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Gene Editing and Gene Therapy: Entering Uncharted Territory in Veterinary Oncology

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Abstract: With rapid advances in gene editing and gene therapy technologies, the development of genetic, cell, or protein-based cures to disease are no longer the realm of science fiction but that of today's practice. The impact of these technologies are rapidly bringing them to the veterinary market as both enhanced therapeutics and towards modeling their outcomes for translational application. Simply put, gene editing enables scientists to modify an organism's DNA *a priori* through the use of site-specific DNA targeting tools like clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR/Cas9). Gene therapy is a broader definition that encompasses the addition of exogenous genetic materials into specific cells to correct a genetic defect. More precisely, the U.S Food and Drug Administration (FDA) defines gene therapy as "*a technique that modifies a person's genes to treat or cure disease*" by either (i) replacing a disease-causing gene with a healthy copy of the gene; (ii) inactivating a disease-causing gene that was not functioning properly; or (iii) introducing a new or modified gene into the body to help treat a disease. In some instances, this can be accomplished through direct transfer of DNA or RNA into target cells of interest or more broadly through gene editing. While gene therapy is possible through the simple addition of genetic information into cells of interest, gene editing allows the genome to be reprogrammed intentionally through the deletion of diseased alleles, reconstitution of wild type sequence, or targeted integration of exogenous DNA to impart new function. Cells can be removed from the body, altered, and reinfused, or edited *in vivo.* Indeed, manufacturing and production efficiencies in gene editing and gene therapy in the 21st century has brought the therapeutic potential of *in vitro* and *in vivo* reprogrammed cells, to the front lines of therapeutic intervention (Brooks et al., 2016). For example, CAR-T cell therapy is revolutionizing hematologic cancer care in humans and is being translated to canines by us and others, and gene therapy trials are ongoing for mitral valve disease in dogs.

Keywords: Gene Editing; Gene Therapy; Oncology; Comparative Medicine; One Health

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Introduction

Gene editing and gene therapy are increasingly used in the human clinic today and their application in companion animals is beginning to emerge. About 700 monogenic diseases have been reported in various dog breeds, with at least 200 of these diseases harboring known causative mutations (Switonski, 2020). About 400 of these spontaneous diseases in dogs are considered potential models for human disorders (https://omia.org/home). This makes the dog a particularly attractive animal model for accelerated commercial deployment of gene editing and gene therapy candidates with applications in cancer, hemophilia, lysosomal storage diseases, ophthalmology, immune-mediated disorders, muscular dystrophy, and others (Acland et al., 2001, Acland et al., 2005)., These indications may share genetic drivers, physiology, and presentation with their human counterparts. This creates bidirectional value, where on one-hand human experience and research may derisk similar innovations in veterinary patients. On the other-hand accelerated application of the underlying therapeutic in veterinary medicine can answer translational questions not effectively answered in pre-clinical studies or even human trials. Ultimately, regulatory incentives to develop parallel (veterinary and human) drug development programs would present an opportunity to streamline, accelerate, and improve pharmaceutical research and development in veterinary and human oncology.

In cancer research, gene editing and gene therapy applications in canines have garnered considerable interest since, in contrast to mice, cancers develop spontaneously in dogs (i.e., without genetic manipulation) and in the context of intact immune system with a syngeneic host and tumor microenvironment (Gordon et al., 2009). As more information on the dog genome is being released, multiple studies have demonstrated significant homologies between canine and human cancer-associated genes, including MET, mTOR, KIT and TRAF3 (Paoloni and Khanna, 2008). As such, biological and genomic similarities between canine and human cancer provides an impetus for parallel development of novel drug candidates (including gene therapy and gene editing) in canine and human clinical trials (Schneider et al., 2018). Specifically, ample literature has established similarities between the pathologic, biologic, immunophenotypic, and genetic components of Diffuse Large B-cell Lymphoma (DBCL) in dogs and humans (Richards et al., 2013; (Mochel et al., 2019). Based on these similarities, preliminary proof-of-concept studies from Dr. Nicola Mason's group are the first public reports of CAR-T cell therapy being used in companion animals (Panjwani et al., 2016). Even more recently, Sakai et al. have generated second and third-generation canine CAR-T cells using retroviral gene transduction with RetroNectin and showed positive cytotoxic responses against CD20-positive cells *in vitro* (Sakai et al., 2020).

Besides applications in companion dogs, descriptions of gene editing in veterinary medicine have been reported in various species, including horses and cats. In horses, CRISPR/Cas9 was used to correct a deleterious point mutation associated with Glycogen Branching Enzyme Deficiency in primary fibroblasts (Pinzon-Arteaga et al., 2020). Likewise, lentivirus-delivered CRISPR/Cas9 directed gene editing was used in a series of *in vitro* experiments to modulate the proviral load and production of virions of the Feline Immunodeficiency Virus (FIV) (Murphy et al., 2020). In this proof-of-concept study, the authors reported a reduction of cell-free viral RNA in gene-edited cells

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relative to control. The reduced infectious potential of this new construct was later confirmed by infecting feline naïve T-cells with cell-free FIV harvested from FIV-infected and CRISPR/Cas9 lentivirus-treated cells.

Our knowledge on the role of genetic variation in disease continues to evolve and developments in the Dog Genome Annotation Project (DoGA) are rapidly aiding in comparative medicine efforts. Advances in DoGA combined with faster, cheaper, and more efficient gene editing and gene therapy methods will lead to even greater emphasis on translational modeling and parallel drug development efforts using spontaneous dog diseases. In this chapter, through a translational lens based on successes and failures in human medicine, we will present imminent application of gene therapy and gene editing in companion animals; how we got to the use of gene editing and gene therapy in companion animals; how these technologies may bring curative outcomes to canines with cancer and other diseases; and how the future of companion animal medicine as a whole will undoubtedly include genetic cures to disease.

How we got here: a journey from past to present

In considering how gene therapy and gene editing are now mentioned alongside routine veterinary medicine applications, one must understand how abrupt and revolutionary the advances in these enabling technologies have been (**Figure 1**). Advances in the design, safety, and transduction efficiencies of gamma retroviral- and lentiviral-based gene delivery in the mid-90's and early 2000's were paramount to the pioneering study of CD19 targeting CAR-T cells in Phase I human trials in 2009 (Hucks and Rheingold, 2019) and the plethora of ongoing gene and cell therapy FDA approvals and trials using viral vectors (Shahryari et al., 2019). Moreover, recent analyses demonstrated the cost for a single pair of the first readily re-engineered, modular gene editor, Zinc-Finger Nucleases, was $25,000 as recently as 2012 (WareJoncas et al., 2018). Today, generating gene editing reagents like CRISPR/Cas9 in academic labs costs well under $100 per experiment. This substantial reduction in operating cost, democratizing access to enabling technologies and enhancing the efficiency of desired outcomes, has led to over 22,000 peer-reviewed publications mentioning CRISPR/Cas9 and over 29,000 peer-reviewed publications mentioning gene therapy in the last decade (PubMed search, January 2021). Here, we summarize seminal studies that have led to modern day gene therapy and gene editing, and their current applications in basic research and translational science, emphasizing dog models and/or applications where appropriate.

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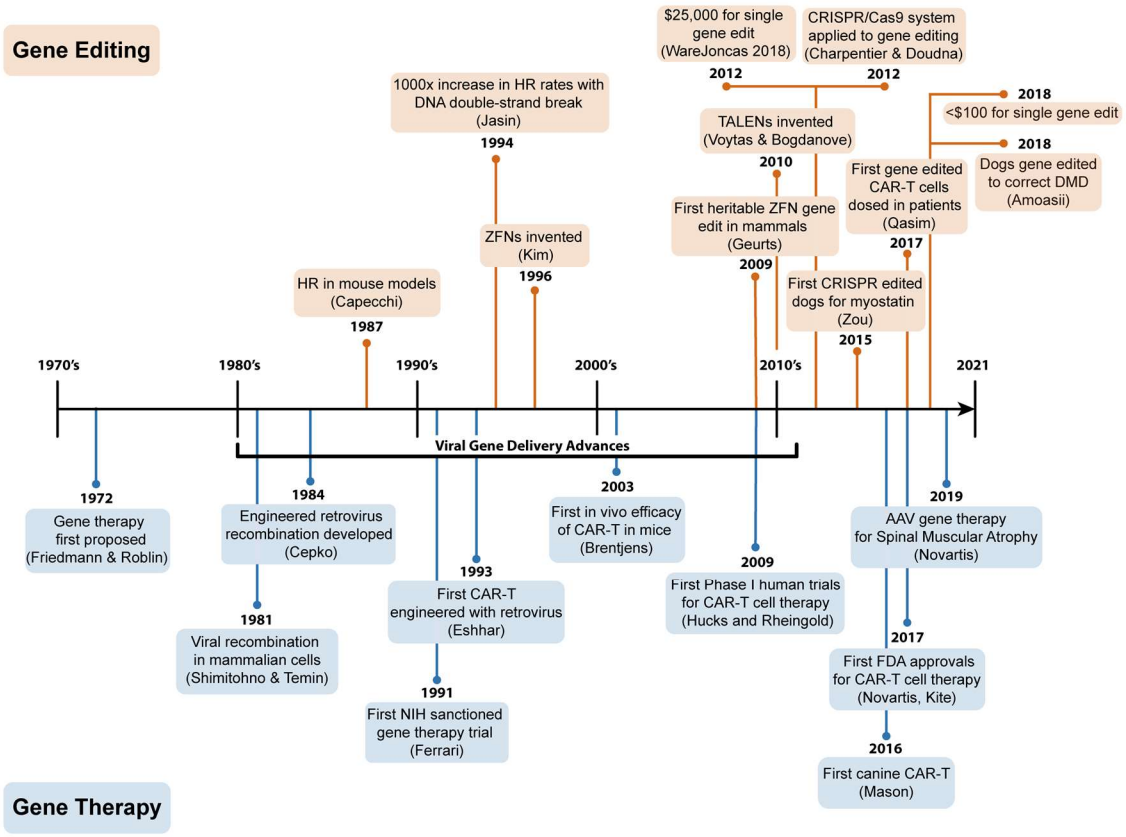

Figure 1: Seminal discoveries in gene editing and gene therapy leading to the proposal and application of these technologies in veterinary medicine.

