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Use of CRISPER for Gene Editing in Mosquito that Transmit Malaria

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ABSTRACT

Malaria is one of global silent and thoughtful medical concern, caused by Plasmodium parasites which is spread via the bites of female mosquitos specifically Anopheles gambiae. In spite of current and advanced vector control measures and therapeutic precautions, the development of insecticide-resistant mosquitoes encourages the requirement for new approaches for its management like herbal products or gene-editing. CRISPR/Cas9, a new genome-manipulating technique, provides incomparable precision and competence for genetic modulation that makes it a promising option for suppressing malaria-carrying populations of mosquito. The CRISPR/Cas9 system contains of the Cas9 nuclease and a guide RNA, which work collectively to make alterations in targeted DNA. In mosquitos, this technique has been used to decrease number of malarias spreading vectors by targeting its productiveness or viability genes. Active transport of CRISPR/Cas9 composite into mosquito cells is crucial for effective gene editing, and more than a few techniques have been developed and improved. Microinjection is a frequently employed method that contains injecting Cas9 protein, mRNA, and guide RNA straight into embryos of vector. Receptor-Mediated Ovary Transduction of Cargo, which updates the transfer procedure by inserting Cas9-peptide complexes into adult female mosquitos. These short-proteins fix to specific ovary receptors, permitting the carriage for removal. Improving CRISPR/Cas9 delivery methods is important for actual and active gene editing in vector mosquitos. These advances can aid to shape effective preventive measures.

INTRODUCTION

Malaria remains the most common parasitic disease affecting humans worldwide, with 228 million infections and 405,000 deaths each year (Ashour & Othman, 2020). Although well-controlled in many places, it threatens over a billion people in poor areas, mainly in southern Africa. Current success rates are based on artemisinin-based combination therapy (ACT), indoor residual spraying of insecticides, and insecticide-treated mosquito nets. Malaria control measures have become ineffective due to the emergence of multidrug-resistant parasites and

insecticide-resistant mosquitos, resulting in a halt or even reverse in the reduction of malaria infections. (Moyes et al., 2020; Shaw & Catteruccia, 2019). Resistance to frontline artemisinin-based treatments is on the rise in Southeast Asia's Greater Mekong Subregion, raising concerns. Artemisinin-resistant genotypes have emerged in Africa, potentially jeopardizing global malaria control efforts. Indeed, there is an increasing amount of evidence of pesticide resistance in malaria-transmitting vectors, which is endangering the viability of malaria vector control initiatives (Mekuriaw et al., 2019). Vector transmission control is usually mediated by two major strategies: lowering the vector competence for infections, referred to as 'population replacement', or spreading sterility among vector populations, referred to as 'population suppression'(Crampton et al., 1994).

The human malaria transmission system has four components: (i) the protozoan parasite Plasmodium, (ii) the human host, (iii) the mosquito vector, and (iv) the environment. Controlling the vector has significantly reduced malaria incidence in Africa (Bhatt et al., 2015). The current vector control programs rely mainly on the use of chemical-based insecticides through the insecticide-treated nets (ITNs) which includes pyrethroids and the indoor residual spraying (IRS) with organophosphates and carbamates. The purpose of these conventional tools is to reduce vector density below the threshold required for transmission or to prevent human-vector contact (Karunamoorthi, 2011). Specifically, the Anopheles mosquitoes are among the most important malaria vectors in endemic areas. In African countries, the leading Anopheles vectors of human malaria are *Anopheles gambiae, Anopheles arabiensis, Anopheles coluzzii*, and *Anopheles funestus*, as well as *Anopheles stephensi* (Sinka et al., 2020).

The adaptability of CRISPR-Cas9 nucleases has enhanced the expansion of homing-based gene-drive systems with enormous promise for control of mosquitos (Gantz et al., 2015). Considerable hard work has been put into the progress of such gene-drive systems, including appraisals of potential impediments with decrease in resistance emergence being a major barrier to be overcome (Hammond et al., 2017). Before to open-field testing, it is also crucial to analyze and manage any threats that may occur, including those are associated with cleavage at the off-target locations by the CRISPR (Hammond et al., 2017). A nuclease-induced change could become common if it improves the mosquito's fitness and thus is absolutely selected, or if off-target cleavage occurs at such a high rate at that site that the break itself that results in a high frequency of the mutation. Other mutations may harm the fitness of genetically modified mosquitoes, influencing the efficacy of gene drive (Esvelt et al., 2014; Hammond & Galizi, 2017). Although not expected, mutations resulting from off-target cleavage events have the potential to affect epidemiologically important traits such as insecticide resistance or pathogen susceptibility, and the risk of these unintended consequences should be assessed on an individual case basis.

The CRISPR/Cas9 system uses programmable sgRNA to locate and attach to specific sections of the genome, where the Cas9 nuclease causes double-strand breaks (DSBs) at the guide sequence-specified places. Defective endogenous genes can then be repaired by deleting specific portions of the target gene or adding an exogenous strand of DNA, depending on the DSB repair mechanism (Sharma et al., 2021).

This study aims to improve vector mediated control of malaria by merging and compiling existing research and focusing on using CRISPER/Cas9 delivery system to introduce new version of mosquitos which play an important role in malaria transmission.

