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Applying gene silencing technology to contraception

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Contents

Population control of feral animals is often difficult, as it can be dangerous for the animals, labor intensive, and expensive. Therefore, a useful tool for control of animal populations would be a nonsurgical method to induce sterility. Our laboratories utilize methods aimed at targeting brain cells *in vivo* with vehicles that deliver a payload of either inhibitory RNAs or genes intended to correct cellular dysfunction. A useful framework for design of a new approach will be the combination of these methods with the intended goal to produce a technique that can be used to noninvasively sterilize cats and dogs. For this approach to succeed it has to meet several conditions: The target gene must be essential for fertility; the method must include a mechanism to effectively and specifically silence the gene of interest; the method of delivering the silencing agent must be minimally invasive, and finally, the silencing effect must be sustained for the lifespan of the target species, so that expansion of the population can be effectively prevented. In this article we discuss our work to develop gene silencing technology to induce sterility; we will use examples of our previous studies demonstrating that this approach is viable. These studies include: a) the use of viral vectors able to disrupt reproductive cyclicity when delivered to the regions of the brain involved in the control of reproduction, and b) experiments with viral vectors that are able to ameliorate neuronal disease when delivered systemically using a novel approach of gene therapy.

Keywords

RNA interference; Adeno-associated virus; microRNA

Introduction

An inexpensive noninvasive method to permanently sterilize dog and cats would be of great benefit to both humans and animals. In the case of feral populations of animals such a sterilant would provide a means of population control without euthanizing the adult animals. By reducing the population of feral animals, there would be less stress on the environment and the animals would hopefully suffer less from malnutrition and disease. Further, the chances of serving as a vector for human disease would also be reduced. In the case of pet

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Conflicts of Interest

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populations, an inexpensive sterilant would hopefully result in increased pet sterilization and a corresponding decrease in the number of unwanted pets, strays and abandoned dogs and cats which are often euthanized.

Reproductive success, in all mammals requires the pulsatile release of GnRH from the hypothalamus. Thus the control of GnRH release is a natural control point that can be exploited for the purpose of producing a sterilant. The seminal event underlying the initiation and progression of puberty is the increase in the pulsatile release of GnRH (Ojeda et al., 2010); once firmly established, GnRH pulsatility is central to the normalcy of reproductive cyclicity in females and fertility in both sexes. The GnRH neuronal network is mostly located in the medial basal hypothalamus in humans, monkeys, sheep, dogs and cats (Barry and Dubois, 1975; Belda et al., 2000; Heger et al., 2007; Dissen et al., 2012a). Neuronal (Kordon et al., 1994; Ojeda and Terasawa, 2002) and glial (Ojeda and Terasawa, 2002; Ojeda et al., 2003) inputs to GnRH neurons provide coordination to the network. Neuronal inputs act trans-synaptically to regulate GnRH secretion using either excitatory or inhibitory neurotransmitters/neuromodulators (Terasawa and Fernandez, 2001; Ojeda and Terasawa, 2002; Plant and Witchel, 2006), while the glial component is predominantly facilitatory, and is to a significant extent represented by growth factors that directly or indirectly stimulate GnRH secretion (Ojeda et al., 2003; Ojeda and Skinner, 2006; Lomniczi and Ojeda, 2009). The episodic release of GnRH is a mode of hormonal output that is essential for reproduction, and is driven by a subset of neurons located in the arcuate nucleus of the medial basal hypothalamus. These cells produce Kisspeptin, Neurokinin B (NKB), and Dynorphin (Wakabayashi et al., 2010; Navarro et al., 2011); because of this, they are known as KNDy neurons (Lehman et al., 2010; Navarro et al., 2011). The release of NKB from KNDy neurons stimulates kisspeptin release from the same type of neurons by a specific receptor mediated activation (Wakabayashi et al., 2010; Navarro et al., 2011). Key to this system is a phase-delayed inhibitory feedback of dynorphin on NKB release to produce an oscillatory release of NKB and kisspeptin (Wakabayashi et al., 2010; Navarro et al., 2011). Deletion of the gene or receptor for kisspeptin or NKB has shown that both are required for normal fertility (de Roux et al., 2003; Funes et al., 2003; Semple et al., 2005; d'Anglemont, X et al., 2007; Clarkson et al., 2008; Topaloglu et al., 2008; Silveira et al., 2010; Topaloglu et al., 2012). The absolute requirement of kisspeptin and NKB for fertility makes them targets for knockdown by RNA interference (RNAi) that would result in infertility and potentially sterility.

