Gene Silencing Potential for Sterilization of Cats and Dogs

Scientific Think Tank
October 15-17, 2009
Denver. CO

Overview

On October 15-17, 2009, a think tank on the use of gene silencing approaches to sterilize cats and dogs was held at the Harrison Memorial Animal Hospital in Denver, Colorado. The meeting was convened by the Alliance for Contraception in Cats and Dogs (ACC&D), with support from the Found Animals Foundation (FAF) and the Animal Assistance Foundation (AAF), to discuss the potential of using RNAi or other gene silencing approaches in the pursuit of a non-surgical sterilant for cats and dogs.

The mission of the ACC&D is to expedite the successful introduction of non-surgical methods to sterilize cats and dogs and to support the distribution and promotion of these products. ACC&D's motivation is to reduce animal death and suffering worldwide by enhancing the tools available to humane population control programs. Non-surgical approaches are presumed to be less expensive and less labor-intensive options for sterilization, allowing far more animals to be treated quickly and safely.

For most markets, it is preferable for non-surgical contraceptive or sterilization treatments to suppress female estrus cycles, and hormone-based behaviors such as spraying for male cats and marking for male dogs. These are judged to be behavioral challenges to pet keeping and contribute to nuisance behavior in free-roaming animals. The veterinary field in many parts of the world also strongly supports surgical sterilization of female dogs and cats to reduce risks of mammary cancer and reproductive health issues such as pyometra and wants to retain that benefit in methods of birth control.[1]

Shirley Johnston of FAF provided an overview of the Michelson Grants in Reproductive Biology, which has pledged to fund up to $50 million in research in the pursuit of a "safe, effective, and practical non-surgical sterilant for use in cats and dogs." The Michelson Prize in Reproductive Biology will award $25 million to the first entity to provide a safe, effective and practical non-surgical sterilant that works in cats and dogs, both males and females. The Michelson Prize and Grants Program offers the most research funding ever available to spur research in this field, as funding for control of reproduction in cats and dogs has been generally unavailable from major comparative biomedical research funding agencies.

Gene silencing was chosen by the ACC&D board and scientific advisors as one of two research areas that present promise for achieving the goal of a non-surgical sterilant.

[1] ACC&D advisor Steve Zawistowski, PhD, CAAB, notes the complexity of the issues surrounding non-reproductive effects of surgical sterilization and hormone suppression on health and behavior. For more information, see the entire session *Non-reproductive Effects of Spaying and Neutering*, ACC&D's Third International Symposium Proceedings, 2006, or *Benchmarking Surgical Sterilization: A Review of the Safety, Efficacy, and Non-reproductive Effects of Surgical Sterilization, as a Benchmark for Assessing Non-surgical Approaches,* I. Reichler, ACC&D's Fourth International Symposium Proceedings, 2010.

As described by the moderator, Carl Johnson, the think tank would seek to:

1. Define what is known about the subject.
2. Determine what additional information is needed to understand how gene silencing could be applied to meet the goal.
3. Decide how best to obtain the needed information.

Joyce Briggs described the hoped-for outcomes of the think tank to include a general research agenda for this field of exploration. This agenda will be presented at the ACC&D Symposium to be held in April 2010.

Attendees

(See www.acc-d.org for bios of participants.)

Foundation and Nonprofit Representatives:

Joyce Briggs, MS	President, Alliance for Contraception in Cats and Dogs, Portland, Oregon
Shirley Johnston, DVM, PhD	Director of Scientific Research, Found Animals Foundation, Los Angeles, California
Kevin Morris, PhD	Director of Research, Animal Assistance Foundation, Denver, Colorado

Scientific Panel:

Note: Dr. Carl Johnson, executive director for science of the Hereditary Disease Foundation, moderated the think tank.

