administered by introducing into the cell or culture medium a nucleic acid molecule that encodes the acid ceramidase (or acid ceramidase precursor protein, as described above) (JOSEPH SAMBROOK & DAVID W. RUSSELL, 1-3 MOLECULAR CLONING: A LABORATORY MANUAL (3d ed. 2001); SHORT PROTOCOLS IN MOLECULAR BIOLOGY (Frederick M. Ausubel et al. eds., 1999); U.S. Patent No. 4,237,224 to Cohen & Boyer; each of which is hereby incorporated by reference in its entirety). Suitable nucleic acid molecules include those set forth in Table 1, supra. This includes introducing into the culture medium a cell that contains (and expresses) the nucleic acid molecule, and which secretes the acid ceramidase/acid ceramidase precursor protein into the culture medium.

[0049] Nucleic acid agents for use in the methods of the present invention can be delivered to a cell in a number of ways known in the art. For example, the nucleic acid can be contained within a vector, e.g., a vector that can be transferred to the cell(s) and provide for expression of the nucleic acid therein. Such vectors include chromosomal vectors (e.g., artificial chromosomes), non-chromosomal vectors, and synthetic nucleic acids. Vectors include plasmids, viruses, and phages, such as retroviral vectors, lentiviral vectors, adenoviral vectors, and adeno-associated vectors.
[0050] Nucleic acid agents can be transferred into the cell(s) using ex vivo methods, as will be apparent to the skilled artisan. For example, nucleic acids and vectors can be delivered to cells by physical means, e.g., by electroporation, lipids, cationic lipids, liposomes, DNA gun, calcium phosphate precipitation, injection, or delivery of naked nucleic acid.

[0051] As an alternative to non-infective delivery of nucleic acids as described above, naked DNA or infective transformation vectors can be used for delivery, whereby the naked DNA or infective transformation vector contains a recombinant gene that encodes the acid ceramidase/acid ceramidase precursor protein. The nucleic acid molecule is then expressed in the transformed cell.

[0052] The recombinant gene includes, operatively coupled to one another, an upstream promoter operable in the cell in which the gene is to be expressed and optionally other suitable regulatory elements (i.e., enhancer or inducer elements), a coding sequence that encodes the nucleic acid, and a downstream transcription termination region. Any suitable constitutive promoter or inducible promoter can be used to regulate transcription of the recombinant gene, and one of skill in the art can readily select and utilize such promoters, whether now known or hereafter developed. The promoter can also be specific for expression in the cell(s) whose survival is to be promoted. Tissue specific promoters can also be made inducible/repressible using, e.g., a TetO response element. Other inducible elements can also be used. Known recombinant techniques can be utilized to prepare the recombinant gene, transfer it into the expression vector (if used), and administer the vector or naked DNA to the cell. Exemplary procedures are described in SAMBROOK & RUSSELL, 1-3 MOLECULAR CLONING: A LABORATORY MANUAL (3d ed. 2001), which is hereby incorporated by reference in its entirety. One of skill in the art can readily modify these procedures, as desired, using known variations of the procedures described therein.

[0053] Any suitable viral or infective transformation vector can be used. Exemplary viral vectors include, without limitation, adenovirus, adeno-associated virus, and retroviral vectors (including lentiviral vectors).

[0054] Adenovirus gene delivery vehicles can be readily prepared and utilized given the disclosure provided in Berkner, "Development of Adenovirus..."


Retroviral vectors which have been modified to form infective transformation systems can also be used to deliver a recombinant gene encoding a desired nucleic acid product into a target cell. One such type of retroviral vector is disclosed in U.S. Patent No. 5,849,586 to Kriegler & Perez, which is hereby incorporated by reference in its entirety. Lentivirus vectors can also be utilized, including those described in U.S. Patent No. 6,790,657 to Arya, and U.S. Patent Application Publication No. 2004/0170962 to Kafri et al. and U.S. Patent Application Publication No. 2004/0147026 to Arya, each of which is hereby incorporated by reference in its entirety.

Acid ceramidase treatment can be carried out as frequently as required and for a duration that is suitable to promote survival of the cell(s). For example, treatment can be carried out once, or multiple times.

The amount of acid ceramidase to be administered will, of course, vary depending upon the particular conditions. Generally, the acid ceramidase is administered to achieve an amount effective for improving survival of the cell(s). The amount required to obtain the desired effect may vary depending on the cell type, culture conditions, and duration for which it is desired that cell survival be promoted. Effective amounts can be determined empirically by those of skill in the art. For example, this may involve assays in which varying amounts of acid ceramidase are administered to cells in culture and the concentration effective for obtaining the desired result is calculated.

In a preferred embodiment, the acid ceramidase/acid ceramidase precursor protein is introduced into a culture medium, and the one or more cells are exposed to the culture medium before or after the acid ceramidase/acid ceramidase precursor protein is introduced.

Promoting survival according to this and all aspects of the present invention refers to any increase in the survival rate of the cell(s), including
increasing the time it takes the cell(s) to die, and completely preventing the death of the cell(s).

[0059] A second aspect of the present invention relates to a method of promoting in vivo survival of one or more cells in a female mammalian subject. This method involves administering to the female mammalian subject acid ceramidase under conditions effective to promote survival of one or more cells in the female mammalian subject.

[0060] Mammals according to this aspect of the present invention include, without limitation, humans, monkeys, mice, rats, guinea pigs, cows, sheep, horses, pigs, dogs, and cats.

[0061] Cells according to this aspect of the present invention include those identified above. In a preferred embodiment, the one or more cells are eggs (fertilized or unfertilized).

[0062] It is predicted that acid ceramidase can protect the oocytes/embryos of women undergoing radiation and/or chemotherapy treatments, since these treatments are known to induce apoptosis in eggs via the ceramide pathway (Jurisicova et al., "Molecular Requirements for Doxorubicin-mediated Death in Murine Oocytes," Cell Death Differ. 13:1466-74 (2006); Tilly & Kolesnick, "Sphingolipids, Apoptosis, Cancer Treatments and the Ovary: Investigating a Crime Against Female Fertility," Biochem. Biophys. Acta 1585:135-8 (2002), each of which is hereby incorporated by reference in its entirety). Thus, in a preferred embodiment, the cells are eggs and the subject is a female human subjected to chemotherapy after administering the acid ceramidase.

[0063] An another preferred embodiment, the method according to this aspect of the present invention is carried out to prevent cell death of oocytes in vivo to enhance breeding efficiency of agricultural animals (e.g., horses, cows, sheep, pigs), domestic animals (e.g., dogs, cats, guinea pigs, hamsters), and/or laboratory animals (e.g., monkeys, mice, rats, guinea pigs, hamsters).

