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Vectored gene delivery for lifetime animal contraception: Overview and hurdles to implementation

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ABSTRACT

There is a need for permanent, non-surgical methods of contraception for many animal species. Here we discuss the hypothesis that transgene-mediated expression of fertility inhibiting molecules such as monoclonal antibodies, ligands for cell surface receptors, receptor decoys, or small RNAs can provide such a method, which we term vectored contraception. We outline the technologies involved, progress made, and discuss challenges to implementation.

1. Introduction

Permanent methods of bringing about infertility in animals include surgery, and intra-testicular injection of zinc gluconate or calcium chloride [1]. Surgery is the only method available for females. Given the high cost and time required for surgery and recovery, and the large unmet need for fertility control in captive and free roaming animals, there is a need for permanent non-surgical methods of contraception that are fast and cost-effective [2–6]. In particular, there has long been interest in contraceptive alternatives to surgery that involve only a single encounter with the animal, ideally in the form of an injection [7,8]. Here we discuss a strategy - vectored contraception (VC) - that has the potential to achieve this goal. In brief, we argue that methods of gene delivery involving a single injection can be used to drive expression and secretion into the circulation of monoclonal antibodies or other proteins that inhibit fertility through interaction with specific membrane bound or extracellular proteins. Gene delivery that results in the cell type-specific expression of small RNAs that silence the expression of mRNAs that encode these or other proteins provides a complementary approach to the same end. Several proof-of-principal experiments in mice demonstrate the potential for this approach and highlight some outstanding issues [9,10]. Below we provide an overview of VC and the challenges that must be overcome. Target populations of interest include pets (owned cats and dogs), community or feral cats and dogs, production animals (pigs, cattle poultry), wildlife (deer, wild horses), and zoo animals.

1.1. Reproduction targets

Reproduction requires multiple hormones, gamete production, follicle maturation, sperm maturation, sperm motility and activation, as well as ovulation, fusion of sperm and egg, and embryo implantation and development. Molecules required for each of these processes are possible points at which fertility can be inhibited (Fig. 1). Many molecules required for these processes are located in the extracellular space or at the cell surface, and can therefore interact with antibodies or other proteins secreted into the general circulation in vivo. Other proteins, such as the components of intracellular signaling cascades and mRNAs that encode intracellular, transmembrane and secreted proteins can in principal be targeted for silencing through expression of small RNAs such as

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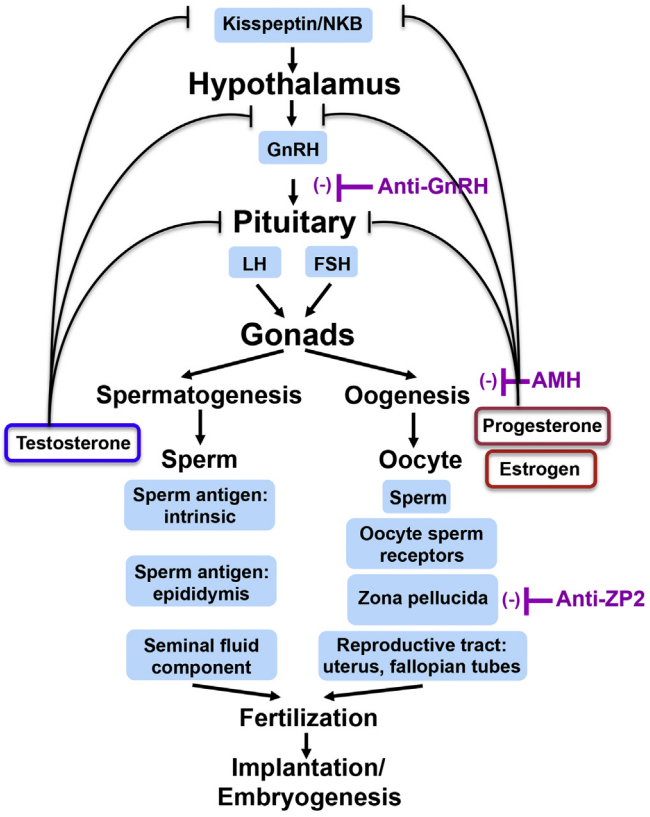

Fig. 1. Some potential targets of interest for vectored contraception. Some, but not all, points at which antibodies, ligands, or small RNAs could interfere with fertility are indicated. Proteins or structures of particular interest are indicated in the blue boxes. Receptors for GnRH, FSH, and LH, located on target tissues are also of interest, but are not indicated. Negative feedback pathways mediated by steroid hormones are complex. They are indicated in simplified form by the black lines with a bar on the end. Antibodies and molecules targeted by Li et al. [9] and Kano et al. [10] are indicated in purple. Many targets of interest, including molecules required for embryo implantation and development, are untested. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microRNAs (miRNAs) or small interfering RNAs (siRNAs). Several targets, gonadotropin hormone releasing hormone (GnRH) and the zona pellucida (ZP), have drawn particular interest over the years because of their universal involvement in reproduction of all vertebrates. GnRH is a highly conserved peptide hormone required for gamete and sex steroid production in males and females. The zona pellucida is a species-specific glycoprotein matrix. It surrounds and supports the developing oocyte, serves as a critical binding site for sperm, and is therefore required for female, but not male fertility. A number of other proteins and pathways have been considered as targets, and many have been tested for contraceptive effect in one system or another [3–5,11–15]. One in particular, known as Anti-Mullerian Hormone or Mullerian Inhibiting Substance (AMH/MIS) will be discussed in more detail below [10].

1.2. The problem of bringing about lifetime infertility: immunocontraception as an example

Permanent, non-surgical contraception can in principal be achieved through the delivery of toxins that kill hormone producing or responding cells, gametes or their support cells (e.g. Refs. [1,16,17]). Alternatively, individuals can be made to continuously express molecules that inhibit fertility through interaction with specific targets. Much work has gone into this latter idea, over many decades, in the form of traditional immunocontraception, which seeks to stimulate the individuals' own immune system to continually produce antibodies that inhibit fertility. Immunocontraception has intuitive appeal as an approach to lifelong contraception since vaccination provides a catalytic stimulus that can sometimes provide lifelong protection against pathogens. Vaccination with proteins, peptides or cells important for reproduction has long been known to cause infertility in a number of animals, including humans [3–5,11,18]. In many cases this is thought to result from the production of antibodies that bind to one or more self-antigens. Many proteins have been tested in these systems, and these studies are reviewed elsewhere [3–5,11,18]. A number of other points at which it may be possible to intervene so as to inhibit fertility have not been explored. These include epididymal and seminal fluid components required for sperm maturation, and viability and activity in the female reproductive tract; molecules present in the female reproductive tract required for sperm motility and movement to the egg; and molecules required for maternal-fetal interactions needed to support implantation and subsequent development (Fig. 1).

