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IMPROVED RATE OF TARGETED GENE KNOCK-IN OF *IN-VITRO* FERTILIZED BOVINE EMBRYOS

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SUMMARY

Variables for achieving targeted gene knock-ins using CRISPR/Cas9 mediated gene insertion in bovine embryos following in-vitro maturation were tested to evaluate the rate of integration at a target genomic location, and the level of mosaicism. Guide-RNAs (gRNA) were developed targeting downstream of the Zinc Finger X-linked (ZFX) gene located on the bovine X-chromosome. One gRNA (ZFXg3) was found to cut with high frequency in-vivo (82%). Donor vectors utilizing different endogenous repair pathways: homologous recombination (HR) or homology-mediated end joining (HMEJ), were then designed to insert the sex determining region on the Y-chromosome (SRY) gene into the target cut-site of ZFXg3 to produce bulls that would sire all male offspring (XY males, and $X_{SRY}X$ males). CRISPR/Cas9 reagents were introduced into either MII oocytes, or six hours after in-vitro insemination (hpi). The HMEJ donor vector (hmejSRYp) showed a significantly higher insertion rate compared to the HR donor vector (hrSRYp) (32.5% vs. 0%; $p < 0.0001$). Additionally, of those that were positive for the insert, 23.4% were non-mosaic hemizygous (males) or homozygous (female) knock-ins There was no significant difference in the level of mosaicism when injecting hmejSRYp in mature oocytes as compared to six hours post in-vitro insemination (hpi), although to date a limited number of blastocysts injected 6hpi have been analyzed. Finally, there was no significant difference between the knock-in efficiency, or the level of mosaicism when comparing XX and XY embryos ($p > 0.05$). Utilizing the HMEJ pathway in bovine embryos resulted in a significantly higher rate of CRISPR-mediated gene knock-in as compared to HR, and approximately a quarter of these X chromosome knock-ins were non-mosaic (hemizygous males or homozygous females) by PCR.

INTRODUCTION

Genome editing technologies have the potential to have a positive impact on livestock genetic improvement (Van Eenennaam and Young 2019). However, for these tools to be implemented, they must seamlessly integrate into existing breeding program designs to maintain or accelerate the rate of genetic gain. Obtaining high rates of targeted gene knock-ins through homology-directed repair (HDR) using site-directed nucleases in the presence of a repair template has proven difficult in livestock embryos, often resulting in a low integration rate and/or mosaic individuals (Georges *et al*. 2018). The primary method that has been trialed for HDR-mediated knock-ins in bovine embryos is the homologous recombination (HR) pathway. However, the primary method for double-strand break (DSB) repair in gametes and the early zygote is the end-joining pathway (Rothkamm *et al*. 2003). The HDR pathway is primarily restricted to actively dividing cells (S/G2-phase) and only becomes highly active towards the end of the first round of DNA replication in the one-cell zygote (Hustedt and Durocher 2017). Consequently, gene knock-ins in livestock in livestock have typically been achieved by HR in cell culture, followed by somatic cell nuclear transfer (SCNT) cloning of the edited cell line. However, this method can be costly and inefficient (Tan *et al*. 2016). We describe an approach to achieve improved rates of knock-ins in developing bovine embryos using the alternative homology-mediated end joining (HMEJ) DSB repair pathway, and a method to screen for non-mosaic founder individuals prior to embryo transfer, thereby avoiding the need for SCNT to obtain knock-in founders, and allowing the opportunity to edit the next generation of animals in a breeding program in a single step.

MATERIALS AND METHODS

Four single–guide RNAs (sgRNAs) were designed for high specificity and limited off-target potential using the online tools sgRNA Scorer 2.0 (Chari *et al.* 2017) and Cas-OFFinder (Bae *et al.* 2014), respectively. *In-vitro* fertilized bovine embryos were produced using methods previously described (Bakhtari and Ross 2014). The sgRNAs (ZFXg1-4) Cas9 individually injected by laser-assisted cytoplasmic injection (Bogliotti *et al.* 2016) of a solution containing 67ng/µL of each sg-RNA alongside 167ng/µL of Cas9 protein (PNA Bio, Inc., Newbury Park, CA) as ribonucleoprotein complexes (RNP) in three replicates of 30 embryos per guide. Embryos that reached blastocyst stage were collected, lysed, and analyzed using PCR (Table 1), followed by Sanger sequencing.