Our goal in this study is to develop a permanent method of sterility, by combining gene silencing with a gene therapy. The gene silencing technique will specifically target the gene of interest; while the gene therapy delivery system will target the specific brain region that is critical for fertility. Because the gene therapy vector is expected to remain active indefinitely, permanent sterility would be the outcome of a single systemic administration.

RNA interference induced gene silencing

The cellular mechanism known as RNAi results in post-transcriptional gene regulation in both plants and animals; the most basic unit of the RNAi system is the microRNA (miRNA) (Krol et al., 2010). In order to harness the RNAi system, artificial miRNAs known as small interfering RNAs (siRNAs) have been developed (Provost et al., 2002; Cullen, 2005). Natural miRNAs and siRNAs are similarly short double stranded RNAs (~22 nt) (Cullen, 2005). The miRNA is synthesized as part of a structure known as a primary miRNA containing stem-loop regions. The primary miRNA is processed by the enzyme Drosha, an RNase III, releasing a stem-loop pre-miRNA structure (Lee et al., 2003). A RNA molecule known as the short hairpin RNA (shRNA) is the artificial version of the stem-loop pre-miRNA. A second RNase III enzyme known as Dicer acts on both the pre-miRNA and the

shRNA to release the natural miRNA or the artificial siRNA (Lee et al., 2003; Gregory et al., 2004). The natural miRNA and the artificial siRNA enter the cellular machinery in an identical manner. The antisense or guide strand of the miRNA is complementary to the mRNA and is incorporated into a ribonucleoprotein RNA-induced silencing complex (RISC) (Khvorova et al., 2003; Schwarz et al., 2003; Cullen, 2005). The degree of complementarity of the miRNA or siRNA to the target mRNA determines the subsequent processing pathway. High complementarity results in the cleavage of the mRNA and suppression of transcription and low complementarity and imperfect binding leads to translational repression and mRNA destabilization (Guo et al., 2010; Boudreau et al., 2011). This type of interaction often occurs in the 3'-untranslated region (UTR); it is thought that pairing with as few as 6–7 nucleotides of the miRNA is sufficient to achieve silencing (Lewis et al., 2005; Boudreau et al., 2011). As the scientific community learns more about how miRNAs are naturally produced and how they achieve gene silencing, miRNA mediated RNAi is becoming a more useful tool to silence virtually any gene for therapeutic purposes.

Using RNAi to disrupt fertility

Suppression of a gene known as Enhanced at Puberty 1 (*EAP1*) using RNAi demonstrated that EAP1 is required for normal cyclicity in both rats and non-human primates, and simultaneously provided evidence that RNAi can be exploited to disrupt fertility. In female rats a region-specific decrease in EAP1 expression, induced by RNAi, delayed puberty and disrupted adult reproductive cyclicity (Heger et al., 2007). The shRNA complementary to *Eap1* mRNA was inserted in the 3'-long terminal repeat of a lentiviral (LV) vector. The shRNA-LV vector was administered to juvenile 23-day-old female rats by bilaterally microinjections into the preoptic area (POA) (Heger et al., 2007). In rats, most of the GnRH neurons involved in the hypothalamic control of gonadotropin secretion by the pituitary are located in the POA-anteroventral periventricular nucleus (AVPV) and an intact AVPV is required for the recurrent surges of gonadotropin released during the rat estrous cycle (Simerly, 2002; Herbison, 2006). LV particles devoid of inhibitory shRNAs and containing an enhanced green fluorescent protein (eGFP)-reporter gene were injected into control animals (Heger et al., 2007). Cells located along the lateral borders of the AVPV were transduced and exhibited expression of eGFP. The morphological appearance of infected cells was that of neurons, and some of the infected cells were identified by immunohistochemistry as GnRH neurons. The shRNA was shown to be effective in suppressing *Eap1* expression by the reduced content of EAP1 immunoreactive material observed in infected cells vs. non-infected cells.

