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REVIEW

Genetics of canine behavior: A review

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Abstract

The past decade has seen rapid progress in the field of canid behavioral genetics. The recent advances are summarized in this review. The identification of the genes responsible for tameness in silver foxes is the culmination of a half century of behavioral testing and, more recently, genomic investigation. There is agreement that domestic dogs evolved from wolves, but when and from which population remains controversial. The genetic differences between wolves and dogs identified include those for neurotransmitters and digestion. Breed differences in behavior are well known, but only recently have the genetics underlying these differences been investigated. The genes responsible for flank sucking in Doberman Pinschers and for several other obsessive compulsive problems in other breeds have been identified. Aggression is the least desirable canine trait, and several laboratories have detected differences in neurotransmitters and their receptors between aggressive and non-aggressive dogs. In English Cocker Spaniels, the genes linked to aggressive behavior code for dopamine, serotonin, and glutamate receptors. A dopamine transporter gene has been associated with impulsive behavior in Malinois.

Key words: Dog; Wolf; Fox; Canine aggression; Dopamine; Serotonin; Canid

Core tip: This review incorporates the latest findings in the rapidly moving field of canine behavioral genetics. The genes involved in tameness of foxes and in domestication of dogs from wolves are discussed. The genes involved in several obsessive compulsive behaviors such as flank sucking and circling are mentioned. The genetic and physiological differences between aggressive and non-aggressive dogs of various breeds are emphasized.

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INTRODUCTION

This review is a summary of recent research focusing on the current knowledge of the genetic contribution to behavior in the Canidae family. We first review the farm fox experiment and how this long-term study has led to greater understanding of the process of canine domestication at the phenotypic and molecular levels. We then turn our attention to the relationship between dogs and wolves and canine breed differences in behavior. Finally, we review the current knowledge of the genetic basis of aggressive behavior in dogs.

TAME FOX EXPERIMENT

The farm-fox experiment constitutes a major milestone in canid behavioral genetics, clearly demonstrating the genetic basis of behavior. No discussion of the genetics of canine behavior would be complete without summarizing some of the groundbreaking research performed at the Institute of Cytology and Genetics (ICG) of the Russian Academy of Sciences[1-3]. For more than 50 years,

scientists at the ICG in Novosibirsk, Russia have been reconstructing experimentally the domestication process in farm-bred silver foxes (a variant form of the red fox, Vulpes vulpes) as a novel model for studying the genetic basis of canine domestication and behavior. In 1959, scientist Dmitry Belyaev and his team began an intensive selective breeding program of silver farm foxes to isolate the trait of tame behavior towards humans[2,4]. After several generations of selective breeding in a controlled environment, Belyaev succeeded in attaining a subset of tame foxes. During fifty years of continuous selective breeding, the farm-fox experiment has tested over 52000 foxes for tameness, with the resultant tame population of foxes showing friendly dog-like responses to humans as early as one month of age[2,3,5].

The goal of selective breeding of the farm foxes was limited strictly to behavioral criteria related to tameness. However, physical, developmental, physiological, and other behavioral differences also emerged in the tame foxes compared to the original farm-bred foxes. The selection for tameness led to numerous physical changes in the foxes, including piebald coats, floppy ears, and curly tails, despite no selection criteria for these traits[2]. In addition, the socialization period elongated from approximately 45 to 60 d in the selected tame foxes, similar to the socialization period in the domestic dog[1]. Tame foxes also developed a novel repertoire of affiliative vocalizations towards humans to promote interaction[6]. Hare *et al*[7] found that tame fox kits are as skillful as puppies in using human point and gaze gestures for finding hidden food, demonstrating that domestication has led to improved social cognitive ability. Physiological differences also were found with hormonal assays showing that tame foxes do not experience stress when in contact with humans. A comparative study of hypothalamic-pituitary-adrenal axis (HPA) function in tame *vs* unselected foxes showed that in tame foxes, basal and stress-induced blood cortisol levels were respectively three- and five-fold lower than in the unselected foxes[2,8].

In the 1970s, a second parallel strain of farm foxes began to be bred selectively at the Institute for Cytology and Genetics-those with aggressive behaviors towards humans. Fifty farm-bred silver foxes with the most aggressive responses towards humans were selected and used as the basis of the aggressive population[9]. Criteria for measuring behavior in the aggressive population were the critical distances between the experimenter and the caged animals at which the animals first demonstrated aggression and the intensity of the aggressive responses[10].

From the evolution of these tame and aggressive populations of foxes, much information has been learned about the changes that can occur with intensive behavior selection pressures. Because the fox-farm domesticated foxes were created in only a few decades through intense selection and by focusing exclusively on certain behavioral traits, it seemed reasonable to assume that a small number of genetic loci determined the behavioral traits[11]. A rudimentary map of the fox genome with karyotype and some linkage groups was available by the late 1990s; however, a meiotic linkage map of the fox was needed to determine which loci were implicated in tame behavior[9]. Fortunately, the fox and the dog share a close evolutionary and genetic relationship, and since the dog genome was sequenced by 2005[12], available canine genomic information then could be utilized to develop the necessary fox meiotic map[3,11]. The availability of high resolution canine genome maps and sequence data aided in the creation of the fox meiotic linkage map, with the high genomic sequence identity between dog and fox permitting the adaptation of canine microsatellites for genotyping and meiotic mapping in foxes. Using 320 such markers, Kukekova *et al*[3] constructed the first meiotic linkage map of the fox genome. This first mapping covers 16 fox autosomes and the X chromosome. After alignment with a canine genome sequence of similar length, high conservation of marker order between homologous regions of the two species was apparent[11]. Utilizing and adapting scoring systems (for tameness and aggression phenotypes) developed by the fox-farm experiment over the years for the selective breeding process, Kukekova *et al*[3] created a new principal-component analysis of fox behavior with selected traits. This new scoring system effectively reduced 311 binary scoring behaviors to fifty of the most important traits that would serve as quantitative phenotypes (and continuous variables) to represent heritable differences in behavior among individual foxes and the fox populations and permit quantitative genetic analysis[10]. By interval mapping using fox and canine meiotic maps, a locus for tame behavior on fox chromosome VVU12 was identified. This locus is orthologous to a genomic region implicated in canine domestication[13]. Tameness as the defining trait of domestication is a complex "phenotype" consisting of many behavioral variables. In fact, when genome-wide association studies were performed by Kukekova *et al*[3], the resulting data suggested that at least two VVU12 loci are associated with tame *vs* aggressive behavior and active *vs* passive behavior. Moreover, differing mapping characteristics of specific behavioral traits were found, suggesting different genotype/phenotype relationships; for example, floppy *vs* erect ears are associated with different regions of VVU12 and vary between tame and aggressive foxes. Expression of the VVU12 loci thus appears to depend on interaction with other parts of the genome and on individual fox parents[13].

At the molecular level, the development of transcriptome sequencing significantly enhances genetic study without the need for a fully sequenced genome. The comparison of transcriptome sequencing from the prefrontal cortices of a tame and an aggressive fox is in the preliminary stages at this time[9]. Thus far, preliminary analysis of "comparison of transcriptome sequences of the same genes between the tame and aggressive fox samples has identified a large set of informative single nucleotide polymorphism (SNP) markers and begun a catalogue of gene-specific sequence variants between the

two strains"[13].

The farm-fox experiment demonstrates that over generations, intensive selection for tame behavior in foxes can serve as a reliable model for studying the genetic basis of canine domestication. The identification of genetic loci that both influence tame behavior in foxes and are homologous to regions in the dog genome supports the hypothesis that domesticated behavior in dogs and foxes may have similar genetic bases. These recent advances will help identify more genes implicated in fox behavior that can be correlated to dog domestication.

WOLVES TO DOGS

The complete sequencing of the dog genome has greatly expanded general knowledge of the processes of genome evolution and the genetic basis of phenotypic traits in dogs and other animals. However, the evolutionary path leading from wild ancestor to domesticated dog continues to remain elusive. Comparative genomics utilizing the completed dog genome has confirmed the close relationship of dogs to such other canidae as foxes, coyotes, and wolves. It appears that modern canids share a common ancestor dating back approximately ten million years; the closest relatives to the dog such as the gray wolf and coyote share a common ancestor dating to approximately three to four million years ago[14]. Like the dog, all wolf-like canids have 78 chromosomes and can mate with one another to produce fertile offspring. Thus, wolf-like canid species are among the strongest candidates for the ancestors of today's dog. Moreover, molecular genetic data from the past two decades[12,15,16] strongly support the origin of the dog from the gray wolf in particular[17]. Molecular evidence also suggests that divergence of dog from wolf and the beginning of the dog's relationship with humans occurred as recently as 15000 years ago[9,14]. Other studies looking at genomic variation in wolves, Chinese indigenous dogs, and modern breeds point to an even earlier beginning to domestication, possibly about 30000 years ago, prior to the development of an agricultural human society[18,19]. Very early domestication may have involved the intentional taming of small groups of wolves who, less fearful of humans and motivated by hunger, scavenged the camps of Mesolithic human hunters-gatherers[20].

Where canine domestication originated also is debatable. While DNA genomic data suggest a Middle Eastern origin, analyses of mitochondrial DNA and Y-chromosome markers from various dog breeds and from geographically-dispersed wolf populations suggest that canine domestication originated in East Asia[9]. Wang *et al*[18] used whole-genome sequencing to compare gray wolves, Chinese indigenous dogs, and modern breeds. They found that the genetic variation between the three canid groups generally decreased step-wise from wolf to Chinese dog to modern dog breed. Based on these findings, they speculate that the Chinese indigenous dog may represent the link between wolf and dog and the progenitor of today's diverse modern dog breeds. They identified 311 genes that appear to have been selected in dogs compared to wolves and that have functions affecting sexual reproduction, digestion/metabolism, neurological processes, and cancer. The fact that these particular genes overlap to a great extent with those also selected in humans suggests a parallel evolutionary process in dogs and humans, especially in the realm of neurological processes. They note that: As domestication is often associated with large increases in population density and crowded living conditions, these "unfavourable" environments might be the selective pressure that drove the rewiring of both species. Positive selection in neurological pathways, in particular the serotonin system, could be associated with constant need for reduced aggression stemming from the crowded living environment[18].

Another study employed mitochondrial DNA sequencing, showing a closer relationship of dogs to gray wolves from East Asia[21]. VonHoldt *et al*[22] sought to identify the primary source of genetic diversity for domestic dogs and conducted an extensive genome-wide survey of over 48000 SNPs in dogs and gray wolves. Their data, however, showed that dogs share a greater percentage of multi-locus haplotypes unique to gray wolves from the Middle East rather than from East Asia[22].

Although genetic data support the theory that the process of canine domestication began in East Asia over 15000 years ago, a recent study compared the complete mitochondrial genome sequences of 18 European prehistoric canids to a comprehensive panel of modern dogs and wolves. The researchers found phylogenic relatedness between the modern dogs and the ancient canids of Europe dating back to more than 30000 years ago, thus suggesting that canine domestication first may have occurred in Europe rather than in Asia[23].

