Published in final edited form as:

Biotechniques. ; 64(3): 118–124. doi:10.2144/btn-2017-0116.

Single step production of *Cas9* mRNA for zygote injection

Bethany K Redel[1], **Benjamin P Beaton**[1,2], **Lee D Spate**[1], **Joshua A Benne**[1], **Stephanie L Murphy**[1], **Chad W O' Gorman**[1], **Anna M Spate**[1], **Randall S Prather**[1], and **Kevin D Wells**[1]

[1]Division of Animal Science, National Swine Resource and Research Center, University of Missouri, Columbia, MO, U.S.A.

[2]Current address, Genus plc, DeForest, WI, U.S.A.

Abstract

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.

METHOD SUMMARY

A new *Cas9* mRNA that contains an EMCV IRES and triple helical tail structure can be utilized 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 modifications as commercially available *Cas9* mRNA.

Keywords

Genetic modification; Cas9; zygote

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*

To request reprints and other inquiries: Kevin D Wells, wellskev@missouri.edu.

AUTHOR CONTRIBUTIONS

B.K.R., B.P.B, R.S.P., and K.D.W designed the experiments. B.K.R performed the experiments. L.D.S, J.A.B., and S.L.M. assisted with IVF and injections. C.W.O. and A.M.S. assisted in developing gene assays. B.K.R. and R.S.P. wrote the manuscript with contributions from all authors.

COMPETING INTERESTS

The authors declare no competing interests.

mRNA that creates modifications at the same efficiency as commercially available *Cas9* mRNA. In 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 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 1). 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". 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) 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]; a brief description follows. 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 and Integrated DNA Technologies (Coralville, IA, USA) synthesized the guide. 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 1). *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 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 experiment one 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 concentration 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.

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.

REFERENCES

References

1. Whitworth KM, et al., Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos. Biol Reprod, 2014 91(3): p. 78. [PubMed: 25100712]
2. Whitworth KM, et al., Zygote injection of CRISPR/Cas9 RNA successfully modifies the target gene without delaying blastocyst development or altering the sex ratio in pigs. Transgenic Res, 2017 26(1): p. 97–107. [PubMed: 27744533]
3. Sheets TP, et al., Somatic Cell Nuclear Transfer Followed by CRIPSR/Cas9 Microinjection Results in Highly Efficient Genome Editing in Cloned Pigs. Int J Mol Sci, 2016 17(12).
4. Wang X, et al., Generation of gene-modified goats targeting MSTN and FGF5 via zygote injection of CRISPR/Cas9 system. Sci Rep, 2015 5: p. 13878. [PubMed: 26354037]
5. Wang Y, et al., Efficient generation of gene-modified pigs via injection of zygote with Cas9/sgRNA. Sci Rep, 2015 5: p. 8256. [PubMed: 25653176]
6. Marzluff WF, Metazoan replication-dependent histone mRNAs: a distinct set of RNA polymerase II transcripts. Curr Opin Cell Biol, 2005 17(3): p. 274–80. [PubMed: 15901497]
7. Wilusz JE, et al., A triple helix stabilizes the 3' ends of long noncoding RNAs that lack poly(A) tails. Genes Dev, 2012 26(21): p. 2392–407. [PubMed: 23073843]
8. Cong L, et al., Multiplex genome engineering using CRISPR/Cas systems. Science, 2013 339(6121): p. 819–23. [PubMed: 23287718]
9. Ran FA, et al., In vivo genome editing using Staphylococcus aureus Cas9. Nature, 2015 520: p. 186. [PubMed: 25830891]
10. Spate LD, et al., PS48 can replace bovine serum albumin in pig embryo culture medium, and improve in vitro embryo development by phosphorylating AKT. Mol Reprod Dev, 2015 82(4): p. 315–20. [PubMed: 25776657]