In rats in which expression of the *Eap1* gene was knocked-down by shRNA in the POA, the time of puberty (defined by the time of first ovulation) was delayed as compared to LV eGFP-injected rats (Heger et al., 2007). Rats also exhibited a disrupted estrous cycle following *Eap1* gene knock-down including prolonged episodes in estrus, and reduced plasma LH, FSH and estradiol levels.

Using non-human primates, a species closer to humans, EAP1 was also shown to be required for reproductive cyclicity. Improvements in RNAi technology allowed the production of an artificial primary-miRNA, which permitted a more natural processing of the inhibitory RNA expressed from the LV construct. Because the LV construct employed contains the reporter gene eGFP, the cells in the monkey hypothalamus that had been transduced were identified by eGFP immunohistofluorescence (Dissen et al., 2012a). Throughout the medial basal hypothalamus-arcuate nucleus region cells positive for eGFP were found in animals receiving correctly-placed injections. There were no signs of an inflammatory reaction in the transduced area, as the distribution of cell populations

identified by Hoechst staining of cell nuclei was normal and astrogliosis was absent (Dissen et al., 2012a). Similar to the observation in the rat, cells infected with LV particles carrying RNAi had a lower content of EAP1 immunoreactive material than non-infected cells.

Normal reproductive cycling monkeys were used for this study. Animals that received the control miRNA LV particles continued to cycle following the injections. Menstrual cyclicity was completely arrested in monkeys that received LV particles containing the EAP1 miRNA, within the target area, (Dissen et al., 2012a). The importance of transducing the correct region of the brain was demonstrated by the finding that animals in which the microinjections were misplaced continued to cycle after the injection.

The studies described above clearly demonstrate that RNAi is capable of disrupting female reproductive cyclicity. However, the LV vector delivering RNAi had to be directly targeted to the intracerebral location of interest to ensure effectiveness. This delivery method is not only undesirable, but also impractical when the goal is to induce infertility in dogs and cats. Because one of our groups succeeded in targeting genes to the brain vasculature using a novel gene delivery method (see below), our laboratories have joined efforts to devise an approach that can be effectively used to overcome this major limitation of gene delivery.

Novel RNAi delivery vehicle

The aforementioned studies might suggest that *EAP1* would be a good target for RNAi-mediated silencing of reproduction, however, *EAP1* expression is widespread throughout the body (Su et al., 2004) (database accessed via <http://en.wikipedia.org>), thereby limiting the specificity of a systemic approach to gene silencing. The target gene for RNAi mediated suppression of fertility should be essential for fertility, exhibit tissue-specific expression, and not be required for processes other than reproduction. A gene that meets these criteria is the *Kiss1* gene (de Roux et al., 2003; Funes et al., 2003; Semple et al., 2005; Clarkson et al., 2008; Topaloglu et al., 2012). *Kiss1* has a limited tissue distribution, including the placenta (Su et al., 2004) (database accessed via <http://en.wikipedia.org>), the hypothalamus (Gottsch et al., 2004; Clarkson et al., 2009) and the ovary (Castellano et al., 2006; Gaytan et al., 2009). Confirmation that *Kiss1* is an ideal target candidate comes from studies of mice lacking *Kiss1*, which are infertile and yet exhibit no other gross abnormalities (d'Anglemont, X et al., 2007).

For permanent RNAi-based, viral-mediated gene therapy incorporation of the viral genome into the host genome, as is the case of LV vectors, is advantageous. However, there is also a risk of genomic alterations that could lead to cancer or other gene mutations. A vector that does not incorporate into the host genome, such as the recombinant adeno-associated virus (AAV) vector would be a better choice for an RNAi mediated sterilitant. Recombinant AAV differs from its wild-type counterparts in that the viral genome takes up residence in the cell nucleus as a double stranded episome which does not insert into the host genome. In spite of existing as an episome, the AAV genome can drive transgene expression for long periods of time. Transgenes from AAV vectors can be detected in many different tissues of rodents throughout the lifespan of the animal (Kaplitt et al., 1994; McCown et al., 1996; Xiao et al., 1996); in nonhuman primates, they have been detected for as long as 10 years (Dissen et al., 2012b). Another factor that has contributed to the acceptance of the AAV vector for gene therapy is that the vector is derived from a non-pathogenic virus (Mueller and Flotte, 2008; Mingozzi and High, 2011).