Behavior differences between dogs and wolves are the most striking result of the domestication process, even more than the marked differences in physical size and shape. In fact, the canine breeds in existence today have diverse physical characteristics that distinguish them from one another just as much as from wolves. However, the fact that all breeds of domestic dog as a group are more similar in behavior when compared to one another than when compared to the wolf suggests that genetic selection for behavior drove the domestication process. It is logical to hypothesize that ancestral wolves initially may have experienced natural selection for tame behavior, permitting coexistence with humans. Based on the findings of the fox-farm experiment where genetic loci influencing tame behavior in foxes are homologous to regions in the dog genome and also related to selection differences between dogs and wolves, it is plausible to suggest that domesticated behavior in dogs and foxes share a similar genomic basis[9].

Several studies before and after the complete sequencing of the dog genome in 2005 have attempted to target, at the molecular level, the genetic basis of behavioral differences between the domestic dog and its wolf progeni-

tor. A study by Saetre *et al*[24] used microarray technology to evaluate mRNA expression levels of 7762 genes in the post-mortem brains of dogs, wolves, and coyotes. They found markedly altered gene expression of two neuropeptides, CALCB and NPY, in the dogs as compared to the wolves and coyotes. These neuropeptides, present in all mammalian brains, are implicated in energy control and feeding behavior, neuroendocrine stress response *via* the HPA axis, and possibly play a role in anxiety and depression. The findings of species-specific differences in the elaboration of the neuropeptides suggest that selection for behavior during domestication may have resulted in modification of mRNA expression patterns in genes located in the hypothalamus of the dog[24]. Björnerfeldt *et al*[25] postulate that domestication of dogs created a new lifestyle that changed selective forces acting on the species, in turn affecting the dog's genome. Using mitochondrial DNA sequencing in 14 dogs, 6 wolves, and 3 coyotes, they showed that dogs have accumulated into their genome non-synonymous changes in mitochondrial genes at rates faster than in wolves. In turn, this results in elevated levels of protein variations in the dog as compared to the wolf. Björnerfeldt *et al*[25] conclude that an important consequence of domestication is a "relaxation of selective constraint on dog mitochondrial DNA" that also could have affected other parts of the dog genome to facilitate "the generation of novel functional genetic diversity"[25]. Cruz *et al*[26] compared the genome of the dog to that of the gray wolf to examine the effect of domestication. Using whole-genome SNP data, they compared the variation in dog and wolf genes. They also found increased frequency in the trend for non-synonymous mutations in dogs as compared to their wild canid counterparts. They concluded that the increase in mutation rate could have myriad effects, some deleterious, and may indicate that the process of domestication in the dog led to an increase in functional genetic variation that has contributed to the markedly diverse physical and behavioral phenotypes characteristic of dog breeds, as well as to the prevalence of pathology in modern breeds[26].

Li *et al*[27] studied the expression profiles of a specific subset of developmental genes believed to be implicated the evolution of dog domestication. They ran comparative genomic analyses by assaying the SNP genotypes in Chinese native dogs (believed to have the genetic structure most similar to that of ancient dog), German Shepherd (purebred) dogs, and gray wolves to detect a genetic basis for the behavior transformation from wolf to primitive dog to modern purebred dog[27]. Genomic regions that have undergone strong selection in the recent past should show extended haplotype homozygosity[28]. Following this line of reasoning, Li *et al*[27] detected four regions of high extended haplotype homozygosity that contained only a single highly differentiated SNP located within a single gene. Comparison of candidate genes between the Chinese native dogs and wolves showed a high bias for expression localized in the brain's prefrontal cortex, the center for complex cognitive-type behaviors. However, candidate genes showing large population differentiation between the Chinese dogs and German Shepherds did not demonstrate significant expression bias. Thus, the finding that wolves and dogs have highly differentiated brain-based genes suggests that behavioral transformation most likely was key to the onset of domestication and that "this rapid evolution likely was driven by artificial selection during the primary transition from wolves to ancient dogs, and was consistent with the evolution of dog-specific characteristics, such as behavior transformation, for thousands of years"[27,28].

Other recent studies have taken a closer look at the genetic processes underlying physiological and behavior differences resulting from dog domestication. Utilizing whole-genome resequencing of wolves and dogs, Axelsson *et al*[29] identified 36 genomic regions that likely are implicated in selection during the domestication of the dog. It is of interest that more than half of the regions play roles in brain function with 8 regions in particular involved in neurophysiologic pathways that may underlie behavioral changes characteristic of dog domestication. Moreover, they identified 10 genes with selection signals that play key roles in starch digestion and fat metabolism. In terms of starch digestion, three genes (*AMY2B*, *MGAM*, and *SGLT1*) that facilitate the digestion of starches show evidence of being selected for during the process of dog domestication. These findings may indicate that, unlike in carnivorous wolves, genetic mutations found in modern dog facilitate the adaptation to and even thriving on a diet available in cohabitation with humans[29].

BREED DIFFERENCES

Over the past hundreds of years, the selective breeding of domestic dogs has given rise to more than 400 modern dog breeds with many unique differences in both physical appearance and behavior characteristics[30]. The physical differences among the dog breeds mostly are obvious to the naked eye, and the behavior differences between breeds also are distinctive and diverse[31]. Humans have exerted genetic pressure on dogs by selecting various traits to create breeds better adapted to utilitarian purposes such as herding, guarding, or hunting. The modern dog's extraordinary diversity in phenotype, behavior, and ability to perform tasks is unmatched by any other species on earth[32]. A study by McGreevy *et al*[33] investigated the relationship between height, bodyweight, and canine cephalic index (CI: the ratio of skull width to skull length) and how these values correlated with certain behavior traits using the Canine Behavioral Assessment and Research Questionnaire (C-BARQ). It is of interest that certain canine morphotypes were associated reliably with particular behavior profiles. For example, brachycephalic skull shape (high CI) may be a by-product for human selection of neotenous behavioral characteristics, and dolichocephalic skull shape is a product of human selection for hunting and chasing ability. The authors note that it is unclear if these associations between mor-

phology and behavior represent functional co-adaptations or accidental by-products of allometric change. Therefore, the relationships noted in this study could be either genetically or environmentally driven or both[33].

With its wealth of phenotypic diversity, the dog clearly is a valuable genetic model for studying both breed-specific behaviors and abnormal behaviors. The persistence of such breed-specific behaviors as herding, pointing, tracking, and hunting in the absence of training or motivation suggests that these behaviors are, at least in part, controlled at a genetic level[34,35].

Prior to the completion of the dog genome in 2005, genetic studies used mitochondrial sequencing to reveal a large amount of variation in relatively short sequences. Although some breed clustering could be demonstrated, researchers found that mitochondrial sequences were more successful at distinguishing between species than between breeds[36]. Early genetic studies also utilized microsatellite-based marker sets to study the genomes of a small number of breeds. Differences in allele frequencies occurred in different breeds supporting the hypothesis that there was less variation within breeds than across the species[36]. Parker *et al*[37] investigated the relationships among 85 breeds using 96 microsatellite markers, demonstrating marked population stratification within the dog species and establishing that the breeds were indeed genetically separate. Once the whole genome of the dog became available, use of SNPs became favored over microsatellites due to the ease of genotyping bialleles and analyzing thousands of markers in a single assay[36]. SNP genotyping chips were derived from the over 2 million SNPs in the dog genome[38]. Both analytic techniques are useful; clustering analysis using mitochondrial DNA demonstrates hybridization among groups, while SNP analysis results in a phylogenetic tree that show the unique placement of a breed within a group[36]. Moreover, SNP analysis corroborates earlier research showing that genetic variation among breeds is greater than that among individuals. A study by Vonholdt *et al*[22] demonstrated a 4% overall variation between breed clusters.

In the 1950s through the 1960s, Scott and Fuller pioneered research on identifying heritable differences in behavior and cognition in the dog using five different breeds in a laboratory model setting[39]. More recent studies have assessed heritability of behavior in working and/or pet dog populations outside of the laboratory setting[40].

In 1989, the Swedish Dog Mentality Assessment (DMA) was initiated as a tool for selective breeding in working dogs. The test originally was developed as a tool for selective breeding of working dogs, but it is used today as a general behavioral test by many breeding clubs in Sweden. The DMA has been applied to over 24000 dogs representing more than 180 breeds. Using this data set and the pedigrees of German Shepherds and Rottweiler dogs, Saetre *et al*[41] noted that the genetic correlation of the score on one test was not independent of the score on another test. In fact, their analysis provides evidence that there may be substantial shared genetics underlying most of the behavioral response in all of the test situations except for aggression which tended to be distinct. Saetre *et al*[41] identified "shyness-boldness" as a generalized trait underlying many behavioral scores with a heritability of 0.25-0.27.

Recent research has shown that the genetic similarities among different breeds may not correlate well to characteristic behavior traits attributed to historical functional breed groups such as herders and hunters[30,42]. Turcsan *et al*[30] investigated whether or not behavioral traits historically believed to characterize certain breed categories actually correlated with genetic relatedness. Using online questionnaires submitted by 5733 dog owners of 98 breeds, they looked at trainability, boldness, calmness, and dog sociability. They found that the breeds differed to a great extent in the four traits and that breed-specific behavior in trainability and boldness appeared to be determined partly by genetics. However, breeds that were similar in behavioral characteristics per report of the owners did not correspond well to recognized functional/conventional breed classification nor to genetic breed clusters. The authors state that this lack of correlation between the questionnaire results and commonly acknowledged breed or functional group traits could be associated with cross-breeding with breeds of dissimilar behavioral traits or could represent differences in socialization and/or relationship with owners. The authors conclude "…the behavioural breed clusters showed poor correspondence to both the functional and genetic categorization, which may reflect the effect of recent selective processes. Behavioural breed clusters can provide a more reliable characterization of the breeds' current typical behaviour"[30]

Meyer *et al*[43] estimated the heritability and correlation of 7 behavioral traits in German Shepherd Dogs in Switzerland using data from 4855 animals that underwent the standardized behavior test of the German Shepherd Dog Club of Switzerland between 1978 and 2010. The traits tested were self-confidence, nerve stability, hardness, temperament, sharpness, defense drive, and reaction to gunfire. Sex, year of testing, judge, place of testing, and age at testing were found to have significant effects on the outcome of the test. Overall, estimated heritability of the traits was low, ranging from 0.05 (5%) to 0.21 (21%). It also is of interest that some traits were highly correlated; self-confidence and nerve stability had a genetic correlation of 0.98 and sharpness and defensive drive, 0.93. Meyer *et al*[43] suggest that while the heritability of behavioral traits is generally low, genetic evaluation of behavior can be helpful as a basis for selection of a given trait, with the caveat that precise definition of the desired traits along with accurate scoring of the dog's behavior are requisite[43]. Mehrkam *et al*[44] recently reviewed the current state of knowledge regarding canine breed differences in behavior, finding scientific evidence for differences both between breeds as well as within-breed differences[44].