Modern gene therapy

Gene therapy was introduced in biomedical sciences as early as 1972 by Theodore Friedmann and Richard Roblin after Stanfield Rogers came up with the idea to replace defective DNA for the treatment of inherited diseases (Friedmann and Roblin, 1972). While eukaryotic viral vectors were developed from DNA viruses in animals, none of these viruses provide solutions to recombine the viral genome into the host genome. Thus, the idea for gene therapy was not realized for decades due to limiting technologies for the introduction of foreign DNA into cells. In 1981, Shimotohno and Temin reported the first viral recombination of foreign DNA into mammalian cells using retrovirus; however the production of these retroviruses was inefficient, limiting wide adoption of the technology (Shimotohno and Temin, 1981). A pioneering study in 1984 demonstrated the first efficient technology for production of infectious virions and subsequent transfer of foreign DNA into mammalian cells using engineered retrovirus vector systems (Cepko et al., 1984), though the cost and scale of this approach did not allow broader use of the technology.

As research into using viruses as DNA delivery vectors continued to progress, warranted apprehension arose regarding the use of these infectious agents in the clinic. Therefore, modern gene therapy approaches that rely on viral vectors for DNA delivery utilize highly engineered viral

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particles wherein only a minimal amount of viral genome is retained. Non-replicative and tissue-specific viruses have been developed using such genetic engineering approaches which continue to drive innovations in safety, quality control, and efficacy of modern gene therapy.

Within the past 50 years, technological innovations in recombinant DNA technology have rapidly propelled advancements in gene therapy. Along with continuous gains in our understanding of molecular genetics and gene regulation brought about by the genetic revolution, methods to safely and efficiently deliver foreign genes into cells remains an active area of research. In particular, the development of viral vectors as gene delivery systems capable of stably integrating genetic cargo into the genome has shown clinical efficacy as years of incremental advances in the production and efficiency of retroviral recombination led to the first NIH-sanctioned gene therapy clinical research study in 1991 (Ferrari et al., 1991). In this pioneering study, two children afflicted with adenosine deaminase (ADA) deficiency were given retroviral transduced T cells containing a functional copy of the adenosine deaminase gene to rescue a severe combined immunodeficiency (SCID) phenotype.

Gene therapy-based interventions utilizing both viral and non-viral vectors in humans have continued to progress over the past 30 years with important implications for veterinary medicine. We will here discuss the foundational biology of retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses with special emphasis on the technological innovations and genetic interventions that have allowed these infectious agents to become some of the most widely utilized gene delivery systems for gene therapy. Examples of non-viral methods of gene delivery will be briefly discussed in the latter portion of this section. **Figure 2** summarizes these main mechanisms for introduction of genetic, protein, or small-molecule based material into living cells.

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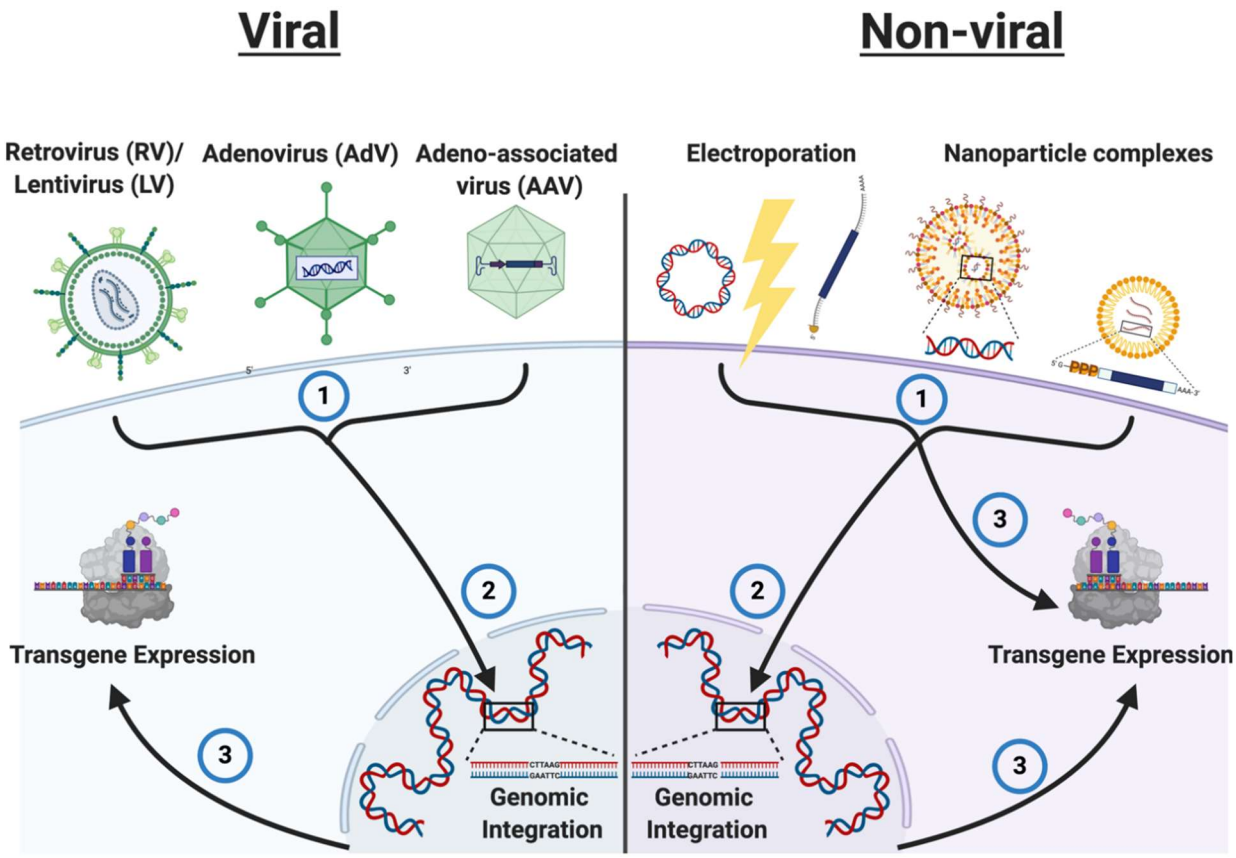

Figure 2: Shown are examples of viral (left) and nonviral (right) transgene delivery methods. **Left:** (1) Virus-based delivery using +ssRNA retrovirus (RV) or lentivirus (LV), dsDNA Adenovirus (AdV), or ssDNA Adeno-associated virus (AAV) mediate cellular entry through specific cell surface ligands or ubiquitously expressed proteoglycans. (2) Genomic integration of the transgene is mediated by processes exclusive to the viral vector utilized for delivery. Though these integration events mostly occur at random locations throughout the genome, in the case of AdV, cargo DNA is replicated and transcribed but not stably integrated. (3) Transgenes are expressed as functional proteins via mRNA translation by ribosomes. **Right:** (1) Nonviral delivery can be accomplished via temporary disruption of the cell membrane by physical methods, such as electroporation for the introduction of free nucleic acids, or with lipid nanoparticle complexes encompassing nucleic acids that enter cells indiscriminately through electrostatic interactions with glycoproteins and proteoglycans. (2) Transgene integration can be accomplished using enzyme-mediated processes that cleave genomic DNA and may include transposases or nucleases, such as CRISPR/Cas9. (3) Transgene expression via mRNA translation by ribosomes can occur transiently through direct inoculation of mRNA into cells or as a result of transcribed DNA which has been integrated into the genome or from an episomal source.

Viral gene delivery

RNA virus vectors: retroviruses and lentiviruses

RNA virus vectors derived from retroviruses (RVs) were one of the first viral delivery systems used for gene therapy and remain among the most used today. They are represented by a large family of enveloped viruses encompassing 7-12 kb plus-sense RNA genomes. Lentiviruses (LVs), including human immunodeficiency virus (HIV), represent a class of retrovirus often used for

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biomedical research and require the same basic components as other RVs for successful cellular entry and viral genome integration (Verma and Weitzman, 2005).

To employ the natural ability of RVs to facilitate gene transfer for either *ex vivo* or *in vivo* gene therapy applications, notable modifications to the viral genome were required to maximize safety and ensure efficacy before clinical benefit could be evaluated. RVs bring natural tissue tropism, like HIV's propensity to infect T cells, and are replicative in nature. Accordingly, first-generation lentiviral vectors were modified to include the envelope protein of vesicular stomatitis virus, VSV-G, which functions to broaden cellular tropism by targeting the low-density lipoprotein (LDL) receptor which is ubiquitously expressed (Finkelshtein et al., 2013). However, these first-generation vectors retained much of the original HIV genome and warranted broad safety concerns. Thus, known virulence factors were removed from the viral genome to produce second-generation lentiviral vectors (Vannucci et al., 2013). Third-generation lentiviral vectors were then developed to further improve safety wherein multiple modifications were incorporated to eliminate the possibility of producing replication-competent lentiviruses (Dull et al., 1998).

DNA virus vectors: adenoviruses and adeno-associated virus

Adenoviruses (AdVs) and adeno-associated virus (AAV) are the most prominent DNA viruses used for modern gene therapy applications. Although both viruses encapsulate a DNA genome within and nonenveloped icosahedral protein capsid, the AdV genome consists of ~36 kb dsDNA while AAV possesses a much smaller genome, ~4.7 kb, and is comprised of ssDNA. Similar to RVs and LVs, plasmids encoding essential viral components can be introduced into packaging cell lines to generate AdV and AAV vectors for transgene delivery; however, critical differences in virus replicative cycles necessitate unique modifications for their production and implementation as gene therapy agents (Verma and Weitzman, 2005).

