Single step production of Cas9 mRNA for zygote injection

Bethany K Redel¹, Benjamin P Beaton¹,², Lee D Spate¹, Joshua A Benne¹, Stephanie L Murphy¹, Chad W O’Gorman¹, Anna M Spate¹, Randall S Prather¹ and Kevin D Wells¹*

¹Division of Animal Science, National Swine Resource and Research Center, University of Missouri, Columbia, MO, USA
²Current address, Genus plc, DeForest, WI, USA

Keywords: genetic modification; Cas9; zygote

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 (IncRNA), it is among the most abundantly expressed IncRNAs 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 pCRT™-TOPO 9 (Invitrogen, Thermo Fischer, Grand Island, NY, USA) (pBKR1). pBKR1 was cut with Ncol to BstEII, gel purified, and cloned into a T7 IRES expression plasmid (pSF-T7-EMCV, Oxford Genetics, Oxford, UK) at Ncol to BstEII (pBKR2). Cas9 was cut Ncol to EcoRI from pX330 [8], gel purified, and cloned into pBKR2 at Ncol to EcoRI to make pBKR3 (Figure 1A). The pBKR3 plasmid was linearized with BsmBI and in vitro transcription was completed by using the mMessage mMMachine 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, USA).

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.
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 promotor 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 MEGAscript (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.

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

a,b denotes p < 0.05.

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

<table>
<thead>
<tr>
<th>Oligo or Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>gRNA1</td>
<td>GCTATGGCAGAACAAAACTA</td>
</tr>
<tr>
<td>gRNA2</td>
<td>GTCTACCGCCATCCAAGAAA</td>
</tr>
<tr>
<td>Forward</td>
<td>CTTGTCTTGCTTTCAGTTCTTC</td>
</tr>
<tr>
<td>Reverse</td>
<td>CACAGAACACGCCACTTAT</td>
</tr>
</tbody>
</table>
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, respectively. 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-

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

<table>
<thead>
<tr>
<th>Cas9 Source</th>
<th>% of Blastocysts Modified ±SE</th>
<th>Number of Blastocysts Assayed</th>
<th>% of Blastocysts Modified ±SE</th>
<th>Number of Blastocysts Assayed</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 ng/μL Cas9</td>
<td>49%±8%</td>
<td>N=29</td>
<td>47%±5%</td>
<td>N=37</td>
</tr>
<tr>
<td>20 ng/μL MU Cas9</td>
<td>49%±9%</td>
<td>N=21</td>
<td>38%±14%</td>
<td>N=35</td>
</tr>
</tbody>
</table>

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.

The Geno/Grinder® is a high-throughput plant & animal tissue homogenizer with an adjustable clamp that accommodates up to six deep-well titer plates, or multiple sample tubes from 2mL to 50mL. It is specifically designed for rapid cell disruption, lysis and tissue homogenization.

The new design incorporates a new front loading clamp and a programmable touch screen control panel that saves up to 500 protocols.
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.

Acknowledgements
This work was supported by the National Swine Research and Resource Center (NSRRC) U42 OD011140. This paper is subject to the NIH Public Access Policy.

Competing interests
The authors declare no competing interests.

References

First draft submitted: 7 November 2017; Accepted for publication: 19 February 2018.

Address correspondence to Kevin D Wells, Division of Animal Science, National Swine Resource and Research Center, University of Missouri, Columbia, MO, USA. E-mail: wellskev@missouri.edu

To purchase reprints of this article, contact: s.cavana@future-science.com