Single step production of Cas9 mRNA for zygote injection

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Production of Cas9 mRNA *in vitro* typically requires the addition of a 5' cap and 3' polyadenylation. A plasmid was constructed that harbored the T7 promoter followed by the EMCV IRES and a Cas9 coding region. We hypothesized that the use of the metastasis associated lung adenocarcinoma transcript 1 (*Malat1*) triplex structure downstream of an IRES/Cas9 expression cassette would make polyadenylation of *in vitro* produced mRNA unnecessary. A sequence from the *mMalat1* gene was cloned downstream of the IRES/Cas9 cassette described above. An mRNA concentration curve was constructed with either commercially available Cas9 mRNA or the IRES/Cas9/triplex, by injection into porcine zygotes. Blastocysts were genotyped to determine if differences existed in the percent of embryos modified. The concentration curve identified differences due to concentration and RNA type injected. Single step production of Cas9 mRNA provides an alternative source of Cas9 for use in zygote injections.

With the rise of the CRISPR/Cas9 technology, we have constructed a new Cas9 mRNA that does not possess a poly(A) tail but rather a triple helical tail originating from the *mMalat1* gene. The use of the triple helix tail lends itself to produce an unlimited, inexpensive Cas9 mRNA that creates modifications at the same efficiency as commercially available Cas9 mRNA. In the absence of polyadenylation, the resulting mRNA has a defined, fixed length that facilitates assessment of mRNA degradation.

Since the development and use of the CRISPR/Cas9 system, genetic engineering in zygotes of mammals is becoming highly efficient [1-5]. However, *in vitro* production of Cas9 mRNA has its limitations. After *in vitro* transcription, it is difficult to assess RNA degradation due to the variable length of the poly(A) tail. An alternative source of stable, assessable Cas9 mRNA may be more useful and more efficient for laboratories that produce their own Cas9 as compared to commercially produced

Cas9 mRNA that can be expensive and difficult to evaluate.

The majority of mRNAs are polyadenylated to possess a poly(A) tail. This poly(A) tail generally contains around 200 adenosine (A) nucleotides and is critical for the transcript to be exported out of the nucleus, translated, and is important for RNA stability. Similar to *Malat1*, histone mRNAs end in a six-base stem and four base loop that functionally replaces the poly(A) tail [6]. Surprisingly, recent research found that the 3' end of a nuclear noncoding RNA, *Malat1*, can also support the translation of a GFP reporter gene just as efficiently as the poly(A) tail [7]. While *Malat1* is a long non-coding RNA (lncRNA), it is among the most abundantly expressed lncRNAs and the A rich triple helical structure is able to bind to the ribosome and protect it from exonucleases. Addition of a triple helical structure to the Cas9 instead of the poly(A) tail simplifies mRNA production.

To create the triple helical structure on Cas9, a triple helical sequence from the *mMalat1* gene was cloned into the plasmid vector pCR™4-TOPO 9 (Invitrogen, Thermo Fischer, Grand Island, NY, USA) (pBKR1). pBKR1 was cut with *NcoI* to *BstEII*, gel purified, and cloned into a T7 IRES expression plasmid (pSF-T7-EMCV, Oxford Genetics, Oxford, UK) at *NcoI* to *BstEII* (pBKR2). Cas9 was cut *NcoI* to *EcoRI* from pX330 [8], gel purified, and cloned into pBKR2 at *NcoI* to *EcoRI* to make pBKR3 (Figure 1A). The pBKR3 plasmid was linearized with *BsmBI* and *in vitro* transcription was completed by using the mMessage mMachine kit (Thermo Fisher). The kit utilizes an anti-reverse cap analog during transcription. The RNA was purified with a MEGAclear kit (Thermo Fisher). The Cas9 RNA that contains a triple helical tail is referred to as 'MU Cas9 mRNA' (Figure 1B). Alternatively, an uncapped mRNA was also produced by using the linearized pBKR3 vector and completing *in vitro* transcription by using a ribonucleotide solution mix (NEB, Ipswich, MA,

METHOD SUMMARY

A new Cas9 mRNA that contains an EMCV IRES and triple helical tail structure can be used for zygote injections. This mRNA can be produced in a single step without the addition of a poly(A) tail and maintains the same efficiency of modification as commercially available Cas9 mRNA.

Figure 1. Diagrams of the components of the new method. A. Schematic diagram of a linearized pBKR3 vector. B. MU *Cas9* mRNA_NC with specific elements identified. C. *Cas9* containing a 5' cap and modified with pseudouridine and 5-methylcytidine mRNA containing a 3' poly(A) tail (TriLink Biotechnologies).

USA) and an RNA polymerase (NEB), and then the resulting mRNA was purified by using the MEGAclear kit. This uncapped mRNA was named MU *Cas9* mRNA_NC. All guide RNAs (gRNAs) were produced as in Whitworth et al. [1]. Briefly, the 20 bp guides were designed to target sequences next to an *S. pyogenes* protospacer adjacent motif [9]. A gBlock containing a T7 promoter sequence was added upstream of the guide, which was synthesized by Integrated DNA

Technologies (Coralville, IA, USA). Each gBlock was PCR amplified and then purified by using a Qiagen (Valencia, CA, USA) PCR purification kit. The purified gBlock amplicons were used for *in vitro* transcription by using the MEGAshortscript (Ambion, Thermo Fisher) kit. The resulting RNAs were then purified by using the MEGAclear Transcription Clean-Up Kit. Single guide RNA and *Cas9* mRNA were diluted in nuclease-

free water and stored at -80°C until zygote injection.