[0064] As discussed above with respect to ex vivo delivery, active acid ceramidase can be directly administered to the subject, and/or it may be delivered in the form of an acid ceramidase precursor protein and/or a nucleic acid encoding
the acid ceramidase/acid ceramidase precursor protein. Exemplary proteins and nucleic acids include those set forth in Table 1. infra. The conjugated and chimeric proteins or polypeptide agents described above are also suitable in this aspect of the present invention.

As will be apparent to one of ordinary skill in the art, administering may be carried out using generally known methods. Exemplary methods are set forth below.

Administration can be accomplished either via systemic administration to the subject or via targeted administration to affected tissues, organs, and/or cells. The therapeutic agent (i.e., acid ceramidase, acid ceramidase precursor protein, nucleic acid encoding acid ceramidase/acid ceramidase precursor protein) may be administered to a non-targeted area along with one or more agents that facilitate migration of the therapeutic agent to (and/or uptake by) a targeted tissue, organ, or cell. Additionally and/or alternatively, the therapeutic agent itself can be modified to facilitate its transport to (and uptake by) the desired tissue, organ, or cell, as will be apparent to one of ordinary skill in the art. Preferred target tissues in the case of promoting survival of eggs include ovarian tissue and uterine tissue.

Any suitable approach for delivery of the agents can be utilized to practice this aspect of the present invention. Typically, the therapeutic agent will be administered to a patient in a vehicle that delivers the therapeutic agent(s) to the target cell, tissue, or organ.

Exemplary routes of administration include, without limitation, by intratracheal inoculation, aspiration, airway instillation, aerosolization, nebulization, intranasal instillation, oral or nasogastric instillation, intraperitoneal injection, intravascular injection, topically, transdermally, parenterally, subcutaneous ly, intravenous injection, intra-arterial injection (such as via the pulmonary artery), intramuscular injection, intralekle instillation, intraventricularly, intralesionally, by application to mucous membranes (such as that of the nose, throat, bronchial tubes, genitals, and/or anus), or implantation of a sustained release vehicle.

Typically, the therapeutic agent will be administered as a pharmaceutical formulation that includes the therapeutic agent and any
pharmaceutically acceptable adjuvants, carriers, excipients, and/or stabilizers, and can be in solid or liquid form, such as tablets, capsules, powders, solutions, suspensions, or emulsions. The compositions preferably contain from about 0.01 to about 99 weight percent, more preferably from about 2 to about 60 weight percent, of therapeutic agent together with the adjuvants, carriers and/or excipients. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage unit will be obtained.

The agents may be orally administered, for example, with an inert diluent, or with an assimilable edible carrier, or they may be enclosed in hard or soft shell capsules, or they may be incorporated directly with the food of the diet. For oral therapeutic administration, these active compounds may be incorporated with excipients and used in the form of tablets, capsules, elixirs, suspensions, syrups, and the like. Such compositions and preparations should contain at least 0.1% of the agent.

The percentage of the agent in these compositions may, of course, be varied and may conveniently be between about 2% to about 60% of the weight of the unit. The amount of the agent in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, capsules, and the like may also contain a binder such as gum tragacanth, acacia, corn starch, or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, or alginic acid; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose, or saccharin. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a fatty oil.

Various other materials may be present as coatings or to modify the physical form of the dosage unit. For instance, tablets may be coated with shellac, sugar, or both. A syrup may contain, in addition to active ingredient(s), sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye, and flavoring such as cherry or orange flavor.

The agents may also be administered parenterally. Solutions or suspensions of the agent can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in
glycerol, liquid polyethylene glycols, and mixtures thereof in oils. Illustrative oils are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, or mineral oil. In general, water, saline, aqueous dextrose and related sugar solutions, and glycols such as propylene glycol or polyethylene glycol, are preferred liquid carriers, particularly for injectable solutions. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol), suitable mixtures thereof, and vegetable oils.

The agents according to this aspect of the present invention may also be administered directly to the airways in the form of an aerosol. For use as aerosols, the compounds of the present invention in solution or suspension may be packaged in a pressurized aerosol container together with suitable propellants, for example, hydrocarbon propellants like propane, butane, or isobutane with conventional adjuvants. The materials of the present invention also may be administered in a non-pressurized form.

in Vitro and in Vivo,” Biochim. Biophys. Acta 802:259-73 (1984), each of which is hereby incorporated by reference in its entirety), transdermal patches, implants, implantable or injectable protein depot compositions, and syringes. Other delivery systems which are known to those of skill in the art can also be employed to achieve the desired delivery of the therapeutic agent to the desired organ, tissue, or cells in vivo to effect this aspect of the present invention.

Administration can be carried out as frequently as required and for a duration that is suitable to provide effective promotion of cell survival. For example, administration can be carried out with a single sustained-release dosage formulation or with multiple daily doses.

The amount to be administered will, of course, vary depending upon the treatment regimen. Generally, an agent is administered to achieve an amount effective for improving survival of the cell(s). Thus, a therapeutically effective amount can be an amount which is capable of at least partially preventing apoptosis of the cell(s), including delaying the onset of cell death. The dose required to obtain an effective amount may vary depending on the agent, formulation, cell type, the duration for which it is desired that cell survival be promoted, and individual to whom the agent is administered.

Determination of effective amounts may also involve in vitro assays in which varying doses of agent are administered to cells in culture and the concentration of agent effective for promoting cell survival is determined in order to calculate the concentration required in vivo. Effective amounts may also be based on in vivo animal studies. A therapeutically effective amount can be determined empirically by those of skill in the art.

A third aspect of the present invention relates to a kit for promoting ex vivo primary cell survival. The kit includes a cell culture medium and an acid ceramidase.

Suitable cell culture media according to this aspect of the present invention include, without limitation, M2 for oocytes and embryos, RPMI and DMEM for many primary cells (including fibroblasts), and B27 for neurons.
The acid ceramidase according to this aspect of the present invention includes those identified above. The acid ceramidase can be in protein form, and/or in the form of a nucleic acid molecule encoding acid ceramidase.

The kit according to this aspect of the present invention may optionally include one or more cells in the culture medium, including any of the cells identified above. As will be apparent to the skilled artisan, where more than one cell is included, the cells can be homogeneous (i.e., the same cell type from the same species) or heterogeneous (i.e., different cell types and/or cells from different species).

A fourth aspect of the present invention relates to a method of predicting in vitro fertilization outcome. This method involves providing a sample of serum or follicular fluid from a female subject. The sample is screened for acid ceramidase activity level, and the acid ceramidase activity level is correlated to a prediction of the outcome of in vitro fertilization for the female subject.