Development of the Malaria parasite in the Mosquito

Plasmodium, an apicomplexan parasite, causes malaria, which is vectored by anopheles' mosquitos. The parasite is haploid for the most of its life cycle and causes disease of malaria in the vertebrate host during the asexual stage of red blood cells (Frischknecht & Matuschewski, 2017). During its replicative phase, some parasites discriminate into sexual cells, male and female gametocytes, which are halted during the G0 phase of the cell cycle. When a mosquito intake a blood meal, the male (micro)gametocyte activates and undergoes three rounds of DNA replication and mitosis to produce eight haploid microgametes

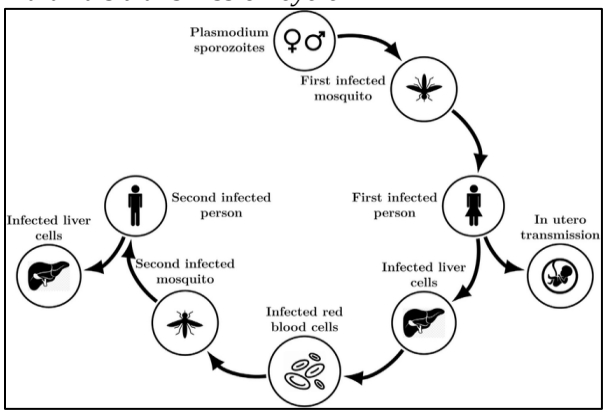
(Ganter et al., 2017). These microgametes then fertilize activated females (macrogametes). The diploid zygote goes through the first stage of meiosis, which is a single round of DNA replication that results in a tetraploid cell. This cell then divides and elongates into a mature ookinete, which enters the mosquito midgut wall and develops into an oocyst. After numerous rounds of endomitotic division and cytokinesis, hundreds of haploid sporozoites (known as sporogony) are liberated from the oocyst and travel to the salivary glands for transmission to the vertebrate host during the mosquito's next blood meal (Gerald et al., 2011).

Different proliferative stages exhibit two unique types of mitosis and a single phase of meiosis.

Mitosis has three stages includes pre-erythrocytic schizogony in liver hepatocytes, blood stage schizogony in host erythrocytes, and sporogony in mosquitos (Aly et al., 2009). During this asynchronous, closed endomitotic division before cytokinesis, genome duplication and segregation of the chromosomes to occur through a spindle within an intact nuclear membrane, without the traditional morphological features of mitosis, such as chromosome condensation (Aikawa & Beaudoin, 1968). Male gametocytes have abnormal mitosis, with three rounds the endo-reduplication creates an octoploid nucleus, which is then followed by chromosomal condensation and nuclear budding to create haploid gametes (Guttery et al., 2012). Meiosis remains a mystery, with the tetraploid ookinete lacking chromosomal condensation despite having four nuclear poles.

Figure 1

Malaria's transmission cycle



Sexual Differentiation and Proliferation

Mosquito gametocytes are activated by environmental conditions such as a reduction in temperature (to 20-25°C), a rise in pH (Billker et al., 1997), and the presence of xanthurenic acid (XA). Microgametocytes quickly form a tetrad of kinetosomes (Zeeshan et al., 2019), which create microtubule-organizing centers (MTOCs) and kinetochores, anchoring mitotic spindles and axonemes. Within 12 minutes, the microgametocyte completed three rounds of endomitotic DNA replication, increasing its DNA content from 1N to 8N (Zeeshan et al., 2021). Following karyokinesis and atypical cytokinesis, eight microgametes exit the parasite body (exflagellation), leaving behind leftover nuclear and cytoplasmic aggregates (Sinden, 2015).

Fluorescently tagged proteins, including as kinesin-8B, NDC80, and kinesin-5, have revealed unique aspects of male gametogenesis. Kinesin-8B is a basal body marker found on kinetosome tetrads that helps with axoneme formation, whereas NDC80 is a kinetochore marker for chromosome segregation. Fluorescence microscopy reveals that Kinesin-5 is located on spindles near kinetochores (NDC80). Electron microscopy is the most reliable approach for investigating the subcellular location of cell organelles. Electron micrographs of the basal body, nuclear pole, and kinetochore supplement live-cell imaging. Electron micrographs reveal the normal 9+2 arrangement of microtubules in flagella, albeit Plasmodium flagellum formation is different. Male gametocytes lack an intraflagellar transport (IFT) system, therefore all flagellum constituents are formed in their cytoplasm. The lack of IFT may contribute to the inefficiency of the process, resulting in incompletely produced axonemes. Several proteins orchestrate male gametogony, resulting in a quick and highly coordinated sequence. In P. falciparum, XA activates guanylyl cyclases and phospholipase C (PLC), leading to an increase in cyclic GMP (cGMP) and activation of protein kinase G (PKG) (McRobert et al., 2008). This process is characterized by transient and reversible protein phosphorylation. In P. berghei, XA activates phosphoinositide (PI)-PLC and hydrolyzes phosphatidylinositol 4,5-bisphosphate, leading to a fast increase in calcium from internal and external reserves. CDPK4 begins DNA replication and

