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GABRIELLA BORBA DE OLIVEIRA

STRATEGIES FOR GENE EDITING USING THE CRISPR SYSTEM FOR THE GENERATION OF GENETICALLY ENGINEERED LIVESTOCK

PORTO ALEGRE (RS), Brasil

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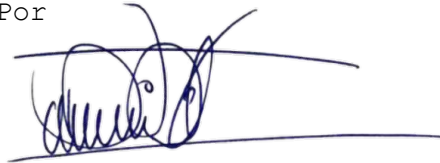para obtenção do Grau de

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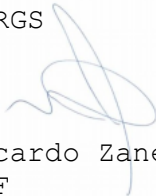
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Estratégias para edição gênica utilizando o sistema CRISPR para a geração de animais de produção geneticamente modificados[1]

Autora: Gabriella Borba de Oliveira

Orientador: Dr. Marcelo Bertolini

Resumo: O melhor entendimento dos procedimentos de biologia molecular tem possibilitado o aprimoramento de estratégias de edição gênica, como o sistema CRISPR, possibilitando a modulação de genes em locais específicos do genoma, incluindo modificações de marcações epigenéticas, temas que foram abordados no **Capítulo I**. Os objetivos desta tese foram comparar diferentes estratégias utilizando o sistema CRISPR (a) para promover a reprogramação celular parcial de fibroblastos suínos utilizando o sistema de ativação com CRISPR (CRISPRa); e (b) avaliar a sobrevivência e a viabilidade de embriões bovinos após a microinjeção de zigotos com o sistema CRISPR/Cas9 e modelos de reparo de DNA para promover recombinação homóloga em *safe harbor loci* (SHL) em embriões bovinos produzidos por fecundação *in vitro* (FIV). No **Capítulo II**, as eficiências de duas nucleases de fusão com domínios de ativação (dCas9-VPR e dCpf1/Cas12a-VPR) foram comparadas para permitir a ativação da expressão transitória de genes alvo de reprogramação (*Oct4, Myc, Klf4, Sox2* e *Lin28a*), e para alterar a transcrição de genes relacionados à senescência celular em células de suínos em passagens avançadas. A dCas9-VPR regulou positivamente genes únicos de forma mais eficaz do que a dCpf1-VPR, também usando menor número de gRNAs por gene, com maior nível de expressão para os genes *Myc* e *Lin28a*. Por outro lado, a dCas9-VPR não foi efetiva na regulação de múltiplos genes concomitantemente, embora tenham sido observados efeitos possivelmente relacionados aos genes-alvo, como a expressão dos genes *p53* e *Dkc1*. O sistema CRISPRa promoveu a reprogramação *in vitro* parcial de células suínas em cultivo, apesar de em um nível menor do que o esperado. No **Capítulo III**, a sobrevivência *in vitro* e o desenvolvimento de embriões bovinos de FIV foram avaliados após a microinjeção citoplasmática (MI) do sistema CRISPR/Cas9 e de oligonucleotídeos de reparo de DNA em embriões no estádio de 1-célula, tendo como alvo os SHL H11 e Rosa26. Após a MIV por 20 h, CCOs bovinos foram fecundados *in vitro* por 8 h (grupos tratamento) ou por 18 h (grupo intacto). Grupos de zigotos foram

parcialmente desnudados 8 h pós-fecundação (hpf) e, em seguida, segregados em grupos tratamento: Semi-desnudo (Semi), controle sem MI; grupo MI com CRISPR/Cas9; e grupos SHL, MI com CRISPR/Cas9, gRNA para cada SHL e uma das duas doses de oligonucleotídeos de reparo de DNA (5 ng/µL ou 20 ng/µL). Os embriões foram cultivados *in vitro* até o estádio de blastocisto, avaliando-se as taxas de sobrevivência pós-MI (D1), clivagem (D2) e de blastocisto (D7). A sobrevivência não foi afetada pela injeção do sistema CRISPR/Cas9, nem pelas doses ou os *loci*-alvo, embora a remoção parcial das células do *cumulus* com 8 hpf, ou a microinjeção de oligonucleotídeos de reparo de DNA com o sistema CRISPR/Cas9 reduziram o desenvolvimento a blastocisto (inferior a 20% na maioria dos grupos) em comparação com os controles (acima de 20%), independentemente da dose injetada ou do *locus*-alvo. A microinjeção com oligonucleotídeos de reparo de DNA com o sistema CRISPR/Cas9 se demonstrou viável para experimentos de recombinação homóloga em embriões bovinos de FIV, apesar da redução no desenvolvimento embrionário. Em conclusão, as estratégias utilizando o sistema CRISPR para auxiliar na edição gênica em cultivo de células somáticas suínas ou em embriões bovinos de FIV foram viáveis e relativamente eficientes. Por outro lado, a realização de outros experimentos será necessária para avaliar a viabilidade do uso de células de suínos reprogramadas para a clonagem, e a eficiência por análise genômica dos resultados das estratégias utilizadas para a recombinação homóloga em embriões bovinos.

Palavras-chave: Edição de genes; reprogramação epigenética; recombinação homóloga; sistema de ativação CRISPR; células somáticas; embrião; suínos; bovinos.