1.3. Traditional immunocontraception is inherently challenging because the immune response to vaccination shows individual variability

Following vaccination with an antigen of interest, some individuals never mount a strong immune response, while others require multiple boosts to achieve adequate antibody titers. Antibody titers often, though not always, decrease over time, resulting in restoration of fertility. However, one cannot generally predict if or when this will occur. Finally, vaccination can also result in more permanent reproductive tissue damage (which may or may not be a desired outcome) if specific antibody effector- and/or T-cell-mediated pathways are activated [3–5,11,19,20]. This variability in immune response is not surprising. First, the antigens to which an immune response is desired are self-antigens, and therefore immunological tolerance must be broken. Second, immunoglobulin selection and production in populations of individuals with substantial genetic diversity in immunoglobulin, T-cell, and MHC repertoires, and immunological history, is inherently stochastic and variable.

Variability in the immune response is of course not unique to immunocontraception, and also occurs with vaccination to prevent infectious disease. Here also vaccines often require multiple boosts for effective protection, with some individuals never mounting an effective response (e.g. Ref. [21]). However, for infectious disease some frequency of failure in individual protection is often acceptable (e.g. Ref. [22]), particularly if herd immunity of a successfully vaccinated majority still provides individuals that did not mount an immune response with some protection. In contrast, a good lifetime contraceptive must cross a much higher bar: the failure rate should be comparable to that of surgical procedures such as tubal ligation, vasectomy, spay or neuter. One variant of traditional immunocontraception being explored seeks to mount and maintain an immune response to specific antigens through the use of a live virus as a vehicle for providing more continuous antigen presentation [23–28]. While the use of a live virus is an attractive option in some respects because of its potentially self-sustaining nature, use of a replication-competent virus, even one that has been attenuated, raises a number of ecological and regulatory issues relating to possible recombination between the modified virus and wild counterparts, the host range of the virus, and the possibility of virus spread to other individuals and/or outside the area in which fertility control is desired.

1.4. Contraceptive molecules can be provided directly to the individual in the form of a transgene

One can bypass reliance on the natural immune system in a target species by providing a monoclonal antibody or other protein derived from the target species directly to the members of that species, in the form of a transgene designed to express and secrete the protein into the general circulation. This should, possible host polymorphisms in the target epitope notwithstanding, bind the same target in the same way in all individuals of the relevant sex. A number of gene delivery platforms are under development. Modified mRNAs can provide transient gene expression [29,30]. RNA virus-based expression systems are also being explored for longer-term expression, though this work is at an early stage [31]. DNA-based systems can in principal be used to bring about very long-term expression of extracellular proteins. Each of these systems can also be used to bring about expression in specific cells of small RNAs that silence the expression of mRNAs encoding secreted or intracellular molecules.

Here we focus on DNA-based expression systems as they are the farthest along in development, and can provide expression for years. One approach utilizes a replication-competent episome (plasmid) that can maintain copy number in cells undergoing divisions [32–35]. A second approach utilizes retroviral vectors or transposons, which can integrate into the genome of dividing or long-lived non-dividing cells such as muscle or neurons [36]. Genomic integration provides a basis for lifelong expression, and large cargo genes can be introduced. However, insertions also have the potential to alter expression of nearby genes, which could be oncogenic. Ongoing experiments designed to characterize insertion site specificity and phenotypes of cells with integration events will provide important information on the safety of this approach [36–38]. Finally, forms of DNA that lack the ability to replicate, and that remain largely episomal, can be introduced into non-dividing cells, where they can persist for years. Below, we focus on this group of DNA-based systems, as they are farthest along in terms of clinical development.

The simplest non-replicating episomal forms of DNA vectors are plasmids, minicircles, and closed linear DNA [39]. For each of these vectors the DNA is packaged with some sort of transfection reagent that protects it from digestion in serum and promotes entry into the cell. Following entry, the DNA then must make its way to the nucleus where it is transcribed, all the while avoiding activation of the innate immune system, which promotes inflammatory responses and degradation of cytosolic DNA [40]. The appeal of non-viral DNA delivery systems is that both the vectors and the transfection reagents needed for the DNA to gain entry into the cell cost relatively little to prepare. Their downside is that they are generally perceived as providing lower levels of expression, for shorter periods of time, than can be obtained with the viral-derived systems described below [39,41–44]. That said, non-viral delivery methods are rarely compared head to head with viral systems in vivo, leaving it unclear how great the differences in expression are. In addition, ongoing work has identified multiple variables, such as GC content, the fraction of the DNA that is not transcribed, and specific DNA motifs, that can bring about increased levels of transcription and decreased gene silencing [45,46]. Thus, there remains hope that low cost and efficient non-viral DNA delivery systems will someday be available.

A number of viral or virus-derived methods of DNA delivery are also under development [47]. The current method of choice for delivery of non-replicating, episomal DNA (though not without its limitations, discussed below) is the recombinant adeno-associated virus (rAAV; a virus-derived system) [48]. AAVs are non-pathogenic, naturally replication-deficient parvoviruses. A productive wildtype AAV infection requires co-infection in the same cell with helper viruses such as Adenovirus or Herpes Simplex virus. AAV consists of a linear single-stranded DNA genome surrounded by a non-enveloped capsid. The genome consists of rep and cap genes flanked by two inverted terminal repeats (ITRs). Recombinant AAV (rAAV) vectors only carry the ITRs, with the transgene and regulatory sequences being located between them (Fig. 2). When a rAAV vector is grown in cell culture the rep and cap functions and the essential Adenovirus or Herpes genes are provided from other sources, often plasmids [49] (Fig. 2). Thus, rAAV are prevented from replication through several different mechanisms when introduced into a host cell. Most copies of the rAAV genome remain episomal, and can bring about stable levels of transgene expression in non-dividing cells such as muscle for many years [50–52]. An important limitation of rAAV is that its packaging capacity is restricted to roughly 5 Kb. This creates a “bad new, good news” situation in that it limits the kinds of transgenes that can be expressed, but it also makes it physically impossible for recombination between rAAV and wildtype AAV to create a rAAV that carries a transgene and viral genes essential for replication (replication of which would still require co-infection with Adenovirus or Herpes Simplex). In consequence, while a small fraction of a rAAV introduced into an organism can be shed into the environment for a short period of time, the encapsidated transgene has no ability to increase in copy number if it somehow makes its way into another organism. These features, in conjunction with the fact that wildtype AAV is not associated with human or animal disease, are reflected in the classification of rAAV as a Risk Group 1 agent for lab and animal studies in the US [53]. Finally, the recent approval by the EU of Glybera, a rAAV engineered to express lipoprotein lipase in muscle for lipoprotein lipase deficiency in humans provides a template for how regulatory requirements can be met [54,55]. These positive points notwithstanding it is important to note that producing rAAV requires the use of mammalian or insect cells. Economies of scale will be needed to make production of clinical grade rAAV competitive for a market such as contraception of feral and wild animals where cost per individual needs to be low. Efforts are under way to develop rAAV-based antibody therapies as long-term prophylactics for common human infectious diseases such as HIV, dengue, malaria and influenza [56–58]. It will be informative to follow these projects as they face related cost issues.