regulates efficient axoneme assembly with PF16 and SAS6 (Straschil et al., 2010). CDPK1 initiates gametogony, while MAP2 and SRPK regulate axoneme motility and cytokinesis, resulting in eight microgametes. Cell division cycle 20 (CDC20) and anaphase-promoting complex/cyclosome (APC/C) member APC3 are important proteins in cytokinesis. Deleting CDC20 and conditionally knocking down APC3 leads to loss of chromatin condensation at the nuclear spindle/kinetochore stage (Wall et al., 2018). Exflagellation involves the metallo-dependent protein phosphatase PPM1, cyclin-dependent kinase-related kinase 5 (CRK5), and Ca2+-dependent calcineurin A. However, the exact timing of their action is uncertain. After DNA replication, axonemes move and exflagellate by egressing basal bodies from the main cellular body. This is facilitated by actin-II and kinesin-8B, which are microtubule-based motors. After detachment, PF16 regulates microgamete motility until it connects to a female gamete via male-specific 6-cysteine proteins P48/45 and P230 (van Dijk et al., 2001) and female-specific 6-cysteine protein P47. The plant-sterility gene HAP2/GCS1 stimulates gamete fusion (Mori et al., 2010), while the histone chaperone FACT promotes chromatin transcription. Exposure to mosquito-derived factors in the mosquito midgut activates female gametocytes, causing PKG activation, cell rounding, and emergence from the erythrocyte. Female-specific proteins, such as PfG377, MDV1, and PPLP2, are secreted and have a biolytic function. During female gamete development, hundreds of proteins undergo translational suppression to prepare for the zygote-to-ookinete transition (Balestra et al., 2020).

Asexual Proliferation and Transmission

The zygote-to-ookinete transition and oocyst formation are critical stages in the parasite life cycle. Only 50 to 100 out of thousands of ingested gametocytes mature into ookinetes. Only 10% will complete oocyst formation (Whitten et al., 2006), while the majority (>80%) of ookinetes are killed (Shiao et al., 2006). The longest step of the life cycle is oocyst maturation, which lasts about 14 days. After 21 days, mature sporozoites infiltrate the mosquito salivary glands and are ready for transmission during the next blood meal. After passing through the peritrophic matrix and midgut epithelium, an ookinete attaches to the basal lamina and differentiates into an oocyst (Sinden, 2002). This process is believed to occur through the interaction of P25/28, CTRP, and SOAP with laminin. During sporogony, the parasite genome replicates numerous times, causing the oocyst to grow to 50-60 μm in diameter. NDC80, a kinetochore marker, was utilized to track endomitosis and identified several foci near nuclear DNA.

Sporogony involves a diversity of proteins, with obtaining nutrients from host, avoiding the immune system of mosquito and regulation of DNA replication and its metabolic processes takes place in parasite (Stanway et al., 2019). Plasmodium specific P-type cyclin (CYC3) plays a important role in endomitosis during oocyst growth. In absence of it, the plasmalemma fails to to generate the sporoblasts. Deleting some genes for the repair of DNA the protein meiotic recombination 11 (MRE11) and 3-hydroxyacyl-CoA dehydratase (DEH), which plays a role in production of fatty acid, that leads to degenerative oocysts and no sporogony (Guttery et al., 2020). Functional studies of Plasmodium FAS have shown that deleting components of FAS II pathway leads to deterioration of oocysts and sporogony (van Schaijk et al., 2014). Protein kinases GAK and PK7, as well as PPM5, have been associated to oocyst formation. Adult sporozoites exit the oocyst into the hemocoel 16-21 days after a blood meal, facilitated by ECP1 and Circumsporozoite Protein (CSP). The sporozoites move to the salivary glands and enter the tissue using TRAP, UOS3, CRMPs, and MAEBL (Thompson et al., 2007).

Molecular Components Involved in Parasite Proliferation in Mosquito

Molecular and genome based useful shades have helped to determine crucial factors in growth of malarial parasite in craniate hosts and non-craniate like mosquito as vectors. Two of the functional screens exposed that more than half of the Plasmodium genome is important for development of blood stage (Bushell et al., 2017). Additionally, over 450 genes that are dispensable at this stage are necessary for effective transmission through mosquitos (Stanway et al., 2019). The mosquito parasite develops through two main processes: gene expression control and reversible protein phosphorylation. Here, we discuss key studies that

have elucidated the role of transcription factors, posttranslational modification by reversible protein phosphorylation, and the function of motor proteins in regulating parasite development, proliferation, differentiation, and invasion.