Strategies for gene editing using the CRISPR system for the generation of genetically engineered livestock[1]

Author: Gabriella Borba de Oliveira

Supervisor: Dr. Marcelo Bertolini

Abstract: The better understanding of molecular biology procedures has enabled the improvement of gene editing strategies, such as the CRISPR system, making it possible to modulate genes at specific sites in the genome, including changes in epigenetic marks, subjects that were addressed in **Chapter I**. Therefore, the aims of this thesis were to compare different strategies using the CRISPR system (a) to promote partial cellular reprogramming in pig fibroblast cells using the CRISPR activation system (CRISPRa); and (b) to evaluate embryo survival and viability after zygote microinjection with CRISPR/Cas9 system and DNA oligonucleotide templates to promote homologous recombination into safe harbor *loci* (SHL) in bovine embryos produced by *in vitro* fertilization (IVF) procedures. In **Chapter II**, the efficiencies of two nucleases fused to activation domains (dCas9-VPR and dCpf1/Cas12a-VPR) were compared in enabling the transient upregulation of reprogramming target genes (*Oct4, Myc, Klf4, Sox2* and *Lin28a*), and for the ability to alter transcription of downstream genes related to reprogramming of porcine somatic cells at advanced passages. The dCas9-VPR more effectively upregulated single genes than dCpf1-VPR, also using lower number of gRNAs per gene, with highest expression levels for *Myc* and *Lin28a* genes. On the other hand, dCas9-VPR failed to upregulate multiple genes concomitantly, although downstream effects were detected in the expression of *p53* and *Dkc1* genes. The CRISPRa system promoted partial reprogramming in pig somatic cells *in vitro*, although at lesser extent than expected. In **Chapter III**, the *in vitro* survival and developmental outcome of IVF bovine embryos were assessed after cytoplasmic microinjection (MI) of CRISPR/Cas9 system and DNA templates at the 1-cell stage embryo, targeting the SHL H11 and Rosa26. Bovine COCs were *in vitro* matured for 20 h and fertilized for either 8 h (treatment groups) or 18 h (Intact Group). Groups of presumptive zygotes were partially denuded 8 h post-fertilization (hpf), and then segregated into treatment groups: Semi-denuded (Semi), non-MI control; group MI with CRISPR/Cas9; and SHL groups, MI with CRISPR/Cas9, gRNA for each SHL, and

one of two doses of repair oligonucleotide templates (5 ng/µL or 20 ng/µL). Embryos were *in vitro* cultured up to the blastocyst stage, evaluating post-MI survival (D1), cleavage (D2) and blastocyst (D7) rates. Survival was not affected by the injection of either the CRISPR/Cas9 system, the doses, or the target *loci*, although the partial *cumulus* cells removal at 8 hpf, or the microinjection of donor oligonucleotides and the CRISPR/Cas9 system reduced development to the blastocyst stage (lower than 20% in most groups) in comparison to controls (above 20%), irrespective of the injected dose or the targeted *locus*. The microinjection with repair templates and CRISPR/Cas9 system was feasible for homologous recombination experiments in bovine preimplantation IVF embryos, despite the reduction in embryo development. In conclusion, the strategies using CRISPR approaches to assist in gene editing pig cells in culture or early bovine IVF embryos were feasible and rather efficient. On the other hand, the performance of other experiments will be necessary to evaluate the feasibility of using reprogrammed pig cells for cloning, and the efficiency by genomic analyses of the strategies used for homologous recombination in bovine embryos.

Keywords: Gene editing; epigenetic reprogramming; homologous recombination; CRISPR activation system; somatic cells; embryo; pig; cattle.

SUMMARY

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LIST OF ABBREVIATIONS

Ads   Activation Domains
COCs/CCOs   *Cumulus*–Oocyte Complexes/ Complexos *cumulus*-oócito
CRISPR   Clustered Regularly Interspaced Short Palindromic Repeat
CRISPRa   CRISPR activation
crRNA   CRISPR RNA guide
dCas9   Deactivated Cas9
dCpf1/Cas12a   Deactivated Cpf1/Cas12a
DMEM   Dulbecco's Modified Eagle's Medium
DSBs   Double-Strand Breaks
ESCs   Embryonic Stem Cells
FBS   Fetal Bovine Serum
GMOs   Genetically Modified Organisms
gRNA   Guide RNA
HDR   Homology-Directed Repair
HR   Homologous Recombination
iPSCs   Induced Pluripotent Stem Cells
IVC   *In Vitro* Culture
IVF/FIV   *In Vitro* Fertilization/Fecundação *In Vitro*
IVM/MIV   *In Vitro* Maturation/Maturação *In Vitro*
IVP   *In Vitro* Production
KI   Knock-in
KO   Knock-out
MI   Microinjection
MST   Multiplex Single Transcript
NHEJ   Nonhomologous End Joining
NT   Nuclear Transfer
PAM   Protospacer-Adjacent Motif
PSCs   Pluripotent Stem Cells
qRT-PCR   Quantitative Reverse Transcription PCR
RNP   Ribonucleoprotein
RVD   Repeat Variable Di-residue
SCNT   Somatic Cell Nuclear Transfer
SHL   Safe Harbor *Loci*
ssDNA   Single Stranded DNA
TALENs   Transcription Activator-Like Element Nucleases
TE   Tris-EDTA
TF   Transcription Factor
tracrRNAs   Trans-Activating crRNAs
TSS   Transcription Start Site
VPR   VP64-p65-Rta
ZFNs   Zinc Finger Nucleases

CHAPTER I: THE GENE EDITING ERA

1. INTRODUCTION

The development and improvement of biotechnology and molecular biology procedures have enabled a better understanding of biological processes and gene networks involved in important traits in farm animals. With such advances, the genetic manipulation of organisms and production of transgenic animals has expanded, making it possible to modulate features and traits in a customized way, through addition (Knock-In), deletion (Knock-out), and any other modulation (e.g., Knock-down) that changes genes and gene functions at specific sites into the genome. The development of methods to genetically modify organisms (GMOs) and to produce transgenic animals has been based on many purposes, mainly for agriculture (e.g., disease resistance in livestock) and human health (e.g., expression of recombinant proteins in milk) applications (Wheeler, 2003). The main technologies that have largely been used to produce transgenic animals have been pronuclear microinjection of foreign DNA into zygotes (Gordon and Ruddle, 1981; Brinster *et al.*, 1985; Hammer *et al.*, 1985) and cloning by nuclear transfer (NT), using genetically modified cells of somatic or embryonic origins (Schnieke *et al.*, 1997, Cibelli *et al.*, 1998, Wheeler, 2003). By genetic engineering cells, it is possible to screen and to select cells to produce genetically modified cloned animals, either through the insertion (transgenesis) or deletion of DNA sequences into the host genome (Murray and Maga, 2016). Although cloning by somatic cell NT (SCNT) has been successful in the production of transgenic animals, it has not yet been possible to reach the maximum potential due to technical limitations. Such low overall efficiency also depends on the ability of the nuclear donor cell to be fully reprogrammed to a totipotent state, which must occur in differentiated cells for proper embryo development (Oback, 2008).