An important feature of the recombinant AAV is that it can be administered in a non-invasive manner such as intravenous injections. Systemically administration of AAV is minimally invasive, but there are no known serotypes that target the brain selectively. Serotypes are defined as isolates of AAV that do not exhibit cross-reaction with neutralizing

sera from any other known serotype (Wu et al., 2006). Importantly, AAV serotypes have been found to exhibit unique tropism for different tissues throughout the body. The various tropisms are achieved by the use of different cell surface receptors to gain entry into the host cell (Wu et al., 2006). The most extensively studied serotype is AAV2; hundreds of publications have detailed both pre-clinical and clinical uses of AAV2 to deliver transgenes to the liver (the primary target for AAV2) and various other tissues throughout the body (Mueller and Flotte, 2008). Unfortunately, very little of the virus crosses the blood brain barrier and enters the brain. The AAV2 vector is targeted to specific cell types, via a heparin sulfate proteoglycan (HSPG) binding domain on the surface of the AAV capsid protein (Bartlett et al., 2000).

Modification of the AAV capsid to target the brain

The tropism of AAV2 can be redirected by targeting the HSPG binding domain of the capsid protein. This modification has been achieved by inserting a peptide sequence at the arginine 588 of the capsid protein (Muller et al., 2003). The new tropism of the virus is dependent on the peptide sequence inserted. A technique termed biopanning has shown great promise for selecting such peptide sequences (Work et al., 2002). The essence of biopanning is that a random peptide library is displayed on the binding domain of a bacteriophage; this phage-display library is injected into the vasculature of the target species, then the tissues of interest are collected and the adherent phage is extracted (Work et al., 2006; Chen et al., 2009). The biopanning technique was used by one of our groups to select an epitope that directs the binding of AAV2 to the vasculature of the brain (Chen et al., 2009). The virus carrying this epitope showed targeting of the brain several orders of magnitude greater than the wild-type virus, and a viral reporter gene was detected in cells positive for the neuronal marker NeuN (Chen et al., 2009). The colocalization of the reporter gene, with the NeuN marker suggests that the virus not only targeted the brain vasculature but also entered and transduced the underlying neuropil.

Generally the insertion of a peptide at arginine 588 alters the capsid receptor binding characteristics without compromising virus viability (Muller et al., 2003; Perabo et al., 2006; Work et al., 2006). In previous studies the entire brain was used for the biopanning experiment resulting in several candidate peptides that were used for targeting the virus to the brain (Chen et al., 2009). In current studies, our groups used biopanning to identify a peptide epitope able to redirect the tropism of AAV2 to the medial basal hypothalamus, the region where neurons expressing *Kiss1* are located. This region of the brain is more accessible to systemically delivered molecules via the vasculature because it does not appear to be isolated by a fully functional blood brain barrier, a feature that allows significant transfer of macromolecules from the bloodstream to the hypothalamic parenchyma (Broadwell and Brightman, 1976; Herde et al., 2011).

Improved design of siRNAs to enhance specificity

The ability of siRNAs to specifically suppress expression of target mRNA determines the effectiveness of RNAi as a gene silencing tool. A negative feature of siRNAs is the suppression of unintended mRNAs, an effect known as off-target silencing (Chi et al., 2003; Jackson et al., 2003; Semizarov et al., 2003). This type of silencing likely occurs when the region of the siRNA known as the “seed” (nucleotides 2–8 of the antisense strand) specifically pairs with the 3′-UTR sequences of mRNAs other than the intended target and results in destabilization or repression of those transcripts (Birmingham et al., 2006; Anderson et al., 2008). Off-target silencing has even been associated with, and thought to result in, toxic phenotypes (Fedorov et al., 2006). The magnitude of siRNA off-targeting has been shown to be directly related to the frequency of hexamer seed complements present

in the 3'-UTR transcriptome (Boudreau et al., 2011). As a result of these observations, an siRNA design scheme has been developed to prioritize seed specificity (known as safe-seeds) as a means to improve the safety profile of therapeutic RNAi sequences. The safe-seeds method of sequence selection proved successful in identifying siRNAs which silenced target gene expression, induced minimal seed-related off-targeting and were well-tolerated in mice (Boudreau et al., 2011). We have now used the safe-seeds method to design siRNAs against *Kiss1*. In ongoing studies we are determining if one of these siRNAs can be delivered by a peptide-modified AAV2 vector to the hypothalamus and interfere with fertility in rats.