The sample may be provided using methods that will be apparent to the skilled artisan. For example, serum can be obtained by standard blood draw. Follicular fluid is obtained during oocyte retrieval during assisted fertilization.

Suitable subjects according to this aspect of the present invention include those identified above. In a preferred embodiment, the subject is a human.

The sample can be screened for acid ceramidase activity by methods that will be apparent to the skilled artisan. Suitable methods include, for example, AC activity assays (Eliyahu et al., "Acid Ceramidase Is a Novel Factor Required for Early Embryo Survival," *FASEBJ*. 21(7): 1403-9 (2007), which is hereby incorporated by reference in its entirety), western blotting to determine the relative amount of AC present in the sample (where a higher amount of AC protein correlates to a higher AC activity level) (Eliyahu et al., "Acid Ceramidase Is a Novel Factor Required for Early Embryo Survival," *FASEBJ*. 21(7): 1403-9 (2007), which is hereby incorporated by reference in its entirety), and RIA (Ferlinz et al., "Human Acid Ceramidase: Processing, Glycosylation, and
It is expected that females with low AC in serum and/or follicular fluid have a higher percentage of apoptotic eggs, and thus a poorer predicted outcome for in vitro fertilization. Therefore, the AC activity level of the sample can be correlated to the predicted outcome by comparing that level to a standard level. The standard can be determined using population data from females of various ages (see, for example, Example 14, infra).

The present invention may be further illustrated by reference to the following examples.

EXAMPLES

Example 1 — Mouse Egg and Embryo Collection.

All experiments involving animals were approved by, and performed in strict accordance with, the guidelines of the appropriate institutional animal care and use committees. Seven to 8-week-old 129-SV/IMJ and C57-Black/6 female mice (Jackson Labs, Bar Harbor, Maine) were superovulated with 10 international units ("IU") of pregnant mares’ serum gonadotropin ("PMSG;" Syncro-part, Sanofi, France), followed by 10 IU of human chorionic gonadotropin ("hCG;" Sigma, St. Louis, MO) 48 hours later. Mature and old MII stage eggs were collected from the oviducal ampullae 16 hours or 46 hours, respectively, after injection of hCG. Cumulus cells were removed by a brief exposure to 400 IU/ml of highly purified hyaluronidase (H-3631; Sigma) in Todd-Hewitt medium (Eliyahu & Shalgi, "A Role for Protein Kinase C During Rat Egg Activation," Biol. Reprod. 67:189-95 (2002), which is hereby incorporated by reference in its entirety). For 2-cell embryo collection, superovulated females were caged with males of proven fertility and sacrificed 46 hours after injection of hCG. Embryos were isolated from the oviducal ampullae and cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.
Example 2 — Single Cell Long Nested PCR Genotyping.

[0091] DNA from individual embryos was subjected to PCR amplification using a mixture of two sets of long and short (nested) primers. Long\textsuperscript{1st} and short\textsuperscript{2nd} PCR amplification of the wild-type \textit{Asahl} allele was performed using forward and reverse primers (5'-ACCCAGGTTCCATCGTTGCACACATTTCATC-3' (SEQ ID NO: 1), 5'-ATGCCACATGGGAATACTGTCCAAAGCAGAA-S' (SEQ ID NO: 2), and 5'-CACACAAACACATGTATGTGCACACGTGAA-S' (SEQ ID NO: 3), 5'-GCTGCCCTGGAACTCACTCACTCT-S' (SEQ ID NO: 4)) to produce ~9-kb and 180-bp DNA fragments, respectively. Amplification of the mutated \textit{Asahl} allele using forward and reverse primers (5'-ATGCCACATGGGAATACTGTCCAAAGCAGAA-3' (SEQ ID NO: 2)', 5'-GAGGAGTAGAAGGTGGCGCGAAGGGG-S' (SEQ ID NO: 5), and 5'-GCTGCCCTGGAACTCACTCACTCT-S' (SEQ ID NO: 4), 5'-GCTGCCCTGGAACTCACTCACTCT-S' (SEQ ID NO: 4)') produced ~7-kb and 255-bp DNA fragments, respectively.

Example 3 — Western Blot Analysis.

[0092] Eggs and embryos were subjected to lysis in buffer containing 50mM Tris-HCL, 150mM NaCl, 2mM EDTA, 1% NP-40, 1mM Vanadate, 5mM Naf, and 1\mu g/ml aprotinine (pH 7.4). Proteins were separated by SDS-PAGE using 10% or 12% pre-cast Nupage Bis/Tris gels under reducing conditions and MES running buffer (Invitrogen), and transferred onto a nitrocellulose membrane (Amersham Biosciences) using a semi-dry transfer apparatus (BioRad) and Nupage-MOPS transfer buffer. For immunoblot analysis, blots were blocked with TBS/Tween containing 5% dry milk, and then were incubated with Goat IgG against acid ceramidase ("AC") (specific for the $\beta$-subunit). Bound antibodies were recognized by secondary antibodies conjugated to horseradish peroxidase. Detection was performed by an enhanced chemiluminescence detection reagent (Amersham Biosciences). Approximate molecular masses were determined by comparison with the migration of pre-stained protein standards (BioRad).
Example 4 — Acid Ceramidase Activity Assay.

Eggs were subjected to lysis in 0.25% sucrose solution. Total cell extracts were incubated for 22 hours at 37°C with O.1ng/ml BODIPY-conjugated C12-ceramide in 0.1M citrate/phosphate buffer (pH 4.5), 150mM NaCl, 0.05% BSA, and 0.1% Igepal CA-630. After the reactions were complete, 5µl of the assay mixtures were removed and added into 95µl of ethanol, mixed, and then centrifuged for 5 minutes at 10,000 x g. The supernatants were then transferred to a Waters glass sampling vial, and 5µl (2.5% of the original reaction mixture) were auto-sampled by a WIPS 712 (Waters) autosampler onto a high performance liquid chromatograph equipped with a reverse-phase column (BetaBasic-18, 4.6 x 30 mm, Keystone Scientific Inc., Bellefonte, PA), and eluted isocratically with methanol/water (95:5 v/v) at a flow rate of 1 ml/min. Fluorescence was quantified using a Waters 474 fluorescence detector set to excitation and emission wavelengths of 505nm and 540nm, respectively. The undigested substrate (i.e., BODIPY-conjugated C12-ceramide) and product (i.e. fatty acid) peaks were identified by comparing their retention times with standards, and the amount of product was calculated using a regression equation that was established from a standard curve using BODIPY-conjugated C12 fatty acid.

Example 5 — Immunohistochemistry.