More recently, the clustered regularly interspaced short palindromic repeats (CRISPR)-based system became a tool of choice for gene editing (Jinek *et al.*, 2012), allowing an increase in efficiency for genetic manipulation of cells in culture used for SCNT cloning or of embryos by direct cytoplasmic microinjection (Navarro-Serna *et al.*, 2020). Although the latter procedures have been efficient in promoting gene modifications (deletions, knock-outs) at a rather high rate, precise DNA insertions

(knock-ins) using the CRISPR system and exogenous DNA into early developing embryos has been a challenge.

The use of genome-integrating methods using viral transduction remains a gold standard in induced Pluripotent Stem Cells (iPSC) generation. However, new methods, so-called "non-integrating techniques", are being extensively developed and evaluated (González *et al.*, 2011; Schlaeger *et al.*, 2015). One of such methods is the modified version of CRISPR system, used for transcriptional activation, leading to a fully transgene independent reprogrammed cell without persistent expression of exogen reprogramming factors (Gilbert *et al.*, 2013; Chavez *et al.*, 2015). Such cells, once reprogrammed, may be used for cloning at a potentially higher efficiency rate. However, some barriers must be overcome, from the initial genetic construction itself, to the limitations of techniques to produce genetic engineered animals. Therefore, studies involving gene editing and transgenesis become necessary in order to increase production efficiency and overcome such limitations, contributing to research advances in several areas.

2. LITERATURE REVIEW

2.1 Genetically Engineered Livestock

The improvement of animal reproduction technologies combined with the methodologies for DNA edition in farm animals enabled the development of rather efficient methods for the genetic manipulation of organisms and the production of genetically modified animals, which includes procedures for the deletion or the insertion of bases or DNA sequences (transgenesis). The first success to produce transgenic livestock was attained in 1985 (Hammer *et al.,* 1985) and since then, many farm animals have been genetically modified for application in agriculture and biotechnology (Kues and Niemman, 2011). However, genetic manipulation in livestock generally faces some challenges. The exogenous DNA integration in the germ line of farm animals has been proven challenging and often inefficient over the transgenic animal generation process (Niemann and Kues, 2003; Meng *et al.*, 2013; Chi *et al.*, 2019; Lamas-Toranzo *et al.,* 2019). Such difficulties usually lie on the random transgene integration and the control of transgene copy number, which can lead to an unpredictable phenotype of protein expression (Ruan *et al.*, 2015). Due to the low efficiency of the process in livestock and the increased demand for novel transgenic animal models, the classical methods have been improved and new complementary techniques have been developed for more efficient production of transgenic animals.

Historically, the main advanced reproductive technologies used to produce genetically modified animals have been pronuclear microinjection of exogenous DNA into zygotes (Gordon and Ruddle, 1981; Brinster *et al.*, 1985; Hammer *et al.*, 1985), somatic cell nuclear transfer (SCNT), or genetically modified embryonic stem cells for production of chimeras (Schnieke *et al.*, 1997; Cibelli *et al.*, 1998; Wheeler, 2003). Although such procedures have been successful in the production of transgenic animals, it has not yet been possible to reach the maximum production potential due to the limitations of the techniques themselves and the low rate of integration of exogenous DNA in specific sites into the genome (Hodges and Stice, 2003; DeMayo *et al.,* 2012).

Although great steps have been made in generating transgenic large animals, most of the events in the genome editing process cannot be fully controlled. For this reason, research is continuing, and novel approaches are progressively being

developed, aiming to insert transgenes in specific sites into the genome, improving the homologous recombination process in large animals, and allowing gene modification and selection of transformed more reprogrammable cells in culture that could be cloned to produce fertile animals (Schnieke *et al.,* 1997; Murray and Maga, 2016).

2.1.1 Microinjection procedures

The first approach to generate transgenic livestock was made by pronuclear DNA microinjection (Hammer *et al.*, 1985). Although such technique has been successfully used in mice, pronuclear microinjection is not cost-effective in large animals due to its low efficiency and high costs for generating many offspring with a low rate of transgenesis (Galli *et al.*, 2012). Despite the inefficiency of the pronuclear microinjection procedures, many transgenic farm animals have been generated in such way (Murray *et al.*, 1989; Baldassarre *et al.*, 2003; Uchida *et al.,* 2001).