The human AdV family contains over 50 unique serotypes with a wide tissue tropism. The majority of these viruses utilize the Coxsackie-adenovirus receptor for cellular attachment (Philipson and Pettersson, 2004) and internalization is facilitated through an interaction with cellular integrin αv receptors (Wickham et al., 1993). AdV DNA is not normally integrated into the host genome (Pombo et al., 1994) so, in terms of gene therapy applications, only transient transgene expression can be achieved with AdV vector systems. To ensure safety, essential viral replication genes were deleted in the first-generation AdV vectors which could incorporate transgenes ranging from 4.7-4.9 kb (Bett et al., 1993), and transgenes up to 8.3 kb where later cloned into second-generation AdV vectors wherein additional viral genes had been deleted. Although, it is noteworthy that recent developments towards third-generation AdV vectors have further enhanced the potential for efficient gene delivery and long-term transgene expression, high immunogenicity remains a hurdle that must be overcome before AdV vectors are to be widely used for gene therapy *in vivo (*Crystal, 2014).

AAV serotype 2, a nonpathogenic human parvovirus, is the best characterized AAV vector to date. AAV2 utilizes ubiquitously expressed heparan sulfate proteoglycan for cell attachment (Summerford and Samulski, 1998) and membrane internalization is facilitated by either the fibroblast growth factor receptor or integrin αvβ5 (Summerford et al., 1999) (Qing et al., 1999).

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In gene therapy studies, AAV vectors have demonstrated transduction in the muscle, retina, brain, liver, and lungs where a slow rise in transgene expression plateaued after a few weeks *in vivo* (Xiao et al., 1996). Although AAV vectors have been successfully used for gene therapy applications *in vivo* in animal models, a relatively small transgene capacity ~4 kb and a high prevalence of neutralizing antibodies directed against the viral capsid proteins represent significant limitations (Verma and Weitzman, 2005).

Non-viral methods of gene delivery

It is important to note that non-viral methods indeed exist for delivery of proteins and nucleic acids into cells, however they are not typically considered part of the "gene therapy" class, as they are all currently utilized *ex vivo* for cell programming. Although viral vectors are currently the most efficient approach used to deliver DNA cargo into host cells *in vivo*, non-viral approaches are becoming much more common due to a better potential safety profile and technical advantages in use and production. From a biosafety perspective, viral vectors can be highly immunogenic and lead to adverse inflammatory reactions in patients such that, in 1999, the first gene therapy-related fatality was reported in a clinical trial due to an inflammatory reaction in response to an adenovirus (AdV) vector (Lehrman, 1999). A phenomenon known as 'insertional mutagenesis', wherein the chromosomal insertion of viral DNA unintentionally results in cellular transformation by either disrupting the expression of a tumor suppressor gene or activating an oncogene, represents another potential safety concern regarding the use of viral vectors in gene therapy. Additionally, as viral vector-mediated integration into the genome can occur at random loci, each individual engineered cell is theoretically different. Ultimately, this leads to heterogeneity of the resulting therapy with potential consequences resulting from differential expression of the cargo gene. For these reasons, non-viral vectors are widely considered to be a safer alternative (Ramamoorth and Narvekar, 2015) and, due to advances in nanotechnology, *in vivo* applications for non-viral delivery systems have recently been realized in the context of gene therapy (El-Sayed and Kamel, 2020).

The flexibility of non-viral delivery systems is a notable advantage as they can be used to introduce various types of nucleic acids into cells including chemically synthesized small DNA molecules (Oligodeoxynucleotides), large DNA molecules (plasmid DNA), various RNA molecules such as ribozymes, small interfering RNAs (siRNA), and messenger RNAs (mRNA), and even proteins directly into cells. Although many nucleic acids can be passively delivered to cells via endocytosis, physical methods of gene delivery can help facilitate the introduction of genetic material into cells by temporarily disrupting the cell membrane using physical forces. Common physical methods of gene delivery include microinjection, electroporation, sonoporation, particle bombardment, and magnetofection (Ramamoorth and Narvekar, 2015). Chemical carriers can also be used to deliver nucleic acids into cells. These delivery systems are most commonly made of a nucleic acid complexed with either cationic lipids (Lipoplexes), cationic polymers (Polyplexes) or a combination of cationic lipids and polymers (Lipopolyplexes) (Midoux et al., 2009). These chemical complexes function to protect encapsulated nucleic acids from degradation and enhance their intracellular uptake via electrostatic interactions with glycoproteins and proteoglycans on the cell membrane. Nucleic acid complexes may also influence intracellular trafficking of nucleic acids. For instance, the cationic polymer polyethylenimine (PEI), disrupts

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endosomal membranes resulting in the translocation of the complexed nucleic acid into the cytosol and cationic peptides comprised of basic residues, such as lysine and/or arginine, can be employed to target specific cell surface receptors or provide nuclear localization signals to facilitate the nuclear entry of cargo DNA (Al-Dosari and Gao, 2009).

Modern gene editing

Gene editing allows the mutation or alteration of DNA at specific locations in the genome *a priori* (Yeh et al., 2019). Gene editing can be used as a basic scientific tool to heighten understanding of disease pathophysiology using cell systems and animal models, including complex disease like cancer, as well as monogeneic disorders such as cystic fibrosis, hemophilia, sickle cell disease, heart disease, and human immunodeficiency virus (HIV) infection (Doudna, 2020). Multiple approaches to gene editing have been developed in the 21st century, but the crux of the technology requires the ability to direct enzymatic activity to a chosen locus in the genome. To achieve this, scientists use “programmable nucleases” that induce DNA damage in the form of a double-strand break or nick at specific loci in the genome. Whereas gene editing is the broader concept, programmable nucleases are the critical tool for the modern development of cell-based therapies and gene therapies. Programmable nuclease-induced DNA double-strand breaks can result in random mutations around the break, specific alterations of endogenous base pairs using homology templates, or the targeted integration of exogenous DNA via an engineered donor template.

What makes programmable nucleases so powerful is their ability to induce DNA repair at their target sites, allowing scientists to rewrite the genetic code at these genomic loci. Though oversimplified, an effective analogy converts the genome to a word document, the nucleases to a mouse cursor, and donor DNA templates to a keyboard. Scientists can “click” the genome, and “delete” or “rewrite” the paragraph surrounding the cursor. Sometimes referred to as “genome writing”, this technology gives scientists endless possibility towards inducing targeted mutations, correcting genetic defects, or giving cells an ability to perform functions that nature did not evolve (Doudna, 2020).

Brief history of gene editing tools

Modern gene editing ideals can be traced back to seminal works by Dr. Mario Capecchi and Dr. Maria Jasin. Dr. Capecchi, who later won the 2007 Nobel Prize of Physiology and Medicine for his work, pioneered strategies using the DNA repair pathway homologous recombination to specifically engineer mouse embryonic stem cells and mouse models using targeted DNA integration (Thomas and Capecchi, 1987). Dr. Jasin’s pioneering discovery demonstrated that targeted DNA integration, that is the addition of exogenous DNA to a locus of interest through homology-directed repair, can be stimulated 100-1000 times over Capecchi’s work by causing a double-strand DNA break at the locus of interest in the presence of a repair template (Rouet et al., 1994). This finding has served as a seminal discovery in the field of gene editing. However, the nuclease used in this case, I-SceI, is a large restriction enzyme whose endogenous DNA binding activity cannot be reprogrammed to target DNA outside of its restriction site, as possible with modern programmable nucleases like Transcription Activator Like Effector Nucleases (TALENs) (Joung and Sander, 2013), and CRISPR systems (Jinek et al., 2012). Zinc-Finger

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Nucleases, as well as some homing endonucleases like I-CreI (MacLeod et al., 2017), pre-date TALENs and are used today in clinical trials (Tebas et al., 2014), but limitations in their genomic targeting range and expense to engineer generally relegate them to use in industry settings and they will not be discussed here.

TALENs were invented in 2010 by Bogdanove, Voytas, and colleagues (Christian et al., 2010), after fusing the FokI nuclease to a bacterial transcription factor, called a TAL effector. In nature, TAL effectors evolved to bind to promoters and activate host genes in plant:pathogen interactions (Moscou and Bogdanove, 2009). TAL effectors rely on highly conserved, modular repeats containing 34-35 amino acid loops repeated 12-20 times, with 2 amino acid residues in each loop specifying near 1:1 nucleotide binding. Thus, through the addition of the FokI nuclease, individual TALENs can be engineered to bind short stretches of DNA to induce a double-strand DNA break. The FokI used is an obligate heterodimer that requires another TALEN to be supplied to the target site spaced 12-20 base pairs opposite of the first. TALENs require relatively complex, multi-day cloning reactions to engineer a single TALEN pair for gene editing. Even still, TALENs have been used to target DNA breaks, inducing mutations, or used for exogenous gene integration in basic research, translational science, plant biotechnology, and clinical settings for a decade.

The most widely adopted programmable DNA binding agent, and 2020 Nobel Prize of Biochemistry winning technology, is called CRISPR/Cas9, short for clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9. Evolved as a bacterial immune system to catalog and resist bacteriophage, CRISPR systems are now utilized to target DNA double-strand breaks, DNA nicks, targeted nucleotide edits, or exogenous gene integrations to precise loci in genes of interest on a routine basis. While TALENs and ZFNs require the engineering of entirely new proteins to target different genes, the power of CRISPR systems is that the genomic target site is specified by a short, ~20 base pair RNA molecule (called a guide RNA or gRNA) which, with the help of Cas enzyme DNA melting, is able to bind 1:1 to a homologous match through simple RNA:DNA base pairing. Engineering CRISPR to bind new genomic target sites is thus as simple as *in vitro* transcription of gRNAs specifying a new target site or ordering an RNA primer from a nucleic acid synthesis company. While CRISPR/Cas9 technology specifically won a 2020 Nobel Prize, studies have shown that up to 40% of bacteria and 90% of archaea contain at least 1 functional CRISPR system (Westra and Levin, 2020). These systems differ in their CRISPR-associated proteins, guide RNAs, nucleic acid binding kinetics, and nucleolytic activities, but all accomplish the same feat; targeting enzymatic activity to a genomic locus of interest to induce gene editing.