Greg Dissen, PhD	Staff Scientist, Oregon National Primate Research Center, Beaverton, Oregon
Norman Hecht, PhD	William Shippen, Jr., Professor of Human Reproduction, Center for Research on Reproduction and Women's Health, Department of Obstetrics and Gynecology, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania
John Herr, PhD	Professor of Cell Biology and Director of the Center for Research in Contraceptive and Reproductive Health, Department of Cell Biology, University of Virginia, Charlottesville, Virginia; CEO of ContraVac, Inc. (www.ContraVac.com)
Michelle Kutzler, DVM, PhD	Associate Professor of Theriogenology, Oregon State University College of Veterinary Medicine, Corvallis, Oregon
Dennis Lawler, DVM	Retired Veterinary Clinical and Research Scientist, St. Louis, Missouri
R. Michael Roberts, PhD	Curators' Professor of Animal Science and Biochemistry, University of Missouri, Columbia, Missouri
John Rossi, PhD	Chair and Professor of Molecular Biology, Dean of the City of Hope Graduate School of Biological Sciences, Duarte, California
Michael Skinner, PhD	Professor in the School of Molecular Biosciences and Director of the Center for Reproductive Biology, School of Molecular Biosciences, Washington State University, Pullman, Washington

Topics of Discussion

Sterility in dogs and cats could result from interfering with several basic processes, including:

- The release of master reproductive hormones in the hypothalamus and pituitary
- The production and/or maturation of germ cells
- Detection of pheromone signals that promote mating behaviors
- Fertilization
- Embryo implantation
- Trophoblast/embryo support

The bulk of the think tank discussion revolved around the first two processes, with focus on the various tissues and molecules that are important to the production of sperm and ova.

Overview of the tissues and molecules of interest

The tissues and cell types discussed as potential targets to cause sterilization include:

1. Hypothalamic neurons, responsible for the release of gonadotropin releasing hormone (GnRH)
2. Pituitary cells, which release the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH)
3. Gonads
    a. Female: Ovary
        i. Granulosa cells, which convert testosterone to estrogen
        ii. Theca cells, which secrete androgens (as well as progesterone) in response to LH
        iii. Germ cells
    b. Male: Testis
        i. Sertoli cells, which support gametogenesis and contain FSH receptors on their basal surface
        ii. Leydig cells, which secrete testosterone in response to LH
        iii. Differentiating germ cells
        iv. Stem cells

An important consideration is that the overarching goal is a permanent sterilant, as opposed to a contraceptive with limited duration of effect. Several of the potential target cell types (such as Leydig cells, and the population of primary spermatogonia) are regenerating, which is a challenge for an approach in which a single treatment is expected to have a permanent effect.

Genes that are potential targets include those required for the survival or function of cells important to reproduction, but also include genes that through an indirect or epigenetic effect can permanently interfere with the function of a reproductive tissue. Since RNAi is usually a transient phenomenon (the exception being a viral construct that can permanently integrate into the genome), the challenge is to arrive at a permanent change via a transient silencing event. This might be achieved, for example, through activation of the apoptosis pathway within a gonadal stem cell population, eliminating the entire ovarian or testicular germ cell reserve, or activating apoptosis within all of the granulosa or Sertoli nurse cells.

The discussion regarding potential targets is outlined below.[2]

Think tank participants, deep in discussion

[2] ACC&D scientific advisors note that this document is a summary of a single discussion at a point in time, and does not constitute a comprehensive review of the literature. Other targets are of interest, including hypothalamic peptides that control GnRH, such as kisspeptin and GnIH. (See *Endogenous GnRH Control: Kisspeptin and FnIF,* D. Fellmann, C. Pralong and P.Y. Risold, ACC&D Third International Symposium Proceedings, pp. 51-52, 2006.)