1.5. Vectored contraception can work, at least in mice

1.5.1. The zona pellucida

The ZP consists of three or four (depending on the species) glycoproteins (ZP1-4) that are synthesized by the growing oocyte, surrounding it and early embryo. The ZP functions as a binding site for sperm, and this interaction is required for sperm to ultimately penetrate the ZP and fuse with the egg plasma membrane [59]. Because inhibition of sperm-ZP interactions can bring about infertility, but does not (in many but not all cases) alter the cyclicity of hormonal patterns in females, the ZP is an interesting target when the goal is to bring about female infertility while leaving hormonally driven behaviors intact. Many studies have shown that vaccination of animals with solubilized ZP or isolated ZP proteins or peptides results in female infertility with normal ovarian and hormonal cyclicity [5,19,60]. Cats have proved somewhat more recalcitrant sterilization by ZP vaccination [61]. However, given the universality of the ZP and its role in fertilization, this may simply reflect the nature of the feline immune response (antibodies produced), resulting from vaccination with ZP proteins derived from other species. Often the effects on fertility of ZP vaccination are transient. In others they may be more permanent, and result in loss of cycling. These latter effects (which could be desirable in some contexts) may be due to antibody effector activities, T-cell-

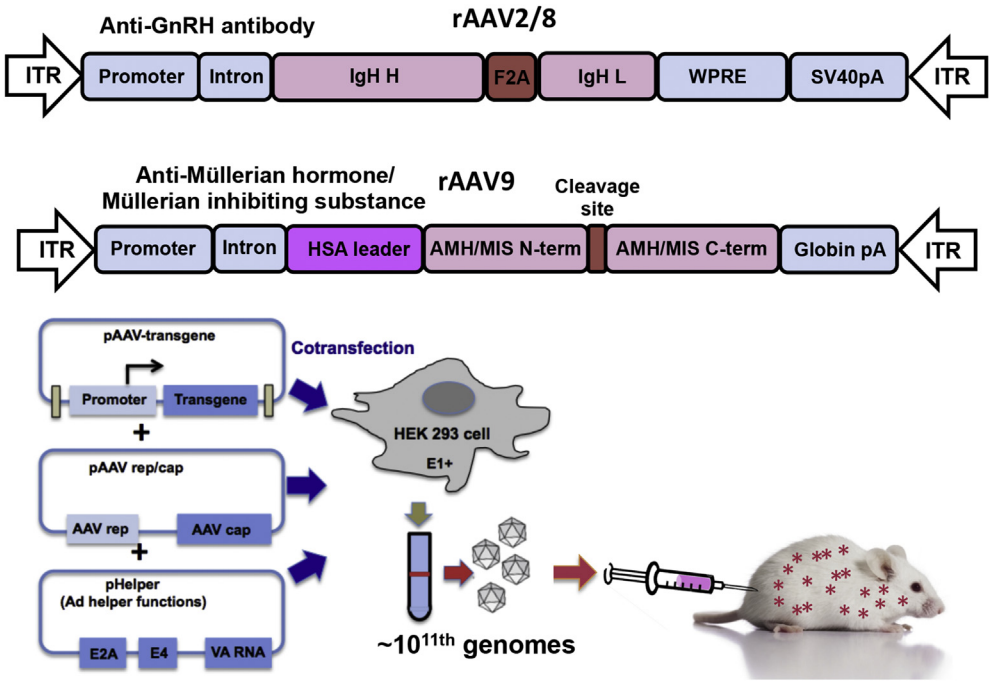

Fig. 2. Outline of vectored contraception using recombinant adeno-associated virus (rAAV). The overall structure of the rAAVs used to express a recombinant antibody (top) or AMH/MIS (middle), are indicated. Key components of the rAAV used to produce recombinant antibody include the following: AAV2 inverted terminal repeats (ITRs); the promoter used to drive transgene expression; an intron, which enhances expression, and can serve as a location for enhancer sequences; the immunoglobulin heavy (IgG H) and light (IgG L) chains; the F2A ribosome skipping sequence which results separation of the IgG H and IgG L chains during protein synthesis; the WPRE element, which promotes transgene expression; and the SV40 PolyA sequence. This rAAV is packaged using AAV8 capsid proteins. For expression of AMH/MIS the components are similar and include the following: AAV9 ITRs; a promoter; an intron; the human albumin leader sequence; the N-terminal region of AMH/MIS; a modified internal cleavage site; the C-terminal region of AMH/MIS; the globin PolyA sequence. This AAV is packaged using AAV9 capsid sequences. Recombinant AAV genomes carrying a gene of interest are most easily prepared as shown in the lower left. For both AAVs, injection of roughly 10^{11} viral genomes results in levels of expression of an effector (Eff) sufficient to induce infertility in males and females (anti-GnRH) or females (anti-ZP2 and AMH/MIS) [9,10].

mediated cytotoxicity or disruption of follicle development caused by defects in ZP assembly [5,19,20,62–64].