Outcomes of gene editing

For simplicity, we will narrow our focus of gene editing to the use of CRISPR tools, but the ideas that follow generally hold true for other programmable nucleases. In most applications, these tools are combined with gene delivery as discussed above:

After CRISPR induces a double-strand DNA break, cells work to repair it using one of two major pathways generally called “non-templated repair” or “templated repair” (**Figure 3**). These pathways differ in their outcome pending the availability of a repair template either supplied by a

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sister chromatid for endogenous repair or a donor DNA template for genome writing applications. Two specific pathways for non-templated repair are non-homologous end joining (NHEJ), which results in random insertions or deletions (indels) and microhomology mediated end joining (MMEJ), which results in short deletions but can be predicted (Bae et al., 2014) (Ata et al., 2018). While viral gene therapy brings the ability to randomly supply diseased cells with donor DNA carrying a corrected version of defective proteins to treat diseases, CRISPR mutations are rarely therapeutic in and of themselves and require templated repair. Indeed, gene editing technologies rely on precision control of the DNA repair outcomes after CRISPR targeting to alter single or few nucleotides surrounding the target site or to integrate a cargo DNA for reverse genetics and functional genomic applications and for therapeutic benefit in the clinic.

When programmable nucleases cut their target DNA site, enzymes specific to sub-pathways of DNA repair compete to heal the double-strand DNA break (**Figure 3**) These pathways hinge upon the initial recognition of the double-strand DNA break and subsequent processing of the DNA ends for no, short-range, or long-range end resection.

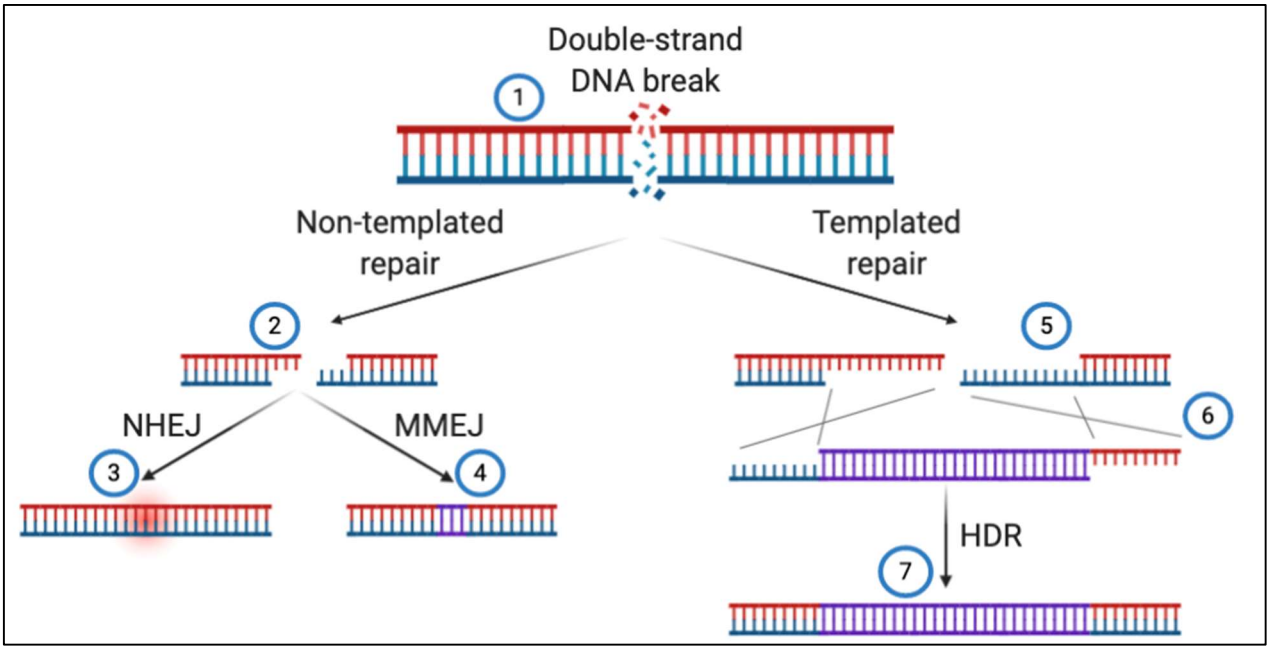

Figure 3: Shown are examples of how DNA double-strand breaks are corrected in gene editing applications. **(**1) A DNA double-strand DNA break is induced using a programmable nuclease, like TALENs or CRISPR/Cas9. (2) In non-templated DNA repair, little to no end-resection takes place. (3) Non-homologous end joining (NHEJ) resolves DNA breaks by short insertions or deletions (indels) represented by the error cloud. (4) microhomology meditated end joining is used when 2-5 bp homologies are present in DNA ends, resulting in a short deletion of the intervening sequence and one of the homologous sequences (displayed in purple). (5) In templated DNA repair, long range end resection results in >100bp ssDNA ends that are used as substrates for homology directed repair (HDR), either with a sister chromatid or, in gene editing, an exogenously supplied template. (6) Exogenously supplied templates, like double-stranded DNA, plasmids, AAV vectors, or ssDNA are built with homology to match the DNA break site. (7) Pending the donor template and changes to be introduced (SNPs or entire transgenes), HDR will proceed through either homologous recombination (HR), single-strand annealing (SSA), or MMEJ-like mechanisms to recombine exogenous DNA precisely into the genome.

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Non-Homologous End Joining (NHEJ)

The most common non-templated DNA repair outcome is called non-homologous end joining (NHEJ), which results in short insertions/deletions (indels) at the target site. In research settings, CRISPR is most often used in reverse genetics applications to generate indel mutations in the genome in order to abrogate gene function and study resulting phenotypes, though CRISPR is also particularly powerful for forward genetic screens. Indeed, CRISPR gene knockouts have been used in canine reverse genetics to study cardiovascular disease (Feng et al., 2018), Duchenne's muscular dystrophy (Mata Lopez et al., 2020), and even to generate germline edits of the myostatin gene (Zou et al., 2015). NHEJ activity requires KU70/80 to stabilize the DNA ends, preventing end resection, and DNA-PKcs (Chang et al., 2017). These proteins recruit other components, importantly DNA ligase IV which seals the broken DNA ends. Competition with end resection enzymes and more complicated DNA break structures (like 5' and 3' overhangs) result in the random indels associated with NHEJ.

Microhomology-Mediated End Joining (MMEJ)

Microhomology-mediated end joining (MMEJ) is another non-templated outcome of CRISPR-induced DNA breaks and competes directly with NHEJ (Black et al., 2019). The core difference between them is that MMEJ requires end resection to reveal short 3' overhangs. Short stretches (commonly 2-5 nucleotides) of homology are found by Polymerase Theta and LigIII is used to seal the DNA break, thus resulting in small deletions of the intervening sequence and one copy of the microhomology. Interestingly, CRISPR target sites can be chosen *a priori* to induce MMEJ, thus resulting in predictable DNA repair outcomes, increasing the likelihood of an outcome of interest (Martinez-Galvez et al., 2021). The ability to select CRISPR target sites that will result in predictable outcomes is invaluable in research settings to ensure that a resulting mutant will carry an out of frame deletion and early translational stop codon to abrogate gene function. More importantly, in therapeutic settings, the ability to select MMEJ sites in somatic tissue editing brings predictable outcomes to clinical applications. For example, 1 in ~1000 cases of human Limb-Girdle Muscular Dystorphy 2G (LGMD2G) contain an 8bp duplication in exon 1 of *TCAP* (Nigro and Savarese, 2014). Using CRISPR/Cas9, this mutation has been successfully reprogrammed to WT in up to 57% of alleles in patient derived iPSCs using MMEJ repair (Iyer et al., 2019). Not all diseases, however, carry such defined genomic signatures to be exploited for reversion to wild type. In these instances, scientists could use diseased cell- and tissue-based genomics to identify MMEJ-prone CRISPR target sites that may abrogate gene circuits involved in pathologies.

Homology-Directed repair (HDR)

Homology-directed repair, as the name suggests, is the general set of pathways that uses homologous templates to direct DNA break repair (Jasin and Rothstein, 2013). After endogenous DNA breaks e.g., from DNA replication stress or radiation, the cell uses a sister chromatid as template. However, in gene editing applications, scientists supply an exogenous repair template embedding the changes desired. Pending the desired edit, multiple sub-pathways of HDR are utilized:

Templated repair to induce single nucleotide polymorphism (SNP) changes

- Most often, templates for SNP changes are <100bp single-stranded oligonucleotides (ssODNs) containing 50bp homology arms flanking the desired

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SNP. The first demonstrated use of ssODNs *in vivo* was developed using TALENs (Bedell et al., 2012), and subsequent deployment with CRISPR systems has shown this is a universal HDR strategy to achieve single or few nucleotide changes desirable to rescue a mutant phenotype, like the A to T SNP in sickle-cell anemia (Frangoul et al., 2021). The molecular mechanisms of this type of targeted DNA repair change are currently the subject of debate, but most likely involves combinations of the Fanconi Anemia pathway important to stabilization of stalled replication forks, and the synthesis dependent strand annealing (SDSA) pathway (Yeh et al., 2019). In this model, it is speculated that the homology supplied in the ssODN anneals with a matched, resected 3' DNA end, and subsequent DNA polymerase activity incorporates the desired changes before the template dissociates. Whether or not the template itself is incorporated into the chromosome is currently unknown.

- It is also possible to utilize longer homology templates to incorporate single or multiple nucleotide changes into genes of interest. In general, the mechanisms governing this type of templated gene editing fall under homologous recombination (HR). HR is well known for its role in meiosis but can also be induced by supplying donors with >500 bp homology arms flanking an insert of interest (Yeh et al., 2019) (Jasin and Rothstein, 2013). HR proceeds after MRN/CtIP complex-induced long range end resection involving many components, importantly the 5'-3' exonuclease ExoI. After DNA ends are resected, the RAD family controls the precision of repair through RAD51 DNA end stabilization, homologous template search and sampling, RAD52 mediated strand invasion, annealing, and subsequent template polymerization and end capture (Yeh et al., 2019). Many types of donors have been used to induce this type of repair, including adeno-associated viral templates, ssDNA, linear dsDNA, and plasmid DNA. The use of these types of donors and the requirement for homologous recombination is arguably less efficient than the use of ssODN for SNP incorporation.