1. Hypothalamus and GnRH

Gonadotropin releasing hormone (GnRH) is an attractive target for sterilization because it is absolutely required for fertility in males and females, cats and dogs. GnRH is synthesized in the brain by neurons of the hypothalamus and secreted. Upon binding to receptors on the pituitary, GnRH stimulates the synthesis and release of the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH stimulates the maturation of ovarian follicles in females, and supports Sertoli cell function required for spermatogenesis in males. LH acts on Leydig cells in the testes, or theca cells in the ovary, stimulating secretion of testosterone (which is subsequently converted to estrogen in the female) and inducing ovulation and supporting corpora luteal function (essential during the latter half of pregnancy) in females.

Blocking GnRH function as an approach to sterilization and contraception has been pursued for decades, and potent GnRH agonist and antagonist drugs have been developed in long-acting formulations that cause what has been referred to as "medical castration" lasting for many months. The think tank panel discussed the successes and challenges of immunologic and pharmaceutical interference with GnRH signaling, with an eye to how this portends the targeting of this system via gene silencing.

Numerous vaccine approaches have been developed to suppress GnRH, and vaccination against GnRH is used in the regulation of some wildlife populations, and for the treatment of benign prostatic hypertrophy in dogs, and to suppress boar taint in pigs. However, for use as a contraceptive/sterilant, GnRH vaccines have generally suffered from a variable response among immunized animals, and the fact that the response is transient, multiple injections are often required, and the length of time until fertility returns is unpredictable.

Modulation of GnRH via gene silencing would require targeting the gene that codes for GnRH in the hypothalamus. Alternatively, the neuronal regulatory network, consisting of both positive and negative neurotransmitters, could be targeted. Difficulties in targeting hypothalamic neurons via RNAi include the protection provided by the blood brain barrier (though it was also mentioned that the GnRH neuron terminals are outside the blood brain barrier and should be accessible), and the fact that interfering with a more primary "upstream" function, such as hypothalamic neuron function, can result in more unintended consequences than a more focused approach on a single down-stream cell type (such as sperm). With respect to the latter concern, the evidence that GnRH agonists used in the treatment of human prostate cancer have been associated with cardiac side effects[3] led the panel to identify the need for further research characterizing distribution and activity of GnRH receptors in different tissues in the cat and dog, to understand the effects of chronically changing (whether increased or decreased) GnRH levels. Although use of GnRH agonists in dogs has been demonstrated to be safe when used as contraceptives and they are widely used in Europe, Australia and New Zealand, little is known regarding the effects of lifetime use. Surgical sterilization removes feedback inhibition of GnRH, so surgically sterilized cats and dogs provide a model for chronically high GnRH levels. Additionally, it was agreed that the long-term effects of GnRH therapies on tissues such as the heart should be studied.

The characterization of aged GnRH knockout mice, which simulate the lack of GnRH resulting from immune or gene silencing induced reduction of GnRH, was of interest in this regard. Also, looking at familial groupings having GnRH receptor mutations could be informative.

The subject of GnRH prompted discussion of a recurring theme throughout the think tank – the lack of very basic knowledge about the reproductive biology of the cat or dog.

2. Pituitary and LH, FSH

[3] Skinner, D.C., *et.al.* (2009) *J. Neuroendocrinology* 21: 282-292.

LH and FSH were considered more difficult targets, since receptors are present in multiple tissues (such as FSH receptors in the urinary tract), and the pituitary is difficult to target. However, the use of receptors for LH or FSH for targeting of gene silencing constructs was considered a possibility, if the constructs themselves contained specificity for the tissue of interest (a promoter to drive expression only in the desired tissue, for example, or a construct that suppressed the expression of a gene that was only required for survival of the cell type of interest) such that other unintended cell types would not be adversely affected.

3. Germ line

Several genes or RNA species specifically found in the germ line were discussed as possible targets for gene silencing.

Norman Hecht discussed a species of small RNAs, piwi-interacting RNAs (piRNA), which interact with the piwi protein, a member of the Argonaut family. The piRNAs are approximately 30 nucleotides in length and are present in large numbers in germ cells. The sequences of 17-20% of these match known transposon sequences, indicating that they may have a function in controlling transposition, but the other 80% have no known function. Knockouts of the piwi gene results in male sterility, but females are fertile, making it a potential target to induce male sterility, either by gene silencing of the piwi gene, or through inhibition of piwi protein function through use of locked nucleic acids.