Concluding Remarks

Proof-of-principle that RNAi can be used to cause sterility has been provided by studies in which RNAi targeted against a gene involved in the control of reproduction was delivered to the hypothalamus via intracerebral administration of an miRNA encoding LV vector. This procedure disrupted reproductive cyclicity in both female rats and nonhuman primates. Because in nonhuman primates ovulation was blocked, silencing of this single gene appears to cause a state of infertility. Current efforts are aimed at devising a modified AAV2 vector to selectively, or even specifically, target the hypothalamus upon systemic administration. We envision that this method will provide the means to silence genes essential for reproduction in a non-invasive, effective and sustained manner in dogs and cats. Figure 1 is a schematic representation/summary of this experimental plan. We also anticipate that these studies will provide the basis for new delivery strategies to the brain for basic research purposes and emerging therapies.

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References

- Anderson EM, Birmingham A, Baskerville S, Reynolds A, Maksimova E, Leake D, Fedorov Y, Karpilow J, Khvorova A. Experimental validation of the importance of seed complement frequency to siRNA specificity. *RNA*. 2008; 14:853–861. [PubMed: 18367722]
- Barry J, Dubois MP. Immunofluorescence study of LRF-producing neurons in the cat and the dog. *Neuroendocrinology*. 1975; 18:290–298. [PubMed: 765883]
- Bartlett JS, Wilcher R, Samulski RJ. Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *J Virol*. 2000; 74:2777–2785. [PubMed: 10684294]
- Belda M, Covenas R, Narvaez JA, Aguirre JA, Tramu G. Distribution of luteinizing hormone-releasing hormone in the upper brainstem and diencephalon of the cat: an immunocytochemical study. *Brain Res Bull*. 2000; 51:281–291. [PubMed: 10704778]
- Birmingham A, Anderson EM, Reynolds A, Ilsley-Tyree D, Leake D, Fedorov Y, Baskerville S, Maksimova E, Robinson K, Karpilow J, Marshall WS, Khvorova A. 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat Methods*. 2006; 3:199–204. [PubMed: 16489337]
- Boudreau RL, Spengler RM, Davidson BL. Rational Design of Therapeutic siRNAs: Minimizing Off-targeting Potential to Improve the Safety of RNAi Therapy for Huntington's Disease. *Mol Ther*. 2011; 19:2169–2177. [PubMed: 21952166]
- Broadwell RD, Brightman MW. Entry of peroxidase into neurons of the central and peripheral nervous systems from extracerebral and cerebral blood. *J Comp Neurol*. 1976; 166:257–283. [PubMed: 57126]