The genetics underlying racing performance has been

studied in sled dogs[45]. The Alaskan sled dog is considered genetically distinct in that the population has been shaped to create a group of high-performance athletes through selective interbreeding with purebred dogs based on working ability rather than breed physical appearance. New breeds have been introduced gradually into the lines of racing dogs to improve racing performance. Therefore, Alaskan sled dogs provide a unique opportunity to research the impact of trait selection and breed composition and their influence on genomic structure. Huson *et al*[45] genotyped 199 Alaskan sled dogs using 96 microsatellite markers and compared the data to that from 141 genotyped purebred breeds. The breed composition of each sled dog was compared to its performance phenotype, including speed, endurance, and work ethic. It is of interest that the sled dogs separated into two groups that aligned with their racing style-sprint *vs* distance[46]. Huson *et al*[46] then used a set of 7644 ancestry informative marker SNPs to model ancestry in the sprint and distance sled dog populations with four known reference breeds, the Alaskan Malamute, Siberian Husky, German Shorthaired Pointer, and Borzoi. It was found that the distance sled dogs had, on average, highest Alaskan Malamute allele patterns compared to the sprint dogs who had the highest German Shorthaired Pointer allele patterns. In addition, genetic comparison between sprint *vs* distance racing Alaskan sled dogs identified several genomic regions associated with differences in racing style and pinpointed a variant of *MYH9* gene that is associated with increased heat tolerance in sprint dogs[46]. Although variants responsible for improved muscle function are important, those responsible for the motivation to perform are also involved.

There are many genetic differences in behavior, but few of the genes are known. The laboratory of Veterinary Ethology of Tokyo University has located putative genes affecting canine behavior. The researchers have identified polymorphisms in five breeds of dogs (Golden Retriever, Labrador Retriever, Maltese, Miniature Schnauzer, and Shiba) that pinpoint differences in SNPs in genes regulating neurotransmitters, the enzymes that synthesize or destroy the neurotransmitters, and the receptors[47]. SNP (T199C) is located on the putative third exon of the canine monoamine oxidase B gene that causes an amino acid substitution from cysteine to arginine. Takeuchi *et al*[47] also found 4 SNPs in the tyrosine hydroxylase and dopamine beta hydroxylase genes. Ogata *et al*[48] found 2 SNPs in the glutamine transporter gene. The Tokyo University researchers have related the polymorphisms with breed behaviors as identified by Hart *et al*[49], although there is no direct evidence that these could explain interbreed differences[31].

Due to the great diversity of dog breeds, the dog is a valuable genetic model for studying both breed-specific behaviors and abnormal behaviors. At a molecular level, analytic techniques to study breed differences include using mitochondrial DNA to perform cluster analysis that shows hybridization among groups and SNP analysis that develops a phylogenetic tree and places a breed within a group on that tree. Recent studies also have assessed heritability of behavior in both working dog and pet dog populations. The genetic similarities among different breeds may not correlate well to characteristic behavior traits attributed to historical functional of the breed groups. However, behavioral breed clusters may provide a more reliable characterization of the breeds' current typical behavior. Currently, only a few genes that underlie inheritable behavior characteristics are known. Polymorphisms have been identified in five breeds of dogs, pinpointing differences in SNPs in genes regulating neurotransmitters, enzymes acting on neurotransmitter enzymes, and receptors.

GENETICS OF ABNORMAL BEHAVIOR

Flank sucking

Yokoyama *et al*[50] pointed out that genome wide association testing is more profitable than the candidate gene approach to determining the genetics of behavior. Using this approach, the first gene for a specific behavior was found. Flank sucking, a very specific and easily recognized compulsive problem, is a behavior seen almost exclusively in Doberman Pinschers. *CDH2* is the gene associated with this compulsive behavior. Occasionally a blanket or another material can serve as the substrate for sucking. It is not a serious behavior problem because the irritation to the skin is mild. More owners complain about fabric sucking because the material must be replaced. The sucking behavior occurs mostly as the dog is resting prior to sleeping. Using genome-wide analysis, Dodman *et al*[51] found an association of SNPs peak on canine chromosome 7. The most significantly associated SNP is located within the *CDH2* gene. *CDH2* is widely expressed, mediating synaptic activity-regulated neuronal adhesion. Dogs showing multiple compulsive behaviors have a higher frequency of the risk allele than do dogs with a less severe phenotype (60% and 43%, respectively) compared with 22% in unaffected dogs[51].

In an interesting follow-up to the genetic basis of this abnormal behavior, Ogata *et al*[52] found that the brains of flank sucking Dobermans differed from those of unaffected Dobermans. Magnetic resonance imaging revealed higher total brain and gray matter volumes and lower dorsal anterior cingulate cortex and right anterior insula gray matter densities in the affected dogs. The affected Dobermans also had higher fractional anisotropy in the splenium of the corpus callosum, the degree of which correlated with the severity of the behavioral phenotype[52].

Another behavior abnormality, tail chasing, can have multiple etiologies including neuropathic pain, so it is not surprising that there is no association with the *CDH2* gene[53,54]. Single photon emission computed tomography (SPECT) was used with ^{123}I-R91150 and ^{123}I-FP-CIT, in combination with ^{99m}Tc-ECD brain perfusion co-registration, to measure the serotonin (5-HT) 2A receptor, dopamine transporter (DAT), and serotonin transporter (SERT) availability. There was significantly less 5-HT2A

receptor binding in the frontal and temporal cortex of obsessive compulsive dogs. The midbrain SERT also was lower. The DAT differences between normal and compulsive dogs were mixed[53].

More recently the original data from Dodman *et al*[51] was reanalyzed using a new calling algorithm called MAGIC was used to identify genes, in addition to cadherin, that are involved in flank sucking and other obsessive compulsive behavior (OCD). The genome wide association revealed 119 variants in evolutionarily conserved sites that are specific to dogs with OCD. Using small numbers of dogs, (< 16 of each breed), case dogs (exhibiting OCDs), control dogs, and unphenotyped dogs were compared. Four genes have an excess of case-only variation in evolutionarily constrained elements, even after correcting for gene size: ataxin-1 (ATXN1), neuronal cadherin (CDH2), catenin alpha2 (CTNNA2), and plasma glutamate carboxypeptidase (PGCP). CDH2, a neural cadherin, encodes a calcium dependent cell-cell adhesion glycoprotein important for synapse assembly, where it mediates presynaptic to postsynaptic adhesions.

CTNNA2 encodes a neuronal-specific catenin protein that links cadherins to the cytoskeleton. ATXN1 encodes a chromatin binding protein that regulates the Notch pathway[42], a developmental pathway also active in the adult brain, where it mediates neuronal migration, morphology and synaptic plasticity[55]. All three of these genes are involved in synaptic formation. The fourth gene PGCP, encodes a poorly characterized plasma glutamate carboxypeptidase. It may be involved in the hydrolysis of N-acetylaspartylglutamate. One might consider glutamate targeting drugs for treatment of OCD's.

NEUROTRANSMITTERS AND AGGRESSION

Canine aggression has been the subject of many genetics studies because it is the most common behavior presented as a problem and the only one responsible for human injury or even death[56]. Hyperactivity and impulsive (unpredictable) aggression by dogs are problems frequently presented to veterinarians. Since behavior is the consequence of central nervous activity, it is not surprising that differences in neurotransmitters are associated with differences in behavior. These differences can be at any stage in the production and function of the neurotransmitter. The levels of neurotransmitter or their metabolites in brain, blood or cerebral spinal fluid have been investigated, and transporters and receptors of neurotransmitters have been associated genetically with aggression and other behaviors.

Dopamine and serotonin are the neurotransmitters examined most frequently in studies of aggression. Serotonin is produced from tryptophan and is widely believed to be important in the etiology and treatment of mood disorders, including aggression in dogs[57]. It is logical to conclude that serotonin levels in the body fluids or number of serotonin receptors should be measured in normal and abnormal dogs with the prediction that serotonin levels would be lower in aggressive dogs. The results of these studies are summarized below.

Dopamine (D1 and D2) is formed from tyrosine and catalyzed by the enzyme tyrosine kinase. Dopamine has multiple receptors and is inactivated by another enzyme, monoamine oxidase (MAO). Dopamine is transported back into the pre-synaptic neuron *via* a transporter. Studies in dogs exhibiting aggression have examined blood and cerebrospinal fluid levels of dopamine and its expression in the brain. In genetic studies, alleles regulating dopamine transporters, receptors, and dopamine deactivating enzymes have been compared in non-aggressive dogs and dogs exhibiting aggression. The results of these studies are summarized below.

Blood and body fluids

Cakiroğlu *et al*[58] found that serotonin in blood varied with canine disposition. Serum serotonin was 33 ng/mL in non-aggressive dogs and 12 ng/mL in aggressive dogs[58]. In a later study, Leon *et al*[59] found lower levels of serotonin in plasma, serum and platelets in aggressive dogs of various breeds that presented to a behavior clinic than in the control group of Beagles. However, the differing serotonin levels might represent breed differences in serotonin rather than differences between aggressive and non-aggressive dogs.

It is probably more fruitful to look for genetic differences between dogs within the same breed. For that reason, English Cocker Spaniels were studied because dogs of that breed frequently exhibit unpredictable or impulsive aggression towards their owners[60]. Moreover, the prevalence of aggression varies with coat color; red (blonde or buff) spaniels are more aggressive than black ones and solid color spaniels are more likely to be aggressive than parti-colored ones. It is not clear how the production of pheomelanin (yellow pigment) rather than melanin (black pigment) leads to or is related to aggression although melanin and dopamine share a common precursor-tyrosine. This area bears investigation[61]. Amat *et al*[62] compared serum serotonin levels in aggressive English Cocker Spaniels with those of aggressive dogs of a variety of other breeds and found the serotonin levels were significantly lower in the cockers.

MAO-A is an enzyme that catalyzes monoaminergic neurotransmitters such as dopamine and serotonin. A mutation that lowers the amount of MAO-A is associated with incarcerated humans, if they had bad childhood environments[63]. There is evidence in dogs that aggressive individuals have lower cerebrospinal levels of 5-hydroxyindole acetic acid and homovanillic acid, the major metabolites of serotonin and dopamine respectively[64].

Based on current studies, dopamine is the neurotransmitter most involved in aggression. Different breeds appear to have genes that are active at different points in the pharmacodynamics of the catecholamine. For example, compared to their non-aggressive counterparts, aggressive English Cocker Spaniels have significantly different

alleles for a dopamine receptor as well as a serotonin receptor. The gene for a dopamine receptor also appears to affect impulsive behavior in working German Shepherds, and the dopamine transporter appears to be involved in aggression, at least in the Malinois. In addition, the short form of the tyrosine hydroxylase gene appears to be involved in dopamine synthesis in German Shepherds and Siberian Huskies with particular behaviors. These studies will be discussed in detail below.