Norman Hecht noted that MSY2 is a protein expressed only in male and female germ cells and that the homozygous knockout of the MSY2 gene results in sterility in both males and females. Silencing of the MSY2 gene expression might lead to sterilization.

Extensive data exist on gene expression in mouse germ and somatic cells, and gene expression appears to be well conserved between mouse and human, implying that the same genes may be expressed in cat and dog. This high level of conservation led the panel to propose that bioinformatics analyses should be carried out to identify a list of genes predicted to be expressed in the germ line of the cat or dog, facilitating target gene approaches. Discussion later expanded this idea to include bioinformatics of the entire axis from pituitary to gonad.

Many other potential targets are available that are specific to the male or female germ lines.

3a. Male germ line

Michael Skinner provided an overview of spermatogenesis, and presented an argument for targeting the Sertoli cells of the testes as an approach to male sterilization. Sertoli cells are accessible to the bloodstream through their basal surface, making them an easier target than the differentiating germ stem cells that are protected from the immune system within the adluminal compartment. However, spermatogonial cells are also theoretically accessible to systemically delivered drugs since they lie on the blood side of the blood-testis barrier in the basilar compartment below the Sertoli-Sertoli tight junctions. Sertoli cells are also post-mitotic, so a single effective treatment might eliminate them without the possibilities of replacement or regeneration. Since Sertoli cells are required for sperm development, their removal would result in infertility without the need to reach and eliminate all germ line or stem cells. The presence of the FSH receptor on the Sertoli cell basilar membrane provides a potential drug targeting mechanism through the blood stream.

Drs. Hecht and Herr

3b. Female germ line

The female germ line presents unique problems, in that permanent sterility would require that the primordial oocytes be targeted. The Dicer knockout mouse indicates that Dicer function is not essential for oogenesis, and therefore raises the issue that RNAi may not function in

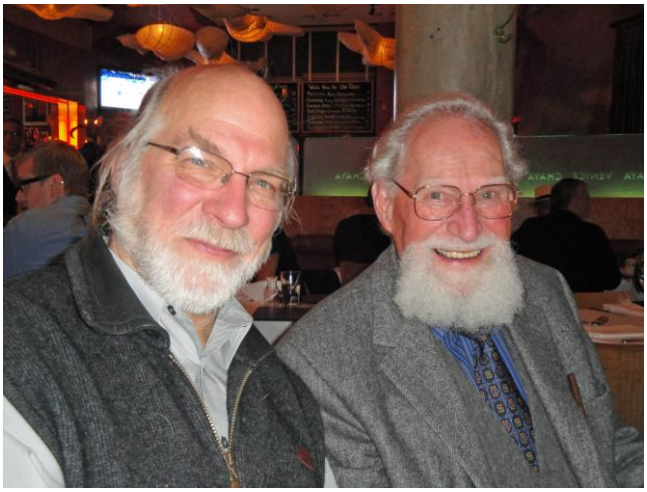
the oocyte. It is also possible that the machinery is there and could be used in a gene silencing approach, but determining if this is true will require future experimentation.

John Herr presented a general overview of the stages of follicle maturation, and it was noted that no specific markers are yet known for the primordial follicle, and identifying such targets would be an important goal of future research. Support cells, or oviductal cilia, were suggested as targets that might generate permanent sterility. Michelle Kutzler noted a paper[4] reporting unique aspects of oocyte maturation in dogs.