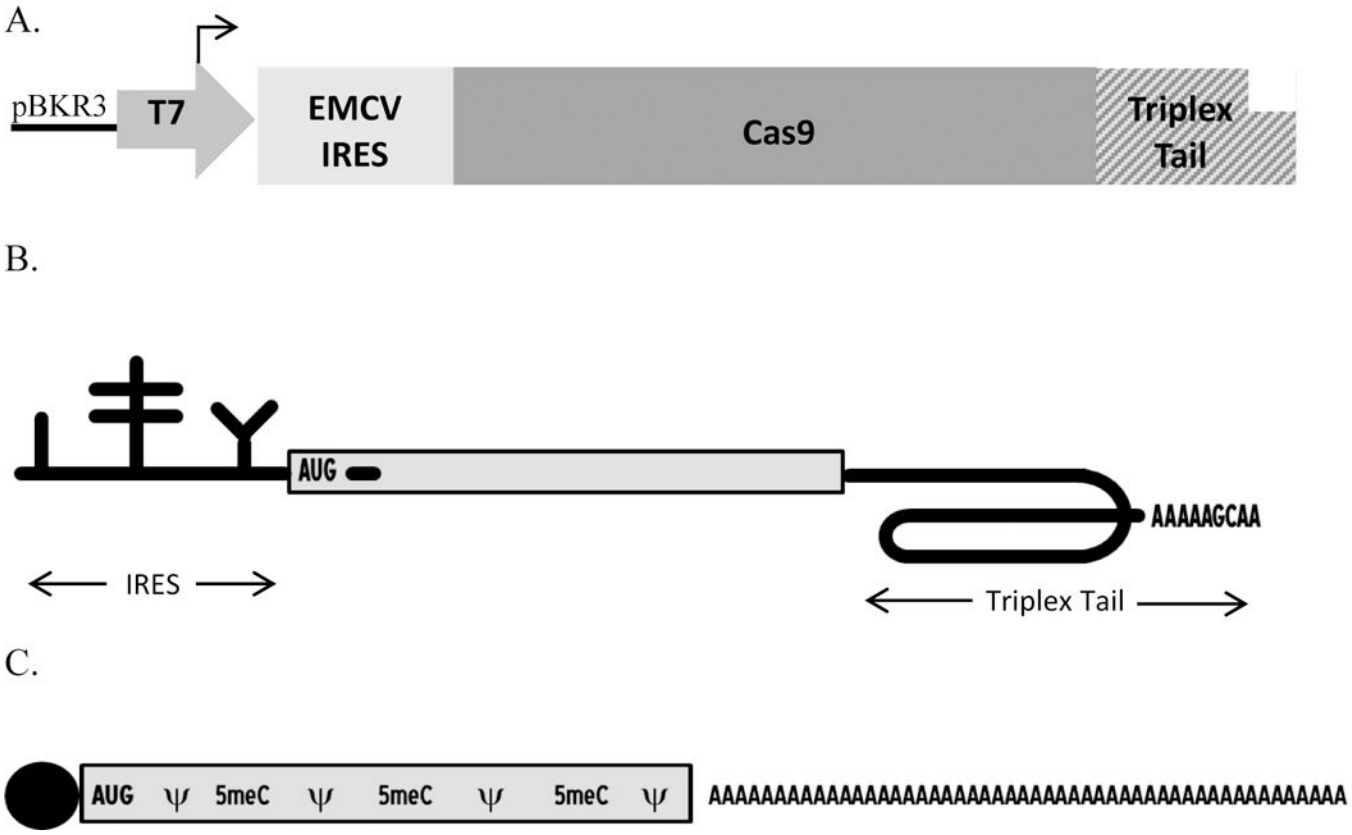

Figure 1.