- Castellano JM, Gaytan M, Roa J, Vigo E, Navarro VM, Bellido C, Dieguez C, Aguilar E, Sanchez-Criado JE, Pellicer A, Pinilla L, Gaytan F, Tena-Sempere M. Expression of KiSS-1 in rat ovary: putative local regulator of ovulation? *Endocrinology*. 2006; 147:4852–4862. [PubMed: 16825322]
- Chen YH, Chang M, Davidson BL. Molecular signatures of disease brain endothelia provide new sites for CNS-directed enzyme therapy. *Nat Med*. 2009; 15:1215–1218. [PubMed: 19749771]
- Chi JT, Chang HY, Wang NN, Chang DS, Dunphy N, Brown PO. Genomewide view of gene silencing by small interfering RNAs. *Proc Natl Acad Sci USA*. 2003; 100:6343–6346. [PubMed: 12730368]
- Clarkson J, d'Anglemont dTX, Colledge WH, Caraty A, Herbison AE. Distribution of kisspeptin neurones in the adult female mouse brain. *J Neuroendocrinol*. 2009; 21:673–682. [PubMed: 19515163]
- Clarkson J, d'Anglemont dTX, Moreno AS, Colledge WH, Herbison AE. Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. *J Neurosci*. 2008; 28:8691–8697. [PubMed: 18753370]
- Cullen BR. RNAi the natural way. *Nat Genet*. 2005; 37:1163–1165. [PubMed: 16254559]
- d'Anglemont, dTX.; Fagg, LA.; Dixon, JP.; Day, K.; Leitch, HG.; Hendrick, AG.; Zahn, D.; Franceschini, I.; Caraty, A.; Carlton, MB.; Aparicio, SA.; Colledge, WH. Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene. *Proc Natl Acad Sci USA*. 2007; 104:10714–10719. [PubMed: 17563351]
- de Roux N, Genin E, Carel J-C, Matsuda F, Chaussain J-L, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci USA*. 2003; 100:10972–10976. [PubMed: 12944565]
- Dissen GA, Lomniczi A, Heger S, Neff TL, Ojeda SR. Hypothalamic enhanced at puberty 1 (EAP1) is required for menstrual cyclicity in non-human primates. *Endocrinology*. 2012a; 153:350–361. [PubMed: 22128022]
- Dissen, GA.; McBride, JL.; Lomniczi, A.; Matagne, V.; Dorfman, M.; Neff, TL.; Galimi, F.; Ojeda, SR. Using lentiviral vectors as delivery vehicles for gene therapy. In: Morozov, A., editor. *Controlled Genetic Manipulations*. Humana Press; Bethesda MD: 2012b. p. 69-96. Springer Science + Business Media in their Neuromethods series formerly
- Fedorov Y, Anderson EM, Birmingham A, Reynolds A, Karpilow J, Robinson K, Leake D, Marshall WS, Khvorova A. Off-target effects by siRNA can induce toxic phenotype. *RNA*. 2006; 12:1188–1196. [PubMed: 16682561]
- Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, Yang S, Monsma FJ, Gustafson EL. The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. *Biochem Biophys Res Commun*. 2003; 312:1357–1363. [PubMed: 14652023]
- Gaytan F, Gaytan M, Castellano JM, Romero M, Roa J, Aparicio B, Garrido N, Sanchez-Criado JE, Millar RP, Pellicer A, Fraser HM, Tena-Sempere M. KiSS-1 in the mammalian ovary: distribution of kisspeptin in human and marmoset and alterations in KiSS-1 mRNA levels in a rat model of ovulatory dysfunction. *Am J Physiol Endocrinol Metab*. 2009; 296:E520–E531. [PubMed: 19141682]
- Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology*. 2004; 145:4073–4077. [PubMed: 15217982]
- Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R. The Microprocessor complex mediates the genesis of microRNAs. *Nature*. 2004; 432:235–240. [PubMed: 15531877]
- Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*. 2010; 466:835–840. [PubMed: 20703300]
- Heger S, Mastronardi C, Dissen GA, Lomniczi A, Cabrera R, Roth CL, Jung H, Galimi F, Sippell W, Ojeda SR. Enhanced at puberty 1 (EAP1) is a new transcriptional regulator of the female neuroendocrine reproductive axis. *J Clin Invest*. 2007; 117:2145–2154. [PubMed: 17627301]
- Herbison, AE. Physiology of the gonadotropin-releasing hormone neuronal network. In: Neill, JD., editor. *Physiology of Reproduction*. 3. Academic Press/Elsevier; San Diego: 2006. p. 1415-1482.