Table 1

Molecules that control parasite proliferation, invasion, and development in the mosquito vector

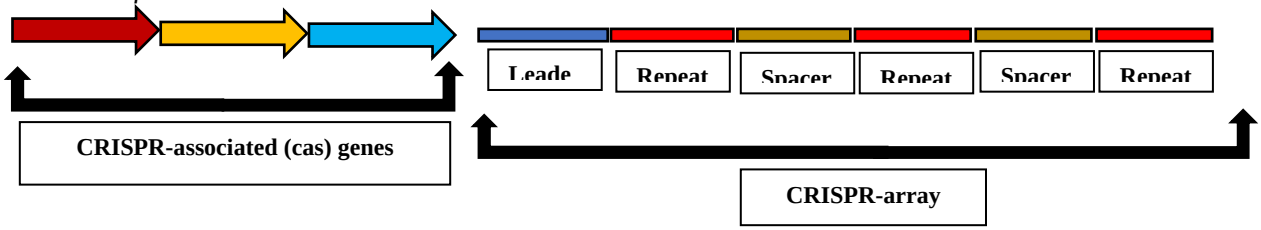
Category	Female	Male	Ookinete	Oocyst	Sporozoite
Transcription Factors	Unknown	Unknown	AP2-O, AP2-O2, AP2-O3, AP2-O4, AP2-O5	None	AP2-SP, AP2-SP2, AP2-SP3
Protein Kinases	NEK2, NEK4	CDPK4, CRK5, SRPK1, MAP2	CDPK3, GAK, PK7	CDLK, CDPK6, PK7	PbKIN
Protein Phosphatases	Unknown	PPM1	PPM2, PPKL, SHLP1	PPM5	Unknown
Kinesins	Unknown	Kinesin-8B, kinesin-13, kinesin-15	Kinesin-13, kinesin-20	Kinesin-8X	Kinesin-5
Myosins	Unknown	Unknown	Unknown	Unknown	MyoE
Cyclins	Unknown	Unknown	Unknown	CYC3	Unknown
Condensins	Unknown	Unknown	Unknown	SMC2, SMC4	Unknown
Anaphase-Promoting Complex	Unknown	CDC20, APC3	Unknown	Unknown	Unknown
Others	P47	Actin-II, SAS6, GEST, HAP2, PF16, P48/45, P230, FACT	DOZI, CITH, CAX, AP2-O, CelTOS, TRAP, PDEδ	GEX, DMC1, MISFIT, C-CAP, DEH, MRE11	LAPs, CDLK, CelTOS, SPECT, SPECT2, TRAP, CSP

CRISPR-Cas9 Technology

CRISPR-Cas9 is an abbreviation for 'Clustered Regularly Interspaced Short Palindromic Repeats-Cas CRISPR-associated Protein 9'. CRISPR-Cas9 technology holds significant potential for genome editing and gene therapy to cure diseases like cancer, infections, and genetic disorders (Doudna & Charpentier, 2014). CRISPR-Cas9 can detect gene activation during disease, correct damaging mutations, and switch cancer-causing or tumor suppressor genes (Abudayyeh et al., 2016).

Figure 2

Structure of CEISPER Locus



Classification of CRISPR–Cas system

CRISPR-Cas organization has two classes, six kinds (I-VI), and 27 subclasses. Many archaea and bacteria have the Class 1 CRISPR-Cas system in their genomes, while the Class 2 system is found in bacteria but not hyperthermophiles (Koonin et al., 2017). Class 1 systems have multi-subunit Cas protein effector complexes, while Class 2 systems have single protein effector modules. The interference stage requires the presence of nuclease effector proteins **Table No 1** denotes the various classification of this system (Hodgkins et al., 2015).

Table 2

CRISPR–Cas system Class 1 and 2 with their effectors

Class	Type	Sub-type	Effector(s)	References
1	I	I-C	Cas5, Cas7, Cas8	(Koonin et al., 2017)

		I-E	Cas5, Cas6, Cas7	
	III	III-A	Cas5, Cas7, Cas10	
		III-B		
	IV		Csf1, Cas5, Cas7	
2	II		Cas9	
	V	V-A	Cas12a (Cpf1)	(Hodgkins et al., 2015)
		V-B	Cas12b (C2c1)	
		V-U	C2c4, C2c5; five subgroups (V-U 1–5)	(Koonin et al., 2017)
	VI	VI-A	Cas13a (C2c2)	(Abudayyeh et al., 2016)
		VI-B	Cas13b (C2c6)	(Smargon et al., 2017)
		VI-C	Cas13b (C2c7)	(Koonin et al., 2017)

Mechanism in the CRISPR-Cas9 system

CRISPR-Cas organization involves three phases: Adaptation, Expression & maturation and Interference.

Adaptation

The adaptation phase involves two steps: first, the bacterium's Cas proteins identify the invader and acquire specific sequences from foreign nucleic acids, known as 'protospacers'. Second, the protospacers are incorporated in the extremity of the leader sequence in the CRISPR array as ‘spacers', causing the first repeat of the CRISPR array to be extended. These spacers create immunological memory for archaea and bacteria to defend against MGEs when they encounter them again. Cas1 and Cas2 are primarily involved in this stage (Yosef et al., 2012).

Expression and Maturation

During the expression and maturation phase, the leader sequence promotes transcription of the CRISPR loci, resulting in a long precursor CRISPR RNA or pre-crRNA. This pre-crRNA is then processed into small and mature crRNA units. The representation of crRNA involves combining a spacer region (complementary to the foreign nucleic acid) at the 5′ end with a repeat sequence at the 3′ end (Barrangou, 2015).