Templated repair to incorporate new genes

- For many gene-edited cell therapy applications, scientists do not wish to fix single nucleotide polymorphisms, but need to add exogenous DNA to educate cells with new functions that nature did not evolve. In research applications, this could be the addition of DNA encoding fluorescent proteins to tag spatiotemporal gene expression patterns in model organisms and cell lines (Wierson et al., 2020). In the clinic, the best example is the addition of DNA encoding chimeric antigen receptors to impart tumor killing activity onto T cells. Whatever the application, the incorporation of multi-kilobase gene cassettes into CRISPR induced double-strand DNA breaks generally requires a repair template carrying homology arms that flank the DNA break. Just like in templated SNP alterations, the homologous recombination pathway of DNA repair is a commonly employed mechanism for these alterations. There are publications demonstrating the efficacy of many types of templates to accomplish this feat; single stranded or double stranded linear DNAs (Roth et al., 2018) (Shin et al., 2014), plasmid DNA (Wierson et al., 2020), or rAAV donors (MacLeod et al., 2017) to mediate site-specific integration of this

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DNA. More recently, scientists have discovered that delivering a donor template with CRISPR sites flanking homology arms and cargo DNA is an efficient way of inducing a sub-pathway of HDR, dubbed homology-mediated end joining, to create engineered model organisms and cell lines (Wierson et al., 2020, Wierson et al., 2019) (Yao et al., 2017) (Hisano et al., 2015).

Base editing for SNP changes

An alternative form of CRISPR-based single nucleotide alterations is accomplished through the fusion of "base editors" to Cas enzymes. Base editors can be used to alter single nucleotides in DNA or RNA. The first base editor, engineered by David Liu's lab in 2016, is a fusion of a Cas9 nickase enzyme (so that only a single-strand DNA break is induced) to the cytidine deaminase enzyme APOBEC1 (Komor et al., 2016). Nicking the non-base edited DNA strand using a Cas9-nickase induces a mismatch repair pathway that effectively "tricks" the cell to use the uracil as a template for DNA replication, thus converting the target cytosine/guanine base pair to a thymine/adenine base pair. Similarly, the Liu lab used directed evolution to engineer an adenosine deaminase that can effectively target adenine/tyrosine base pairs for conversion to guanine/cytosine (Gaudelli et al., 2017). With these two tools and their enhanced versions, 63% of known pathogenic single nucleotide variants could theoretically be corrected. Additionally, an advantage of base editing is that no double-strand DNA break has to be generated, as emerging concerns with DNA breaks have been noted (Cullot et al., 2019) (Kosicki et al., 2018) (Ihry et al., 2018). An elegant review was recently published on the advances and opportunities for furthering base editing technology (Porto et al., 2020).

Armed with the toolboxes of viral integration, gene editing, and non-viral gene delivery, scientists now have control over the genome at scale and resolution never before imagined to engineer living therapies.

Revitalizing cells, tissues, and organs *in vivo*

Human clinical gene therapy

Numerous experimental gene therapy trials have been initiated with varying degrees of success, leading to the first FDA-approved gene therapy in humans in 1998, Vitravene (Fomivirsen®) (Stein and Castanotto, 2017). This antisense oligodeoxyncleotide was indicated for the local treatment of cytomegalovirus (CMV) retinitis in immunocompromised patients but was later removed from the market in 2002 and 2006 in the EU and U.S, respectively (Stein and Castanotto, 2017). In Europe, the very first gene therapy ever approved was based on a recombinant adeno-associated virus (rAAV) vector to treat familial lipoprotein lipase deficiency (Alipogene tiparvovec, marketed under the trade name Glybera®) (Yla-Herttuala, 2012). Since 1998, 22 gene therapy products, including naked nucleic acids, non-viral and viral vectors, as well as cell-mediated therapy have been approved for commercialization and are elegantly described in a 2020 review by Ma and colleagues (Ma et al., 2020).

Advantages of canine models for gene therapy

Animal models serve a critical role in biomedical research both as basic science tools for elucidating molecular mechanisms as well as for the preclinical evaluation of novel therapies.

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Dogs play an integral role in modern society and enhance the lives of countless individuals by providing both mental and physical assistance, security, and companionship. The domestic dog has also been recognized as a valuable model of monogenic diseases in humans and possesses key advantages over inbred rodent models classically used for preclinical studies regarding gene therapy. Not only do causative mutations spontaneously occur in dogs as they do in humans, detailed pedigrees and opportunities to evaluate treatments over a long-term, often years, provide crucial advantages in a preclinical setting (Switonski, 2020).

Specifically, immunotherapies present unique opportunities to be tested *in vivo* in dogs before formal evaluation into human clinical trials, since an intact immune system and tumor microenvironment can be adequately modeled in the dog (as opposed to mice). This is particularly relevant for future accelerated development of many novel treatment options, including, among others, immune checkpoint inhibitors, CAR-T cell, and adoptive T-cell transfer therapies in both veterinary and human oncology.

Also, it has been well-established that spontaneous tumorigenesis occurs in dogs through similar mechanisms as what is known to occur in humans. Although preclinical studies in dogs have contributed to the development of cancer therapeutics for human medicine, approaches utilizing gene therapy remain limited in this regard, as gene therapy is not an explicit class of therapeutic options for oncology.

One could ponder how spontaneous disease in dogs could again be of use to test novel hypotheses for gene therapy-based solutions in oncology that correct genomic risks in somatic tissues. One such hypothesis involves hijacking a cancer cell's reliance MMEJ. As MMEJ is inherently mutagenic, cancers carry many genomic hallmarks of MMEJ-based re-arrangements (Alexandrov et al., 2020), as well as overexpression of protein components involved in MMEJ repair (Lemee et al., 2010). Drugs are currently under development that can induce lethality, synthetic or otherwise, at the protein level. Theoretically, as tissue specific *in vivo* gene delivery continues to advance, one could imagine a scenario where gene therapy is combined with gene editing to exploit MMEJ to correct driver mutations or to induce synthetic lethality in cancer cell populations. As discussed at length in the cell therapy section below, we are most interested in the use of gene edited cell therapies (not explicitly "gene therapies") in oncology. However, we are interested in testing these hypotheses in spontaneous cancers via collaboration with *in vivo* gene delivery and gene therapy experts. Given the few formal descriptions of gene therapy that have been described in veterinary oncology (Thamm, 2019), examples of translational medicine regarding gene therapy outlined herein apply specifically to monogenic diseases.

A special issue of *Human Genetics* published in 2019 highlights many recent discoveries regarding monogenic diseases in dogs that have significant implications for human health (Shaffer, 2019). For instance, variants in the *TTN* gene are known to contribute to cardiomyopathies in humans and a missense mutation in *TTN* was discovered in Doberman pinscher dogs with diagnosed dilated cardiomyopathy (Meurs et al., 2019). Another group studying amelogenesis imperfecta (AI) in Parson Russell terriers and Akita dogs identified variants in the *ENAM* and *ACP4* genes which are implicated in the pathophysiology of human AI,

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further highlighting relevant physiological similarities between humans and dogs (Hytonen et al., 2019).

There have been numerous successful proof-of-concept gene therapy studies in dogs, both as a model for human translational application and to solve pressing needs in canine health. As of 2017, hundreds of gene therapy trials have been undertaken to deal with monogenic diseases and researchers have recently been able to shed light on the molecular mechanisms underpinning many disorders to pave the way for novel interventions utilizing gene therapy. Beginning in 1993, the First International DogMap Meeting in Oslo, Norway sought to gain a more in-depth understanding of the genetics driving approximately 700 monogenic diseases known to afflict dog

Table 1: Description of monogeneic diseases in dogs, their counterpart in humans, genetic drivers, and any dog or human gene therapies developed for the disorders.

Dog Disease	Human counterpart	Known genetics	Breeds affected	Gene therapy
Congenital stationary night blindness (CSNB)	CSNB/Type-2 Leber's congenital amaurosis (LCA2)	Recessive *RPE65* (Aguirre et al., 1998)	Briard	AAV delivery of WT *RPE65* (Acland et al., 2005), Human trials (Testa et al., 2013) (Schimmer and Breazzano, 2015) led to FDA approvals for humans in 2017
Muscular dystrophy	Duchenne's muscular dystrophy	*DMD* mutations (Yiu and Kornberg, 2015).	Retrievers, Pointers, Rottweilers, Spaniels, Corgis, Terrier	CRISPR/Cas9 to restore *DMD* function (Amoasii et al., 2018)
Hemophilia A and B	Hemophilia A and B	*Coagulation factor VIII* and *Coagulation factor IX*	Setters, Schnauzers, Pointers, Lhasa Apso, Retriever, Terrier/Beagle	Viral delivery of *FVIII* and *FIX* in dogs (Nichols, et al., 2016), AAV gene therapy in humans (Peyvandi and Garagiola, 2019)
Severe combined immunodeficiency (SCID)	SCID	Mutations in DNA-PK, RAG1, IL2RG (Switonski, 2020)	Terriers, Frisian Water Dogs, Hounds, Corgis	Retrovirus gene replacement in dogs (Ting-De Ravin et al., 2006)

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Leukocyte adhesion deficiency (LAD)	LAD I, LAD III/CLAD I, CLADIII	Mutation in *ITGB2* (Zimmerman et al., 2013), *FERMT3 (*Hugo and Heading, 2014)	Setters, German Shepherds	Retrovirus gene replacement of *ITGB2* in dogs (Bauer et al., 2013)
Lysosomal storage diseases	MPS I, MPS IIIB, MPS VI, and MPS VII	Mutations in *GUSB*	German shepherds, mixed	Retrovirus gene replacement of MPS VII (Xing et al., 2013)

breeds (Switonski, 2020). Although analysis of genome sequence variations between different dog breeds is currently underway (Ostrander et al., 2019), known causative mutations are continuously being identified and approximately 430 monogenic diseases in dogs have the potential to serve as preclinical models for homologous human diseases (Switonski, 2020). In fact, gene therapy has shown efficacy in treating many monogenic diseases that afflict both canines and humans and positive results in preclinical canine studies have resulted in the initiation of multiphase clinical trials. These diseases are represented among multiple organ systems and include ocular diseases, muscular dystrophy, hemophilia, severe combined immunodeficiencies, leukocyte adhesion deficiencies, and lysosomal storage diseases (Table 1).