- Herde MK, Geist K, Campbell RE, Herbison AE. Gonadotropin-releasing hormone neurons extend complex highly branched dendritic trees outside the blood-brain barrier. *Endocrinology*. 2011; 152:3832–3841. [PubMed: 21791557]
- Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS. Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol*. 2003; 21:635–637. [PubMed: 12754523]
- Kaplitt MG, Leone P, Samulski RJ, Xiao X, Pfaff DW, O'Malley KL, During MJ. Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. *Nat Genet*. 1994; 8:148–154. [PubMed: 7842013]
- Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. *Cell*. 2003; 115:209–216. [PubMed: 14567918]
- Kordon, C.; Drouva, SV.; Martínez de la Escalera, G.; Weiner, RI. Role of classic and peptide neuromediators in the neuroendocrine regulation of luteinizing hormone and prolactin. In: Knobil, E.; Neill, JD., editors. *The Physiology of Reproduction*. 2. Vol. 1. Raven Press; New York: 1994. p. 1621-1681.
- Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet*. 2010; 11:597–610. [PubMed: 20661255]
- Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN. The nuclear RNase III Drosha initiates microRNA processing. *Nature*. 2003; 425:415–419. [PubMed: 14508493]
- Lehman MN, Coolen LM, Goodman RL. Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology*. 2010; 151:3479–3489. [PubMed: 20501670]
- Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005; 120:15–20. [PubMed: 15652477]
- Lomniczi A, Ojeda SR. A role for glial cells of the neuroendocrine brain in the central control of female sexual development. In: Parpura, V.; Haydon, P., editors. *Astrocytes in (Patho)Physiology of the Nervous System*. Springer; NY: 2009. p. 487-511.
- McCown TJ, Xiao X, Li J, Breese GR, Samulski RJ. Differential and persistent expression patterns of CNS gene transfer by an adeno-associated virus (AAV) vector. *Brain Res*. 1996; 713:99–107. [PubMed: 8724980]
- Mingozzi F, High KA. Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. *Nat Rev Genet*. 2011; 12:341–355. [PubMed: 21499295]
- Mueller C, Flotte TR. Clinical gene therapy using recombinant adeno-associated virus vectors. *Gene Ther*. 2008; 15:858–863. [PubMed: 18418415]
- Muller OJ, Kaul F, Weitzman MD, Pasqualini R, Arap W, Kleinschmidt JA, Trepel M. Random peptide libraries displayed on adeno-associated virus to select for targeted gene therapy vectors. *Nat Biotechnol*. 2003; 21:1040–1046. [PubMed: 12897791]
- Navarro VM, Gottsch ML, Wu M, Garcia-Galiano D, Hobbs SJ, Bosch MA, Pinilla L, Clifton DK, Dearth A, Ronnekleiv OK, Braun RE, Palmiter RD, Tena-Sempere M, Alreja M, Steiner RA. Regulation of NKB Pathways and Their Roles in the Control of Kiss1 Neurons in the Arcuate Nucleus of the Male Mouse. *Endocrinology*. 2011; 152:4265–4275. [PubMed: 21914775]
- Ojeda SR, Lomniczi A, Loche A, Matagne V, Kaidar G, Sandau US, Dissen GA. The transcriptional control of female puberty. *Brain Res*. 2010; 1364:164–174. [PubMed: 20851111]
- Ojeda SR, Prevot V, Heger S, Lomniczi A, Dziedzic B, Mungenast A. Glia-to neuron signaling and the neuroendocrine control of female puberty. *Ann Med*. 2003; 35:244–255. [PubMed: 12846266]
- Ojeda, SR.; Skinner, MK. Puberty in the rat. In: Neill, JD., editor. *The Physiology of Reproduction*. 3. Academic Press/Elsevier; San Diego: 2006. p. 2061-2126.
- Ojeda, SR.; Terasawa, E. Neuroendocrine regulation of puberty. In: Pfaff, D.; Arnold, A.; Etgen, A.; Fahrbach, S.; Moss, R.; Rubin, R., editors. *Hormones, Brain and Behavior*. Vol. 4. Elsevier; New York: 2002. p. 589-659.
- Perabo L, Goldnau D, White K, Endell J, Boucas J, Humme S, Work LM, Janicki H, Hallek M, Baker AH, Buning H. Heparan sulfate proteoglycan binding properties of adeno-associated virus