Brain receptors for neurotransmitters

The amygdala is a structure in the brain that is associated with fear. The basolateral nuclear group of the amygdala is involved directly in the modulation of aggressive behavior in dogs. This structure has an increased volume and a higher number of neurons in aggressive dogs[65]. Serotonin 1B receptors act as auto-receptors regulating serotonin release. Indirect immunohistochemistry revealed that aggressive dogs had a higher number of serotonin 1B receptors than non-aggressive dogs. One might have expected the number to be lower in aggressive dogs, but one possible explanation is that a lower serotonergic activity is present in aggressive dogs because stimulation of presynaptic serotonin-1 autoreceptors causes a reduction of the serotonin release[65].

Substance P is a neuropeptide that stimulates defensive aggression in cats[66] and mice[67]. It binds preferentially to neurokinin receptors. Using immunohistochemistry, Jacobs *et al*[65] found that although the brains of aggressive dogs had more neurokinin reactivity in the amygdala than did normal dogs, the numerical densities and fractions of receptor-positive neurons did not differ significantly between the two groups. As noted above aggressive dogs have 27% more neurons in the amygdala than do normal dogs[65].

Vermeire *et al*[68] found differences in serotonin 2A receptors in the brains of impulsively aggressive dogs compared to normal dogs. Aggressive dogs had higher binding indexes for serotonin 2A receptors in the frontal and temporal cortex as revealed by SPECT following a 5-hydroxytryptophan (5-HT) antagonist radioligand injection. Although expensive and technically difficult, SPECT could be used to confirm a diagnosis of impulsive aggression[68].

The brains of aggressive German Shepherds were compared with those of non-aggressive dogs of the same breed for beta adrenergic and serotonergic receptors using radioligand binding assays[69]. More binding of low affinity 5-HT (serotonergic) receptors were found in the whole brains of aggressive dogs. High affinity 5-HT was greater only in the hypothalamus and thalamus of the aggressive dogs. One might have expected 5-HT receptors to be decreased in aggressive dogs however, the increase in the number of 5-HT receptors may be due to a decrease in physiological serotonin levels at synaptic clefts or to an altered turnover of the neurotransmitter[69].

It is not surprising that the adrenergic neurotransmitter norepinephrine might be involved in aggression. Badino *et al*[69] found that beta adrenergic binding was decreased in the frontal cortex, hippocampus, and thalamus of aggressive dogs. The decrease in beta adrenergic concentrations observed in these brain regions of aggressive dogs may be explained by a prolonged stimulation exerted by the high catecholamine levels resulting in beta adrenergic receptor down-regulation[69].

In summary, there are differences in the brain, blood and cerebrospinal fluid between aggressive and non-aggressive dogs. Serotonin and its metabolites have been investigated most thoroughly. In general, blood serotonin levels are low and its metabolites are lower in aggressive dogs. The studies of receptors in the brain present a more complicated picture with serotonin receptors higher in aggressive dogs.

HUMAN-DIRECTED IMPULSIVE AGGRESSION

Heritability

Pérez-Guisado *et al*[70] investigated the heritability (the percent variability due to genetics) of aggression in English Cocker Spaniels. They found that in addition to sex and coat color, nurture also influenced whether or not a dog was aggressive. The variance due to the sire heritability of aggression was only 0.2 (20%) whereas that due to the dam was 0.46 (46%) indicating a maternal-environmental effect[70].

Although commonly perceived as gentle, non-aggressive dogs, Golden Retrievers can be aggressive, especially in European populations. Linamo *et al*[71] used the Restricted Maximum Likelihood method to determine heritability of aggression based on the dog owner's impression of the animal's human and dog-directed aggression or the responses on C-BARQ[71,72]. They found heritability of 0.77 for human-directed aggression and 0.81 for dog-directed aggression. There is little correlation between the two types of aggression indicating separate genetic causes of the traits. There were high heritability estimates on several C-BARQ items such as strange dog approaching leashed dog (0.85), family member grooming dog (0.83), family member removing food (0.95), and stranger trying to touch dog (0.99)[71]. The next step in researching the etiology of aggression is to determine which mutations in the neurotransmitter, its receptor, or its transporters might be involved in aggression or other behavior abnormality.

Genes

van den Berg *et al*[73] did an extensive study of the genetic differences in four candidate genes affecting serotonin in aggressive and non-aggressive Golden Retrievers. They used mutation screens, linkage analysis, an association study, and a quantitative genetic analysis. There were no systematic differences in the coding DNA sequence of the candidate genes in aggressive and non-aggressive Golden Retrievers. An affected-only parametric linkage analysis revealed no strong major locus effect on human-

directed aggression related to the candidate genes. An analysis of 41 SNPs in the 1 Mb regions flanking the genes in 49 unrelated human-directed aggressive and in 49 unrelated non-aggressive dogs did not show association of SNP alleles, genotypes, or haplotypes with aggression at the candidate loci. They completed their analyses with a study of the effect of variation in the candidate genes on a collection of aggression-related phenotypic measures. The effects of the candidate gene haplotypes were estimated using the Restricted Maximum Likelihood method, with the haplotypes included as fixed effects in a linear animal model. They found no effect of the candidate gene haplotypes on a range of aggression-related phenotypes[73].

Hejjas *et al*[74] genotyped police and pet German Shepherd Dogs and diagnosed hyperactivity and impulsivity based on questionnaires. They compared the dopamine D4 receptors subtypes 2/2 with 2a/3a and 3a/3a (combined because 3a/3a is rare) with the behaviors. There was no difference in the activity-impulsivity scores between dogs with 2/2 genotype *vs* the 2/3a and 3a/3a combined genotype group either in the total sample or in the pet dog group. In contrast, police dogs with 2/2 genotype showed significantly lower activity-impulsivity scores compared with police dogs with 2/3a or 3a/3a genotype[74].

Kubinyi *et al*[75] found that German Shepherds with the short form of the tyrosine hydroxylase (*TH*, the enzyme involved in dopamine formation) gene were more active and impulsive. Wan *et al*[76] also found that Siberian Huskies with the short form of the *TH* gene were more impulsive. They also reported that Siberian Huskies possessing at least one short dopamine D4 allele displayed greater activity-impulsivity in the behavioral tests than did those with two long alleles; dogs with the short allele tended to receive higher ratings on the activity-impulsivity scale of the questionnaire[76].

Våge *et al*[77] have used English Cocker Spaniels, a breed in which aggressive behavior has been noted for the past forty years. By using one breed, breed differences in the genotype can be eliminated so that any differences found should reflect differences in temperament. In a study comparing non-aggressive English Cocker Spaniels with English Cockers that had bitten and broken skin, there were significant associations between aggression and four SNPS in the region of the dopamine D1 receptor (DRD1), two SNPs in the serotonin ID receptor (HTR1D), and five SNPS in a glutamate receptor (SLC6A1)[77].

The same laboratory later identified 62 SNPs occurring in or in the close vicinity of 16 neurotransmitter-related genes. Allelic associations with aggression were identified for DRD1, HTR1D, HTR2C (5-HT receptors D1 and 2C) and SLC6A1 (solute carrier family 6 neurotransmitter transporter gamma amino acid member). Risk or protective haplotypes for aggressive behavior based on 2-5 SNPs were identified. The frequency of aggressive dogs varied significantly between the haplotypes within loci, and the odds ratios of aggression in dogs with risk haplotypes compared with protective haplotypes varied from 4.4 (HTR2C) to 9.0 (SLC6A1). No haplotypes in complete association with the recorded phenotypes were identified, supporting a complex inheritance of aggression. Gene *SLC6A1* on chromosome 20 should be investigated in association with aggression in other breeds, and use of benzodiazepines which bind with gamma amino acid receptors should be investigated further as treatments for aggression[78].

Most dogs are homozygous for the dopamine transporter-variable number tandem repeat two-tandem-repeat allele (2/2). The one-tandem-repeat allele is over-represented in American Malinois, both as heterozygotes and homozygotes (1/2 or 1/1). All American Malinois with reported seizures were 1/1 genotype. Those with at least one "1" allele (1/1 or 1/2 genotype), were more likely display hypervigilance and exhibit episodic aggression as well as more fearful postures[78].

Methylation

Although the genome acts as a blueprint for the production of observable morphological, physiological, and behavioral characteristics (*i.e.*, the phenotype), the expression of these traits may vary in different social or ecological contexts and in generations. Environmentally-induced phenotypic variation resulting from differential gene expression may be regulated by processes that that do not include the DNA sequence itself (*i.e.*, "epigenetic mechanisms"). DNA methylation is one such epigenetic mechanism that allows organisms to respond to environmental change *via* changes in gene expression that alter the phenotype. DNA methylation during development and early life can have long-term consequences for gene expression, physiology, and behavior in many vertebrates. This is a completely uninvestigated subject in canine behavior.

CONCLUSION

In the last ten years, the field of canine behavioral genetics has experienced rapid and exciting scientific advances, especially after completion of the sequencing of the dog genome. Although the history of dog domestication in terms of time and location is still debated, the divergence of dogs from wolves based on friendliness towards humans clearly has been outlined and experimentally repeated in the tame fox experiment. Genetic research also has focused on the great diversity of dog breeds, the genetic differences between breeds, and normal and abnormal behavioral traits. While much progress has been made in elucidating the genetics underlying aggression in dogs, future scientific studies will continue to examine this most serious problem threatening the human-canine bond and expand our knowledge about the genetic basis of canine behavior.

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REVIEW

Molecular genetics of gastric adenocarcinoma in clinical practice

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Abstract

The molecular genetics of gastric carcinoma (GC) dictates their biology and clinical behavior. The two morphologically distinct types of gastric carcinoma by Lauren classification, *i.e.*, intestinal and diffuse cell types, have a significant difference in clinical outcome. These two types of GC have different molecular pathogenetic pathways with unique genetic alterations. In addition to environmental and other etiologies, intestinal type GC is associated with *Helicobacter pylori* (*H. pylori*) infection and involves a multistep molecular pathway driving the normal epithelium to intestinal metaplasia, dysplasia, and malignant transformation by chromosomal and/or microsatellite instability (MSI), mutation of tumor suppressor genes, and loss of heterozygosity among others. Diffuse type shows no clear causal relationship with *H. pylori* infection, but is commonly associated with deficiency of cell-cell adhesion due to mutation of the E-cadherin gene (*CDH1*), and a manifestation of the hereditary gastric cancer syndrome. Thus, detection of *CDH1* mutation or loss of expression of E-cadherin may aid in early diagnosis or screening of diffuse type GC. Detection of certain genetic markers, for example, MSI and matrix metalloproteinases, may provide prognostic information, particularly for intestinal type. The common genetic alterations may offer therapeutic targets for treatment of GC. Polymorphisms in Thymidylate synthase to metabolize 5-fluorouracil, glutathione S-transferase for degradation of Cisplatin, and amplification/overexpression of human epidermal growth factor receptor 2 targeted by monoclonal antibody Trastuzumab, are a few examples. P13K/Akt/mTOR pathway, c-Met pathways, epidermal growth factor receptor, insulin-like growth factor receptor, vascular endothelial growth factor receptor fibroblast growth factor receptor, and micro RNAs are several potential therapeutic biomarkers for GC under investigation.