Monoclonal antibodies that bind mouse ZP2 (IE3) [65] or ZP3 (IE10) [66] were shown many years ago to transiently block sperm binding and fertility without obvious ovarian pathology when passively introduced into female mice. IE3 is particularly interesting as it recognizes the N-terminus of mouse ZP2 [65], which serves as critical ligand for sperm binding [67], and is cleaved post-fertilization as an essential part of the block to polyspermy [68]. To determine if vectored expression of anti-ZP antibodies could bring about long-term infertility, Li et al. [9] determined the sequence of IE3. The IE3 coding region, and transcriptional and translational regulatory sequences designed to provide high levels of expression and secretion from muscle [69], were then introduced into an AAV2/8 vector (containing the inverted repeats from AAV2 and the capsid protein from AAV8). Intramuscular injection of 5×10^9-$1x10^{10}$ viral particles carrying this vector resulted in high antibody titers (between 2.8 and 19μg/ml), which were maintained over more than six months (when the experiments were ended). Slightly more than half the females were infertile when tested at 5 weeks post injection. The others showing reduced litter sizes and subsequently became infertile. Importantly, no defects in follicle growth and ovulation were observed, suggesting that normal ovarian and hormone cyclicity was maintained. Interestingly, oocytes from IE3 treated females showed gaps and tears in the ZP not present in controls. This suggests that long-term exposure to IE3 over the course of oocyte development disrupts ZP assembly or integrity. It is possible that this disruption plays a role in inhibition of fertility. This last result highlights the fact that there may be multiple ways of bringing about infertility through targeting of the ZP: by direct inhibition of sperm binding, as inferred from the rapid infertility induced when IE3 is passively infused [65], and by disrupting assembly of the ZP over time, which may also prevent productive interactions with sperm and/or cause defects in follicle development [9]. The structure of the ZP has been inferred through a variety of techniques [70]. It will be interesting to determine if monoclonal antibodies that target other regions within this ordered multi-protein meshwork can bring about infertility at lower titers through either of the above mechanisms, or through recruitment of specific antibody effector activities (see below).

1.5.2. GnRH

The other target that has been most often considered for immunocontraception in animals is GnRH. GnRH is produced by neurons of the hypothalamus, where it is processed from a larger precursor to an active peptide of 10 amino acids, of identical sequence in all mammals examined to date, with the exception of the guinea pig [71]. Mature GnRH is released in regular pulses into the median eminence. From here it enters into the hypophyseal portal capillary system, which carries it a short distance to the anterior pituitary, where it binds its receptor, stimulating the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the general circulation. FSH and LH are required in multiple roles in various cells of the gonads to promote the formation of gametes and the production of sex steroids. Thus, loss of GnRH results in male and female infertility, and loss of other steroid sex hormone-dependent traits in otherwise healthy animals [72,73]. Similar phenotypes are observed in animals treated continuously with GnRH agonists, which inhibit (following an initial surge in LH and FSH) GnRH-dependent signaling through

receptor downregulation [74,75]. Inhibition of GnRH is considered an attractive strategy when the goal is to inhibit fertility and steroid sex hormone-dependent behavioral traits such as mounting and urine marking. Immunocontraception through vaccination with GnRH inhibits fertility in a number of species, but these effects are temporary, and often require multiple boosts for maximum activity [3,4].

To determine if vectored expression of anti-GnRH antibodies can bring about long-term infertility, Li et al. [9] first determined the protein sequence of a high affinity anti-GnRH monoclonal antibody known as SMI41 [76]. This was recoded back in to DNA and introduced into the same AAV2/8 vector utilized for the anti-ZP experiments described above. Twenty-one males and 42 female mice were given intramuscular injections with from 10^9 to 10^{11} particles. Titers of ~10^{11} particles resulted in complete infertility (52 weeks) in continuous mating experiments if antibody titers reached ~200μg/ml or more in females, or ~100μg/ml in males. All age-matched control males (n = 18) and females (n = 12) were fertile. The lowest titer sufficient to inhibit male fertility was unclear given the range of titers observed. Importantly, both male and female gonads were atrophied, and testosterone was undetectable in males. These phenotypes are similar to those of animals that lack GnRH [72] or that are treated continuously with GnRH agonists [74,75].

These positive points notwithstanding, the titers of SMI41 needed to inhibit fertility in the mouse are high. It remains to be determined what anti-GnRH antibody concentration is required to inhibit fertility in species with larger body size, but it could be comparable. Of course any version of SMI41 used in another species would need to be engineered to utilize antibody framework and constant regions from the species of interest so as to be seen as immunological self; the equivalent of humanizing an antibody from another species for use in humans [77] [78]. A possible reason for the high titers of SMI41 needed to inhibit fertility has to do with the fact that GnRH acts within roughly a minute of its release from the hypothalamus [79], in a very local environment, the portal capillary circulation, and is then rapidly degraded [80]. Thus, the only anti-GnRH antibody relevant for inhibition of fertility is the tiny fraction present in the portal capillary circulation during a very brief window of time. In consequence, serum antibody titers need to be high. In addition, we hypothesize that contraceptive efficacy of an anti-GnRH antibody also benefits from having a fast antibody-GnRH binding on rate. This would facilitate antibody binding to GnRH released into the portal capillary circulation before GnRH has time to interact with its receptor. Evidence consistent with this hypothesis comes from other observations by Li and colleagues utilizing a second anti-GnRH antibody, HB9094 [81], which they also cloned, characterized, and introduced into mice in the same rAAV vector. They found that the equilibrium binding constants of HB9094 and SMI41 were comparable (Fig. 3) [82], but that the kinetics of binding and dissociation were very different. In particular, SMI41 has a 20X faster on rate than HB9094, while HB9094 has a much slower off rate. SMI41 was an effective contraceptive in mice, as discussed above [9]. However, HB9094 at similar titers was not