Eggs were isolated and fixed in 3% paraformaldehyde. Zonae pellucidae ("ZP") were removed post-fixation by pronase (Sigma), and the ZP free eggs were permeabilized by NP-40. The eggs were then incubated with different primary and secondary antibodies (Eliyahu & Shalgi, "A Role for Protein Kinase C During Rat Egg Activation," Biol. Reprod. 67:189-95 (2002), which is hereby incorporated by reference in its entirety). The fluorescent reagents were visualized and photographed with a Ziess confocal laser-scanning microscope. For apoptosis detection, live 2-cell embryos were labeled using an Annexin V Apoptosis Detection Kit (Santa Cruz Biotechnology, Inc.).

Example 6 — mRNA Quantification by Polymerase Chain Reaction.

Total mRNA was extracted from equal numbers of eggs and embryos, and reverse-transcribed according to the manufacturer's instruction
and mac137r (5'-TTGACCTTTGGTAACATCCATC-S' (SEQ ID NO: 8)) were used for murine AC PCR amplification with QuantiTect SYBR Green PCR kit (QIAGEN). Changes in AC mRNA levels in old eggs and 2-cell embryos were assessed relatively to the level of AC mRNA in young eggs using the formulas $2^{\Delta(Ct_{\text{young}} - Ct_{\text{old}})}$ and $2^{\Delta(Ct_{\text{young}} - Ct_{2\text{-cell embryo}})}$, respectively. Housekeeping proteins actin beta, glyceraldehyde-3-phosphate dehydrogenase, and ribosomal protein SII (RPS11) were used as internal controls for embryonic mRNA expression.

**Example 7 — Data Presentation and Statistical Analysis.**

[0096] All experiments were independently replicated at least three times with different mice. The combined data from the replicate experiments were subjected to a t-test analysis, and results were considered statistically significant at $P < 0.005$. Graphs represent the mean ± s.e.m. of combined data from the replicate experiments. Representative photomicrographs are presented for the egg morphology, Annexin V labeling, and immunohistochemistry assays.

**Example 8 — Asahl-I- Mouse Embryos Are Formed, but Die During the 2-4-Cell Transition.**

[0097] To gain insights into the pathological mechanism underlying the lethal phenotype of Asahl-I- mice, a single-cell, long nested ("SCLN") PCR genotyping method was developed. This technique allowed for the genotyping of individual embryos immediately after fertilization. Two- to 8-cell stage embryos were collected from Asahl+/- intercrosses 36-60 hours after human chorionic gonadotropin injection, and cultured in M2 media. DNA was obtained from single embryos and subjected to SCLN PCR amplification using a mixture of long and nested PCR primers, as described in Examples 1-2. Genotyping of 196 embryos from these intercrosses revealed that Asahl-I- embryos could be formed, as shown in Figure 1. However, no Asahl-I- embryos were identified beyond the 2-cell stage, as shown in Table 2, suggesting that the lack of AC activity led to embryo death during the 2-to 4-cell transition. Notably, more Asahl+I- embryos were identified at the 4- to 8-cell stage than predicted, i.e., the predicted wild-type
to heterozygote ratio was 1:2, while the actual ratio was ~1:2.8, as shown in Table 2. This was attributed to the fact that older Asahl-I- male mice were used to produce these embryos, and that mutant sperm from such mice have a fertilization advantage compared to wild-type sperm.

Table 2. Embryo Genotyping Results.

<table>
<thead>
<tr>
<th></th>
<th>+/+</th>
<th>+/-</th>
<th>-/-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-cell embryos</td>
<td>40</td>
<td>72</td>
<td>32</td>
<td>143</td>
</tr>
<tr>
<td>4-8-cell embryos</td>
<td>14</td>
<td>39</td>
<td>0</td>
<td>53</td>
</tr>
</tbody>
</table>

2-8-cell embryos from Asahl-I- intercrosses were subjected to genotyping by SCLN PCR. The data represent genotyping of 100% of the embryos obtained from 8 female mice.

Example 9 — Asahl-I- Mouse Embryos Undergo Apoptotic Death During the 2-Cell Stage.

[0098] Ceramide-mediated signaling often leads to apoptosis (Spiegel et al., "Signal Transduction Through Lipid Second Messengers," Curr. Opin. Cell. Biol. 8:159-67 (1996), which is hereby incorporated by reference in its entirety). Therefore, one consequence of inactivating the Asahl gene might be the increase of ceramide in embryos, leading to cell cycle arrest or apoptosis (Hannun, "Function of Ceramide in Coordinating Cellular Responses to Stress," Science 274:1855-9 (1996); Spiegel et al., "Signal Transduction Through Lipid Second Messengers," Curr. Opin. Cell. Biol. 8:159-67 (1996), which are hereby incorporated by reference in their entirety). It has been shown that ceramide levels in eggs are increased during in vivo and in vitro aging (Perez et al., "A Central Role for Ceramide in the Age-related Acceleration of Apoptosis in the Female Germline," FASEB J. 19:860-2 (2005), which is hereby incorporated by reference in its entirety). These data support the conclusion that AC plays an important role in egg/embryo survival by removal of ceramide.

[0099] To further investigate the involvement of AC during development, and to characterize the mechanism leading to the death of Asahl-I- embryos, the possibility of apoptotic death was assessed by Annexin V staining (Chan et al., "Plasma Membrane Phospholipid Asymmetry Precedes DNA Fragmentation in Different Apoptotic Cell Models," Histochem. Cell Biol. 110:553-8 (1998), which is hereby incorporated by reference in its entirety). To perform this analysis, 86
live 2-cell embryos from Asahl+I- intercrosses were collected and designated numbers. Each embryo was examined independently for apoptotic morphology and Annexin V binding using laser-scanning confocal microscopy, and then genotyped by SCLN PCR. The outcome of these analyses revealed that all of the Asahl-I- embryos had apoptotic morphology, as shown in Figures 2D and 2F, and positive Annexin V staining, as shown in Figures 2E-F, while wild-type embryos had normal morphology, as shown in Figures 2A and 2C, and no Annexin V staining except of the apoptotic polar body, as shown in Figures 2B-C. As shown in Table 3, the percentages of apoptotic wild-type (11%) and heterozygous (5%) embryos were negligible compared to Asahl-I- embryos (100%) (t-test, PO.00001).

Table 3. Asahl-I- Embryos Undergo Apoptotic Death During the 2-Cell Stage.