The pronuclear microinjection is based on the introduction of linear DNA sequences into the fertilized zygote through microinjection into the female and/or male pronuclei. Exogenous DNA must be integrated into the genome before the first cleavage and duplication of genetic material so that the animal can present the transgene in all cells and in all cell lineages (Fig. 1). However, such technique has low efficiency and a low success rate in transmitting the transgene to germ cells, with the occurrence of mosaicism in most produced animals (Kubisch *et al.*, 1995; Eyestone, 1999; Hodges and Stice, 2003; Meng *et al.*, 2015). More recently, the cytoplasmic microinjection has re-emerged as an alternative to gene transfer into zygotes, especially with the advent of the new gene editing tools. Compared to the pronuclear injection, such procedure is simpler, not requiring visualization and injection into the pronuclei, not even requiring pronuclear stage embryos for its use. However, it is essential that the injection of exogenous DNA occurs at the exact time the genome is exposed to facilitate transgene integration (Fig. 1; Meng *et al.*, 2015).
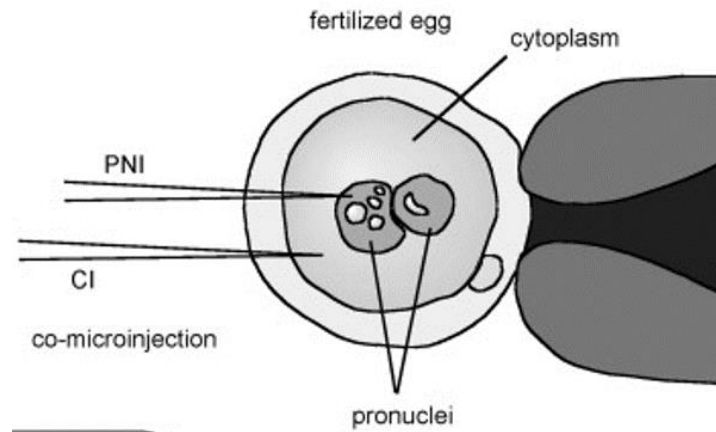
Figure 1. Cytoplasmic and pronuclear microinjection scheme. PNI: pronuclear injection. CI: cytoplasmic injection. Source: Sumiyama *et al.* (2010).

2.1.2 Cloning by Nuclear Transfer (NT) procedures

In the 1990's, a breakthrough in livestock transgenesis came from the development of cloning by somatic cell nuclear transfer (SCNT), with the birth of Dolly the sheep (Wilmut *et al.*, 1997), which was quickly translated into the production of transgenic sheep (Schnieke *et al.*, 1997). So far, cloning by SCNT remains among the cutting-edge options for transgenesis in farm animals (Bertolini *et al.*, 2016). The nuclear transfer technique allows the production of cloned animals, by introducing the genetic material of an animal cell (nucleus donor) into an enucleated oocyte, through micromanipulation (Fig. 2). Thus, genetic engineering of the genome is carried out *in vitro*, in cell cultures, with subsequent selection of cell colonies that have the DNA of interest integrated in its genome (Bertolini *et al.,* 2016; Galli *et al.*, 2012). This process is followed by molecular screening of selected colonies, which allows the determination of copy number and chromosome location of the new DNA in the host genome (Lin *et al.,* 2014).

Cloning by SCNT made it possible for major technical advances in the development of transgenic animals, with advantages related to the convenience of producing transgenic cloned embryos, with more precise molecular characterization of cell lines prior to cloning, when compared to microinjection, with all born animals being of the selected genotype. Moreover, this technique has brought even more flexibility to researchers, as these cells are easily cultured and can be frozen for later use in cloning and transgene integration studies (Bressan *et al.*, 2008).
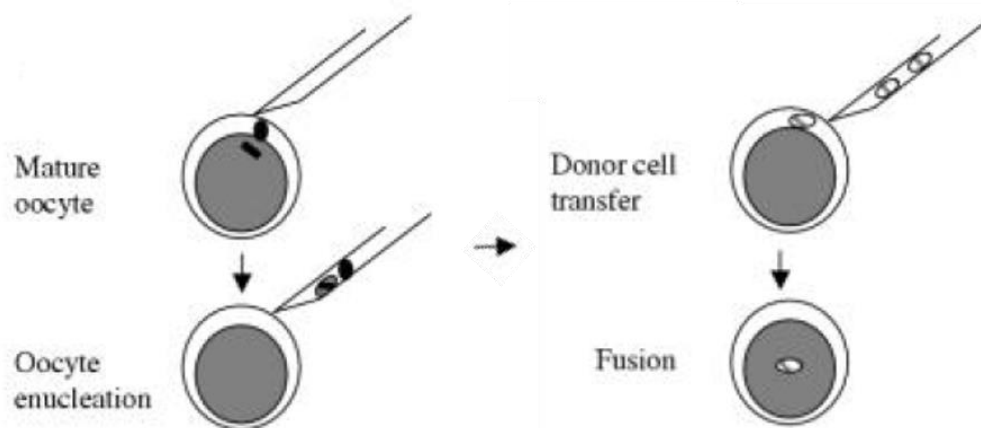
Figure 2. Simplified cloning scheme by nuclear transfer procedures. A matured oocyte (MII phase) is enucleated (cytoplast) and a transgenic somatic cell (gray-striped cell, karyoplast) is transferred into the perivitelline space, under the zona pellucida. An electrical pulse is then given to fuse the two cell membranes, transferring the cell nucleus into the oocyte. Source: Hodges and Stice (2003).

Although SCNT cloning is a feasible procedure for the development of genetically modified animals, other problems still need to be addressed. One of the great cloning limitations, in addition to its low efficiency (Bressan *et al.*, 2008), is related to epigenetic reprogramming that must occur in the differentiated cell for proper embryo development (Niemann and Lucas-Hahn, 2012; Simmet, Wolf and Zakhartchenko, 2021). Some studies have shown that SCNT cloned animals may develop abnormalities in development due to faulty epigenetic reprogramming and gene expression (Fletcher *et al.*, 2007; Chavatte-Palmer *et al.*, 2012). Moreover, primary fibroblast cells, the main and more widely cell type used for SCNT cloning, have a limited lifespan in culture, which is usually decreased by cell transfection, colony selection and screening after genetic modifications, which commonly hinder their use as nucleus donors for cloning (Laible and Alonso, 2009; Galli *et al.*, 2012). Despite the problems, by implementing the cloning approach, the efficiency of generating functional transgenic animals is increased, mainly when compared to embryo microinjection.