Engineering living cells *ex vivo* for therapeutic use

Mainstream application of viral transduction, gene editing technologies, and most recently non-viral gene delivery into cells *ex vivo* has led to a massive shift in the way scientists think about disease treatment. It is now routine to “educate” cells *ex vivo* with genetic information to impart therapeutic benefits with the intention of transplanting them back into diseased patients. However, only in a handful of research groups are developing these strategies for dogs, with only two clinical reports to date (Panjwani et al., 2016) (Panjwani et al., 2020). Here, we will focus on the enabling technologies and their use in humans, knowing that translation of this technology to canines is sure to follow.

In the context of oncology, the most commonly engineered cell used to date is the T cell. Though there are many subtypes of T cells, CD8+ “cytotoxic” T cells are naturally equipped to rid the body of virally infected cells and even some malignant cells through MHC Class I peptide recognition with the T cell receptor (Kumar et al., 2018). Armed with this knowledge, scientists long sought to reprogram the T cell receptor to engineer T cell specificity and killing. Indeed, the idea to use genetically reprogrammed T cells as therapy began in 1992 with a pioneering study by Michel Sadelain reporting the use of retroviral transduction to engineer T cells with exogenous DNA. Later, in 1993, Zelig Eshhar generated the first chimeric antigen receptor (CAR) by fusing an antibody domain to the CD3ζ domain of a T cell receptor in what has become known as a “1st-generation CAR”. Studies in the late 1990’s and early 2000’s set the field ablaze by generating “2nd-generation CAR-T cells” containing a costimulatory domain in addition to the antibody and

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CD3ζ signaling domain (Maher et al., 2002), which culminated in the first report of *in vivo* efficacy of CAR-T cells by targeting CD19 on leukemic B cells in a mouse model (Brentjens et al., 2003).

Numerous subsequent reports and clinical trials utilized CD19-targeting, autologous CAR-T cells generated from T cells isolated from the patient's peripheral blood mononuclear cells (PBMCs) (Hollyman et al., 2009). These efforts culminated in two FDA approvals in 2017 for relapsed and refractory acute lymphoblastic leukemia (ALL) and diffuse large B cell lymphoma (DLBCL) and one FDA approval for mantle cell lymphoma (MCL) in 2020 (Neelapu et al., 2017) (Maude et al., 2018) (Wang et al., 2020). To date, the FDA approved CAR-T cell therapies are engineered via lentiviral transduction of nucleic acid encoding the CAR and are all autologous, using no further gene editing. However, there are numerous FDA-sanctioned clinical trials ongoing with gene-edited CAR-T cells, both autologous and allogeneic, with various alterations to the manufacturing process and resultant gene edited cells. As of January 2020, over 900 potential Investigative new Drug (IND) applications are in the queue for cellular and/or gene therapy applications https://www.fda.gov/news-events/press-announcements/fda-continues-strong-support-innovation-development-gene-therapy-products.

Proof of concept CAR-T cell therapy in dogs

As noted in this chapter and described at length in [Nicola Mason's chapter], the only peer reviewed literature for CAR-T cell therapy in canines is via work from the University of Pennsylvania with two publications by Panjwani et al., (Panjwani et al., 2016, Panjwani et al., 2020). In these seminal studies, the authors used either mRNA or retroviral transduction of canine T cells to generate doses of autologous CAR-T cell therapy designed to target CD20 on B cell lymphomas. The first proof-of-concept experiment showed limited effect, and while the CAR-T cells did traffic to the lymph nodes and reduce tumor burden, this effect was only transient, primarily due to the nature of mRNA-based CAR expression, which are not stably integrated.

In the second publication by Panjwani et al., 5 dogs were treated with stably transduced autologous CAR-T cells, though the doses of CAR-T cells varied in every dog and were all far lower than the target 1-3x10^6 CAR-T cells/kg used in humans. Canine anti-mouse antibodies were detected, likely in response to the murine-derive CAR itself, which the authors postulated triggered rejection of the CAR-T cells and subsequently limited clinical benefit. In any case, the authors concluded that CAR-T cells were detectable in dogs, had modest anti-tumor activity, and, in some instances, selectively forced CD20 antigen loss on malignant B cells, indicating that the dog as a model can faithfully recapitulate pitfalls previously described in human CAR-T cell therapy studies (Majzner and Mackall, 2018) (Maus et al., 2013) (Enblad et al., 2015).

In addition to canine CAR-T cell therapy being developed for B cell lymphoma, there is a report of this modality being applied to the treatment of glioma in dogs (Yin et al., 2018). In this study, scientists aimed to develop a novel antigen binding protein to develop CAR-T cells that target IL-13Rα2, commonly found on both human and canine gliomas, but not on healthy tissues. Solid tumors like glioma are historically harder to treat with CAR-T cell therapy than liquid cancers, such as leukemia and lymphoma (O'Rourke et al., 2017). Yin et al., engineered human cells expressing a cross-reactive CAR for both human and canine IL-13Rα2 and showed it effectively targeted IL-

13Rα2 in both species *in vitro* and was effective *in vivo* towards an orthotopic mouse model of canine glioma. Interestingly, the authors engineered both canine and human cells with IL-13Rα2 CARs, and showed that both elicit killing of IL-13Rα2 positive cells, indicating that human T cells can kill canine cells when armed with a CAR specific for a canine antigen. The authors concluded that they are moving forward to enroll a pilot trial to use this technology towards fighting canine gliomas as a preclinical model of the approach in humans (Herranz et al., 2016).

It is notable to discuss here that there are currently multiple industry groups in the United States offering autologous tumor vaccinations or "adoptive T cell transfer" to fight differing cancers, which is quite different than CAR-T cell therapy. Respectively, dogs are either vaccinated with their own tumors to induce a cellular immunity response to the cancer, or T cells are isolated from dogs, grown in the presence of tumor neo-antigens, and reinfused in the hopes that the cells naturally acquire tumor antigen specific killing properties (O'Connor and Wilson-Robles, 2014, O'Connor et al., 2012). Data on these approaches in dogs is currently scant with various stages of regulatory approvals ongoing, however it is well established in humans that genetically reprogramming cells with anti-cancer properties (i.e., CAR-T) is absolutely a more effective approach to induce durable disease remissions.

Gene-edited cell therapies in dogs

With Dr. Mason's pioneering work, the translation of CAR-T cell therapy from humans to companion dogs emerging. While the work in the Mason lab is so far the only peer-reviewed literature for *in vivo* trials of CAR-T in dogs, publicly available knowledge for awarded federal and foundation grants gives us a glimpse of what is to come in this area. These include federal grant awards to Dr. Nicola Mason (3U24CA224122-02S1), Dr. Carl June and Dr. Gerald Linette (1U54CA244711-01) and other colleagues at the University of Pennsylvania (5R01AR075337-03), foundation awards to Dr. Heather Wilson-Robles and colleagues at Texas A&M University (AKC-CHF #1418), and a Small Business Innovation Research award to the biotechnology company LifEngine Animal Health Laboratories (LEAH Labs, NSF #2006130). In all instances, the scientists note the need for better canine therapies and seek to use the high-impact potential of spontaneously occurring disease in a large animal model system to study the effects of novel cell therapies. As mentioned above, the opportunity to support parallel development of novel drug candidates in veterinary and human oncology through a One Health approach could increase availability of revolutionary treatment options in animal health, while reducing attrition rates in Phase II clinical programs. The public summary statements of these grants indicate that gene editing is being used in the development of novel cell-based
therapies; however, to date, there are no peer-reviewed reports of gene-edited cell therapies for dogs.

One of the biggest challenges to overcome in regards to successful translation of CAR-T cell therapy into canines is the cost to deliver the therapy to patients. Notably, the current cost of FDA-approved CAR-T cells in human medicine ranges from $373,000 to $475,000 represents a large barrier to effective translation of this technology to canines. This cost is largely due to the need for individualized manufacturing, lentiviral preparations, FDA regulation, and GMP manufacturing standards. In considering autologous cell therapy in dogs, apheresis to obtain PBMC populations

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from which to generate cell therapies costs >$2,000. This feature, coupled with the labor-intensive processes of isolating, culturing, engineering, and expanding canine T cells, will likely result in a therapy that costs well over $10,000, vastly limiting adoption of this breakthrough therapy to the broader veterinary community. Gene editing could alleviate much of this cost burden by allowing the engineering of allogeneic CAR-T cell therapy, such that one manufacturing run would essentially generate enough material to dose multiple, if not 10 or 100 dogs with a single batch. Fortunately, research and clinical trials for human CAR-T cell therapy is paving the way for allogeneic cell therapy to be possible in dogs (Jung and Lee, 2018).

However, as these challenges are overcome and as ongoing cell therapy trials in humans for a litany of oncologic disorders progress, one can envision how the use of gene editing technology will surely allow the translation of these technologies to companion dogs for the same types of cancers (**Figure 4**). Moreover, as will be discussed below, canines may serve as an important model for these and new cell therapy types as different treatments for new indications emerge in humans.