<table>
<thead>
<tr>
<th></th>
<th>+/+</th>
<th>+/-</th>
<th>-/-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-cell embryos (genotype)</td>
<td>27</td>
<td>42</td>
<td>17</td>
<td>86</td>
</tr>
<tr>
<td>Annexin V positive and/or abnormal morphology</td>
<td>3 ± 1 (11%)</td>
<td>2 ± 1 (5%)</td>
<td>17 ±0 (100%)</td>
<td>22</td>
</tr>
</tbody>
</table>

The data represent analysis of 100% of the embryos obtained from 4 female mice.

Thus, these findings reveal that the absence of functional AC causes apoptotic death during the 2-cell stage, and provides direct in vivo evidence that AC activity is essential for the 2- to 4-cell transition. This developmental period marks the beginning of embryonic genome activation.

Example 10 — Acid Ceramidase Expression in Unfertilized Mouse Oocytes.

Since AC appears to be required for the earliest stages of embryo development, it was hypothesized that the enzyme must be provided to newly formed embryos by the donor egg before embryonic genome activation ("EGA") in order for these embryos to survive. To examine this hypothesis, cell extracts were prepared from 400 pooled, unfertilized Mil eggs (collected 16 hours after hCG injection), and analyzed by western blot to identify the AC protein. As can be seen in Figure 3, the AC precursor protein (55kDa) and β-subunit (40kDa) are expressed in the egg before fertilization. The presence of the processed β-subunit indicates that some of the AC was likely to be active. Cell extracts were therefore
prepared from an additional 65 pooled, unfertilized eggs, and subjected to AC activity assays. As shown in Figure 3, these analyses revealed a high enzymatic activity (t-test, p<0.005), confirming the western blot results.

To obtain information about the subcellular location of AC in eggs, immunohistochemistry was performed using anti-AC specific antibodies combined with anti-LAMP-I staining for late endosome/lysosome detection. The fluorescence distribution of the AC and LAMP-I signals was visualized at the equator and cortex of the egg, and photographed with a Zeiss confocal laser-scanning microscope, as shown in Figures 4A-G. These studies reveal that AC is localized mainly at the egg cortex, as shown in Figures 4A and 4D, and co-localizes with LAMP-I in the late endosomes/lysosomes, as shown in Figures 4B and 4D-F.

**Example 11 — Normal Mouse Embryos Express Acid Ceramidase at Embryonic Genome Activation.**

The death of AC-deficient embryos during the 2-cell stage implies that in normal embryos AC gene expression occurs as early as the EGA to sustain survival. To confirm this hypothesis, changes in AC mRNA levels in old, unfertilized Mil eggs and 2-cell embryos (both collected 46 hours after hCG injection) were assessed relative to the levels in young, unfertilized eggs (collected 16 hours after hCG injection). Total mRNA was extracted from equal numbers of eggs and embryos, and quantified using the QuantiTect SYBR Green PCR kit (see Example 6). Housekeeping proteins β-actin, glyceraldehyde-3-phosphate dehydrogenase, and ribosomal protein S11 were used as internal controls for embryonic mRNA expression. As shown in Figure 5, of AC mRNA decreased significantly in old versus young unfertilized eggs (t-test, p<0.0003). This would predictably result in ceramide increase and apoptotic cell death. On the other hand, as shown in Figure 5, AC mRNA levels were enhanced in fertilized, healthy 2-cell embryos (t-test, p<0.0005), suggesting AC gene activation during EGA.

To confirm the PCR findings, AC protein levels were assessed in 140 pooled unfertilized eggs in comparison to 140 2-cell embryos by western blot analysis, followed by densitometric analysis, using actin as a control. The level of
AC precursor was increased in 2-cell embryos as compared to unfertilized eggs, consistent with the mRNA findings. As shown in Figure 5, ribosomal protein S11, one of the first genes expressed immediately after fertilization, was also used as a control to mark the initiation of EGA. Glyceraldehyde-3-phosphate dehydrogenase was used as a negative control, as shown in Figure 5. Overall, the fact that AC was expressed in young eggs before fertilization and AC levels decreased during the aging process, together with the fact that there was enhanced AC expression during EGA, highlights the importance of this enzyme for embryo survival. These data show that embryonic AC gene expression is initiated during the 2-cell stage.

Discussion of Examples 1-11

During normal development eggs proceed to apoptosis unless fertilization occurs. Among the complex regulatory pathways that are needed to control this delicate balance between death and survival, sphingolipid signaling is an important component. Indeed, ceramide accumulation in aging eggs has been shown to result in apoptosis, and the anti-apoptotic lipid, sphingosine-1-phosphate ("SIP"), can counteract the effects of ceramide and promote egg survival (Perez et al., "A Central Role for Ceramide in the Age-related Acceleration of Apoptosis in the Female Germline," *FASEB J.* 19:860-2 (2005); Miao et al., "Cumulus Cells Accelerate Aging of Mouse Oocytes," *Biol. Reprod.* 73:1025-1031 (2005), which are hereby incorporated by reference in their entirety). Other physiological changes in unfertilized eggs and early embryos, including Ca$^{2+}$ oscillations, are also important components of this regulatory decision. Upon fertilization, young, healthy eggs must supply sufficient anti-apoptotic proteins and mRNA to newly formed embryos to overcome the default apoptosis pathway. Afterwards, the newly formed embryo must supply these factors through expression of its own genome at embryonic genome activation ("EGA"). In the mouse, EGA begins during the 2-cell stage (Flach et al., "The Transition from Maternal to Embryonic Control in the 2-Cell Mouse Embryo," *EMBO J.* 1:68 1-6 (1982), which is hereby incorporated by reference in its entirety), whereas in humans the major activation event occurs between the 4- and 8-cell stages (Telford et al., "Transition from Maternal to Embryonic Control in Early Mammalian Development: A
Comparison of Several Species," *Mol. Reprod. Dev.* 26:90-100 (1990), which is hereby incorporated by reference in its entirety). Although anti-apoptotic factors should be among the genes/proteins expressed at EGA, very few such factors have been identified to date.

Consistent with prior evidence showing that increased ceramide levels in aging eggs leads to apoptosis (Perez et al., "A Central Role for Ceramide in the Age-related Acceleration of Apoptosis in the Female Germline," *FASEB J.* 19:860-2 (2005); Miao et al., "Cumulus Cells Accelerate Aging of Mouse Oocytes," *Biol. Reprod.* 73:1025-1031 (2005), which are hereby incorporated by reference in their entirety), it was hypothesized that AC, an enzyme responsible for the hydrolysis of ceramide and the production of sphingosine (the precursor of SIP), might be an essential factor required for embryo survival. It was also hypothesized that in the absence of AC activity, ceramide levels in 2-4 cell AC knockout embryos would increase, leading to apoptosis. While it is not possible to accurately quantify ceramide in individual embryos due to the limited sensitivity of the available techniques and/or the fact that these techniques preclude subsequent genotyping (e.g., immunohistochemistry), the present studies clearly show that embryo-derived AC is one of the first proteins expressed during the 2-cell stage of development in mice, and that its activity is necessary for the subsequent expression of the normal developmental program. In the absence of this activity, embryos undergo apoptotic death.