Interference

During the interference phase, the Cas-crRNA complex forms by recruiting Cas proteins to the crRNA. It recognizes foreign MGEs through Watson-Crick base pairing of complementary sequences and cleaves the targeted region. The Cas-crRNA complex identifies self and non-self-nucleic acids based on the presence of a short-conserved sequence (PAM) next to the target site (Amitai & Sorek, 2016).

Precision of CRISPER/Cas9 in gene editing in vector control

The accuracy of CRISPR-Cas9 in technique of gene-editing is crucial for control of vector, specifically when targeting the mosquitos that transmit the malaria. Precise gene editing ensures that the specific genes that participated in reproduction of mosquito, transmission of disease, or their existence are properly edited while avoiding their unnecessary effects. High specificity lessens accidental genetic variations, which can cause (Kyrou et al., 2018). Just like an example, the precise editing of genes such as double sex in *Anopheles gambiae* has efficiently repressed populations of female mosquito, showing its importance in malaria control. Additionally, precision reduces the possibility of off-target effects, which is vital when as the release of genetically altered organisms into surrounding. CRISPR-Cas9 precision is improved by purified guide RNA design and its delivery technologies that ensures its safety and efficacy in vector control programs (Hsu et al., 2014).

Table 3

List of CRISPR Tools

Name of Tools	Tool Type	Website	Purpose
CRISPResso2	Computational tool	http://crispresso.pinellolab.partners.org/	Analyzes genome editing results, determines differences between experiments, and requires less programming time.
CRISPR-ERA	Computational tool	http://crispr-era.stanford.edu/	Detects sgRNA binding sites, determines binding specificity and efficiency, and is used for genome imaging.

WU-CRISPR	Computational tool	http://crispr.wustl.edu/	Selects genomic gRNA for Cas9 and improves the design competence of CRISPR assays.
CRISPR-P	Web tool	http://crispr.hzau.edu.cn/CRISPR/	Selects target DNA sites with high specificity for Cas9, predicts off-target loci, and determines restriction and off-target sites.
CRISPR-P 2.0	Web tool	http://cbi.hzau.edu.cn/CRISPR2/	Predicts on-target and off-target effects of sgRNA, determines microhomology score and sgRNA secondary structure, and visualizes GC content.
CRISPRseek	Web tool	http://www.bioconductor.org	Bioconductor package that designs gRNA for target sites, studies off-target sites.
COSMID	Web tool	http://crispr.bme.gatech.edu	Recognizes potential off-target sites, detects mismatch bases, and identifies excluded or incorporated bases.
CHOPCHOP v2	Web tool	http://chopchop.cbu.uib.no	Designs sgRNA, predicts potential off-target sites, and targets a wide range of genome sequences.
Cas-Designer	Web tool	http://rgenome.net/cas-designer	Recognizes genome target sites cleaved by Cas9, facilitates gene knockout, and determines gRNA sequences and off-target sites.
E-CRISP	Web tool	http://www.e-crisp.org/	Designs gRNA sequences, identifies complementary target sites, and facilitates dsDNA cleavage by Cas9 endonuclease.

CRISPR-Cas9 derived Technologies in Mosquito

Since 1998, researchers have tried to edit mosquitos genetically specifically genes of ZFN and TALEN 37. As CRISPR-Cas9 gene editing technology has renovated, so has the pace of research. Cas9 protein-nucleic acid complexes can break down and alter mosquito genes. This strategy can provide either random repair or precision repair, which involves in the insertion of plasmids with homologous sequences during the process of editing; the latter technique is commonly used in gene drive technology. Many mosquito species, including *Aedes aegypti*, *Anopheles stephensi*, *Anopheles gambiae*, and *Culex burnetiid*, have had gene editing techniques developed (Jasinskiene et al., 1998).

Microinjection Method

Currently, CRISPR-Cas9-based mosquito editing approaches rely on microinjection of sgRNA, Cas9 protein, or Cas9 mRNA into individual embryos within mosquito eggs(Meuti & Harrell, 2020). This procedure is technically complex and expensive, necessitates precise experimental equipment and experimenter handling, and has extremely low injection success rates, resulting in irreversible harm to mosquito eggs(Adelman et al., 2002). To overcome these challenges, Lule-Chávez employed a novel particle inflow gun technology to inject Cas9 protein and sgRNA into Aedes aegypti and Anopheles gambiae eggs using particle bombardment. This technique improves transformation efficiency and survival rates while also being faster, simpler, and less expensive than micro-injection (Lule-Chávez et al., 2021).

ReMOT Control Method

The Chaverra-Rodriguez research team pioneered the development of a technique called receptor-mediated ovary transduction of cargo (ReMOT Control), which removes the requirement for mosquito egg-embryo injection. For introduction of specific transferable mutations, significant volumes of Cas9 sgRNA complexes are directly injected into female *Aedes aegypti* hemolymph 24 hours after blood feeding (Chaverra-Rodriguez et al., 2018). Notably, the Cas9 protein used in ReMOT Control is not a local protein, but rather a Cas9 complex protein with a *Drosophila melanogaster* yolk protein tag (DmYP), which shows to be effectively targeted the particular insect ovaries. This fusion of Cas9 protein has superior pointing properties than commercial Cas9 proteins, making it more effective in gene editing. This approach was used to change the Kmo, Kh, ECFP, and DsRed genes in *Anopheles stephensi* (Li et al., 2021).