Overall, Michelle was concerned that extrapolating primarily from what we know about human and mouse biology will be inadequate to illuminate promising approaches in the cat and dog. Cat and dog reproductive biology are known to differ from other species in several ways. For example, the morphology of the dog oocyte differs from that of the mouse and human, where the former is rendered virtually opaque due to the accumulation of dark brown yolk granules whereas the latter oocytes present a much more transparent cytoplasm. Such morphological differences may point to targetable pathways unique to the dog oocyte. Unfortunately, there are gaps in knowledge about dog and cat oogenesis due to chronic lack of funding for basic research in cat and dog reproduction.

Because the oocyte might be difficult to target, alternative possible targets in the female include the uterine glandular epithelium, since disrupting its function would prevent embryonic development. Targeting the zona pellucida was discussed, as vaccination against ZP has been used to contracept horses and several wild species. Though studied for years in female dogs and cats, albeit with very limited funding, this approach has not been successful in dogs or cats due to issues that may be species specific. In female cats, vaccination of the queen against ZP has not resulted in immunocontraception, despite development of anti-SIZP antibodies. Since ZP protein is synthesized in the oocyte, it is important to determine whether RNAi functions in the oocyte if ZP is targeted.

[4] N. Songsasen and D.E. Wildt (2007) *Anim Reprod Sci*. March 98(1-2): 2–22.



Dr. Johnson, moderator, with Dr. Jöchle, ACC&D advisor

Gene silencing technologies

John Rossi presented an overview of gene silencing approaches. RNA interference (RNAi) results from the introduction of small interfering RNAs (siRNAs) into the cell. The siRNAs are short, generally 21 or 22 nucleotides long, double-stranded RNAs that when introduced into cells interfere with the translation of mRNAs containing complementary sequences. One strand of the siRNA is incorporated into a protein complex known as the RNA-induced silencing complex (RISC) and then the nuclease activity of the Argonaut component of RISC cleaves mRNAs containing complementary sequences. Techniques for biasing the choice of which of the two RNA strands in the double-stranded siRNA is incorporated into RISC have been developed.

Double-stranded RNAs longer than 21-22 nucleotides are processed by the nuclease Dicer into siRNAs. Dicer will preferentially choose one strand as the guide strand, making it possible to design DsiRNAs to minimize non-specific interactions.

Micro RNAs (miRNAs) are 21-23 nucleotide double-stranded RNAs that are generated by the sequential action of the nucleases Drosha and Dicer on larger transcripts, called pre-miRNAs, generated in the nucleus. One strand of the miRNAs is subsequently incorporated into the RISC complex and used to regulate gene expression by targeting mRNAs by mechanisms similar to siRNAs.

Additional agents used for gene silencing include a number of chemically modified oligonucleotides with nuclease resistant backbones (morpholinos, locked nucleic acids, peptide nucleic acids) that bind to complementary sequences in DNA and RNA and disrupt their transcription or translation. Antisense oligonucleotides can provide long-lasting effects since they are not degraded by nucleases. These oligos are transported to the nucleus and interfere directly with gene expression or splicing.

Delivery

Delivery of a gene silencing siRNA or antisense oligonucleotide to the desired tissue and cells is a significant challenge. To efficiently get a siRNA or oligonucleotide agent into a cell, it will need to be bound to or encapsulated within structures that can enter the cell either actively or passively. The resulting construct or formulation must be characterized with respect to its ability to reach and accumulate in the target tissue, and to deliver efficiently the silencing nucleotide to the correct subcellular compartment. Several possible delivery options were discussed:

1. Nanoparticles with surface ligands that direct a specific interaction with the tissue of interest. These are attractive because they can be created to contain multiple siRNAs per particle, allowing multiple genes to be targeted per cell. Nanoparticles are currently used to improve the uptake of poorly soluble anti-cancer drugs, and RNAi has been demonstrated in mice using chitosan nanoparticles to deliver siRNAs.

2. Antibodies conjugated to a positively charged nucleic acid binding protein, protamine, which binds the siRNA. When bound to the surface of the target cell, the complex is internalized via endocytosis. Theoretically, release from the endosome could be a limiting factor, but according to John Rossi, to date release from the endosome has not been problematic.