Key words: Molecular genetics; Lauren classification; Intestinal type gastric cancer; Diffuse type gastric cancer; Molecular Biomarker

Core tip: Intestinal and diffuse cell types of gastric carcinoma have a significant difference in clinical outcome with different molecular pathogenetic pathways. Intestinal type gastric carcinoma (GC) is associated with chromosomal and/or microsatellite instability, mutation of tumor suppressor genes, and loss of heterozygosity. Diffuse type GC is commonly associated with mutation of the E-cadherin gene, and a manifestation of the hereditary gastric cancer syndrome. Detection of certain mutations may aid in early diagnosis, screening, and prognostication of GC, and common genetic alterations may offer therapeutic targets for treatment. Furthermore, potential therapeutic biomarkers for GC are under investigation and may hold future promise.

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INTRODUCTION

Gastric carcinoma (GC) is the second major leading cause of cancer-related death and fourth most common cancer worldwide[1]. The relatively unfavorable outcome is largely attributable to complex biology and marginal effectiveness of treatment options, including surgical resection, chemotherapy, and multidisciplinary approach. Even with use of multimodality approaches, overall survival continues to be poor with 30%-36% 5-year survival rates[2,3]. Chemotherapy is the main treatment in cases of metastatic disease, and the median survival time is only 9 to 14 mo[3,4]. However, not all of GCs have the same outcomes. The biological behavior and clinical presentation of GCs differ with their histological and molecular features.

The current World Health Organization classification (2010 edition), classifies GC into many different types based upon the histology combined with molecular genetic information. However, the traditional Lauren classification, purely on histologic basis, is the most commonly used system. It classifies GC into three different groups: (1) intestinal type with glandular differentiation or pattern; (2) diffuse, or poorly differentiated type, including a signet ring cell histology; and (3) mixed or indeterminate type[5]. The first two types of GCs (intestinal and diffuse types) have distinct histogenesis as well as clinical characteristics. Recent data suggest that these two groups largely differ in their molecular genetics. This paper will mainly review the molecular characteristics of non-hereditary intestinal and diffuse types of GC to understand the molecular pathways involved in GC development and to identify molecular targets for diagnosis, therapy, and prognostication.

MOLECULAR BASIS OF PATHOGENESIS

Genetic predisposition of GC

Certain genetic polymorphisms are predisposed to an increased risk for gastric cancer. These polymorphisms were found in genes involved in the inflammatory response to *Helicobacter pylori* (*H. pylori*) infection[6-8], prevention of DNA to oxidative damage[9], and mucosal protection against *H. pylori* infection[10-12], and detoxification[13,14]. Polymorphisms of the interleukin 1 (*IL-1β*) gene consistently show strong association with GC[15]. The association is also seen with other genes, including IL-1 receptor antagonist genes[15-19], tumor necrosis factor-alpha gene[18,20,21], rs11556218 T/G polymorphism of the *IL-16* gene[8], and genes encoding glutathione-S-transferase (GST) (GSTT1 and GSTM1)[22,23]. Many hereditary tumor syndromes increase the risk to develop GC. The high risk association is well-documented in Hereditary diffuse-type gastric cancer syndrome, familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer, Peutz-Jeghers syndrome, Juvenile polyposis and Li-Fraumeni syndrome[24,25].

Molecular pathogenesis of intestinal cell type of GC

The major etiology of the intestinal type includes dietary, environmental factors, and *H. pylori* infection[26]. There are many good reviews that have discussed the role and significance of dietary habits and environmental factors in gastric carcinogenesis, but *H. pylori* infection has also been shown to emerge as an important carcinogen in the stomach. The bacterial virulence factors of *H. pylori* contributing to GC risk include vacuolating cytotoxin A (vacA), blood group antigen binding adhesion 2, outer inflammatory protein, and cytotoxin-associated gene product (*cagA*) genes[27,28]. Infection with a cagA-positive *H.pylori* strain in comparison with a cagA-negative strain increases the risk for development of GC[29]. CagA is translocated into host cells and induces a growth factor-like response in gastric epithelial cells by forming a physical complex with the Src homology 2 domain-containing tyrosine phosphatase in a phosphorylation dependent manner[30,31]. In addition, aberrant expression of activation-induced cytidine deaminase, a gene originally linked to immunoglobulin class switching and B lymphocyte hypermutation, results in accumulation of mutations in the p53 tumor suppressor gene[31,32]. A second virulence gene, the vacA, induces gastric epithelial cell apoptosis and interferes with T cell activation which suppresses local immune response[33]. Chronic inflammation also causes genetic instability through the generation of reactive oxygen and nitrogen species which can directly damage the genomic and mitochondrial DNA[31,34].

The development of GC is a multi-step process. Chronic or atrophic gastritis may lead to intestinal metaplasia, subsequently dysplasia, and eventually carcinoma in some patients[26,35]. During the above progression, a series of genetic alterations occur. The inactivation of tumor suppressor gene p53 is involved in early carcinogenesis, and is found in 38% of intestinal metaplasia, 58% of dysplasia, and 38%-71% of GC[36-38]. Mutations occur more commonly in CpG sites of p53 and transition of G:C to A:T at these sites is the most common type of mutation irrespective of the histologic type of GC[39]. Mutation of p73, a member of the p53 family, is indicated in the carcinogenesis of GC associated with *H. pylori* infection in the mouse model[40].

Chromosomal instability in intestinal-type GC includes gains at 8q, 17q, 20q and losses at 3p and 5q[28,41,42]. Microsatellite instability-high is often seen in intestinal type of GC, largely due to epigenetic effect, (*i.e.*, hypermethylation of the promoter regions of mismatch repair genes, most commonly mutL homolog 1 and mutS homolog 2, and in small percentage of cases, gene mutations[43-45]. The CpG island methylator phenotype was found in 24%-47% of GC, similar to colorectal cancer[43,46-48]. Recent studies have conducted a genome-wide search to identify novel methylation-silenced genes in GC[49,50]. GC cell lines were treated with a demethylating and/or deacetyling agent and were screened for epigenetically silenced genes using oligonucleotide microarrays[49]. The gene encoding serine proteases inhibitor Tissue Factor Pathway Inhibitor 2 was found to be highly methylated (81%) in GC, and its methylation was a significant and independent prognostic indicator in GC[31,49].

Loss of heterozygosity (LOH) or mutation of Adenomatous polyposis coli (*APC*) gene may be found in approximately 25% of the cancer precursors, adenomas, and in up to 60% of intestinal-type GC[51-53]. Mutation of *CTNNB1*, which encodes β-catenin, appears to be exclusive to the mutations that inactivate APC protein[54]. β-catenin accumulates in the cytoplasm, binds to members of the Tcf/Lef family of transcription factors, and is translocated to the nucleus where the Tcf/β-catenin complex activates target genes such as MYC and cyclin D1 gene[31,54]. The incidence of *CTNNB1* mutations in intestinal- *vs* diffuse-type GC remains unclear. One study reported no mutations in diffuse-type GC but 27% incidence in intestinal-type GC[55]. Clements *et al*[56] 2002 found that 26% of tumors with β-catenin nuclear staining contained *CTNNB1* mutations, with no difference between diffuse- and intestinal-type GC.

LOH at the bcl-2 locus and amplification of cyclin D1 and E genes are also associated with intestinal-type GCs[57,58]. The oncogene ErbB2 (Her-2/neu) is amplified in approximately 20% of intestinal type GCs[59]. E-cadherin gene (*CDH1*) mutations have an insignificant association with the development of intestinal-type GC, in contast to diffuse type GC[60,61].

RUNX3 is now accepted as a tumor suppressor gene and first reported in gastric epithelial cells of RUNX3 knockout mice in 2002[62]. Approximately 45%-60% of human GCs show loss of RUNX3 expression due to hemizygous deletion and hypermethylation of the promoter region, and RUNX3 hypermethylation is seen in *H. pylori* infection, intestinal metaplasia, and gastric adenoma[31,62,63]. In response to transforming growth factor (TGF)-β, RUNX3 inhibits gastric epithelial proliferation by inducing the CDKN1A (*p21*) gene[64] and also upregulates the expression of proapoptotic gene BCL2L11 (Bim) in gastric cancer cells treated with TGF-β[65]. Restoration of RUNX3 also strongly inhibited peritoneal metastases of GC in an animal model[66]. RUNX3 inhibited the expression of vascular endothelial growth factor A (VEGF-A) and suppressed angiogenesis and metastasis of GCs[67].

Molecular pathogenesis of diffuse type GC

Little is known about the etiology of diffuse type GC. Epidemiological studies did not link *H pylori* infection to diffuse type of GC. Its association with hereditary gastric cancer predisposition syndrome is well-documented[68]. The unique molecular genetics of this type of GC, in contrast to intestinal-type, is deficiency of the cell-cell adhesion due to genetic or epigenetic inactivation/down regulation of E-cadherin gene (*CDH1*). Approximately 50% of diffuse-type GC harbor this mutation or inactivation[24,69]. The abnormality of *CDH1* gene can be found in early stage of diffuse GC development and loss of E-cadherin expression is seen in invasive and *in situ* carcinomas[70]. In a model proposed by Carneiro *et al*[70], the development of diffuse GC in E-cadherin mutation carriers encompasses *in situ* signet ring carcinoma with pagetoid spread of signet ring cells as pre-invasive lesions. In early hereditary GC, the wild-type CDH1 allele is suppressed or lost in tumor cells with a second hit caused by promoter hypermethylation of CDH1 in at least 50% of cases[71]. Promoter methylation is also part of the major mechanism underlying E-cadherin downregulation in sporadic diffuse gastric cancers[60]. Chromosomal instability in diffuse-type GC include gains at 12q, 13q and losses at 4q, 15q, 16q, and 17p[28,42,72,73]. The diffuse type GC is also associated with the alterations or mutations in other genes or gene products, including the met proto-oncogene encoding the hepatocyte growth factor receptor and the SC-1 antigen (an apoptosis receptor)[74-76].

Molecular pathogenesis of Epstein-Barr virus-associated GC

Five percent of GC is associated with monoclonal proliferation of Epstein-Barr virus (EBV)-infected epithelial cells and is a specific clinicopathologic subset with characteristics of younger age, male predominance, proximal location, lower rate of lymph node involvement, marked lymphocytic infiltration, and lace pattern within the mucosa[31,77]. EBV maintains its latent infection and expresses viral latent genes which include EBV-determined nuclear antigen 1, EBV-encoded small RNA, latent membrane protein 2A (LMP2A) and Bam H1-A rightward transcripts (BARTs)[31,78]. Frequent loss of p16 (CDKN2A), smad4, Fhit, and CD82 (KAI-1) are seen[79]. Global CpG island methylation in the PTEN promoter region is considered as a characteristic abnormality in EBV-associated GC[80] with viral LMP2A responsible for aberrant hypermethylation by activation of host DNA methyltransferase 1[81]. LMP2A also upregulates Birc5 (survivin) expression through the activation of nuclear factor-κB, activates extracellular signal regulated kinases (ERK/MAPK1), and inhibits TGF-β-induced apoptosis through activation of the Ras/PI3K/Akt pathway[31,82-84].