2.2 Gene Editing Tools

Usually, the insertion of a transgene into the genome occurred in a random fashion though standard genetic manipulation procedures (Clark *et al.,* 2000), making

the regulation of the transgene expression unpredictable. In addition, the site of insertion may also be deleterious to the cell, depending on its location into the genome. The random integration occurs at points into the genome where there are double-stranded breaks (DSB), with the transgene being inserted incidentally during DNA repair. Therefore, the development of gene editing tools allowed a known and precise excision of the DNA, where gene integration occurs, turning the production of genetically modified (GM) and transgenic animals a more efficient process (Bressan *et al.*, 2008). The gene editing technology is based on the use of nucleases formed by sequence-specific DNA domains and non-specific cleavage domains, which induce DSB, which activates the DNA repair machinery, mainly the homologous recombination (HR) or the non-homologous end joining (NHEJ) pathways, enabling precise and specific genetic modifications in the genome (Wyman and Kanaar, 2006; Urnov *et al.*, 2010; Carroll, 2011). Three main systems for gene editing have been progressively developed over the past three decades, starting with Zinc finger nucleases (ZFNs), followed by transcription activator-like element nucleases (TALENs), and finally culminating with RNA-guided endonucleases, mainly represented by the CRISPR/Cas9 system (Jinek *et al.*, 2012; Gaj *et al.*, 2013), a recent disruptive technology of great impact in biology.

2.2.1 Zinc Finger Nucleases (ZFN)

The ZFNs consist of a zinc finger site-specific DNA-binding domain in the N-terminal region, fused to non-specific cleavage domains of the *Fok*I endonuclease, in the C-terminal region (Fig. 3). The ZF motif with specific DNA-binding affinity was discovered as part of a transcription factor IIa in *Xenopus* sp. oocytes (Miller *et al.,* 1985). At least two ZFNs are necessary for use in genetic modifications, as *Fok*I needs to dimerize to excise the DNA, and such feature increases the specificity of the binding to the target sequence (Smith *et al.*, 2000). The two ZFNs molecules bind to the target DNA in a tail-to-tail orientation separated by a 5-7 bp spacer sequence, with the double strand break (DSB) occurring in the region between the molecules (Fig. 3; Petersen, 2017; Gaj *et al.*, 2013).
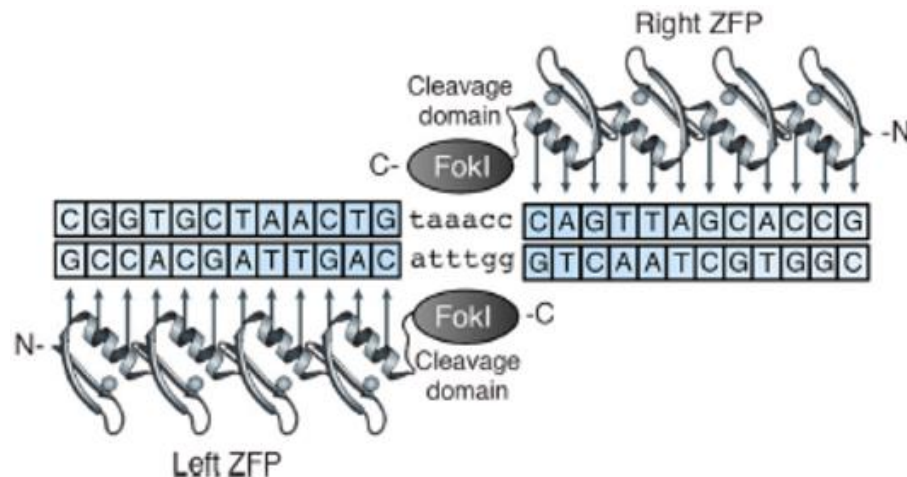
Figure 3.   Dimer of Zinc finger nucleases linked to the target DNA, with the cleavage recognition domain (spacer sequence) by *Fok*I nuclease. Source: Gaj *et al.* (2013).

2.2.2 Transcription Activator-Like Element Nucleases (TALEN)

The TALEs (transcription activator-like effectors) are produced naturally by plant pathogens, such as *Xanthomonas* sp. (Boch *et al.*, 2009). Such molecules can bind to the host's DNA, acting as transcription factors in the activation of plant genes that promote bacterial infection. TALEs consist of repetitions, called RVD (repeat variable di-residue), where each repetition binds specifically to a nucleotide of genomic DNA, establishing a protein-DNA interaction (Boch *et al.*, 2009; Moscow and Bogdanove, 2009). The TALEs repetitions can be used to construct DNA binding domains capable of recognizing endogenous mammalian DNA sequences. By fusing the binding domain in the C-terminal region with a non-specific *Fok*I endonuclease cleavage domain in the N-terminal region, a TALE nuclease (TALEN) is formed, which can be used in dimers to generate specific DSBs (Fig. 4; Li *et al.*, 2011; Gaj *et al.*, 2013; Petersen, 2017).