Additionally, AC activity is not only essential during embryonic development, but during postnatal life as well. In humans, reduced AC activity leads to the lipid storage disease Farber Lipogranulomatosis ("FD"). FD is an extremely rare and fatal lipid storage disorder, and at least two cases of fetal death have been reported (Kattner et al., *Hydropsfetalis: Manifestation in Lysosomal Storage Diseases Including Farber Disease,* *Eur. J. Ped.* 156:292-5 (1997); Van Lijnschoten et al., "Intrauterine Fetal Death Due to Farber Disease: Case Report," *Pediatr. Dev. Pathol.* 3:597-602 (2000), which are hereby incorporated by reference in their entirety). Mutation analysis carried out on surviving FD patients has shown that subtle point mutations account for most of the abnormalities, rather than large gene deletions, rearrangements, or frame-shift mutations, which are likely to cause complete loss-of-function. Indeed, even these subtle point
mutations often lead to a severe clinical condition (Moser et al., "Acid Ceramidase Deficiency: Farber Lipogranulomatosis," in THE METABOLIC & MOLECULAR BASIS OF INHERITED DISEASE 3573-88 (Charles R. Scrver et al. eds., 8th ed. 2001), which is hereby incorporated by reference in its entirety), providing further evidence that AC activity is essential for normal postnatal development. The present Examples demonstrate that mice homozygous for the complete loss-of-function Asahl allele undergo apoptotic death at the 2-cell stage. These findings indicate that complete loss-of-function mutations in FD individuals would lead to early embryonic lethality, and are consistent with the fact that only patients with subtle point mutations survive.

[0108] Historically, AC was classified as a "lysosomal enzyme" because of the appearance of lipid storage vacuoles in FD patients that were reminiscent of lysosomes, as well as the enhanced in vitro activity at acidic pH. The present Examples document the sub-cellular location of AC in unfertilized eggs, and shows the presence of this protein both inside and outside of lysosomes. Although several reports have suggested that ceramide produced in lysosomes does not participate in cell signaling (e.g., Ohanian & Ohanian, "Sphingo lipids in Mammalian Cell Signaling," Cell. Mol. Life Sci. 58:2053-68 (2001), which is hereby incorporated by reference in its entirety), it is important to recognize that AC may contribute to the hydrolysis of non-lysosomal, as well as intra-lysosomal, ceramide pools. In fact, the related lipid hydrolase, acid sphingomyelinase, can hydrolyze sphingomyelin in both lysosomal and non-lysosomal compartments, and rapidly re-locates to the cell surface following various stimuli (Ohanian & Ohanian, "Sphingo lipids in Mammalian Cell Signaling," Cell. Mol. Life Sci. 58:2053-68 (2001), which is hereby incorporated by reference in its entirety).

[0109] The development of a single-cell, PCR genotyping method for AC could potentially facilitate pre-implantation diagnosis of FD embryos for at-risk couples. While this method would have to be adapted from mice to humans, this should not be problematic, since the genes are highly conserved. Furthermore, based on the present findings, physicians could potentially use AC to prolong egg/embryo survival during IVF procedures, facilitating the identification and selection of healthy embryos for re-implantation, especially for older women. In conclusion, these data reveal a new and important role for AC in the earliest
stages of mammalian embryogenesis, and suggest that this enzyme and/or gene may be used to facilitate egg/embryo survival in vitro and/or in vivo.

**Example 12 — Acid Ceramidase Prolongs the Lifespan of Mouse Oocytes.**

[0110] Mil oocytes were collected from superovulated female mice and placed into a fresh media. Oocytes were incubated for 24 hours in a humidified incubator at 37°C in the absence or presence of acid ceramidase. The oocytes were fixed and stained with Hoechst for DNA labeling. The morphology and DNA staining were visualized using laser-scanning confocal microscopy. As shown in Figures 6A-B, addition of acid ceramidase to the incubation media prolonged the lifespan of the oocytes.

[0111] Mil oocytes were incubated for 24 hours in M2 media (with or without AC), media collected from a CHO cell line that does not express AC, or media collected from a CHO cell line that stably expresses and secretes AC. As shown in Figure 7, the rate of apoptosis was significantly reduced in oocytes incubated in media that contained AC.

**Example 13 — Acid Ceramidase Expression and Localization in Human Oocytes and Early Embryos.**

[0112] Oocytes and embryos from women scheduled for in vitro fertilization with intracytoplasmic sperm injection were collected ~32 hours (oocytes) or 3-5 days (embryos) after injection of luteinizing hormone.

[0113] Co-immunohistochemistry assays were performed to detect the localization and possible interaction between AC and lysosome associated membrane protein ("LAMP") (a lysosomal enzyme marker) during human oocyte maturation. Oocytes were triple labeled for AC protein, cellular DNA, and LAMP, and examined for co-localization by immunofluorescence confocal microscopy. As shown in Figures 8A-H, AC is localized mainly in the cortex and membrane, and co-localizes with LAMP, during both the germinal vesicle stage (Figures 8A-D) and the germinal vesicle break down stage (Figures 8E-H). In addition, AC is co-localized with the GV membrane break down, as shown in Figures 8E-H. As shown in Figures 8I-L, AC protein is co-localized with LAMP and with DNA during the MI stage. During the MI stage, AC is homogenously
distributed throughout the cytosol with a marked localization at the membrane and cortex, and co-localization in the spindle, as shown in Figures 8M-P). These data clearly show changes in the developmental pattern of AC expression during human egg maturation. This is the first known study that demonstrates that AC is expressed in human oocytes.

[0114] Co-immunohistochemistry assays were also performed to detect the localization and possible interaction between AC and acid sphingomyelinase ("ASM"), a related enzyme that hydrolyzes sphingomyelin into ceramide, during early embryo development. Embryos were triple labeled for AC protein, cellular DNA, and ASM, and examined for co-localization by immunofluorescence confocal microscopy. As shown in Figures 9A-D and Figures 10A-D, AC is localized in the embryonic fluid and co-localizes with ASM mainly in the inner and outer cell mass. Moreover, high-grade embryos (Figures 10A-D) demonstrate higher expression of AC in the embryonic fluid than do low-grade embryos (Figures 9A-D). Thus, high-grade embryos would be expected to have lower ceramide levels and higher SIP levels than low-grade embryos, and therefore a higher survival rate (due to a lower incidence of apoptosis).