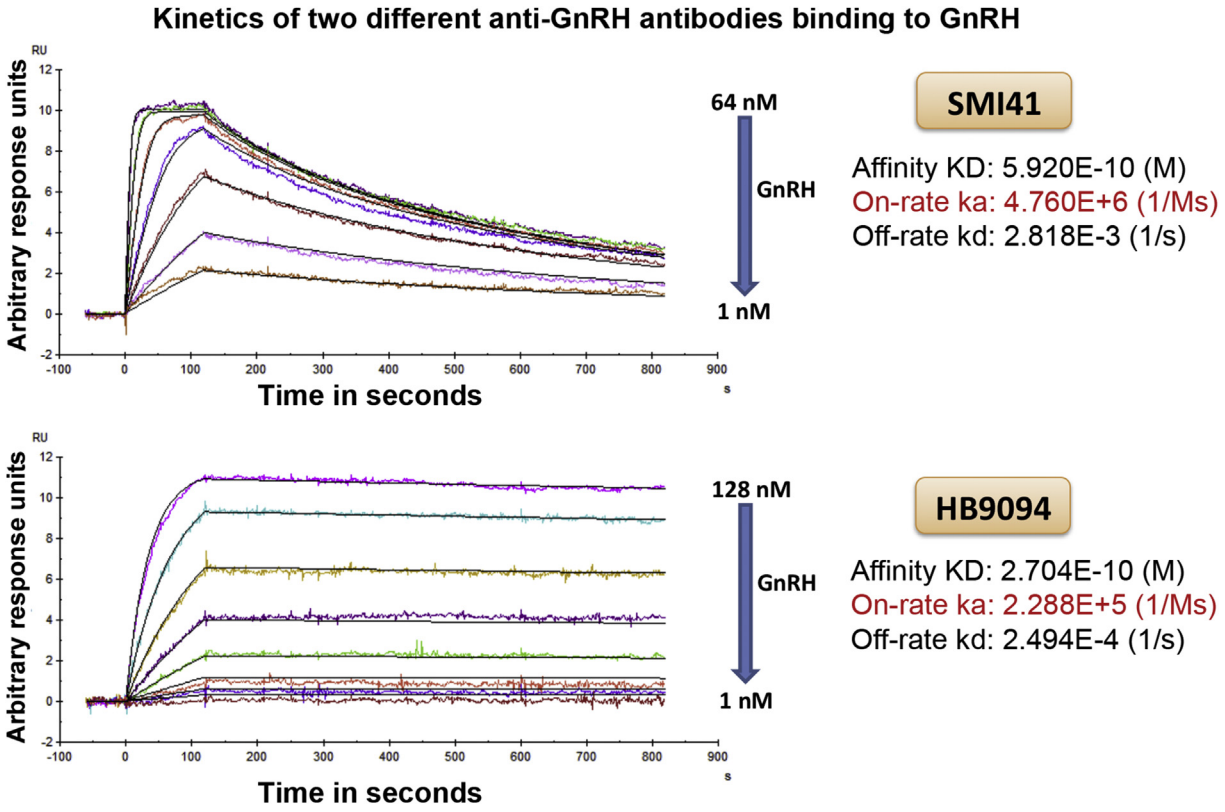

Fig. 3. Binding kinetics of two different anti-GnRH monoclonal antibodies, SMI41 and HB9094. Surface plasmon resonance binding data for GnRH binding to both antibodies is shown, as described in Li et al. [9]. SMI41 data in the upper panel is adapted from Li et al. [9]. HB9094 data in the lower panel is adapted from Ref. [82]. Sensograms in various colors are shown for binding of injected GnRH at various concentrations (128 nM, 64 nM, 32 nM, 16 nM, 8 nM, 4 nM, 2 nM, and 1 nM) to a surface coated with goat-anti-mouse IgG, and subsequently bound with SMI41 or HB9094. Fits for a single-site binding model are shown as black. The Y axis is arbitrary response units. The X axis is time in seconds. For both sensograms binding proceeds for ~100 s, until equilibrium binding is achieved. Unbinding is then visualized as the decrease in signal during the subsequent washout. Note that while SMI41 binds to GnRH very rapidly, it also unbinds rapidly. In contrast, HB9094 binds very slowly, and also unbinds very slowly. The equilibrium binding constant for HB9094 is ~twofold lower than that of SMI41, but HB9094 performs very poorly as a contraceptive in mouse fertility experiments ([82], and Hay and Li, unpublished). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[82]. We hypothesize that this is because the on rate of HB9094 is slow and the off rate is irrelevant given that GnRH has a short half-life and is rapidly rapid diluted into the general circulation.

Other molecules in the GnRH pathway may also be interesting targets for contraception. For example, a high affinity antibody to the GnRH receptor with a slow off rate could be used to block activation of the GnRH receptor. However, to our knowledge antibodies with these characteristics have not been described. Alternatively, antibodies that target FSH and/or LH, released from the pituitary in response to GnRH signaling, could be targeted, as could their receptors Versions of FSH and LH that act as receptor antagonists could also function as contraceptives [83–85]. Finally, transgenes could (in principal) be delivered to neurons of the hypothalamus using engineered versions of AAV that are selectively taken up by neurons in the brain when introduced through an intravenous injection [86] [87]. These transgenes could encode small RNAs that promote GnRH mRNA degradation. Alternatively, or in addition, they could target mRNAs encoding kisspeptin or neurokinin B, which act as positive regulators of GnRH release [88]. Of course, in order for brain delivery-based approaches to work well as a contraceptive a very high percentage of the relevant hormone-secreting neurons will probably need to be transduced. While high level transduction of cortical (69%) and striatal neurons (55%) in mice through a single intravenous injection of 10^{11} rAAV vector genomes has been achieved [86,87], transduction levels of the hypothalamus remain to be determined.

1.5.3. Anti-Mullerian Hormone/Mullerian Inhibiting Substance (AMH/MIS)

The most recent contraceptive target to be tested using VC is AMH/MIS (hereafter referred to as AMH). AMH is a transforming growth factor Beta family member that plays a critical role in male and female reproduction (reviewed in Refs. [89–92]). During male fetal development it is produced by Sertoli cells and blocks the formation of the female Mullerian ducts in the initially bipotential reproductive tract. Levels of AMH in males remain high until puberty, and then decrease dramatically. In females AMH levels are low until puberty, at which point they rise to levels comparable to those of post-pubertal males (reviewed in Ref. [90]). AMH in females plays critical roles in regulating follicle recruitment and growth. In the postnatal ovary AMH and its receptor are both expressed in granulosa cells. Evidence from both loss-of-function and gain-of-function experiments indicates that AMH functions in a paracrine manner to inhibit the recruitment of primordial follicles. AMH can also inhibit later stage FSH-dependent follicle growth (reviewed in Refs. [89,90]). These observations, together with the fact that very high levels of AMH are found normally in prepubertal males, make AMH an interesting candidate female contraceptive.

AMH is, however, a challenging molecule to work with. It undergoes a complex maturation process involving cleavage, dimerization and glycosylation [92]. When the wildtype coding sequence is expressed many inappropriate products are generated, resulting in products with low and variable levels of activity. To overcome these problems, Pepin et al. [93] made several alterations to the protein coding sequence, substituting the endogenous N-terminal leader peptide with that of human albumin, and introducing a modified internal cleavage site. Together, these alterations resulted in a protein that could be expressed at much higher levels than the wildtype protein. In subsequent work a gene encoding this protein was introduced into an AAV9 expression vector (Fig. 2), which was introduced into mice through a single intraperitoneal injection of ~3×10^{11} viral genomes. Expression was observed in muscles throughout the body as well as in other tissues, and high and sustained levels of AMH (~0.25μg/ml) were observed for at least 60 days [94]. In subsequent experiments designed to explore effects on ovarian function, 3×10^{11} viral genomes designed to express either AMH or no transgene were introduced through an intraperitoneal injection into groups of 10 female mice. Mice harboring the AMH vector had serum levels of AMH ranging between 0.075-2μg/ml. Those females with expression levels greater than 0.25μg/ml became completely infertile after 6 weeks (followed for six months), while those with levels under 0.25μg/ml showed greatly reduced fertility [10]. Importantly, in ovaries from other females exposed to 3×10^{11} AMH viral genomes through intraperitoneal injection later stage follicles were progressively depleted over time, as expected based on a mechanism of action that involves prevention of primordial follicle recruitment, while allowing more mature follicles to develop. Finally, because AMH blocks follicle development, it also resulted in a largely acyclic hypergonadotropic hypogonadic state, with levels of FSH and LH being increased due to decreased negative feedback on the hypothalamus by ovarian steroid hormones [10].