3. Aptamers, which are chimeric RNA molecules that can bind protein targets, are internalized via endocytosis, then processed by Dicer to release the siRNA from the aptamer. This approach is attractive because the chimera can be created in a modular way, with multiple siRNAs processed and released from a single RNA. Also, no other components such as proteins or lipids are required, allowing simpler formulation.

4. Cationic liposomes can be used to deliver siRNA to cells via endocytosis. Lipids can be used alone, or in combination with cholesterol or cationic nanoparticles to formulate a delivery vehicle. The addition of polyethylene glycol (PEG) can increase the longevity of the construct in circulation.

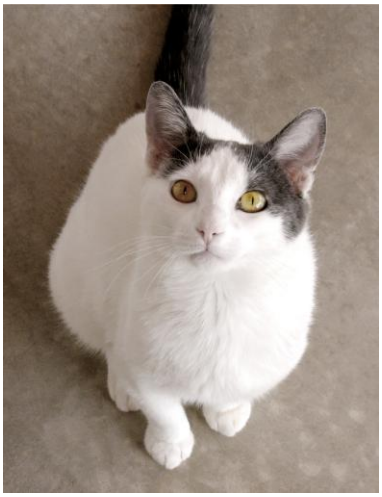
The panel discussed the involvement of toll-like receptors (TLRs) in uptake of double-stranded RNAs. When TLRs do recognize double-stranded nucleic acids, an undesirable interferon response is activated. To avoid this, John Rossi said incorporation of a 2' O-Methyl group into the siRNA would block activation of this response. The panel recommended research to

characterize the conservation of TLRs in cats and dogs.

5. Delivery using viruses is attractive because DNA constructs can be used that direct the desired siRNA to be synthesized within the infected cell. Cell-type specific promoters can be used to drive expression, adding another layer of specificity after the targeting of the virus to the cell of interest. Also, viruses can be created to deliver multiple siRNAs to target multiple genes. Attention must be paid to the promoter used to drive expression, since an overly strong promoter can result in unintended negative side effects. One option presented by John Rossi is the use of chimeric tRNA molecules that can contain multiple small hairpin RNAs that will be cleaved off inside the cell to generate siRNAs. The tRNA promoter is an appropriate strength for gene therapy, though it has the disadvantage of being constitutive,

requiring accurate targeting of the virus to the correct tissue or cell type to avoid expression in unintended tissues.

Several viruses have been used for administration of siRNA, lentivirus, adeno-associated virus (AAV), adenovirus (AV) and herpes virus. AAV has the advantage that infection can result in stable integration of the construct, allowing for permanent effects. Lentivirus was considered to be a more expensive system. Herpes viruses were mentioned to have the ability to infect the reproductive tract, but it was not known whether there exists a virus specific for the dog or cat reproductive tissues.



The panel noted that there is little to no information in the published literature regarding targeting of viruses or other delivery particles to the germ line in male or female animals. The testis in particular, since it is an immune privileged organ, might be expected to be difficult to target via a virus. Greg Dissen reported on recent studies finding that AAV9 can cross the blood brain barrier, and experiments using bacteriophage biopanning to identify epitopes that target the hypothalamus. The panel was very interested in the use of this approach to help in the development of delivery systems specifically targeted to the tissues of interest in the cat and dog.

Companies investigating gene therapies might discard constructs that do target the germ line out of a wish to avoid reproductive side effects. There was support for the idea to contact commercial entities carrying out such studies to find out if they would be willing to share information regarding constructs that were targeted to the germ line, providing a potential starting point for researchers.

The physical administration of the agent was also discussed, with present options being injection or possibly intranasal spray. If injected, attention would need to be placed on the ease of injection, and the possibility for irritation or adverse reaction at the site of injection. At this time, these therapies cannot be administered orally.