Table 1. Sequence of primers used for PCR evaluation and confirmation of SRY knock-in and sex, and guide-RNA sequences (*sequences developed by Gokulakrishnan *et al.* 2012)

	Name	Sequence 5’- 3’	T_m (°C)
PCR primers	ZFXgF	TCCAAGGAGCTATGTCACAGAA	60.8
	ZFXgR	CACTAGCTTTGGGCGATATGA	60.8
	ecZFXknF	CCGCTTCAAATCAGTTTAATCC	58.9
	ecZFXknR	CCCCACCAGGAAAGTACAAA	60.4
	srnckF	TGGTCCTCTGTTAATCAGTTCTTTC	61.3
	srnckR	GGAACTGCTTGGGTACCAAG	62.4
	DDX3-1F*	AGGAAGCCAGGAAAGTAA	55.3
	DDX3-1R*	CATCCACGTTCTAAGTCTC	58.0
Guide RNA	ZFXg1	ACAACCCAAAATGAAGGGGG	-
	ZFXg2	AATACAACCCAAAATGAAGG	-
	ZFXg3	CTCCCATGTCATAACTTCTG	-
	ZFXg4	GATATGAAATTACACTGGAC	-

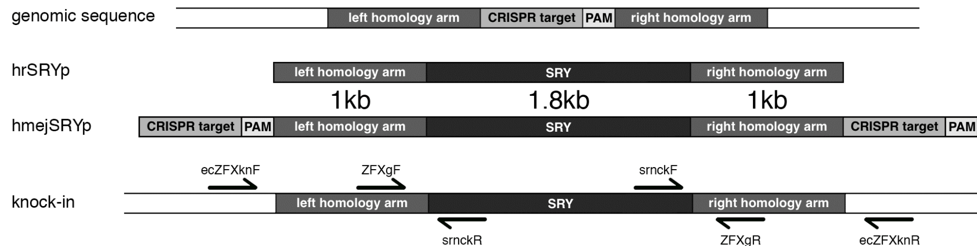

Figure 1. Schematic representation of donor vectors used to test knock-in efficiency in *in* bovine embryos

Donor vectors contained the 1.8kb *Bos taurus* SRY promoter and coding sequence (Accession: U145569), 1kb homology arms flanking each side of the Cas9 cut site, with (hmejSRYp) or without (hrSRYp) the CRISPR target site flanking each homology arm (Figure 1).

Oocytes were collected and *in-vitro* matured for 18 hours prior to injection or *in-vitro* fertilization (Bakhtari and Ross 2014). CRISPR/Cas9 reagents for each donor were introduced by laser-assisted cytoplasmic injection (Bogliotti *et al.* 2016) of a solution containing 67ng/µL of guide-RNA, 167ng/µL of Cas9 protein (PNA Bio, Inc., Newbury Park, CA) and 133 ng/µL of circular plasmid after stripping of cumulus cells from mature oocytes. Injected mature oocytes were in-vitro fertilized and co-cultured with cumulus-oocyte complexes (COCs) for 16 hours. Un-injected in-vitro fertilized embryos were stripped of cumulus cells six hours after fertilization and injected as described above. Injected embryos were scored to developmental stage reached. Embryos that reached blastocyst stage

were collected, lysed and underwent whole-genome amplification using the REPLIg Mini Kit (Qiagen, Valencia, CA), PCR and Sanger sequencing. Data were analyzed with GLM in R to test which variables were statistically different. A χ2 test was used to test whether total knock-in and mosaicism rates differed between donor vector types.

RESULTS AND DISCUSSION

Four sgRNAs (ZFXg1-4) Cas9 ribonucleoprotein complexes (RNP) were individually injected into 90 embryos resulting in *in-vivo* mutation rates of 38%, 57%, 82% and 40%, respectively. Based on these results, we selected sgRNA ZFX3 for the knock-in experiments. Treatment group did not affect overall mutation rate ($P > 0.05$), however embryos injected with ZFX3 RNP and donor hmejSRYp showed a significantly higher rate of total knock-ins (targeted SRY integration) compared to hrSRYp, which showed zero knock-ins (Table 2; P-value < 0.01). When comparing the effect of sex of the embryo, and the time of injection between MII injected oocytes and 6hpi, there was no significant difference on the knock-in efficiency or the level of mosaicism (Table 2; $P > 0.05$). Because we were targeting the X-chromosome, PCR-analysis of embryo biopsies limited our ability to differentiate between heterozygous and mosaic female embryos.

Table 2. Mutation, knock-in, and mosaicism rate of blastocysts after cytoplasmic injection of ZFX3 RNP hmejSRYp or hrSRYp at the MII oocyte, or Embryo (6 hpi) development stage

						Knocked-in subset	
Sex	n	Donor	Time of Injection	%Mutation Rate (n)	%Total Knock-In (n)	%Hemi/ Homo (n)	%Hetero/ Mosaic (n)
Female	78	hmejSRYp	MII oocyte	83^{a} (65)	**40**^{a} (31)	19^{a} (6)	81^{a} (25)
	8		Embryo	88^{a} (7)	**25**^{a} (2)	0^{a} (0)	100^{a} (2)
	6	hrSRYp	MII oocyte	83^{a} (5)	0^{b} (0)	n/a	n/a
	6		Embryo	67^{a} (4)	0^{b} (0)	n/a	n/a
Male	97	hmejSRYp	MII oocyte	70^{a} (68)	**29**^{a} (28)	29^{a} (8)	71^{a} (20)
	14		Embryo	86^{a} (12)	**21**^{a} (3)	33^{a} (1)	67^{a} (2)
	10	hrSRYp	MII oocyte	70^{a} (7)	0^{b} (0)	n/a	n/a
	8		Embryo	75^{a} (6)	0^{b} (0)	n/a	n/a
Total	175	hmejSRYp	MII oocyte	76^{a} (133)	**34**^{a} (59)	24^{a} (14)	76^{a} (45)
	22		Embryo	86^{a} (19)	**23**^{a} (5)	20^{a} (1)	80^{a} (4)
	16	hrSRYp	MII oocyte	75^{a} (12)	0^{b} (0)	n/a	n/a
	14		Embryo	71^{a} (10)	0^{b} (0)	n/a	n/a