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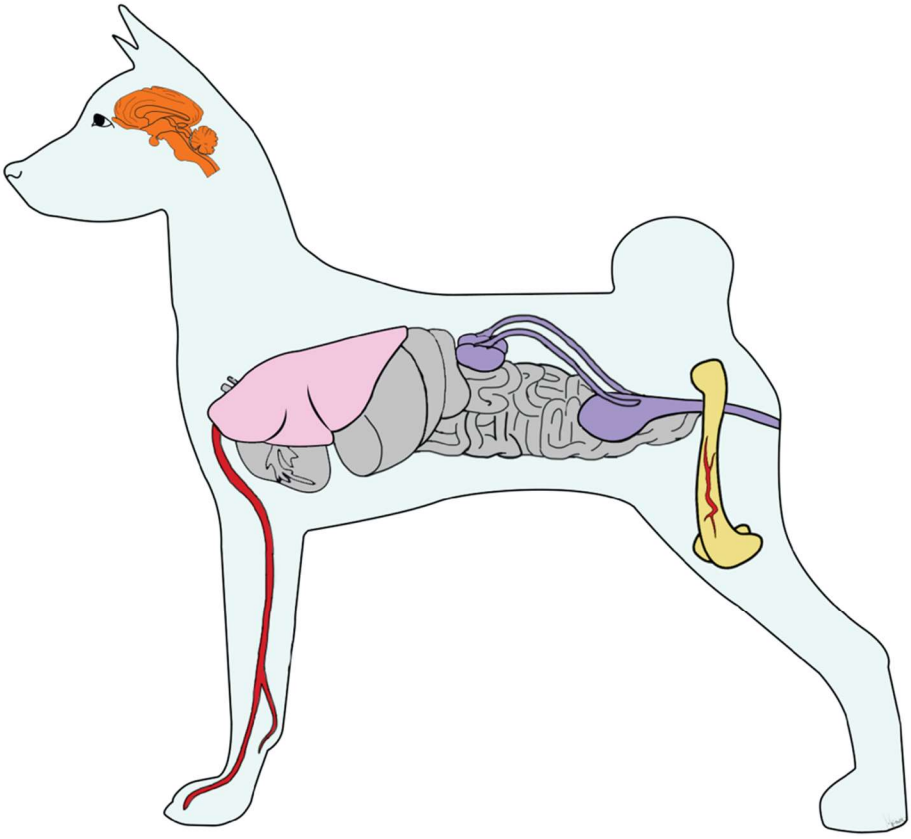

Brain/CNS
Glioma and Glioblastoma

- Shared mutations: PDGFRA, PIK3CA, and EGFR
- Clinical targets: GD2, EGFR, HER2, IL-13Ra
- Trials: GD2 CAR-T (NCT04196413), EGFR CAR-T (NCT01454596), HER2 CAR-T (NCT03500991), IL-13Ra CAR-T (NCT04003649)

Urinary System
Urothelial carcinoma

- Shared mutations: EGFR, HER2, CDKN2A, CDKN2B, BRAF, PIK3CA, BRCA2
- Clinical targets: EGFR and HER2
- Trials: HER2 CAR-T (NCT02713984)

Lung
Pulmonary carcinomas

- Shared mutations: HER2, TP53, PTEN, SMAD4, KRAS
- Clinical targets: CEA and HER2
- Trials: CEA CAR-T (NCT04348643), HER2 CAR-T (NCT02713984)

Blood/Marrow
B cell Leukemia & Non-Hodgkin's lymphoma

- Shared mutations: BCL2, TRAF3
- Clinical targets: CD19 and CD20
- FDA approved CAR-T therapies: Kymriah, Yescarta, Luxturna

Soft Tissue
Mammary carcinoma

- Shared mutations: CDKN2A, PTEN, CDH1 and TP53
- Clinical targets: CEA and HER2
- Trials: CEA CAR-T (NCT04348643), HER2 CAR-T (NCT02713984)

Bone
Osteosarcoma

- Shared mutations: TP53, RB1, PTEN, MYC
- Clinical target: HER2
- Trials: HER2 CAR-T (NCT00902044)

Figure 4: Clinical stage CAR-T cell therapy assets with analogous cancers in dogs. Cancers associated with the highlighted organ systems are analogous to human diseases with shared mutations, clinical targets, and human CAR-T cell therapies listed. Clinical trial information retrieved from *ClinicalTrials.gov*.

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Strategies for allogeneic cell therapy

One of the biggest hurdles in allogeneic CAR-T cell therapy is Graft vs. Host Disease (GvHD) (Gajewski et al., 2009) (Poirot et al., 2015). GvHD is mediated by the T cell receptor (TCR) recognizing non-self through major histocompatibility complex (MHC)/human leukocyte antigen (HLA) interactions. Thus, GvHD is dependent on the TCR. To avoid GvHD, scientists use gene editing-based knockouts of the T cell receptor alpha chain (TRAC), which disrupts the TCR (Qasim et al., 2017). In the first cohort of patients with ALL who were dosed with TALEN-mediated allogeneic CAR-T cell therapy containing TCR knockouts, no GvHD was observed, and clinical remission was induced from one dose of these CAR-T cells (Qasim et al., 2017). In fact, publications have shown that disruption of the endogenous TCR via targeted integration of the CAR at the *TRAC* locus actually enhances CAR-T cell activity *in vitro* and *in vivo* (MacLeod et al., 2017*)*. Additional benefits of targeted integration of the CAR module are better control of transgene expression (vs using an exogenous promotor) and the ability to make a theoretically homogenous therapy where every cell contains the same edit, which are both limitations in the random integration of CAR via lentivirus (Xu et al., 2018).

Indeed, multiple gene editing approaches have been developed towards engineering allogeneic CAR-T cells for human blood malignancies, using viral and non-viral approaches to CAR delivery. Nuclease-based knock-out of the TCR is often combined with lentiviral-based CAR delivery and has been the method used to manufacture allogeneic CAR-T cells in multiple FDA trials. Moreover, adeno-associated virus (AAV) cassettes are relatively efficient vectors to induce homology-directed integration of cargo cassettes at CRISPR cut sites (MacLeod et al., 2017). DNA molecules, either plasmids, dsDNA, or ssDNA, are also used as HDR templates to generate non-viral CAR-T cells (MacLeod et al., 2017) (Eyquem et al., 2017) (Sachdeva et al., 2019) (Roth et al., 2018) (Jung and Lee, 2018). Based on these studies and unpublished work at numerous biotechnology companies and academic centers, there are multiple human clinical trials ongoing for allogeneic CAR-T cell therapy in cancer (clinicaltrials.gov).

Dogs as a unique model for cell therapy development

Many advances have been made in improving efficacy through the inclusion of additional synthetic biology strategies into the CAR construct (Kloss et al., 2013) (Diaconu et al., 2017) or by combining CAR-T cells with monoclonal antibodies (Gargett et al., 2016) (Sterner et al., 2019), or small molecule inhibitors (Giavridis et al., 2018) (Ruella et al., 2016). However, equally important as fine-tuning the CAR-T cells themselves is testing the therapy in an appropriate animal model. Different mouse models—syngeneic, xenograft, transgenic, and humanized—have been implemented in CAR-T research, and some of these models have exposed CAR-T limitations or risks that were not adequately detected in other models (Siegler et al., 2017).

Even though CAR-T cells have been especially successful in treating hematological malignancies, an estimated 30-50% of patients will relapse within one year of treatment (Maude et al., 2018) (Park et al., 2018), arguing for the need to better evaluate preclinical assets before investing in Phase I trials and beyond. The vast majority of preclinical CAR-T studies have been performed in mouse models, which are convenient but do not faithfully reproduce or predict clinical outcomes. Importantly, the incredible outcomes of CAR-T cell therapy in human oncology comes with significant adverse reactions in the form of neurotoxicity (NT) and cytokine release syndrome

(CRS). Thus, there is a disconnect between preclinical models, which display robust antitumor effects and minimal toxicities, and clinical trials, which yield more limited durable responses and often demonstrate serious toxicities such as CRS and NT. Currently, three different mouse models are being utilize to study CAR-T cell toxicities. These include 1) xenograft humanized models (Sterner et al., 2019); 2) humanized mouse models (Giavridis et al., 2018); and 3) syngeneic models (Kueberuwa et al., 2018). These models are limited by the lack of spontaneous tumor development, no relevant tumor microenvironment, absence of immune cells in the case of immunocompromised mice, and use of murine T cells in the case of syngeneic models.

Briefly, syngeneic models are immunocompetent and thus reflect a more realistic tumor microenvironment, but require all components—engineered T cells, tumors, and target antigens—to be of murine origin. Thus, syngeneic models do not provide insight into the functions of human CAR-T cells. Xenograft models are immunocompromised and permit the engraftment of human tumors and T cells, but do not give information into off-tumor toxicities or interactions between tumor and host immune cells. Immunocompetent transgenic mice express a human antigen to better determine off-tumor effects but have limited availability (Chmielewski et al., 2013) (Pegram et al., 2012). Humanized mice recreate components of the human immune system in immunocompromised mice to allow the study of human cells and targets within the context of the human immune system. However, these models are often primitive, with frequent defects in the myeloid compartment, limiting the full recapitulation of human immune interactions with CAR-T cell therapy (Holzapfel et al., 2015) (Zhen et al., 2015).

No less, there have been advances in the understanding of CAR-T cell biology and host responses and methods to abrogate toxicities like CRS and NT. One study used gene editing to make CAR-T cells containing knockouts of granulocyte macrophage colony-stimulating factor (GM-CSF), a protein suggested to be responsible for bridging CAR-T cells with NT and CRS. Indeed, either with a GM-CSF neutralizing antibody (lenzilumab) or CRISPR/Cas9 based GM-CSF knockouts, NT and CRS were abrogated and CAR-T cell function was not impacted (Sterner et al., 2019).

Historically, CAR-T cell therapy has been most successful in hematologic malignancies, with few complete responses induced in solid tumor trials. Solid tumors present a number of hurdles for cell therapy activity that are less prevalent in liquid tumors. One issue to effectively deploying CAR-T for solid tumors is what is known as the “antigen dilemma”; this is essentially because cell types in solid tumors are more heterogeneous, displaying different tumor antigens throughout the tumor. Therefore, if CAR-T cells are designed to target a single antigen, cells without that antigen cannot be targeted by the therapy, a hurdle that can be alleviated by the use of bispecific CARs (Dai et al., 2020).

Another challenge in CAR-T cell therapy for solid tumors lies in the immunosuppressive nature of the tumor microenvironment. One approach to counteracting this for enhanced CAR-T cell activity at solid tumor sites is to use CRISPR to disrupt the PD-1/PD-L1 immune checkpoint blockade that inhibits T cell function (Zhao et al., 2018) (McGowan et al., 2020). At last, *on-target* but *off-tumor* effects can result in the CAR-T cells attacking healthy tissues. In the context of a

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xenotransplant, immunocompromised mouse model, it is nearly impossible to determine how a CAR-T cell's antigen receptor may react to these off-tumor antigens.

Another challenge to overcome for solid tumor targeting is the need to improve CAR-T cell fitness. Even in blood malignancies where targeting single antigens can induce clinical remission, the repeated activation of CAR-T cells via the chimeric T cell receptor can induce T cell exhaustion and lower efficacy in some cases (Blank et al., 2019). An interesting approach to enhance the fitness of CAR-T cells was described by Sachdeva et al., where they generated multiplexed CAR-T cells that can selectively induce secretion of IL-12 upon antigen stimulation to enhance T cell activity (Sachdeva et al., 2019). Again, testing CAR-T cell therapy in spontaneous disease models like dog vs immunocompromised, xenotransplant mice may help scientists understand the gene networks, gene edits, or other strategies to enhance CAR-T cell fitness.