Efficacy

John Rossi noted that gene silencing would likely never reach 100% efficacy, though with optimization a gene may be silenced by as much as 95% in a population of cells, and silencing up to 99% has been reported in organs. The level of silencing that will be required for sterilization is not known, and will likely be dependent on the target.

The panel also discussed the possibility that some fraction of a population of cats or dogs might not respond to a single therapy due to genetic heterogeneity, and that this should be kept in mind when designing gene silencing approaches.

Practical considerations

Discussion touched on the following practical considerations of development of a gene silencing approach to sterilization.

To curtail population, Michael Roberts and Michelle Kutzler emphasized that in a polygynous species such as dog and cat, it is more important to sterilize the female than the male.

Regulatory approval of a gene silencing treatment targeting multiple genes (i.e., a treatment involving multiple RNA targets administered within one virus) may require approval of each of the components separately, followed by approval of the mixture.

Regulatory approval of gene silencing approaches for sterilization will likely be more difficult than for gene therapy approaches to human disease, because in the treatment of disease, there is a willingness to permit protocols that may provide relief to patients who are suffering. However, in the case of dog and cat sterilization, the treatment would be administered to otherwise healthy animals and thus face a higher barrier with respect to proof of safety.

John Herr recommended that FAF consider contacting one or more large pharmaceutical companies working in

this area in the near term, for insight and assistance in future scale-up and distribution.

Conclusions and Recommendations of the Panel

In conclusion, there was broad agreement among the panel that the following future outcomes would be desirable and as such comprise a suggested research agenda:

1. Better characterization of the basic physiology of cat and dog reproduction, including:
    a. Description of basic processes such as the timing of oocyte release
    b. Completion and/or better annotation of the cat and dog genome sequences
    c. Characterization of the genetic diversity within the cat and dog populations
    d. Description of the distribution and function of GnRH receptors in the cat/dog
    e. Characterization of toll-like receptor (TLR) distribution and function in cats/dogs, since these can impact the processing of RNA therapies

2. The creation of a resource of basic cat and dog molecular information and/or samples for interested researchers, including:
    a. Contracting with a biostatistician or bioinformatician to mine the existing data from human and mouse to identify genes specifically expressed in reproductive tissues, and to make this list publicly available
    b. Contracting with a company to prepare RNA libraries from cat and dog tissues, and to make these available to researchers to confirm that their gene of interest is preferentially expressed in reproductive tissues
    c. Similarly, contracting with a company to generate cDNA libraries from tissues of cat and dog, with an emphasis on the cat since few if any cDNA libraries appear to be available.

3. Better characterization of molecular aspects of the cat and dog, male and female germ cells, including:
    a. Identification of markers specific to important cell types, to aid in targeting treatments to these cells (for example, using the serial phage library infection approach)
    b. Identification of genes required for survival of these cell types, to aid in the elimination of these cells
    c. It is noted that the germ cells, being most different from the somatic cells of the body, might be the easiest target for gene therapy approaches

4. Proof-of-principle experiments using mice (such as the mouse model expressing green fluorescent protein, GFP, in the germ line) or other model systems to demonstrate that RNAi or similar therapies can:
    a. Be targeted to the cell types of interest, and/or
    b. Eliminate the targeted cells

5. Characterization of long-term effects of lack of GnRH by using mouse model.
    a. Note existing models are developmental knockouts – the creation of conditional knockouts in which GnRH was removed after completion of development would be attractive

6. Following the ACC&D international symposium in April 2010, there may be additional opportunity for a specially focused symposium to attract experts in various aspects of this research (e.g., gene therapy, stem cell biology) and to present to them what is known about cat and dog biology.

7. A call for proposals with the emphasis:
    a. That any feasible approach will be considered (from targeting the hypothalamus to germ cell to interference with implantation)
    b. That the resulting treatment must be permanent
    c. That the treatment must be safe for the animal, and not cause post-administration reactions

Prepared by Tamara Golden, PhD
Golden Tech Writing

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