Letters that differ in the same column are statistically different (P-value < 0.05)

This increased rate of knock-ins with donor hmejSRYp is likely the result of the DSB repair pathway triggered by the different donor vectors. The hrSRYp donor vector required initiation of the homologous recombination (HR) pathway for integration, which has been shown to have a low activity in early embryos. In contrast, hmejSRYp utilizes the homology-mediated end-joining (HMEJ) pathway (Yao *et al*. 2017). In mice zygotes, this pathway was found to have a significantly higher efficiency of targeted knock-ins as compared to HR, which is consistent with the end-joining pathway being the primary DSB repair mechanism in gametes and pre-S-phase zygotes (Rothkamm *et al*. 2003). It should be noted that the MII injected oocytes were observed to have lower post-fertilization development rates compared to zygotes injected after insemination (12.1% (n=1,584) versus 18.4% (n = 163), respectively), perhaps due to increased rates of polyspermy in the stripped oocytes. Targeting the HMEJ pathway in developing embryos, alongside a method to screen for non-mosaic founder individuals prior to embryo transfer (Figure 2), has the potential to be an alternative to SCNT cloning of genome-edited knock-in cells. The implementation of a gene editing approach such as this

alongside genetic breeding programs could enable the introduction of useful genetic variants such as polled (hornlessness), while maintaining the rate of genetic gain without increasing inbreeding above acceptable levels (Mueller *et al*. 2019). Recent Australian regulation would categorize the use of a donor template to guide the DSB repair to produce a cisgenic knock-in, as detailed in this paper, as resulting in a genetically modified organism (GMO) which may limit the use of this approach in animal breeding programs.

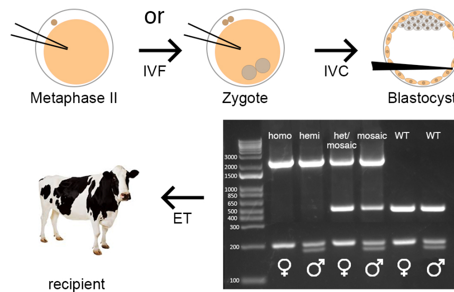

Figure 2. Schematic representation of CRISPR-mediated development of SRY knock-in bovine offspring by cytoplasmic injection (CPI)

Biopsies taken at day 7 and are analyzed via PCR to simultaneously detect sex, success of knock-in, and mosaicism prior to embryo transfer (ET) to synchronized recipients. Upper bands using ZFXgF/R PCR primers: wild type (WT) 520bp, knock-in 2349bp. Lower bands using DDX3-1F/R PCR primers: female 208bp, male 189bp and 208bp. IVF: in-vitro fertilization, IVC: in-vitro culture, het: heterozygous, hemi: hemizygous male, homo: homozygous knock-in female.

CONCLUSION

In-vitro production of bovine embryos combined with CPI of CRISPR Cas9 RNP in MII oocytes or 6 hpi bovine embryos, along with a donor vector designed to target the HMEJ repair pathway, yielded a 32.5% knock-in rate of the 1.8 kb SRY target gene of which 23.4% were non-mosaic, hemizygous (males) or homozygous (females), targeted X-chromosome knock-ins.

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REFERENCES

Bae S., Park J. and Kim J. (2014) *Bioinformatics* **30:** 1473.
Bakhtari A. and Ross P.J. (2014) *Epigenetics* **9:** 1271.
Bogliotti Y.S., Vilarino M. and Ross P.J. (2016) *J. Vis. Exp.* **116**: doi:10.3791/54465
Chari R., Yeo N.C., Chavez A. and Church G.M. (2017) *ACS Synth. Biol.* **6**: 902.
Georges, M., Charlier, C. and Hayes, B. (2018). *Nat. Rev. Genet.* **20**: 135 .
Gokulakrishnan P., Kumar R.R., Sharma B.D., Mendiratta S.K. et al. (2012) *J. Anim. Sci.* **5**: 733.
Hustedt N and Durocher D. (2017) *Nat. Cell Biol.* **19**: 1.
Mueller M.L., Cole JB., Sonstegard T.S. and Van Eenennaam A.L. (2019) *J. Dairy Sci*. **102** 4215.
Rothkamm K., Krüger I., Thompson L.H. and Löbrich M. (2003) *Mol. Cell. Biol*. **23**: 5706.
Tan W., Proudfoot C., Lillico S.G. and Whitelaw C.B. (2016). *Transgenic Res.* **25**: 273-87
Van Eenennaam, AL. and Young, AE. (2019). *Encycl. Food Secur. Sustainability* **3**: 171.
Yao X, Liu Z, Liu J, Zhou H, Shen X, *et al*. (2017) *Cell Res*. **27**: 801.