CTCCAACCAGGTGCTAActgtaaaccatggaaaaggaTTAGCACCTGGTTGGA
GAGGTTGGTCCACGATTgacatttggtaccttttcctAATCGTGGACCAACCT
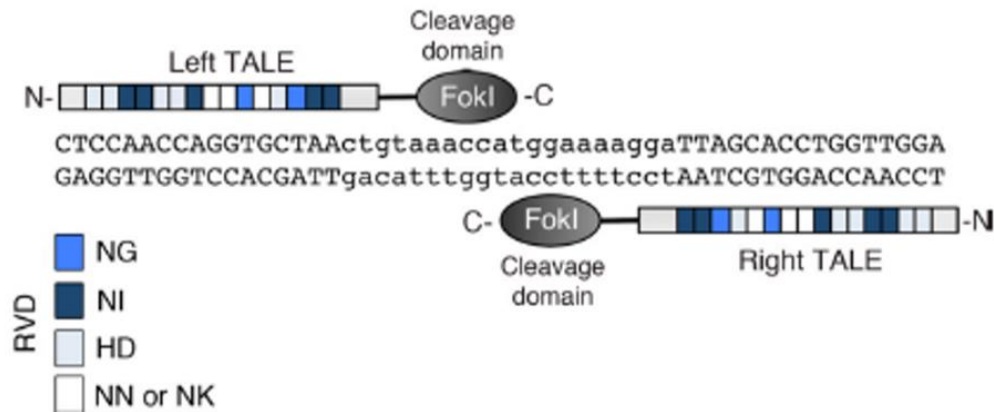
Figure 4. Each transcription activator-like effector (TALE) repeat contains 33-35 amino acid residues that recognize a single nucleotide of the target DNA, through two hypervariable amino acid residues (RVDs). *Fok*I cleavage domain dimers introduce double-strand breaks. Source: Gaj *et al.* (2013).

2.2.3 CRISPR System

The CRISPR system (clustered regularly interspaced short palindromic repeats) is derived from the prokaryotic adaptive immune system, which provides protection against viruses by destroying exogenous DNA, in a sequence-specific manner, encoded by DNA and mediated by RNA (Terns and Terns, 2011; Jinek *et al.*, 2012; Barrangou and Doudna, 2016). The CRISPR/Cas9 system is the most recent method of genomic modification, in which a guide RNA (gRNA) directs the Cas9 nuclease for binding and cleavage of target DNA sequences, generating DSB at specific sites into the genome (Tu *et al.*, 2015). In the type II system, small sequences of exogenous DNA, called protospacers (spacers), are integrated into the CRISPR genomic *locus*, transcribed and processed into small CRISPR RNAs (crRNAs). These crRNAs join with a trans-activating crRNAs (tracrRNAs) and direct site-specific cleavage by Cas (CRISPR-associated) proteins, silencing the pathogen's DNA (Fig. 5; Cong *et al.*, 2013; Gaj *et al.*, 2013; Sander and Joung, 2014).
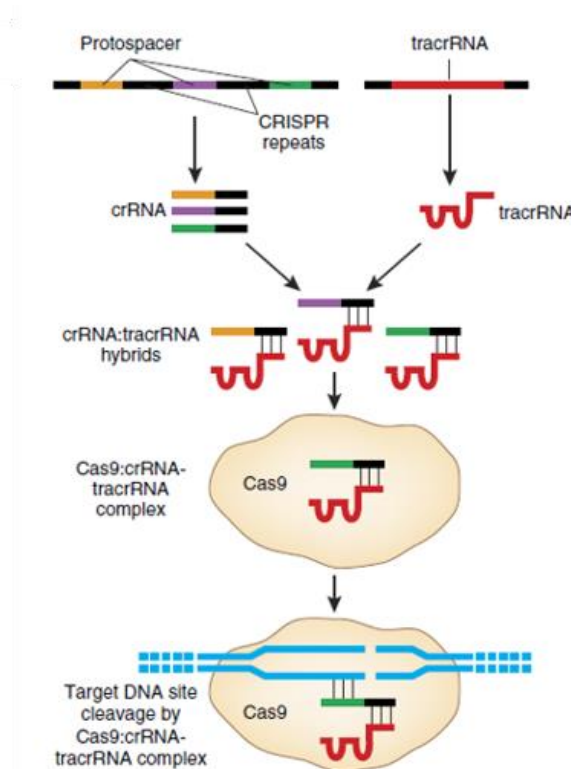
Figure 5. The CRISPR/Cas9 system. The CRISPR system found in bacteria incorporates exogenous DNA sequences into arrays, which then produces crRNAs containing regions of protospacer, which are complementary to the exogenous DNA binding site. The crRNAs hybridize to the tracrRNAs (also encoded by the CRISPR system) and then, the RNA pair may associate with the Cas9 nuclease. The crRNA-tracrRNA/Cas9 complex recognizes and cleaves exogenous DNAs complementary to the protospacer sequence. Source: Sander and Joung (2014).

To simplify the construction process of the CRISPR/Cas9 system and to maintain cleavage efficiency, the crRNA-tracrRNA complex was redefined as a single guide RNA transcript (single-guide RNA or sgRNA) necessary for Cas9 binding and cleavage into the target DNA sequence, which is flanked by a conserved 2-4 bp recognition sequence called protospacer-adjacent motif (PAM), specific for each nuclease, generating DSBs (Fig. 6; Sander and Joung, 2014; Tu *et al.*, 2015). This system allows multiple *loci* to be targeted simultaneously, showing efficiency and specificity similar to ZFNs and TALENs (Gaj *et al.*, 2013; Petersen, 2017). Despite the advantages observed with this system, the correct design of the sgRNA is extremely important to avoid unintended and random mutations (off-targets), due to the non-specificity of DNA cleavage. This non-specificity of the nuclease recognition in the target DNA can generate DSB in undesired sites into the genome, leading to silent mutations or even loss of function of important genes (Ishii, 2017).
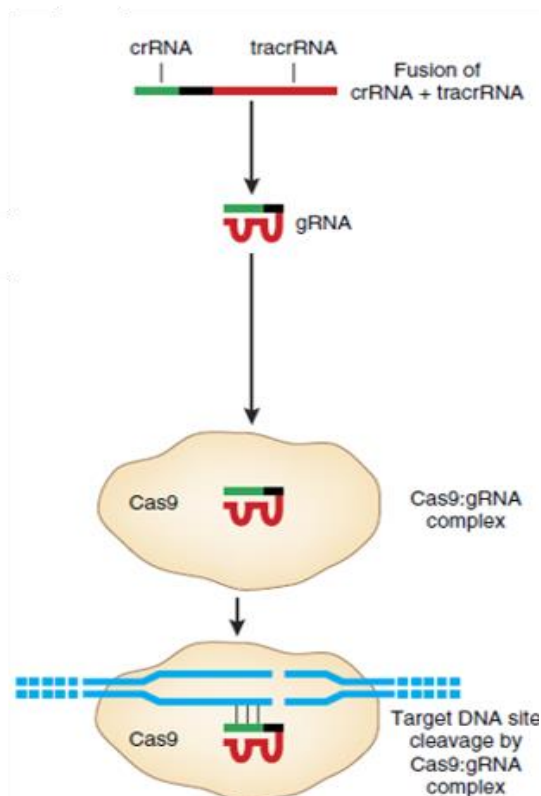
Figure 6. The CRISPR/Cas9 system. The most commonly used CRISPR/Cas system is derived from the fusion between the crRNA and part of the tracrRNA sequence. This unique gRNA forms a complex with Cas9 to mediate the cleavage of target DNA sites that are complementary to the 20 nt of gRNA and which are next to a PAM sequence. Source: Sander and Joung (2014).