- retargeting mutants and consequences for their in vivo tropism. *J Virol.* 2006; 80:7265–7269. [PubMed: 16809332]
- Plant, TM.; Witchel, SF. Puberty in nonhuman primates and humans. In: Neill, JD., editor. *The Physiology of Reproduction*. 3. Academic Press/Elsevier; San Diego: 2006. p. 2177–2230.
- Provost P, Dishart D, Doucet J, Friendewey D, Samuelsson B, Radmark O. Ribonuclease activity and RNA binding of recombinant human Dicer. *EMBO J.* 2002; 21:5864–5874. [PubMed: 12411504]
- Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. *Cell.* 2003; 115:199–208. [PubMed: 14567917]
- Semizarov D, Frost L, Sarthy A, Kroeger P, Halbert DN, Fesik SW. Specificity of short interfering RNA determined through gene expression signatures. *Proc Natl Acad Sci USA.* 2003; 100:6347–6352. [PubMed: 12746500]
- Semple RK, Achermann JC, Ellery J, Farooqi IS, Karet FE, Stanhope RG, O’Rahilly S, Aparicio SA. Two novel missense mutations in G protein-coupled receptor 54 in a patient with hypogonadotropic hypogonadism. *J Clin Endocrinol Metab.* 2005; 90:1849–1855. [PubMed: 15598687]
- Silveira LG, Tusset C, Latronico AC. Impact of mutations in kisspeptin and neurokinin B signaling pathways on human reproduction. *Brain Res.* 2010; 1364:72–80. [PubMed: 20816945]
- Simerly RB. Wired for reproduction: Organization and development of sexually dimorphic circuits in the mammalian forebrain. *Annu Rev Neurosci.* 2002; 25:507–536. [PubMed: 12052919]
- Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, Zhang J, Soden R, Hayakawa M, Kreiman G, Cooke MP, Walker JR, Hogenesch JB. A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc Natl Acad Sci USA.* 2004; 101:6062–6067. [PubMed: 15075390]
- Terasawa E, Fernandez DL. Neurobiological mechanisms of the onset of puberty in primates. *Endocr Rev.* 2001; 22:111–151. [PubMed: 11159818]
- Topaloglu AK, Reimann F, Guclu M, Yalin AS, Kotan LD, Porter KM, Serin A, Mungan NO, Cook JR, Ozbek MN, Imamoglu S, Akalin NS, Yuksel B, O’Rahilly S, Semple RK. TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. *Nat Genet.* 2008; 41:354–358. [PubMed: 19079066]
- Topaloglu AK, Tello JA, Kotan LD, Ozbek MN, Yilmaz MB, Erdogan S, Gurbuz F, Temiz F, Millar RP, Yuksel B. Inactivating KISS1 mutation and hypogonadotropic hypogonadism. *N Engl J Med.* 2012; 366:629–635. [PubMed: 22335740]
- Wakabayashi Y, Nakada T, Murata K, Ohkura S, Mogi K, Navarro VM, Clifton DK, Mori Y, Tsukamura H, Maeda K, Steiner RA, Okamura H. Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. *J Neurosci.* 2010; 30:3124–3132. [PubMed: 20181609]
- Work LM, Buning H, Hunt E, Nicklin SA, Denby L, Britton N, Leike K, Odenthal M, Drebber U, Hallek M, Baker AH. Vascular bed-targeted in vivo gene delivery using tropism-modified adeno-associated viruses. *Mol Ther.* 2006; 13:683–693. [PubMed: 16387552]
- Work LM, Nicklin SA, White SJ, Baker AH. Use of phage display to identify novel peptides for targeted gene therapy. *Methods Enzymol.* 2002; 346:157–176. [PubMed: 11883067]
- Wu Z, Asokan A, Samulski RJ. Adeno-associated virus serotypes: vector toolkit for human gene therapy. *Mol Ther.* 2006; 14:316–327. [PubMed: 16824801]
- Xiao X, Li J, Samulski RJ. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J Virol.* 1996; 70:8098–8108. [PubMed: 8892935]

Figure 1.

Figure Schematic diagram of the research plan summarized in the article. The goal is to use a peptide-modified AAV vector to suppress fertility in cats and dogs. A bacteriophage library is administered by intravenous systemic injection (1) to adult females. The bacteriophage that homes to the hypothalamus is isolated, purified and administered to naïve animals (3). This procedure (termed bio-panning) is repeated until convergence of a single peptide sequence is observed. The DNA coding for the peptide sequence is inserted (4) into the DNA sequence encoding the capsid protein for the AAV vector. An siRNA/miRNA sequence that is the most effective at suppressing expression of the target mRNA in vitro is selected (5), and inserted into the genome of the AAV vector (6). The peptide-modified siRNA/miRNA AAV vector is then administered intravenously (7) to the target species; either cats or dogs. While untreated animals will continue to produce offspring normally, GnRH is reduced in the treated animals resulting in infertility.