The use of AMH as a contraceptive is an exciting development. It is a natural ligand, and in females the receptor shows a restricted pattern of expression, largely though not completely confined to the ovary. This, and the fact that the expression levels needed to bring about female infertility are comparable to those found normally in prepubertal males, raise the possibility (which requires further testing) that the high levels of AMH needed to inhibit female fertility may have few side effects. Finally, AMH introduction can also lead to decreased androgen production in adult male rats [95]. This raises the interesting hypothesis that AMH, alone or in combination with antibodies designed to target GnRH or LH (which is required for testosterone production by Leydig cells of the testes), could have efficacy as a male contraceptive as well. Challenges going forward, as with anti-GnRH and anti-ZP antibodies, revolve around decreasing the number of viral genomes needed to guarantee infertility in animals that are much larger than mice, and have a much longer reproductive lifespan.

1.6. New contraceptive monoclonal antibodies for specific species can be created using existing technologies

As discussed above, many potential contraceptive target proteins are secreted or plasma membrane bound, and thus are targets for inhibition using monoclonal antibodies. However, only a few potentially contraceptive monoclonal antibodies exist: to GnRH, to components of the mouse ZP, and to a carbohydrate epitope of human CD52. All but the anti-CD52 antibody, which was isolated from an infertile woman whose serum had the ability to agglutinate and immobilize human sperm [96], are mouse monoclonal antibodies, which would be seen as foreign in any relevant target species. In short, monoclonal antibodies to most antigens of contraceptive interest have not been generated, and only one (anti-human CD52) was isolated from a potential target species (humans). However, several strategies are now available that should simplify and streamline targeted development of antibodies with contraceptive potential for many species (Fig. 4). First, it is now relatively straightforward to isolate B cells or plasmablasts from vaccinated animals of variious species following a simple blood draw [97–100]. Second, cells expressing antibodies that bind a molecule of interest can then be isolated in several ways. The most straightforward of these involves cell sorting with fluorescently labeled antigen. Third, single cell-based sequencing of the expressed immunoglobulin loci then serves to isolate and clone the relevant antibody [98,101–104]. Finally, rAAV-based fertility experiments such as those described above [9,10], or using passive infusion of recombinant antibody, can then be utilized to identify those with contraceptive activity (Fig. 4).

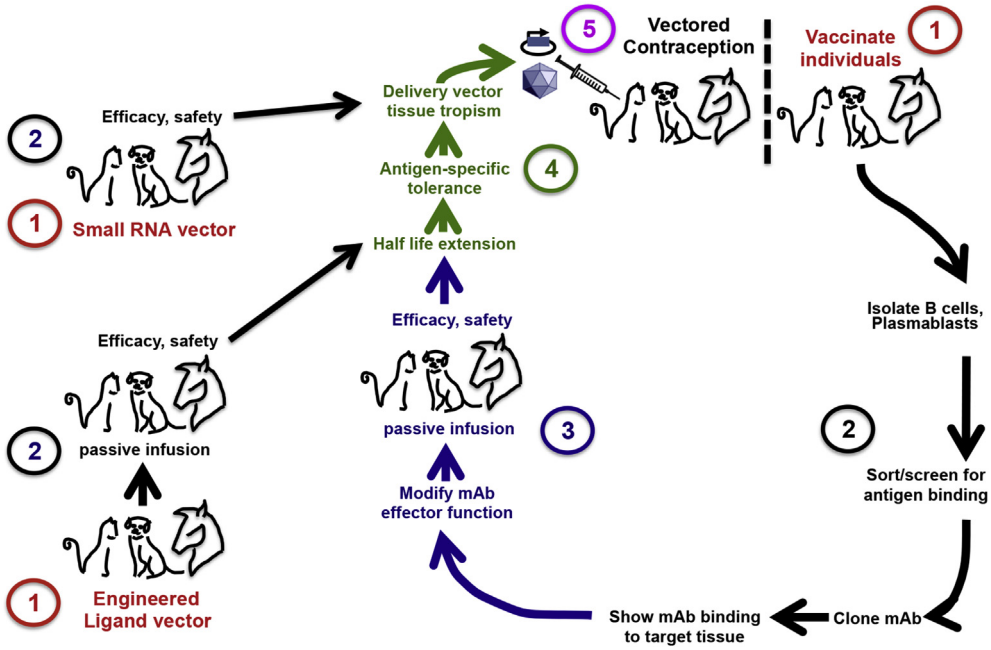

Fig. 4. Overview of key steps in different approaches to vectored contraception. Antibody-based approaches (far right) begin (1) with vaccination of individuals from the species and sex of interest (e.g. males in the case of zona pellucida antigens that are normally expressed only in females). IgG-expressing cells are isolated from these individuals and then screened for those that produce an antibody that recognizes the antigen. The antibody-encoding gene is then cloned, expressed and shown to bind the relevant target tissue in vivo (2). Positives from this process represent candidates for vectored contraception. In step (3) the Fc (constant) region of the antibody-coding region may be modified to enhance or prevent antibody effector activity. The antibody is then tested for efficacy and safety through passive infusion of recombinant antibody. In (4) steps are taken to increase the efficacy of the transgene/ug DNA in vivo. These include genetic engineering to increase the in vivo half-life of the antibody, genetic engineering to decrease the possibility of antigen-specific tolerance, and engineering of the DNA delivery vectors such as AAV to bring about enhanced and targeted delivery of recombinant DNA to a specific target tissue. Delivery of the packaged DNA into the individual to be rendered infertile follows as the final step (5).
Steps involved in the creation of vectored contraceptives using engineered ligands (bottom) and small RNAs (top) are shown at the far left. For both approaches the first step (1) is to create a vector that drives expression of the relevant molecule. In the case of an engineered ligand, efficacy and safety can perhaps (provided it can be made in sufficient quantity) be tested through passive infusion of recombinant protein (2). In the case of small RNAs such testing is likely to require delivery of the DNA that drives small RNA expression given the challenges associated with delivering small RNAs directly to cells in vivo (2). In the case of engineered ligands engineering to extend half-life and maintain antigen-specific tolerance may be useful (4), as with monoclonal antibodies. For both approaches, engineering vector tropism to specific tissues may be useful, particularly for small RNAs, which must act in the cells in which they are expressed (4). Delivery of the packaged DNA into the individual to be rendered infertile follows as the final step (5).