Table 4
CRISPR/Cas9 in mosquito research

Year	Species	Country and Sponsor	Gene(s)	Function in Mosquitoes	Survival and GEF	Reference
2023	*Aedes aegypti*	USA, University of California San Diego	AaRel I	Suppression of DENV2 titer	-	(Bui et al., 2023)
2023	*Aedes aegypti*	China, Chinese Academy of Sciences	OTU7B	Enhanced resistance to fungal infection	-	(Wang et al., 2023)
2022	*Anopheles gambiae*	USA, Johns Hopkins University	CTL-4	Suppression of *Plasmodium falciparum*	–	(Simões et al., 2022)
2021	*Anopheles gambiae*	UK, Imperial College London	Scorpine	Suppression of *Plasmodium falciparum*	7.0%/1.2%	(Hoermann et al., 2021)
2021	*Aedes aegypti*	USA, Texas A&M University	SGS1	Decreased *Plasmodium* sporozoite invasion	19%/1.1%	(Kojin et al., 2021)
2021	*Anopheles stephensi*	USA, Sanaria Inc. Rockville	LRIM I	Suppression of *Plasmodium* sporozoite dev.	3.5%/0.1%	(Inbar et al., 2021)
2021	*Aedes aegypti*	USA, Johns Hopkins University	Obp10/22	Suppression of DENV2 and ZIKV transmission	53.4%/50%	(Dong et al., 2021)
2020	*Anopheles gambiae*	USA, Yale University School of Medicine	mosGILT	Decreased oocyst numbers after *Plasmodium* inf.	15%/4.5%	(Yang et al., 2019)
2020	*Aedes aegypti*	China, National Tsing Hua University	GCTL-3	Decreased DENV-2 infection rate	26%/1.1%	(Li et al., 2020)
2020	*Aedes aegypti*	France, Institut Pasteur	CFAV-EVEs	Enhanced CFAV replication in ovaries	Unknown	(Suzuki et al., 2020)
2018	*Anopheles gambiae*	USA, Johns Hopkins University	FREP I	Suppression of *Plasmodium* infection	10%/3.5%	(Dong et al., 2018)

Electroporation Method

Electroporation is now utilized to transfer nucleic acids to insects in-vivo since its commencement as a microbial alteration technology in the late 1990s. Electroporation-mediated microinjection permits the effective and correct administration of exogenous nucleic acids to produce the gene-silencing or its overexpression in non-embryonic individuals or particular regions in various Lepidoptera (Jamison et al., 2018). This approach has yet to be demonstrated for employing gene function in mosquitoes. This approach includes the ovary absorbing free external nucleic acid fragments in the hemocoel, leading to its genetic modification or inhibition of gene expression in children (KonDo et al., 2017).

RNA-Interfence

RNA interference (RNAi) has quickly innovative our consideration towards insect evolution, its developmental with physiological and molecular biology. RNAi does not cause transferable germline changings, which is a advantage of CRISPR-Cas9 gene editing systems effectively used in mosquitos (Hammond et al., 2016). RNAi is a restricted technique for silencing the gene that does not necessitate the long-term conservation of genetically revised mosquito. RNAi's conditional feature permits researchers to succeed the level of gene silencing, avoiding its problems like embryonic lethality or sterility that can bound the formation and maintenance of heritable mutant strains (Mysore, Hapairai, et al., 2019). Although it is considerable that CRISPRCas9 improvements

via genetic engineering of its non-model insects remains a labor-intensive and expensive procedure. RNA interference (RNAi) is normally used in purposeful genetics inquiries in mosquitoes, despite their concerns about off-site target and variable silencing levels based on the targeted gene and tissue. Most laboratories use long dsRNA (300-400 bp) in their experiments, siRNAs and shRNAs can confirm the phenotypes by targeting multiple 21-25 bp sites in a single a gene. This can lessen concerns about off-site targeting (Mysore, Li, et al., 2019).

Environmental and ecological aspects in mosquitos influence the CRISPER/Cas9 Delivery System

The efficiency and progress of the CRISPR/Cas9 delivery mechanism in mosquitos is operated and controlled by its environmental and ecological factors like temperature, humidity, and the density of mosquitos. All of these factors have an individual impact on outcomes and accuracy of gene editing. Like temperature, effects the mosquito metabolism, development that potentially alters the uptake and activity of CRISPR factors introduced via microinjection or viral vectors (Wang et al., 2024). The ecological factors as habitat type and presence of pathogen can alter mosquito immune responses, thereby decreasing the efficacy of CRISPR/Cas9 delivery and its integration (Kokoza et al., 2000). The genetic variability of mosquitos is shaped by their biological niches, can also effect target-site conservation and effectiveness of RNA (Kokoza et al., 2000). Mosquitoes from different geographic locations, like they are more or less prone to genetic management, demanding specialized delivery mechanisms for CRISPR/Cas9 systems. Besides, the natural interactions like as struggle or its exposure to environmental related stress may increase the chances of its fitness with costs of gene editing and also affecting long-term population control tactics.