Example 14 — Acid Ceramidase Expression and Activity in Human Follicular Fluid.

[0115] Human follicular fluid samples from oocytes assigned for in vitro intracytoplasmic sperm injection were collected during oocyte retrieval. Western blot analysis was used to evaluate the total amount of AC in the follicular fluid. Proteins were separated by SDS-PAGE. A monoclonal mouse anti-human AC IgM was used to detect the AC precursor protein (55kDa). As shown in Figure 11, the AC precursor protein is highly expressed in human follicular fluid.

[0116] An in vitro activity assay was used to evaluate the activity of AC in human follicular fluid. Follicular fluid samples were incubated under acidic conditions for 22 hours at 37°C with BODIPY conjugated C12-ceramide, and then analyzed by HPLC. The results of this activity assay were correlated with patient age using the Pearson correlation test. As shown in Figure 12, there is a trend towards a decrease in AC activity with increasing age. This suggests that the
measurement of AC in follicular fluid can be used as a marker for reproductive aging.

**Example 15 — Acid Ceramidase Prolongs the Life Span of Human Oocytes.**

[0117] Immature oocytes from women scheduled for in vitro intracytoplasmic sperm injection were collected ~24 hours after LH injection and transferred into Quinns Advantage Cleavage Medium with 5% HAS, supplemented with or without human AC, ~32 hours after LH injection. Oocytes were denuded and fixed 24 hours after culture in vitro in 50 µl drop under oil. Oocyte quality was evaluated based on membrane and cytoplasm morphology, and DNA appearance and integrity. DNA integrity was evaluated using the TUNEL staining assay, which detects fragmented DNA, an indicator of apoptosis.

[0118] As shown Figure 13A, oocytes from the control group were more sensitive to fixation, and exhibited membrane blebbing and the beginning of cytoplasm fragmentation, which are indicative of apoptosis. In contrast, oocytes that were cultured in the presence of AC had a stronger membrane and looked intact and smooth after fixation, as shown in Figure 13B. Morphology of the metaphase plate was defective in four out of six Mil oocytes in the control group. As shown in Figure 14A, oocytes cultured without AC have visible, condensed chromatin (presumably due to spindle disruption), an early sign of apoptosis. In contrast, six out of seven oocytes cultured in presence of AC conserved a proper metaphase plate and clearly distinguishable chromosomes (*i.e.*, no condensed chromatin). See Figures 14A-D for an illustration of DNA condensation. This suggests that oocytes cultured without AC are more susceptible to apoptosis than oocytes cultured with AC. TUNEL staining was carried out to more directly confirm this. As shown in Figures 15A-B, oocytes in the control group had a greater extent of DNA fragmentation, presented by stronger TUNEL staining, while oocytes that were cultured in the presence of AC had a lower TUNEL staining. In addition, Figures 15C-D clearly show a breakdown of the membrane and altered morphology of oocytes cultured without AC, while oocytes cultured with AC have a normal, healthy morphology. These results clearly demonstrate that administration of recombinant AC decreases the rate of apoptosis during human oocyte maturation in vitro.
Discussion of Examples 12-15.

[0119] Sphingolipid metabolism and sphingolipid-mediated signal transduction appear to be important in mammalian fertilization and early development. AC is the central enzyme in sphingolipid metabolism, hydrolyzing the pro-apoptotic lipid, ceramide, into the anti-apoptotic lipid, sphingosine-1-phosphate. Thus, AC is a rheostat helping to control a cell's fate between life and death. These Examples demonstrate that administration of recombinant AC enzyme results in a decreased rate of apoptosis in vitro in fresh or aged mouse oocytes, preventing DNA and cytoplasm fragmentation. This confirms the important role of this enzyme for oocyte survival during culture.

Example 16 — Recombinant, Human Acid Ceramidase Protects Primary Rat Neurons from Stress-induced Apoptosis.

[0120] Primary rat hippocampal neurons were grown for 17 hours in the presence of the pathologic Alzheimer's Disease protein, amyloid-β peptide (1 mM), or under the conditions of oxidative stress (i.e., with 50 mM of hydrogen peroxide), with or without recombinant human acid ceramidase present in the culture media (2 mg/ml). Notably, when acid ceramidase was included in the culture media, apoptosis (as assessed by TUNEL staining) was significantly reduced, as shown in Figure 16.

Example 17 — Acid Ceramidase Increases the Survival and Proliferation Rate of Cat Synovial Fibroblasts.

[0121] Primary cat synovial fibroblasts were grown for 24 hours with or without recombinant human acid ceramidase (2 mg/ml), and the levels of the survival factor, sphingosine-1-phosphate ("SIP") were determined. As shown in Figure 17, SIP levels was markedly higher in fibroblasts incubated with AC. This suggests that AC can be used to improve the survival rate of synovial fibroblasts in vitro.

[0122] The proliferation rate of primary cat synovial fibroblasts was determined using the MTS assay (Barltrop et al, "5-(3-Arboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) Tetrazolium, Inner salt (MTS) and Related Analogs of 3-(4,5-Dimethylthiazoly)-2,5-diphenyltetrazolium Bromide
(MTT) Reducing to Purple Water Soluble Formazans as Cell-viability Indicators, "Bioorg. Med. Chem. Lett. 1:611—4 (1991), which is hereby incorporated by reference in its entirety) with or without recombinant acid ceramidase in the culture media (2 mg/ml). As shown in Figure 18, primary synovial fibroblasts cultured in the presence of AC proliferated significantly faster than cells cultured without AC. This confirms that AC can be used to improve the survival rate of synovial fibroblasts in vitro.

Example 18 — Acid Ceramidase Improves the Survival Rate of Mouse Embryonic Stem Cells.

Western blot analysis was used to evaluate the total amount of AC in mouse embryonic stem cells ("ESCs"). Proteins were separated by SDS-PAGE and detected using a polyclonal antibody against the AC beta subunit. As shown in Figure 19, the AC protein is expressed at high levels and active in ESCs. These results suggest that AC could be involved in ESC survival.

The effect of AC on the level of poly(ADP-ribose) polymerase ("PARP") and Bax, two pro-apoptotic factors, was also evaluated. ESCs were incubated for 24 hours in a humidified incubator at 37°C in the absence or presence of AC. The cells were then lysed, and 100 mg of total protein was separated by SDS-PAGE. As shown in Figures 20A-C, the amount of two important pro-apoptotic factors, PARP and Bax, is reduced in the presence of AC. These results suggest that AC can potentially prolong the life-span of ESCs.