1.7. Challenges to implementing VC: efficacy

The proof-of-principal experiments described above in mice demonstrate initial efficacy of transgene-based approaches to inhibition of fertility (~1 year). This time frame is short as compared with the reproductive lifetime of target species of interest. In addition, the mice used are genetically homogenous, and tested under laboratory conditions designed to minimize differences in overall health and immunological history. In contrast, target species are genetically diverse and exposed to many different environments and immunological stimuli throughout their life. In order for vectored contraception to work a number of things need to go right. These include effective delivery of encapsidated DNA to the target cells in vivo, consistent and maintained levels of expression within these cells, and lack of an immune response to the antibody being expressed. We discuss these issues, and some possible solutions, below.

1.7.1. Delivery to target cells

The AAV capsid protein mediates interactions with cell surface receptors that determine vector tissue tropism, and thus expression level in particular cell types. For secreted proteins preferential uptake by particular tissues could lead to higher local concentration in the relevant tissue (the ovary, epididymis, testis, etc), while for small RNA-based strategies cell targeting could increase transduction frequency of relevant cell types while decreasing entry into irrelevant cells. In order to create capsids with very specific tropisms, and/or to identify capsids that result in vector detargeting away from specific tissues, libraries of capsid sequences are generated. These are then screened (in the context of an AAV in which the capsid is encoded) for those that show enhanced targeting to specific cell types in vitro or in vivo. These approaches can be quite sophisticated, and have resulted in the creation of novel rAAVs that show remarkably specific tissue tropisms following intravenous injection. Neurons of the CNS and the brain microvasculature constitute two noteworthy examples [86,87,105–107]. Similar approaches could be used for other tissues such as the ovary or endometrium, though it will be important to ensure that the screening procedures are designed so as to enrich for interactions with long-lived cells rather than those that turn over rapidly.

Capsid engineering is important for another reason as well. Efficacy in gene delivery and transgene expression in humans and other animals such as dogs and horses can also be limited by the presence of pre-existing immunity to the capsid proteins of particular AAV serotypes [109–111]. The presence of these antibodies, which would influence the choice of vector to be tested, can be detected in a variety of ways [111,112]. Various strategies are being developed to reduce or eliminate these effects. These include the identification or creation of novel capsid serotypes, capsid engineering, and the use of capsid decoys [113,114]. Such approaches may suffice when modest numbers of patients are being treated for specific diseases, and need rAAV serotype-specific treatments in order to bring about therapeutic levels of transgene expression. In contrast, rAAV-based contraception requires a vector that

consistently delivers therapeutic levels of the transgene to all members of the target species, regardless of their prior exposure to naturally-occurring AAV serotypes. While capsids with these properties do not yet exit, there are concerted efforts to identify them since they are also required in the context of immunoprophylaxis in humans using AAV-delivered antibodies to prevent common infectious diseases such as HIV, dengue, malaria and influenza [56–58].

1.7.2. Long-term transgene expression

With respect to maintenance of AAV-dependent expression for long periods of time, only modest amounts of information are available. rAAV-dependent expression of proteins for many years has been observed in larger animals such as dogs (8 years), cats (4 years) and humans (10 years) [50–52]. The maximum expression times are unclear, largely because many experiments have not gone on for long enough. However, it remains to be demonstrated that the levels of expression of antibodies, other proteins, or small RNAs needed to achieve infertility can be achieved, and then maintained for the 10–20 year time frames needed for animals such as cats, dogs and horses. Developing more efficacious (higher specific activity/transgene copy number) antibodies, using the approaches noted in the preceding section, provide one approach to this issue, by creating antibodies that remain above the threshold needed to completely inhibit reproduction even at low (sub-microgram/ml) titers. Modification of vector tropism constitutes another.

Several other approaches to increasing efficacy of transgenes may also be considered (Fig. 4). First, the half-life of proteins in the circulation can often be extended by linking them to the constant region of IgG (the Fc domain), to albumin, or to a bacteria-derived albumin-binding domain [115–117]. Each of these strategies results in an increase in the hydrodynamic radius of the protein and promotes recycling by the neonatal Fc receptor (FcRn). FcRn binds the IgG Fc domain, as well as sequences within albumin, and promotes their recycling back to the cell surface following endocytosis, as opposed to entry into intracellular degradation pathways [118]. Other protein fusion partners that extend half-life have also been described, though these can be large, limiting their utility for AAV-based approaches where packaging size is limited [116].

1.7.3. Prevention of an anti-contraceptive immune response

Another phenomenon that can result in reduced efficacy comes from the fact that some individuals make antibodies that neutralize a therapeutic antibody (e.g. Refs. [119–122]) or other therapeutic protein. This situation - lack of immunological tolerance to foreign proteins, even those such as antibodies that are derived from the same species, represents a general problem for therapeutic protein delivery in many settings [122–124]. The problem VC faces is that tolerance must be maintained in all treated individuals. There are a number of approaches to bringing about antigen-specific tolerance [125]. Two that are particularly relevant for vectored expression take advantage of the fact that billions cells die through apoptosis each day, most of which are red blood cells (RBCs) [126]. A therapeutic protein can be linked to RBCs through RBC-binding peptides or proteins [127]. Alternatively, the therapeutic protein can be linked to a phosphatidylserine (PS)-binding domain [128]. PS is a major signal for uptake of apoptotic cells. PS is normally found primarily on the intracellular leaflet of the plasma membrane, but becomes exposed on the cell surface when cells undergo apoptosis. PS exposed on the surface of dying cells acts as a universal “eat me” signal, promoting phagocytosis by cells of the spleen, liver and other tissues carrying receptors that bind plasma-membrane-exposed PS (Segawa and Nagata, 2015). Antigens bound to these dying cells are taken up by phagocytosis along with the dying cell. Importantly, internalized antigens along with apoptotic cells are presented to other cells of the immune system in a way that induces tolerance to them (the antigens) rather than activation of an immune response [129–131]. Thus, with either tagging approach the hope is that by shunting some fraction of the therapeutic protein into association with dying cells each day, antigen-specific tolerance is actively maintained.