GENETIC CHANGES ASSOCIATED WITH MUCIN PHENOTYPIC EXPRESSION IN GC

GC can be classified into four phenotypes according to mucin (MUC1, MUC2 and CD10) expression: gastric or foveolar phenotype (G-type), intestinal phenotype (I-type), intestinal and gastric mixed phenotype and neither gastric nor intestinal phenotype[31,85]. Genetic changes associated with mucin phenotypic expression in GC include *TP53* mutations in I-type GC and microsatellite instability in G-type. Specific epigenetic alterations include methylation of hMLH1 occurring more frequently in MUC2-negative GC and more frequently methylated MGMT in MUC2-positive GC than in MUC2-negative GC[31,85].

MOLECULAR DIAGNOSIS OF GC

Genetic markers associated with the development of GC are numerous. However, very few have diagnostic utility. CDH1 probably is the best candidate marker for such purpose. About 50 of diffuse type of GC have *CDH1*

mutation, either complete or partial deletions of exons, in more than 70% of somatic E-cadherin mutations[24,68]. This unique gene alteration may have a diagnostic potential. *CDH1* mutations can be detected by polymerase chain reaction on paraffin-embedded tissue. Detection of the germline mutation in CDH1 may help identify asymptomatic mutations carriers of hereditary gastric cancer syndrome and provide molecular basis for prophylactic total gastrectomy[86].

Molecular prognostication

Currently clinical stage is considered to be the gold standard to predict clinical behavior and the most valuable prognostic factor for all GC types. However, clinical stage does not address the issue of tumor heterogeneity. Many studies have investigated molecular biomarkers as alternatives or supplements to the current staging system. There is some success in identifying biomarkers potentially useful in predicting prognosis and therapeutic response.

Microsatellite instability-high is commonly seen in GC located in the distal stomach or antrum, usually intestinal type. It is less frequently associated with metastasis to local lymph nodes[45,87,88]. However, it is still controversial whether patients with MSI-H GC have a favorable long term survival than those with microsatellite instability-low or microsatellite stability GCs[89,90].

Overexpression of matrix metalloproteinases (MMPs) is shown to be related to tumor invasiveness and metastasis[91]. MMP-1-overexpression in GCs has a worse prognosis than tumors without MMP overexpression. VEGF overexpression is associated with shorter survival time attributable to its enhancement of tumor angiogenesis. Amplification or overexpression of cyclin E is correlated with aggressiveness[92]. Amplification/overexpression of the ERBB2 (Her-2/neu) oncogene in general is considered to be an independent, poor prognostic factor[93,94]. Overexpression of EGF-R and abnormal expression of E-cadherin and β-catenin decrease survival or have poor prognosis[95-97]. Abnormal gene expression of IGF2, KIAA1093, OCT2, PCOLCW, PFN2, RBP4, and three genes (*BIK*, *Aurora kinase B* and *eIF5A2*) identified in the primary tumor are related to node metastasis[98,99]. Expression of caudal type homeobox transcription factor 2 (CDX2) and combination of normal expression of E-cadherin and negative expression of the transmembrane protein MUC1 predict a better prognosis for patients with GC[100,101]. Down-regulation of a cyclin dependent kinase inhibitor, P27/Kip1, is a negative prognostic factor[102,103]. Loss of expression of tumor suppressor gene Rb is related to worse overall survival or inversely correlates with tumor invasion[104,105]. Mutation or abnormal expression of p53 may have a reduced cumulative survival, lymph node metastasis, and lower chemosensitivity[100,106,107], but its overall prognostic significance is controversial[108]. Protection of telomere expression levels are also higher in advanced GC[28].

Molecular therapeutic predicators

The management of patients with GC, particularly those in late stage of tumors, usually requires chemotherapy or target therapy as single or one of the components of combined modality. The chemotherapy or target therapy is toxic, and the effectiveness is variable with patients. Molecular biomarkers have been proven to be a useful tool to predicate therapeutic response and may be used clinically to select patients or chemotherapeutic regimen for optimal result.

Predictors for Fluorouracil treatment: (1) Thymidylate synthase (TYMS) is a catabolizing enzyme for fluorouracil (5-FU). Polymorphisms in the gene encoding TYMS affect expression and appear to be associated with poorer response with 5-FU (47 marker) levels[109,110]. A specific polymorphism in the 5'-untranslated regions is correlated with low sensitivity to 5-FU based chemotherapy and decreased survival in a retrospective study[111]; (2) Dihydropyrimidine dehydrogenase (DPD) and thymidine phosphorylase (TP) are two regulatory enzymes involved in the degradation of 5-FU. Low levels of DPD and TP have been shown to be associated with better response[24,112,113]; and (3) The role of other genes or products for predicting 5-FU response has been also investigated, but results are inconclusive. These molecular markers include glutathione S-transferase (GST), vascular endothelial growth factor, and apoptosis-related genes and gene products including Bcl-2, Bax and p53[43,114,115].

Molecular predictors for Cisplatin treatment: Unlike for 5-FU, molecular predicting markers for chemosensitivity of Cisplatin are not well established. GST, an enzyme that degrades Cisplatin, is one of the potential markers. Its activity is affected by polymorphisms in the *GSTM1*, *GSTT1*, and *GSTP1* genes, which may in turn cause variable catabolism of Cisplatin and prognosis[111,116,117]. GC with a high LOH rate or MSI-high show a better response to a Cisplatin-based chemotherapy[118].

Molecular targeted therapy: Trastuzumab is a monoclonal antibody targeting HER-2 that has shown an overall survival benefit when combined with palliative chemotherapy in patients with HER-2 amplified GC[119]. HER-2 is currently the only validated therapeutic target in GC with guidelines for HER-2 testing established by the ToGA trial[119].

HER-2 expression may be assessed by immunohistochemistry (IHC), with scoring ranging from 0 to 3+, by gene amplification using fluorescence in situ hybridization (FISH) or by silver in situ hybridization[28,119]. The survival benefit associated with trastuzumab is seen greatest in IHC 3+ or IHC 2+ and FISH-positive patients. Complete membranous staining is not a prerequisite for IHC 2+ or IHC 3+ scores in GC as it is for breast cancer since gastric tumor cells may only show HER-2 staining at the basolateral or lateral membrane regions[28,120].

Other potential candidates for targeted GC therapy include P13K/Akt/mTOR pathway, c-Met pathways, epidermal growth factor receptor (EGFR), VEGF receptor, insulin-like growth factor receptor, and fibroblast growth

factor receptor[121].

Lapatinib is a dual kinase inhibitor of EGFR and HER-2 under investigation in two ongoing phase Ⅲ clinical trials in a select group of patients positive HER-2[121]. These include the Lapatinib Optimization Study in HER-2 Positive Gastric Cancer study with capecitabine and cisplatin in the first-line setting and the TYTAN study in second-line therapy using paclitaxel[121-123].

In terms of other agents, targeting human EGFR in GC remains controversial. Cetuximab is targeted against EGFR and is a recombinant human, chimeric IgG1 monoclonal antibody[121,124]. With combined chemotherapy and cetuximab, promising results have been shown in a phase Ⅱ trial, but when compared to chemotherapy, the EXPAND study (phase Ⅲ) failed in prolonging the progression free survival (PFS) and overall survival[125]. The REAL-Ⅲ trial did not show any advantage of adding panitumumab to a combination of chemotherapy and also showed a worse overall survival and PFS[126-128]. The combination with matuzumab and chemotherapy seems more promising but was evaluated only in phase Ⅱ trials[129]. Thus, additional studies are necessary.

Antiangiogenic therapy has shown minimal effectiveness when compared to existing treatments for GC[130,131]. Biomarkers such as serum VEGF-A and microvessel density still remain unconfirmed as potentially useful predictive markers by phase Ⅲ trials[121]. However, in advanced cases of GC treated with the VEGF inhibitor bevacizumab, plasma VEGF-A and tumor neuropilin-1 are strong biomarker candidates for predicting clinical outcome[132].

Various ongoing trials are testing potential targeting agents addressed to the downstream components of VEGF-R/EGFR, such as inhibitors of mTOR, c-Met, and Histone deacetylase[121]. The phase Ⅲ trial (GRANITE-1) of everolimus, an inhibitor of the P13K/Akt/mTOR pathway, has reported prolonged PFS with a 34% reduction of the risk of progression[121,133].

MICRORNAS AS THERAPEUTIC TARGETS

In recent years, microRNAs (miRNAs) have been investigated as potential markers in treatment of GC. MiRNAs are important regulators of genes with critical roles in cell proliferation, differentiation, and survival[134]. MiRNAs play important roles in the pathogenesis of a variety of malignancies[135-139]. Different miRNA methylation profiles are seen in various cancers. MiR-155 is down-regulated and methylated in GC[140]. MiR-155 is up-regulated in breast cancer[141], colorectal cancer[142] and pancreatic ductal adenocarcinoma[143]. Upregulated miRNAs might act as oncogenes and target tumor suppressors, while down-regulated miRNAs might act as tumor suppressors and target oncogenes[144]. Several miRNAs have been found to be deregulated in GC but the specific molecular mechanisms are unknown[144]. DNA hypermethylation in the miRNA 50 regulatory region accounts for the downregulation of miRNA in tumors[145,146], and many miRNAs have been reported to be down-regulated due to hypermethylation of the CpG islands in GC. MiR-124a-1, miR-124a-2 and miR-124a-3 have been found to be methylation-silenced in GC cell lines[144,147]. Such epigenetic changes are reversible, and make them a potential therapeutic target. Silenced miRNAs in GC could be restored by treating with demethylating agents, such as decitabine (5-aza-20-deoxycytidine), which leads to inhibition of growth, invasion, and metastasis of GC cells[148].

Interestingly, studies have shown that the miRNA methylation levels are positively associated with the clinical stage of GC patients[144]. Low expression of miR-34b and miR-129-3p are associated with a poor clinical outcome of GC patients, and hypermethylation of miR-129-2 and miR-34b CpG islands had a tendency to show poor clinicopathological features[144,149]. Thus, specific miRNA methylation levels may be used in the prognosis of GC patients. However, limitations exist as several factors besides methylation can affect miRNA expression levels. As reported by Tsukamoto *et al*[148], the expression of miR-375 in NUGC3 cells can be significantly increased with either 5-aza-2-deoxycytidine and markedly up-regulated by greater than 20-fold when treated with both 5-aza-2-deoxycytidine and trichostatin A[148]. In addition, *H. pylori* infection can induce aberrant DNA methylation in gastric epithelial cells[150]. Individuals with *H. pylori* had 7.8-13.1-fold higher methylation levels than those without *H. pylori* infection[147,151]. Another limitation is the serious side effects of demethylating drugs. The use of demethylating agents may induce the expression of many otherwise normally silenced genes and cause a variety of diseases. Thus, the use of demethylating agents in restoring the expression of epigenetically silenced miRNA in GC still requires further investigation.