Possible genes to be manipulated via CRISPER/Cas9 to control Malaria

Reducing the mosquito's ability to transmit parasites can effectively diminish or eliminate malaria transmission. To prevent parasite transmission, mosquitoes can be genetically modified for midgut function. Expression of "effector genes" whose products prevent parasite growth. Genetically engineered mosquitoes had significantly reduced ability to spread the parasite (Ito et al., 2002). Subsequent findings from various laboratories using different effector molecules reached similar outcomes. This research demonstrated that genetic modification of the vector mosquito can minimize Plasmodium transmission, serving as proof of concept. Translating these discoveries to the field requires effective methods for introducing anti-malaria genes into wild mosquito populations. Simply releasing transgenic mosquitos is not enough (Moreira et al., 2002).

SM1: Anopheles stephensi was genetically engineered to express the SM1 peptide in its midgut, providing the first proof of concept. The peptide inhibits ookinete invasion by binding to a receptor on the midgut epithelium's luminal surface (Ghosh et al., 2001).

EPIP, or Plasmodium Enolase-Plasminogen Interaction Peptide, slows mosquito midgut invasion by blocking plasminogen binding to the ookinete surface. Single-chain monoclonal antibodies (scFvs) bind to proteins on the surface or released by ookinetes and sporozoites. ScFv 4B7 binds to *P. falciparum* ookinete surface protein Pfs25, 2A10 targets the *P. falciparum* circumsporozoite protein (CSP), anti-Pbs21 single chain antibody targets the *P. berghei* major ookinete surface protein Pbs21, and scFv 1C3 binds a *P. falciparum* secreted enzyme chitinase 1 (Yoshida et al., 2001).

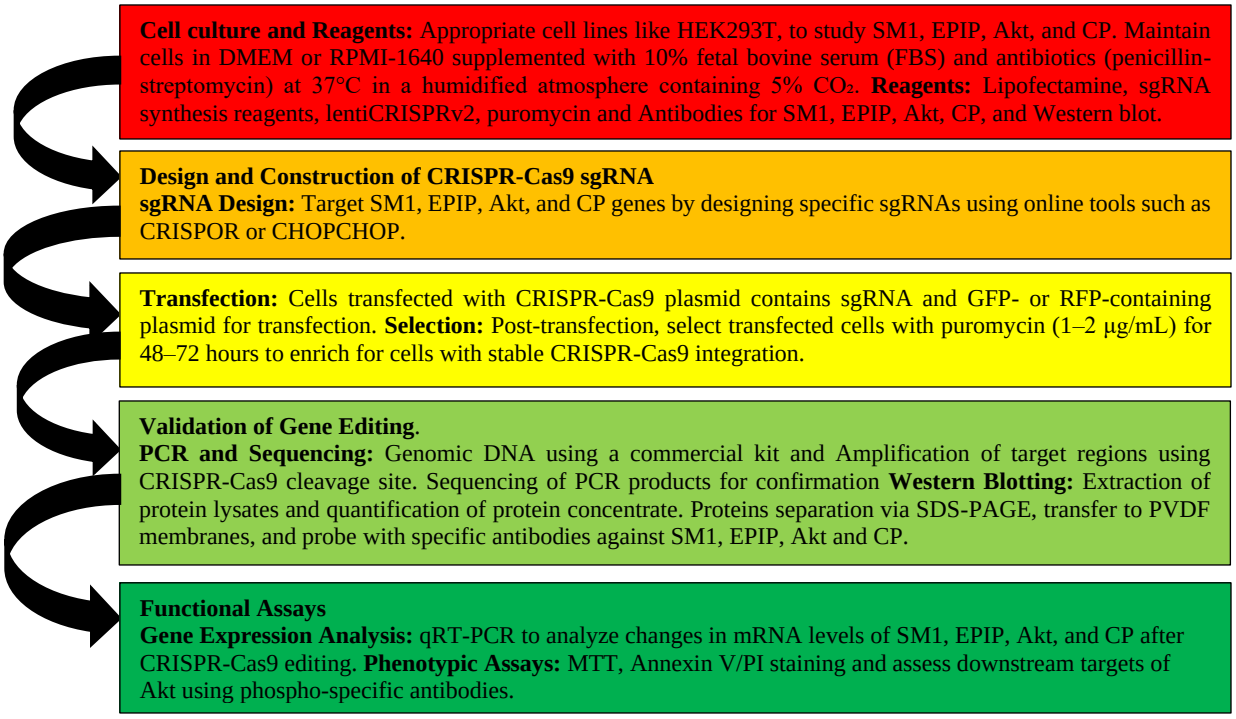
Akt: Blood meal-induced activation of Akt, a critical component in the insulin signaling pathway, makes mosquitos resistant to Plasmodium infection. Overexpression of the IMD pathway mediated transcription factor Rel2 makes mosquitos resistant to Plasmodium infection. Using RNA interference or "smart sprays" to manipulate mosquito immune pathways improves their anti-microbial response (Corby-Harris et al., 2010).