1.8. Challenges to implementing VC: safety

Maintaining immunological tolerance to a contraceptive antibody or an engineered variant of a ligand such as AMH/MIS is also important from a safety perspective. This is because if the antibody or ligand is immunogenic, it can promote the creation of anti-idiotypic antibodies or anti-ligand antibodies that may themselves bind other targets or the natural ligand, respectively [122,124,132]. Finally, antibodies often mediate their effects through recruitment of other components of the immune system (effectors), which can promote tissue damage in various ways. It can be difficult to ensure that antibodies or other therapeutics have no off-target binding. However, in the case of antibodies of the IgG isotype the consequences of such binding can be minimized by introducing mutations into the antibody-encoding gene that block the ability of the mature antibody to recruit effectors that result in complement activation, cell death, phagocytosis, or activation of a cytokine storm [133–137]. The hope is that antibodies so modified will only act as sponges, binding the relevant molecule and blocking its ability to function, or creating some other simple steric barrier to fertility. Similar considerations apply in the case a ligand carries an IgG Fc domain to extend half-life. Conversely, in some cases - where there is good reason to believe off target binding is minimal - it may be possible to increase antibody or ligand efficacy by introducing mutations into the Fc domain that promote specific effector functions such as complement activation that result in target cell killing [117]. Possible examples include antibodies that target sperm or the oocyte plasma membrane.

1.9. Challenges to implementing VC: species specificity

For some targets it may be possible to create antibodies, ligands or small RNAs that recognize targets only in the target species, but not distantly related species such as humans. However, for species more closely related to the target species nucleotide and protein homology may be quite high. Anti-GnRH antibodies constitute a special case, since GnRH is identical in almost all mammalian species. Immunogenicity of contraceptive antibodies utilizing framework and constant regions derived from the target species is likely to limit antibody effectiveness in non-target species through antibody neutralization, as when mouse monoclonal antibodies are rendered ineffective in humans through the creation of anti-mouse antibodies [138]. However, the uncertainties associated with this issue highlight the importance of considering the environmental contexts in which VC would be used on a species-by-species basis. They also argue that DNA used for VC should have multiple barriers to replication in vivo and possible dissemination into other individuals of the same or different species in the environment. They also draw attention to the topic of reversibility.

1.10. Challenges to implementing VC: reversibility

Medical procedures that involve exposure to a drug or device can usually (though often not with surgery) be reversed. In contrast, it is not generally possible to simply “turn off” a vaccine-induced (or otherwise induced, as in autoimmune disease) immune response. As a result, the length of time vaccine-induced antibodies are generated in animals and humans is not typically under human

control. This exception to reversibility on demand notwithstanding, there is little or no precedent for medical procedures designed to permanently and irreversibly alter the repertoire of molecules present in the circulation. The primary goal of spay/neuter is of course to bring about permanent infertility. For most contexts this would also be the goal with VC in animals. However, because of the possibility of adverse events in some individuals, and in consideration of humans who may receive an accidental needle stick, it is useful to consider how VC could potentially be turned off. Below we suggest several methods that could, in principal, be used to achieve this goal. Our focus here is only on technical feasibility. Safety issues associated with each of these options requires separate discussion.

First, a second injection can be carried out at the location where the contraceptive vector was initially injected (as with a localized intramuscular injection). Such an injection could introduce mRNA or protein that results in homing endonuclease or Cas9-mediated cleavage and inactivation of the transgene, or site-specific recombinase-mediated separation of the coding region from regulatory sequences [29,139,140]. A second, local injection could also introduce a vector that drives the expression of transgene-silencing small RNAs. Scenarios in which the contraceptive transgene is introduced systemically through an intravenous or intraperitoneal injection are clearly more challenging to reverse since the likelihood that the second transgene transduces the same cells targeted by the contraceptive transgene may be low. An alternative approach to reversibility would work by promoting the elimination of transgene-expressing cells, taking advantage of skeletal muscle's ability to regenerate following damage [141]. In this scenario the vector carrying the coding region for the contraceptive protein would also carry a transgene encoding a second protein that can, in an inducible manner (following an injection or oral delivery of an otherwise benign compound), cause the apoptotic death of transgene expressing cells. Such systems (suicide genes) are used as a safety feature in genetically engineered T-cells introduced into patients [142]. This family of approaches has the useful characteristic of not requiring a second injection at the same site as the initial contraception-inducing injection. However, it would necessitate the use of a non-AAV-based DNA delivery method, given AAV's limited packaging capacity. Needless to say, such a method could only be envisioned for contexts in which the tropism of the vector prevents transduction of essential tissues such as neurons or heart. In any of these, or other reversal methods, or in the context of monitoring contraceptive titers over time, the fact that all individuals express the same antibody or other therapeutic protein (but not small RNAs) should make it possible to develop low cost assays to monitor progression to infertility, and its reversal, as with home testing for pregnancy [143] or HIV [144]. Such kits should also prove useful in monitoring pets for reduced titers, providing a window of time within which to provide a second injection before an animal returns to fertility.

2. Conclusions

Vectored contraception represents a new path to the development of lifetime contraceptives. It takes advantage of our ever-increasing knowledge as to how reproduction is regulated in mammals. It also take advantage of the large traditional immunocontraception literature, which has made great strides in identifying reproductive antigens and methods of delivery and presentation that result in the generation of a robust and antigen-specific immune response, and inhibition of fertility to varying degrees. The other technologies needed: the ability to isolate in-species antibodies recognizing a specific antigen, to deliver and express their coding regions, or those of other ligands or small RNAs, for long periods of time from DNA- and RNA-based vectors, and to bring about reversibility, already exist in several forms. These technologies will improve as many other researchers work to refine them for the treatment of a number of human diseases. Many remaining challenges relate to the core issues that have faced immunocontraception for decades: how to guarantee long-term efficacy following a single injection, in all treated individuals? In particular, vectored expression of any therapeutic small RNA or protein must face up to several key issues: The first is the fact that gene expression is likely to wane over time (particularly in muscle) as myonuclei in muscle fibers are replaced from a muscle stem cell population in response to wear and tear [145]. Some level of transgene silencing may also occur. A return to fertility can only be prevented if the concentrations of the contraceptive are high enough in young animals that levels of expression greater than those needed to bring about complete infertility are maintained in much older animals. Achieving this goal requires further development of delivery and expression systems. It also requires the development of robust approaches for guaranteeing antigen-specific tolerance in populations of animals with diverse genetics and immunological histories. Some measure of confidence that these issues can be solved comes from the fact that solutions to the same problems are needed more generally in order for gene therapy to contribute to human health. Thus, while vectored contraception is at a very early stage, the stars are aligned for progress to be made.

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