CONCLUSION

The current research has provided some insights to the genetics of gastric cancer. Clinical trials based upon the genetic information have generated promising results. However, up to date, we still do not have an optimal solution for prevention, early diagnosis, and treatment of this disease. The advanced molecular technology, particularly next generation sequencing, may offer hope in deciphering the myth behind the molecular genetics of gastric cancer. Equipped with the advanced technology, together with efforts from clinical oncology and bioinformatics, we have gradually gained much more understanding about the genetic basis of the host-environmental interaction and will have a greater opportunity to identify diagnostic and therapeutic markers for gastric cancer. These advancements have shed light in finding a cure for gastric cancer in the near future.

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REVIEW

Genome engineering using the CRISPR/Cas system

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Core tip: This review introduces the latest information about the genome manipulation technology of the clustered regularly at interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) system to readers. We focus particularly on the application of CRISPR/Cas in mammalian cultured cells and mice. The problems of off-target effects and the prospects for therapeutic applications of CRISPR/Cas in the future are also discussed.

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Abstract

Recently, an epoch-making genome engineering technology using clustered regularly at interspaced short palindromic repeats (CRISPR) and CRISPR associated (Cas) nucleases, was developed. Previous technologies for genome manipulation require the time-consuming design and construction of genome-engineered nucleases for each target and have, therefore, not been widely used in mouse research where standard techniques based on homologous recombination are commonly used. The CRISPR/Cas system only requires the design of sequences complementary to a target locus, making this technology fast and straightforward. In addition, CRISPR/Cas can be used to generate mice carrying mutations in multiple genes in a single step, an achievement not possible using other methods. Here, we review the uses of this technology in genetic analysis and manipulation, including achievements made possible to date and the prospects for future therapeutic applications.

INTRODUCTION

The recent development of site-specific endonuclease technologies for selective genome cleavage has been an important advance in mammalian genome engineering. Zinc-finger nucleases (ZFNs) consist of specific DNA-binding zinc-finger proteins and a nuclease domain of the *Fok* I endonuclease[1-3]. Cleavage with *Fok* I requires dimerization of the protein; therefore, fusion to a pair of zinc-finger proteins provides target specificity, and allows cleavage of the target DNA locus, generating double-strand breaks (DSBs).

On the other hand, transcription activator-like effector (TALE) nucleases (TALENs) are fusions of DNA-binding domain TALE repeats with the cleavage domain of the *Fok* I restriction enzyme. TALE repeats are highly conserved 33-35 amino acid sequences found in naturally occurring TALEs encoded by *Xanthomonas* bacteria[4]. Each TALE repeat binds to a single base pair of DNA and the identities of the amino acids at two posi-

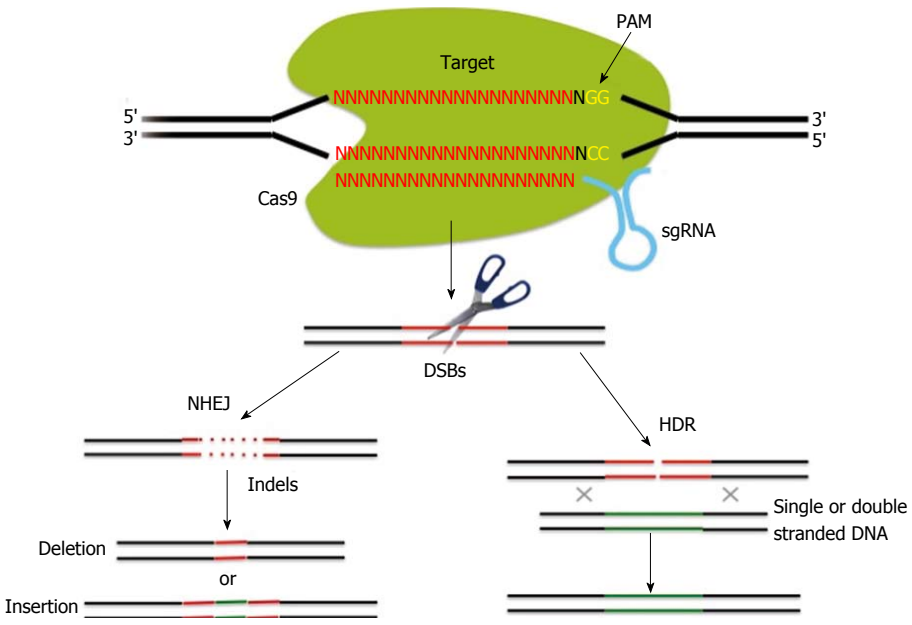

Figure 1 Schematic of the principles of clustered regularly at interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas)-mediated genome editing. The CRISPR associated 9 (Cas9) endonuclease can generate sequence-specific double strand breaks (DSBs) of target DNAs bound to small guide RNAs (sgRNAs). The binding site of a target DNA requires a protospacer-adjacent motif (PAM) (with the sequence NGG). DSBs generated by the Cas9 endonuclease are repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR).

tions have been associated with specificities for different nucleotides[5,6].

These chimeric nucleases enable genome editing by inducing targeted DNA DSBs that are repaired by error-prone, non-homologous end joining (NHEJ) or homology-directed repair (HDR)[7-10]. NHEJ-mediated repair induces small insertions or deletions (indels) at the cleavage site, and results in disruption of gene function by frame-shift mutations. In the presence of a single- or double-stranded DNA template containing homology to the sequences flanking the DSB, mutant alleles with precise-point mutations or DNA inserts can be produced by HDR. However, both ZFNs and TALENs require the design of DNA-binding proteins and the construction of complicated plasmids for expression of these, making these methods time-consuming and laborius.

Recently, a new efficient genome manipulation technology, clustered regularly at interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) system, which uses the RNA-guided nuclease, Cas9, and is derived from the immune system of bacteria and archaea, has been developed. CRISPR/Cas technology has the advantages of a highly efficient mutation rate and simple-to-design target-specific RNA molecules, compared to the complex ZFN and TALEN systems. Therefore, CRISPR/Cas has been rapidly adopted and applied to many species in a short period of time[11-40].

Several reviews about CRISPR/Cas have already been published[41-44]; however, this technology is progressing rapidly, with new reports published weekly. Here, we introduce recent research made possible by CRISPR/Cas technologies and discuss the application of these reagents for genetic analysis and manipulation. We also show the therapeutic potential of CRISPR/Cas and make discussion of future prospects for the field.

THE CRISPR/CAS SYSTEM

CRISPR/Cas is the RNA-based acquired immunity system in bacteria and archaea[45,46]. CRISPR RNA-guided Cas9 nucleases use short RNAs to target and cleave DNA elements captured from foreign invaders (termed "spacers") in a sequence-specific manner. In the type Ⅱ CRISPR/Cas system, a single gene encoding the Cas9 protein and two RNAs, a mature CRISPR RNA (crRNA) which is transcribed from spacers, and a partially complementary transacting RNA (tracrRNA) are sufficient for RNA-guided cleavage of foreign DNAs. For maturation of crRNA, RNase Ⅲ and tracrRNA are necessary[47]; however, this process can be simplified by an engineered small guide RNA (sgRNA) containing a hairpin that mimics the tracrRNA-crRNA complex and short guide sequence[48] with a protospacer-adjacent motif (with the sequence NGG, Figure 1)[49]. Thus, the Cas9 endonuclease can generate sequence-specific DSBs of target DNAs bound to sgRNAs (Figure 1). DSBs generated by the Cas9 endonuclease are repaired by NHEJ or HDR[7-10]. NHEJ-mediated repair leads to the generation of small indels at the targeted site, which results in disruption of gene function *via* frame-shift mutations. In the presence of a single- or double-stranded DNA template with homology to the sequences flanking the DSB, mutant alleles with precise-point mutations or DNA inserts can be produced by HDR.

GENOME EDITING IN CULTURED CELLS

When the CRISPR/Cas system in bacteria and archaea

was elucidated, many researchers expected that it functions in the cells of eukaryotic organisms such as yeast, plants, and even mammals. In January 2013, several papers using the CRISPR/Cas system in human cells were published in succession[50-53]. Cho *et al*[52] showed that combination of Cas9 protein and artificial sgRNAs efficiently cleaved two genomic sites and induced indels with approximately 33% frequencies using human embryonic kidney (HEK) 293T-cells. Two papers published in Science used other cell types or targeting loci[50,51]. For the endogenous AAVS1 safe harbor genomic locus, Mali *et al*[50] suceeded in gene targeting using 293T-cells (10%-25%), human chronic myelogenous leukemia K562 cells (8%-13%), and human induced pluripotent stem (iPS) cells (2%-4%). In addition, they also used HDR to integrate either a double-stranded DNA donor construct (SA-2A-Puro-pA + CAG-GFP-pA) or an oligo donor into the native AAVS1locus, and obtained 293T or iPS clones showing HDR-mediated integration.

CRISPR/Cas also enables NHEJ- and HDR-mediated genome editing in mouse ES cells[54,55]. The high efficiency of the CRISPR/Cas system coupled with the ability to easily create synthetic sgRNAs make it possible to target multiple genes simultaneously, which is not possible using previous methods[54]. Wang *et al*[54] transformed embryonic stem cells using CRISPR/Cas system for three different genes (*Tet1*, *Tet2*, and *Tet3*), and found that > 20% (20/96) of ES cell clones had mutations in all six alleles. To further test the potential of multiplexed gene targeting using the CRISPR/Cas system, sgRNAs targeting five genes (*Tet1*, *Tet2*, *Tet3*, *Sry*, and *Uty*) were ,mixed and co-transfected with a Cas9-expressing vector into ES cells; of 96 clones screened using an restriction fragment length polymorphism assay, 10% carried mutations at all five loci.

The use of the CRISPR/Cas system in combination with haploid ES cells[56-58] provides a powerful platform to manipulate the mammalian genome, because disruption of only one allele can cause loss-of-function phenotypes in haploid ES cells. We have recently reported that co-transfection of mouse haploid ES cells with vectors expressing Cas9 nuclease and sgRNAs targeting *Tet1*, *Tet2*, and *Tet3* results in the complete disruption of all three genes, causing a loss-of-function phenotype with higher efficiency (50%)[59] than that previously reported using diploid ES cells[54]. Thus, the CRISPR/Cas system used in the context of haploid cells will be useful for the efficient disruption of multiple genes.