CP: The carboxypeptidase (CP) promoter and signal sequence have several desired characteristics. A blood meal activates the CP promoter, which secretes the protein into the midgut lumen and initiates Plasmodium growth. We created a synthetic gene (AgCP[SM1]4) with four SM1 units connected by 4-amino-acid linkers, coupled to the CP signal sequence, and driven by

the gut-specific and blood-inducible CP promoter. The gene was put into a piggyBac vector and transferred to the mosquito *Anopheles stephensi* (Catteruccia et al., 2000).

Figure 3

Schematic diagram of procedure to be used during optimization



DISCUSSION

Improving the methods of CRISPR/Cas9 delivery for its precise gene-editing in malaria-transmitting mosquitos is an important and specific scientific step that aims to lowers the malaria's global burden. Malaria that is a life-threatening disease primarily caused by Plasmodium parasites that spreads to people via the bite of infected *Anopheles gambiae*. Although after various precautionary measures like use of insecticide-treated nets with indoor residual spraying and antimalarial medications, the illness due to this disease remains a substantial challenge due to the emergence of resistance in mosquitos with drug resistance in parasites the major factor involved in this disease. Techniques of genetic engineering like CRISPR/Cas9 technology, present exciting paths for tackling this issue by modifying the whole genome sequence of vector to diminish or completely remove their ability to transmit malaria via deleting the genes involved in this process. CRISPR/Cas9, a revolutionary genome-editing technique basically comes from bacteria as part of its immune system in the form of adaptive immune systems allows accurate modification of genetic material. This system is comprising of two main components: the Cas9 nuclease and a single-guide RNA (sgRNA). Double-strand breaks are mended by cellular methods such as non-homologous end joining (NHEJ) or homology-directed repair (HDR), which induce gene disruptions or alters the precise genetic modification. In the context of malaria-transmitting mosquitoes, CRISPR/Cas9 has been used to introduce genetic alteration that will either diminish vector populations or make it incapable of spreading the parasite.

Effective transport of CRISPR/Cas9 components into the genome vector cells is essential for accurate editing of genome. Various approaches for gene-editing in mosquitos have been discovered like microinjection and cargo receptor-mediated ovary transduction (ReMOT Control). Microinjection is one of the most common techniques for delivering CRISPR/Cas9 components to mosquito eggs. This technique includes injecting a solution that consists of three components first one is Cas9 protein while second

is mRNA, and at the last third is sgRNA directly into pre-blastoderm embryos. Microinjection enables true delivery of editing components into germline cells, certifying that genetic changes are transferable from one to next generation. Microinjection is technically problematic and labor-intensive, demanding specialized equipment with trained workers. The low strength of mosquito embryos, combined with the need for precise timing through the injection process, adds to the intricacy. It remains the good standard for introductory proof-of-concept surveys and the creation of genetically engineered vectors.

The ReMOT Control system provides another way of introducing CRISPR/Cas9 components into mosquitos with target site of germline cells. This approach uses a Cas9 protein fusion with a short amino acid sequence that binds to receptors on the mosquito ovary, permitting the editing components to be delivered to adult females by a simple injection. The ReMOT Control system eradicates the requirement of direct embryo manipulation that makes the technique much simpler and requiring less technical skill. This approach has been found to be extremely effective in a variety of mosquito species, including *Anopheles gambiae*. However, the ReMOT Control system may have problems in attaining the accurate temporal and spatial control over the activity of Cas9 that results in off-target effects or mosaicism in the altered mosquitos.

Various other techniques of CRISPER/Cas9 induction also involves like electroporation, which employs electrical pulses to temporarily permeabilize cell membranes for component distribution, and viral vectors, which use viruses' innate ability to infect cells and transport genetic material. These methods show potential, they require more optimization to reach the efficiency and specificity vital for large-scale applications.

The use of CRISPR/Cas9 in malaria-transmitting vectors has centered on two main strategies: one is population suppression and other is population alteration. Population suppression seeks to lower vector populations by targeting genes required for reproduction. For example, CRISPR/Cas9 has been used to damage genes involved in female fertility, such as double sex, that results in sterile female's incapability of bearing the children. While population modification, on the other hand, aims to add specific genes that confer resistance to malaria causing parasites or block their development within the mosquito.

There are various difficulties to overcome when improving CRISPR/Cas9 delivery methods for precise gene editing in malaria vectors. These include growing component delivery efficiency, dropping off-target effects and confirming genetic modifications are stable and transferable. Advances in CRISPR/Cas9 technology, such as the generation of high-fidelity Cas9 variations and improved sgRNA design algorithms, are helping to stunned these issues. Combining CRISPR/Cas9 with some other genetic and molecular tools, like as RNA interference and transposable elements, could advance its value for mosquito gene editing.

CONCLUSION

CRISPR/Cas9 is a durable and adjustable technique for precise gene editing in malaria-carrying mosquitos, with the strength to transform malaria control efforts. Microinjection and ReMOT Control are two important and prominent delivery methods for genome editing experiments, and optimizing them is key for renovating laboratory findings into real-world applications. CRISPR/Cas9-based techniques, which target genes involved in mosquito reproduction and transmission of parasite, can increase existing malaria control measures and play important role to the global objective of malaria exclusion. To ensure their protection, usefulness, and proper tolerability, these technologies must be advanced and deployed in aggregation with strong regulatory frameworks with community engagement and multidisciplinary research.

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