A. Schematic 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).

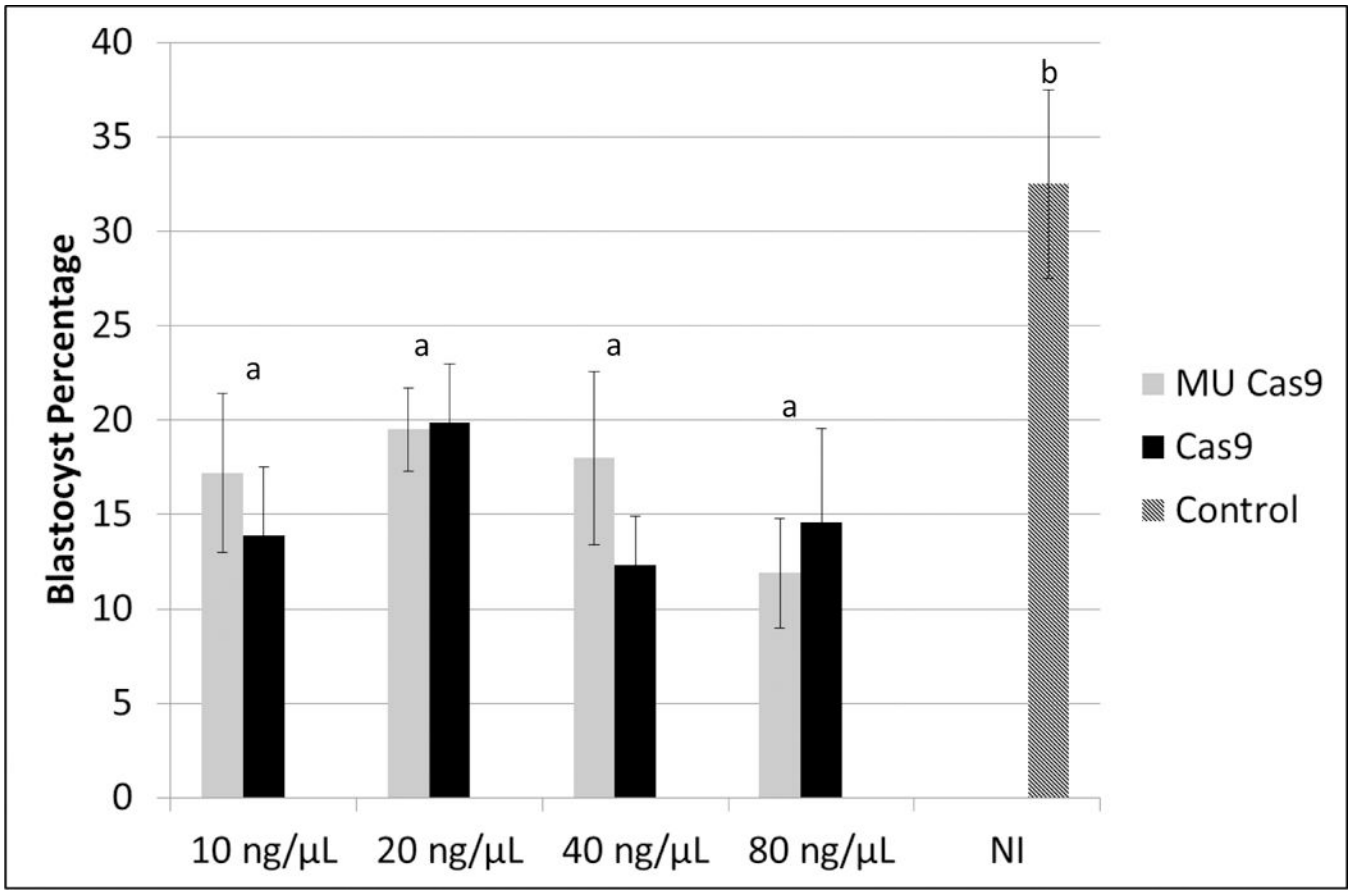

Figure 2.

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

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. [a,b] denotes P<0.05.

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

Table 2.

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

Oligo or Primer	Sequence
gRNA1	GCTATGGCAGAACAAAACTA
gRNA2	GTCTACCGCCATCCAAGAAA
Forward	CTTGTCTTGCTTTCAGTTCTTC
Reverse	CACAGAACACGCCACTTAT

Table 3.

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

	Proprietary Gene X		***PAH***	
Cas9 Source	**% 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