2.2.3.1 CRISPR activation (CRISPRa) system

Modified versions of the Cas9 protein have been engineered by mutating two key amino acid residues within its nuclease domains, generating a deactivated Cas9 (dCas9), a RNA-programmable DNA-binding protein that lacks endonucleolytic activity, while retaining the capacity to interact with DNA (Gasiunas *et al.*, 2012; Didovyk *et al.*, 2016). Catalytically inactivated Cas9 proteins (or also named dead Cas9) can be used to control gene expression by physically blocking transcription or through fusion to transcriptional activation (Ads; e.g., VP64, a viral transcriptional activator) or repression (e.g., KRAB, Krueppel-associated box) domains, enabling Cas9 to serve as a tool for cellular programming at the transcriptional level (Cheng *et al.*, 2013; Gilbert *et al.*, 2013; Maeder *et al.*, 2013).

Activation levels using single Ads fused to Cas9 are generally weak. Consequently, the fusion of multiple Ads per dCas9 molecule may increase transcriptional activation by mimicking the natural cooperative recruitment process (Chavez *et al.*, 2015). Several candidate effectors with known transcriptional roles have been tested, and three different activation domains (VP64, p65, and Rta) presented the most meaningful induction actions (Didovyk *et al.*, 2016). However, such ADs alone were not more effective than VP64, the first generation of transcriptional activators, but when they were fused to form a so-called VPR activator (VP64-p65-Rta), such construction was more effective than a single VP64 fusion (Chavez *et al.*, 2015; Fig. 7).
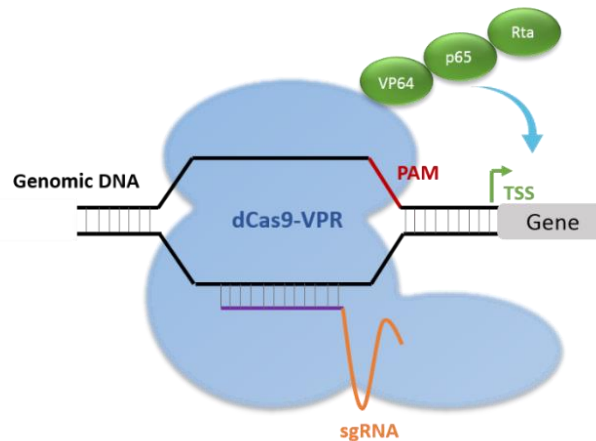
Figure 7. The CRISPR/Cas9 activation system. Cas9 consist of an effector nuclease (colored blue) and a single crRNA (sgRNA) located in the genomic DNA immediately upstream of the protospacer-adjacent motif (PAM) (3′-NGG-5′). The VPR CRISPRa system consists of dCas9 fused to three transcriptional activators (VP64, p65 and Rta19) (dCas9-VPR), which act upstream of the transcriptional start site (TSS) to overexpress a target gene with a single guide RNA (sgRNA). Source: adapted from https://dharmacon.horizondiscovery.com.

Transcriptional activation can also be increased by targeting the gene promoter with multiple sgRNAs (Cheng *et al.*, 2013; Maeder *et al.*, 2013). With dCas9, the process of multiplex sgRNAs requires either relatively large constructs, which is time-consuming, or simultaneous delivery of multiple plasmids, which can also be a problem in terms of efficiency or for *in vivo* applications (Zetsche *et al.*, 2017). Recently, new nucleases with better performances have been discovered to improve CRISPR procedures and to overcome some limitations of the CRISPR/Cas9 system (Kim *et al.*,

2016). The Cpf1 or Cas12a is a smaller endonuclease, similar to Cas9, that also cleaves double-stranded DNA at the recognition site. However, it only requires a shorter (43 nucleotides) and simpler CRISPR RNA (crRNA or gRNA) that consists of a 5′-handle (20 nucleotides) and a guide segment (23 nucleotides), as depicted in Figure 8 (Li *et al.*, 2018).
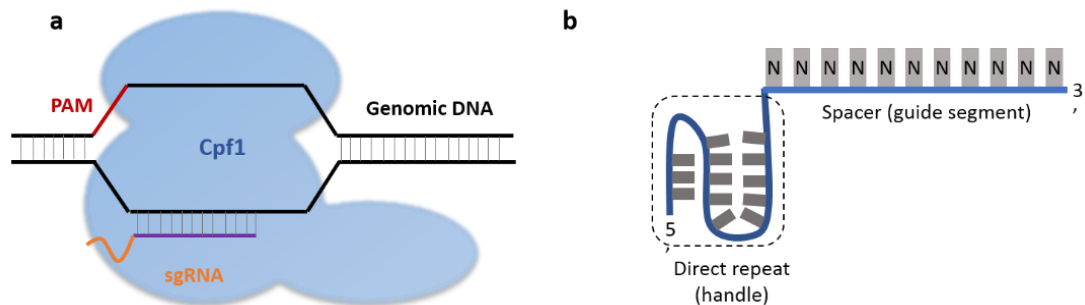
Figure 8. The CRISPR/Cpf1-Cas12a activation system. (a) Cpf1 consist of an effector nuclease (colored blue) and a single crRNA (sgRNA) located in the genomic DNA immediately downstream of the protospacer-adjacent motif (PAM, 5′-TTTN-3′). (b) Structure of the Cpf1 gRNA, composed of a direct repeat (5′ handle) and a spacer (guide segment). Source: adapted from https://dharmacon.horizondiscovery.com; Li *et al.* (2018).