An mRNA concentration curve was completed to determine the optimal concentration of *Cas9* mRNA to be used during zygote injection with respect to both blastocyst development and the percent gene edited. Four proprietary gRNAs that were previously confirmed to create modifications during zygote injections were used to test for the efficiency of modifications. MU *Cas9* mRNA was directly compared with commercially available *Cas9* mRNA that had been polyadenylated and contains 5-methylcytidine and pseudouridine modifications (TriLink Biotechnologies, San Diego, CA, USA) (Figure 1C). *Cas9* mRNA (10 ng/μl, 20 ng/μl, 40 ng/μl, and 80 ng/μl) were each co-injected with all four gRNAs (15 ng/ul). Zygotes were created by using *in vitro* matured oocytes and *in vitro* fertilization [1]. The injected zygotes were cultured in MU2 medium for 7 days [10].

Development to the blastocyst stage was reduced by injecting zygotes with either *Cas9* mRNA compared to zygotes that were not injected (Figure 2), but development was not affected by the type of *Cas9* mRNA or the concentration of *Cas9* mRNA that was injected (Figure 2). The goal was to determine if large deletions existed in the PCR amplicons by agarose gel electrophoresis. There were no differences in the percentage

Table 1. Percentage of modified blastocysts developed from zygote injections of commercially available *Cas9* or MU *Cas9* at varying concentrations. Five replicates of injections were completed to generate this data.

Cas9 Source	Percentage of Modified Blastocysts ± S.E.	Total Number of Blastocysts Assayed
10 ng/μL MU <i>Cas9</i>	72.7%±10.8%	52
10 ng/μL <i>Cas9</i>	73.0%±7.5%	23
20 ng/μL MU <i>Cas9</i>	86.7%±6.1%	61
20 ng/μL <i>Cas9</i>	73.5%±13.4%	26
40 ng/μL MU <i>Cas9</i>	87.0%±7.1%	58
40 ng/μL <i>Cas9</i>	82.2%±6.3%	19
80 ng/μL MU <i>Cas9</i>	90.9%±2.2% ^a	38
80 ng/μL <i>Cas9</i>	58.5%±17.1% ^b	20

a,b denotes p < 0.05.

Table 2. PAH guide oligos and primer sequences used for genotyping single embryos.

Oligo or Primer	Sequence
gRNA1	GCTATGGCAGAACAACAACTA
gRNA2	GTCTACCGCCATCCAAGAAA
Forward	CTTGCTTGCTTTCAGTCTCTC
Reverse	CACAGAACACGCCACTTAT

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Figure 2. The effect of Cas9 type and concentration on blastocyst percentage. This data is derived from 3 replicates containing 196, 178; 198, 175; 204, 180; and 194, 175, injected zygotes respectively. NI refers to the non-injected control. *p > 0.05.

Cas9 Source	Proprietary <i>Gene X</i>		PAH	
	% of Blastocysts Modified ±SE	Number of Blastocysts Assayed	% of Blastocysts Modified ±SE	Number of Blastocysts Assayed
20 ng/μL Cas9	49%±8%	N=29	47%±5%	N=37
20 ng/μL MU Cas9	49%±9%	N=21	38%±14%	N=35

Table 3. Analysis of gene modifications in blastocysts from zygote injections of commercially available Cas9 or MU Cas9 plus gRNAs.

of modifications as seen by gel imaging between the sources of Cas9 mRNA; however there was an increase in the percentage of modifications in the 80 ng/μl concentration of *MU Cas9* mRNA compared to commercial *Cas9* mRNA (Table 1). By assessing if large deletions existed in the PCR amplicons as a way to measure modification efficiency, we underestimated the number of true modifications present in each embryo and did not detect mosaicism.

To confirm our results, we assessed modifications by using gRNAs for two different genes (proprietary *Gene X* and phenylalanine hydroxylase [PAH] Table 2). *MU Cas9* mRNA and *Cas9* mRNA were injected (20 ng/μl) with different pairs of gRNAs and the resulting blastocysts were analyzed for modifications. There were no differences in the percentage of blastocysts that developed after injection by either *MU Cas9* or *Cas9* mRNA with the respective guides. *Gene X* had 16% vs. 14% blastocyst development and PAH had 10% vs 9% blastocyst development for *Cas9* and *MU Cas9*, respec-

tively. There was also no difference in the percent of blastocysts modified by injection of either *Cas9* mRNA with guides (Table 3).

We also wanted to determine if a 5' cap was needed for this mRNA to be functional. We injected the same four guide RNAs from the first experiment and 20 ng/μl *MU Cas9* mRNA_NC (without a 5' cap) into zygotes. On day 7, blastocysts were collected and the gel shift assay showed that 18 of the 19 blastocysts collected were modified (94.7%). This illustrates the functionality of an un-capped *Cas9* mRNA for use in zygote injections.

In summary, making *Cas9* mRNA with a triple helical tail was efficient and results in genetic modification not different from commercial *Cas9* mRNA. When injected into zygotes, there is no difference in blastocyst development compared to the commercial *Cas9* mRNA at the same concentration. There is also no difference in the percent of modifications between the two sources of *Cas9* mRNA, with the exception of the 80 ng/μl concen-

tration where *MU Cas9* mRNA had a higher rate. The successful generation of an alternative *Cas9* mRNA provides an attractive one-step mRNA protocol for use in zygote injections.

Author contributions

BKR, BPB, RSP, and KDW designed the experiments. BKR performed the experiments. LDS, JAB., and SLM. assisted with IVF and injections. CWO and AMS assisted in developing gene assays. BKR and RSP wrote the manuscript with contributions from all authors.

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Competing interests

The authors declare no competing interests.

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