Example 19 — Acid Ceramidase Protects Rat Neuronal Cell Cultures Against Apoptosis.

Chemicals and reagents

Cell culture materials were from Fisher Scientific (Pittsburgh, PA, USA). All other biochemical reagents were from the Sigma Chemical Co. (St. Louis, MO, USA).

Neuronal progenitor cells were isolated from the adult rat hippocampus and cultured in neurobasal A medium consisting of 2% B27, 0.5 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10 ng/ml FGF at 37°C in a humidified 5% CO₂ atmosphere (Chen et al., "Trophic Factors
Counteract Elevated FGF-2-induced Inhibition of Adult Neurogenesis,"
in its entirety). The media was routinely changed every 2-3 days. When the cells
reached ~80% confluency, they were differentiated by replacing FGF with 5 µM
retinoic acid and 10% fetal calf serum. The neuronal cultures were generally used
for experiments after 3-5 days of growth in the differentiation medium. At this
stage, ~80% of the cells expressed the neuronal markers βIII-tubulin and
microtubule-associated protein 2. Less than 5% of the cells expressed the
astroglial marker GFAP or the oligodendrocyte marker 04. Thus, the
differentiated cells were used as a neuronal cell culture model for subsequent
experiments.

Effect of acid ceramidase on ceramide levels and apoptosis in neuronal cell
cultures

[0127] After 3-5 days of growth in the differentiation media, neuronal
cultures were treated with 1 µM of Aβ for 30 minutes with or without 1 hour of
recombinant human AC pre-treatment (1 µg/ml). Ceramide levels were verified
using the DAG kinase method (He et al, "An Enzymatic Assay for Quantifying
Sphingomyelin in Tissues and Plasma from Humans and Mice with Niemann-Pick
Disease," Anal. Biochem. 293:204-1 1 (2001), which is hereby incorporated by
reference in its entirety). Caspase 3 activity was measured using EnzCheck
Caspase-3 assay kit.

[0128] Acid sphingomyelinase activity is significantly elevated in the AD
brain and after amyloid-β peptide treatment of neuronal cultures. As shown in
Figure 21, ceramide levels are also significantly elevated after treating neuronal
cultures with Aβ. More apoptotic cells are also found as determined by caspase 3
activity, as shown in Figure 22. Importantly, however, ceramide levels and
caspase 3 activity did not increase in response to Aβ when purified, rhAC was
included in the culture media, as shown in Figure 21 and Figure 22, suggesting
that AC can be used to protect against ceramide-mediated apoptosis in neuronal
cells. In summary, Figures 21-22 show that ceramide levels and apoptosis are
reduced in the presence of AC.

[0129] Although preferred embodiments have been depicted and described
in detail herein, it will be apparent to those skilled in the relevant art that various
modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.
WHAT IS CLAIMED:

1. A method of promoting the ex vivo survival of cells, said method comprising:
   providing one or more cells ex vivo and
   treating the one or more cells with acid ceramidase under conditions effective to promote survival of the one or more cells.

2. The method of claim 1, wherein the one or more cells are provided in a culture medium and the acid ceramidase is added to the culture medium during said treating.

3. The method of claim 1, wherein the one or more cells are selected from the group of eggs, primary cells, neurons, sperm, synovial fibroblasts, and embryonic stem cells.

4. The method of claim 3, wherein the one or more cells are one or more eggs.

5. The method of claim 4, wherein the one or more eggs are unfertilized.

6. The method of claim 4, wherein the one or more eggs are fertilized.

7. The method of claim 4, wherein the one or more eggs are from a human.

8. The method of claim 1, wherein said treating is carried out with acid ceramidase in protein form.

9. The method of claim 1, wherein said treating is carried out with a nucleic acid encoding acid ceramidase.
10. A method of promoting \textit{in vivo} survival of one or more cells in a female mammalian subject, said method comprising:

administering to the female mammalian subject acid ceramidase under conditions effective to promote survival of one or more cells in the female mammalian subject.

11. The method of claim 10, wherein the one or more cells are one or more eggs.

12. The method of claim 11, wherein the one or more eggs are unfertilized.

13. The method of claim 11, wherein the one or more eggs are fertilized.

14. The method of claim 11, wherein the subject is selected from the group of humans, monkeys, mice, rats, guinea pigs, hamsters, horses, cows, sheep, pigs, dogs, and cats.

15. The method of claim 14, wherein the subject is a human.

16. The method of claim 15, wherein the female mammalian subject is subjected to chemotherapy following said administering.

17. The method of claim 10, wherein said administering is carried out with acid ceramidase in protein form.

18. The method of claim 10, wherein said administering is carried out with a nucleic acid encoding acid ceramidase.

19. A kit for promoting \textit{ex vivo} cell survival, said kit comprising:

a cell culture medium and
an acid ceramidase.

20. The kit according to claim 19 further comprising:
one or more cells in the cell culture medium.
21. The kit of claim 19, wherein the one or more cells are selected from the group of eggs, primary cells, neurons, sperm, synovial fibroblasts, and embryonic stem cells.

22. The kit of claim 21, wherein the one or more cells are one or more eggs.

23. The kit of claim 22, wherein the one or more eggs are unfertilized.

24. The kit of claim 22, wherein the one or more eggs are fertilized.

25. The kit of claim 22, wherein the one or more eggs are from a human, monkey, mouse, rat, guinea pig, hamster, horse, cow, sheep, pig, dog, or cat.

26. The kit of claim 25, wherein the one or more eggs are from a human.

27. The kit of claim 19, wherein the acid ceramidase is in protein form.

28. The kit of claim 19, wherein the acid ceramidase is in the form of a nucleic acid molecule encoding acid ceramidase.

29. A method of predicting in vitro fertilization outcome, said method comprising:
   providing a sample of serum or follicular fluid from a female subject;
   screening the sample for acid ceramidase activity level; and
   correlating the acid ceramidase activity level obtained through said screening to a prediction of the outcome of in vitro fertilization for the female subject.

30. The method of claim 29, wherein the subject is a human.
31. The method of claim 29, wherein said screening is conducted for acid ceramidase in protein form.

32. The method of claim 29, wherein said screening is conducted for acid ceramidase as a nucleic acid encoding acid ceramidase.

33. The method of claim 29, wherein the sample is serum.

34. The method of claim 29, wherein the sample is follicular fluid.
Figure 1

Figures 2A–F
Figure 3

Figures 4A–G

Figure 5
Figures 6A–B
Figure 7
Figures 9A–D
Figures 10A–D
Figure 11

Figure 12

Pearson correlation test = 0.0177
Figures 14A–D
Figure 16

Figure 17
Figure 18

Figure 19

Figures 20A–C