ONE-STEP GENERATION OF GENOME-EDITED ANIMALS

Homologous recombination in mouse ES cells is the most popular method for targeted modifications of the mouse genome; however, generating gene-modified mice through germline chimeras is both time consuming and expensive. Therefore, alternative methods have been developed to accelerate the process of genome modification by the introduction of site-specific nucleases into fertilized embryos to generate DNA DSBs at a target locus in various species. ZFNs and TALENs have been used to produce several gene-modified rodents[60-62]. Although these technologies are widely used in other animals, their use in mice has been limited, principally because the ZFN and TALEN systems are labor-intensive and expensive techniques that do not perform substantially better than ordinary gene knockout technology. On the other hand, CRISPR/Cas-mediated genome editing has successfully demonstrated one-step generation of gene-modified mice, and this technology became widely used within only one year[54,55,63-65]. To understand the functions of genes in families of two or more members, animals carrying multiple mutated genes are required; however, ZFNs or TALENs cannot be multiplexed to generate animals with several targeted loci. In contrast, the CRISPR/Cas system can be used to generate mice carrying mutations in multiple genes in one step[54]. Co-injection of Cas9 mRNA and sgRNAs for *Tet1* and *Tet2* into fertilized embryos led to the generation of mice with biallelic mutations in both genes with an efficiency of 78% (22/28). Wang *et al*[54] also showed that co-injection of Cas9 mRNA and sgRNAs with mutant oligos generated precise-point mutations simultaneously in two target genes with an efficiency of 20% (2/10). Using this "one-step" procedure, Yang *et al*[55] produced mice carrying a tag or a fluorescent reporter construct in the *Oct4, Sox2*, and *Nanog* genes. In addition, *Mecp2* conditional mutant mice with two loxP sites were generated[55]. These results show that a single step by CRISPR/Cas-mediated genome editing can generate mice having NHEJ- or HDR-mediated mutations in multiple genes.

OFF-TARGET MUTATIONS

Compared to ZFNs and TALENs, CRISPR/Cas technology has the advantages of a highly efficient mutation rate and the simplicity of the design of target-specific sgRNAs. It is difficult to compare the off-target effect risk among ZFN, TALEN, and CRISPR/Cas. Although the cleavage of off-target sites has also been observed in ZFN and TALEN systems[66,67], it appears to be less likely because they require two adjacent recognition sites, while the CRISPR/Cas system requires only one. Therefore, it is important to pay careful attention to the specificity of CRISPR/Cas target sequences, because off-target mutations are detrimental to experimental results.

When genome-edited mice are produced using the CRISPR/Cas system, they are rarely influenced by off-target effects. For example, of seven double-mutant mice produced by injection with high RNA concentrations, none showed effects at potential off-target loci using the Surveyor assay[54]. Mashiko *et al*[65] found only one off-target mutation in a total of 144 sites examined. In addition, Fujii *et al*[64] proposed that off-target effects are mostly avoided by the careful control of Cas9 mRNA concentration. Surprisingly, the optimized CRISPR/Cas system has a higher gene targeting rate and a lower occurrence of

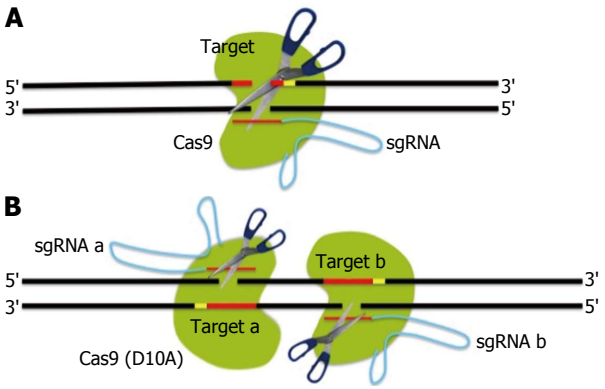

Figure 2 Improvement of site-specificity by double nicking. A: Double strand breaks (DSBs) using wild-type interspaced short palindromic repeats associated 9 (Cas9) endonuclease; B: DSBs using a pair of small guide RNAs (sgRNAs) guiding Cas9 D10A nickases. Using paired nicking can reduce off-target activity because individual nicks that unexpectedly occurred at off-target sites are predominantly repaired by the high-fidelity base excision repair pathway.

off-target effects compared to ZFN[64]. Mutant mouse ES cells generated by the CRISPR/Cas system also showed a very low Cas9-mediated cleavage rate in off-target loci[55]. These reports suggest that the CRISPR/Cas system is highly specific in the "one-step generation" of mutant mice and mouse ES cells.

By contrast, study of the CRISPR/Cas system in human cancer cell lines indicated a widespread occurrence of off-target mutations[68,69]. Cas9-mediated cleavage can be abolished by single mismatches at the sgRNA-target site interface, particularly in the last 10-12 nucleotides located at the 3' end of the 20-nt sgRNA-targeting sequence[48,51]. Using human cell lines (U2OS.EGFP, HEK293, and K562), Fu *et al*[68] found that one or two mismatches are tolerated to varying degrees, depending on their position along the sgRNA-DNA interface. In addition, they easily detected off-target alterations induced by 66% (4/6) of CRISPR/Cas experiments targeting endogenous loci by examination of partially mismatched sites. However, these mismatches were mainly located in the 5' region, with only one base mismatch detected in the last 12 nucleotides at the 3' end of one off-target locus.

Yang *et al*[55] considered several possibilities to explain the lower off-target cleavage rate observed in animals derived from manipulated zygotes compared to the results reported for CRISPR/Cas-treated human cell lines including the following: (1) the cells analyzed in mice and humans are clonal and heterogenous populations, respectively; (2) the transformed human cell lines may have different DNA damage responses, resulting in a different mutagenesis rate compared to normal one-cell embryos; and (3) introduced nucleotides are short-lived RNA or long-lived DNA plasmids in mouse and human systems, respectively, which lead to more extensive cleavage in human cells; however, a definitive explanation has not yet been found.

Several measures to improve the specificity of Cas9-mediated genome editing have been assessed. Firstly, it was hypothesized that cleavage specificity may be improved by increasing the length of the region of base pairing between the sgRNA and its target locus. To test this, Ran *et al*[70] generated sgRNAs with 20 or 30 nucleotides guide sequences; however, they found that extension of the guide sequence did not improve Cas9 targeting specificity. Next, Ran *et al*[70] developed a strategy that combines the D10A mutant nickase version of Cas9[48,51,71] with a pair of offset sgRNAs complementary to opposite strands of the target site (Figure 2A: DSBs using wild-type Cas9 endonuclease; B: DSBs using a pair of sgRNAs guiding Cas9 D10A). Whereas nicking of both DNA strands by a pair of Cas9 nickases leads to site-specific DSBs and NHEJ, individual nicks are predominantly repaired by the high-fidelity base excision repair pathway[72]. As a result, this double nicking method can reduce off-target activity by 50- to 1500-fold and assisted gene knockout without reduction of on-target cleavage efficiency[70,73]. Double nicking allows not only NHEJ-mediated indels but also insertion into the genome *via* HDR in human cells.

In the case of mutant animals produced by CRISPR/Cas, off-target mutations will be eliminated by backcrossing to wild-type animals. Therefore, if researchers do not use F0 pups obtained by CRISPR/Cas for experiments, off-target effects should not be a concern. RNA interference (RNAi) experiment to induce sequence-specific gene silencing is now a standard method for the functional analysis of genes. However, designed small RNA frequently repress translation from unexpected loci[74-76]. To remove this off-target effect, two or more independent small RNAs are generally used in RNAi experiments. In CRISPR/Cas experiment, use of two or more independent sgRNAs for a gene will be also an effective control to remove off-target noise and improve the reliability of the obtained phenotype. Nevertheless, more detailed work will be necessary to determine the frequency of off-target mutations, and improve the specificity in CRISPR/Cas systems.

PROSPECTIVE APPLICATIONS OF THE CRISPR/CAS SYSTEM

Precise genome modifications by CRISPR/Cas system excite the interest of scientists working in both basic science and applied fields, including gene therapy. Undoubtedly, the CRISPR/Cas system is a strong candidate for application in human gene therapy. Several human iPS cell lines have been generated from patients for stem cell-based gene therapy by correction of gene mutations. But, gene targeting in human pluripotent stem cells including ES and iPS cells has been very difficult historically[77]. Nevertheless, ZFNs and TALENs are capable of correcting gene mutations mediated by HDR repair mechanisms in human iPS cells[78-82] and the CRISPR/Cas system has also recently been applied to the gene therapy model[50, 83]. Of course, this application will require a highly efficient gene editing rate and no off-target mutations.

CRISPR/Cas is thought to be applicable for genome

editing based only on NHEJ or HDR; however, nuclease-null Cas9 (Cas9N) can work as a transcriptional activator or silencer without changing DNA sequences[84,85]. Mali *et al*[84] produced a Cas9N directly fused with the VP64 activation domain to generate a Cas9N-fusion protein capable of transcriptional activation. This Cas9N-VP64 protein robustly activated transcription of reporter constructs and endogeneous *REX1*, *OCT4*, *SOX2*, and *NANOG* genes when this fusion protein is combined with sgRNA-targeting sequences near the promoter[84]. This is the example of RNA-guided transcriptional activation. By contrast, a Cas9N-sgRNA complex is specifically able to interfere with transcriptional elongation, transcription factor binding, or RNA polymerase binding[85]. This technology could be applied to genome-wide screens for gene function.

Prior genome-editing technologies, ZFNs and TALENs, suggest new applications for CRISPR/Cas. For example, Konermann *et al*[86] developed a light-inducible genome-editing system, using transcriptional effectors and the customizable TALE DNA-binding domain. They suceeded in transcriptional activation and epigenetic modification of endogenous genes using primary neurons as well as brain of living mice.

Bacterial DNA methyltransferases[87-91] and human DNA metyltransferase 3a and 3b subunits[92-94] have been fused to zinc-finger proteins and successfully demonstrated to perform targeted DNA methylation. Efficient targeting of DNA demethylation was also demonstrated using fusions of TALE repeat arrays and the TET1 hydroxylase catalytic domain (TALE-TET1)[95]. These targeted methylation and demethylation technologies will be applicable for gene therapy of cancer and other epigenetic diseases such as Beckwith-Wiedemann and Angelman syndromes mediated by abnormal DNA methylation, or of Huntington disease, which is caused by by extra repetitive DNA sequences. In addition, Jiang *et al*[96] inserted an inducible *XIST* transgene into chromosome 21 using ZFN in iPS cells derived from a Down's syndrome patient. In this system, chromosome 21 are coated with *XIST* non-coding RNA, followed by stable heterochromatin modifications, DNA methylation and chromosome-wide transcriptional silencing. This successful silencing of trisomy is the first step for chromosome therapy using genome engineering. These applications developed using ZFN and TALEN systems will be also applicable using the CRISPR/Cas technique.

In the future, CRISPR/Cas may be used to target the viral DNA that becomes integrated into the chromosomes of people with lifetime infections (*e.g.*, HIV). If this viral genetic material can be disrupted using the CRISPR/Cas system, this could negate the need for patients to continue taking antiviral drugs throughout their lives.

CONCLUSION

CRISPR/Cas has already been applied to many species in which genome engineering has been difficult, because this technology has the advantages of a highly efficient mutation rate and a simple system for design of target-specific sgRNA. Although improvements in the specificity of CRISPR/Cas will be necessary to eliminate off-target effects, the technique will be indispensable for researchers in both basic and applied science.

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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