While xenotransplants of cancer into immunocompromised mouse models have been the gold standard for preclinical testing of human CAR-T cells for blood malignancies, they cannot faithfully recapitulate these hurdles in bringing cell therapies to human application. The use of spontaneous disease in dog models with an intact immune system could be a game changing new paradigm of testing novel cell therapy strategies, designs, and treatment regimens to increase the confidence of the safety and efficacy of novel cell therapy strategies during the pre-clinical evaluation phase of drug development.

Advanced gene edited cell therapy technologies

Indeed, as the gene editing toolbox continues to grow and more cell types are engineered and shown to work as cancer and other disease therapy, we will continue to see the growth and application of gene edited therapies in dogs.

While 3rd generation, autologous CAR-T cell therapy is leading the field for reprogrammed immune cells, there is considerable attention given to other types of modalities and engineered cells (Yu et al., 2020). T cells can also be engineered with programmed T cell receptors themselves vs the chimeric approach used in CAR-T cell therapy. Indeed, the first clinical application of CRISPR/Cas9 to engineer living cell therapy was a Phase I trial of TCR-engineered T cells with endogenous TCR and PD-1 disruption (NCT03399448). Natural killer cells are also under evaluation in clinical trials after being engineered with CARs to mediate tumor antigen-specific killing and may bring several advantages over engineered T cells, including an abrogated risk of GvHD, reduced rates of NT and CRS due to differential cytokine profiles, and the innate ability to kill tumors in a CAR-independent manner, overcoming limitations in tumor antigen heterogeneity (Xie et al., 2020). Finally, CAR-macrophages are currently under investigation as a potential advance in the ability to penetrate solid tumors and overcome the immunosuppressive tumor microenvironment (Mukhopadhyay, 2020).

A xenogeneic approach to cell therapy in dogs?

In the late 1990's, a group of scientists demonstrated the efficacy of a transformed human T cell line, TALL-104, as a therapeutic option for canine cancer in a series of studies (Visonneau et al., 1997, Visonneau et al., 1999, Cesano et al., 1995, Cesano et al., 1996). Interestingly, the use of

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this cell line in dogs did not result in any toxicity like GvHD and even provided clinical benefit in many cases. This, combined with the supporting evidence of the ability to engineer allogeneic CAR-T cells in research and clinical settings for humans that do not cause GvHD, has generated interest in the use of canine antigen targeting, human CAR-T cells in dogs (Yin et al., 2018).

The use of xenogeneic CAR-T cells for canine therapy would alleviate the burden of isolating, expanding, and engineering canine T cells (methods that have not been broadly studied), and would create a more straightforward strategy to using canine modeling of CAR-T cell therapeutic efficacy. Indeed, the University of Pennsylvania is currently recruiting dogs to participate in a trial with human CAR-T cells engineered to fight canine B cell lymphoma, though it is not publicly known how the CAR module is integrated into the genome in this approach (are they using lentivirus or gene editing approaches?). In industry, LEAH Labs is developing a similar approach using CRISPR to knock-out the TCR while simultaneously integrating CAR DNA using their non-viral GeneWeld platform (Wierson et al., 2020, Wierson et al., 2019). Of note, a combination approach using lentivirus to introduce the CAR transgene and gene editing to knock out the TCR to generate xenogeneic CAR-T cells for canine use may be an effective strategy toward the application of this therapy.

Future applications of gene editing and gene therapy in our canine companions

Improving the cell and gene therapy discovery and validation pipeline

As discussed above, large animal models may better serve preclinical testing of CAR-T cell therapy, as mouse models have the tendency to overstate efficacy and understate toxicity. To date, a few non-human primate models have tested CAR-T safety. However, these studies are expensive and ethically fraught, and no tumors were implanted or spontaneously developed prior to CAR-T administration (Berger et al., 2015) (Kunkele et al., 2017). Canine models of CAR-T cell therapy may circumvent many issues seen in murine and primate modeling (Migliorini et al., 2018). Unlike laboratory mice, dogs are more genetically diverse and develop spontaneous cancers that share similar histology, mutations, microenvironments, and metastases as their corresponding human cancers (Kirkness et al., 2003) (Breen and Modiano, 2008) (Richards et al., 2013).

Humans share a more similar environment and microbiome to pet dogs than they do to laboratory mice, which likely affects tumor development and therapeutic response (Coelho et al., 2018) (Gopalakrishnan et al., 2018). Finally, spontaneous disease in dog models has the potential to serve as relevant models to investigate toxicities after CAR-T cells and other cell therapies. The development of cytokine release syndrome and neurotoxicity following CAR-T cell therapy is caused require the interactions between CAR-T cells, tumor cells and tumor microenvironment (such as myeloid cells). Canine models were instrumental in developing methods for hematopoietic stem cell transplant (Panse et al., 2003) (Graves et al., 2007), and it logically follows that dogs can also be key to bridging the gap between bench and bedside in CAR-T cell therapy.

Interestingly, provisions in regulatory code 9CFR 107.1 allow veterinarians to treat their clients with any therapeutic provided it is manufactured in house with an existing veterinary client patient

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relationship. This code was originally intended to allow for frictionless vaccination of herd animals in the case of pandemics, but also may allow for the use of client owned dogs as n of 1 spontaneous disease models. One could envision a companion animal biotechnology company of the future that develops novel cell and gene therapy strategies that, once validated *in vitro* or in mouse models, could be quickly ported to patient dogs with consenting pet owners as a mechanism for compassionate use. In this way, scientists could glean clinical data in spontaneous disease models that is important for future regulatory submissions with little friction. Additionally, with the One Health model in mind, these data may prove invaluable to human pharmaceutical companies as they seek better models to test their novel cell and gene therapy hypotheses.

Two clinical stage gene therapy examples

As the companion animal market continues to rise, so has funding for university spinouts of high-risk, high-reward intellectual property into companion animal biotechnology companies. Two companies in particular are currently in the clinic with their gene therapy products: Rejuvenate Bio and Scout Bio, spun out of the infamous labs of Dr. George Church and Dr. Jim Wilson, respectively. Academic work out of the Church lab showed that delivery of a combination of AAV payloads containing two genes was successful at treating 4 diseases in a single mouse; obesity, Type II diabetes, heart failure, and renal failure (Davidsohn et al., 2019). As a proof of concept for this therapy working outside of lab models, Rejuvenate Bio is focused first on using this cocktail gene therapy to treat mitral valve disease in cavalier king Charles spaniels. As of this writing, they are currently recruiting patients. Scout Bio is using gene therapy to deliver feline erythropoietin (EPO) in vivo to treat anemia associated with chronic kidney disease in cats. In another study, they are using AAV to deliver GLP-1 to cats as a treatment for diabetes. Without a doubt, both companies are garnering interest from human pharma for their work and each has aspirations to translate their technology to the human clinic.

Fixing diseased alleles for generations through germline editing

Gene editing is not only useful for engineering somatic cells or stem cells to be used as therapy, but also opens the possibility to actually correct alleles that can be passed through the germline. With gene editing, it is theoretically possible to correct the over 700 described monogenetic diseases in dogs and establish new, disease-free dog lines. A review by Pepin et al. (Pepin, 2014) examining 36 breed-associated canine monogenetic diseases reports that single genetic defects are responsible for the vast majority of these disorders, with 91% of the mutations involving coding regions resulting in amino acid substitutions or truncations in the encoded protein. Similar to humans, about half of these diseases involve the nervous and visual system (e.g. cataract in the *Australian Shepherd* (Mellersh, 2014*)*, glaucoma in the *Beagle* (Kuchtey et al., 2013), or spinocerebellar ataxia in the *Parson Russell* (Forman et al., 2013). Additionally, naturally occurring *MDR-1* deletion mutations are common in certain dog herding breeds (e.g. Collies, Ref) (Firdova et al., 2016), making them vulnerable to severe side effects (which can be fatal) from parasiticides, such as macrocyclic lactones (Geyer and Janko, 2012). The *MDR-1* gene codes for P-glycoprotein (P-gp), a drug efflux transporter that plays an important role in cell detoxification and resistance to antimicrobial and chemotherapeutic drugs. Using adult intestinal stem cells-derived enteroids, ongoing work by Dr. Jon Mochel and colleagues focuses on mimicking the wild type (WT) four bp deletion mutation of the *MDR-1* gene. Additionally, by creating the exact WT

deletion, these studies lay the framework to rescue this genotype by simply replacing the HDR template with a template containing the four missing bp in the *MDR-1* gene in the affected canine's germline.

Using our companions as an exploration into gene editing ethics

The long history of canines and others as companion animals showcases their cultural significance well beyond their study as natural models of human disease. Consequently, all work beyond somatic gene editing will undoubtedly bring its own array of important ethical discussions that will have diverse cultural contexts as the rainbow of human-canine relations around the globe, conversations that will undoubtedly presage parallel work in human gene editing.

People have been working to change the germline of dogs since our first domestication from wolves over 10,000 years ago. Selective breeding has developed a rich array of different shapes, colors and dispositions – while causing clustering of genetic-based diseases in certain breeds. Maintaining the unique features of a classical dog breed while removing these negative and unintended consequences of human intervention is challenging and time-consuming when it's possible at all. This genetic complexity also exists in food animals such as cows, represented by the naturally occurring hornless cow trait that is only found natively in a few breeds. Approaches to accelerate genetic exchange to bring in highly valued genetic loci while maintaining other key aspects (such as milk production) has been demonstrated with the *polled* allele (Carlson et al., 2016). Germline gene editing in dogs offers the opportunity to similarly maintain the distinction many value in our different breeds of canine companions while reducing the negative impact of specific disease alleles. Finally, the controversial use of gene editing for enhancing specific non-disease traits in dogs is also a proven reality using CRISPR (Zou et al., 2015). As with nearly all other aspects of gene editing, our 'best friend' will share this complex journey towards our new genetic heritage.

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