The simpler structure of the CRISPR/Cpf1 allows it to encode two or more crRNAs in a multiplex single transcript (MST), which can be processed by the Cpf1 RNase activity (Zetsche *et al.*, 2017). Among the Cpf1-family proteins already evaluated, two Cpf1 orthologs, *Acidaminococcus* sp. Cpf1 (AsCpf1) and *Lachnospiraceae bacterium* Cpf1 (LbCpf1), displayed the best genome-editing activity in a number of organisms, including human cells and mice (Kim *et al.*, 2016; Zhang *et al.*, 2017; Zetsche *et al.*, 2017). Cpf1 is capable of targeting AT-rich promoter regions due to its base pairing-dependent PAM recognition (5′ T-rich PAM; Li *et al.*, 2018). Kleinstiver *et al.* (2016) demonstrated that Cpf1 is highly specific in human cells, showing no detectable off-target effects and efficiencies comparable to those of the SpCas9 nuclease. For these reasons, dead Cpf1 (dCpf1) is an attractive tool for genome regulation and expression amplification in cellular engineering.

2.3 Challenges of the gene editing technology in genetic engineering

The generation of genetically modified animals has undoubtedly become more efficient and specific since its emergence, with the first reports of GM mice in 1974 (Jaenisch and Mintz, 1974), after the microinjection of a DNA sequence into the blastocoel of mouse embryos, and in 1980 (Gordon *et al.*, 1980), by the efforts of Frank Ruddle's group, by microinjecting exogenous DNA into the pronuclei of mouse zygotes. While Jaenisch's animals incorporated the exogenous DNA into the mouse genome but failed to transmit it through the germ cells, Ruddle's mice were true transgenic, as they transmitted the transgene to their progeny. Interestingly, it was Gordon and Ruddle who coined the term "transgenic" for the first time, in 1981 (Gordon and Ruddle, 1981). For decades, the technology for transgenesis was limited, with the cutting and splicing of DNA and insertion of exogenous DNA sequences occurring at random and with concomitant high wastage of animal lives due to its lack of precision and efficiency. The technology of CRISPR system had revolutionized genetic engineering, becoming significantly quicker, cheaper, and easier to modify the genome, providing the knowledge of the genomic sequences, and therefore, it became highly accessible. The advantages of this approach have enabled a significant improvement in the process circumventing some of the previous obstacles in the success of the procedures, such as, for instance, the poor integration efficiency (on-target efficiency) and associated undesired (off-target) effects (Hsu, Lander and Zhang, 2014; Chandrasekaran, Song and Ramakrishna, 2017). The manipulation of the DNA repair machinery to promote homologous recombination (HR), and the identification of safe harbor *loci* (SHL) are strategies currently under intense focus by research groups worldwide in attempts to target genes precisely and individually into genomic sites where transgene integration can be safe for the cell and from gene silencing cellular mechanisms.

Although the efficiency in the gene manipulation processes improved over time, it still remains far from satisfactory, both scientifically and ethically. Currently, it is still difficult to accurately estimate, quantitatively, the efficiency of CRISPR, as estimates vary considerably and are affected by many factors, including the nature of the target site (function and site in the genome) and the chosen CRISPR-associated nuclease (Bailey, 2019). The knock-in efficiencies are still low and highly variable, mostly in research with embryos, but also in cell lines. Regardless of the on-target efficiency, the

off-target effects are a common concern. Unintended mutations induced by the GM process may affect other non-specific sites in the genome, causing low birth rates of animals with the desired genetic modification or may affect the animal's well-being (Fu *et al.*, 2013; Hsu *et al.*, 2013; Hsu *et al.*, 2014; Pattanayak *et al.*, 2013).

In addition to the common use for gene editing, the CRISPR system has undergone many modifications that allow not only DNA editing but also regulation of gene expression without directly modifying DNA sequences, such as gene activation, repression or even chromatin remodeling. The ability to change already altered gene expression is essential for some cell reprogramming experiments, for example, if it is necessary to reprogram somatic cells into iPSCs and then differentiate them into another cell type, which is one of the most promising tools in cell reprogramming (Shakirova, Ovchinnikova and Dashinimaev, 2020). Like other CRISPR-based systems, possible off-target effects should also be taken into account. Non-specific transcription activation/repression can cause altered non-target gene expression and, as a result, disruption of dependent gene cascades (Hsu *et al.*, 2014).

Another important technical aspect of cell reprogramming using CRISPR tools is the sgRNA expression into the cell, which depends on the promoter and may vary according to the concentration of the plasmid. Using specific promoters, it is possible to produce multiple sgRNAs from a single transcript and therefore offer complex control over cell behavior. Nevertheless, multiplexing the sgRNA to increase the scale of reprogramming or screening can result in retroactivity, when different sgRNAs compete for available Cas9 proteins, altering the overall efficiency (Zhang and Voigt, 2018). In addition, the level and duration of gene expression is also of great significance. Therefore, the ability to control the working time of the CRISPR nuclease is crucial in cell reprogramming due to its correlation with cell fate; it also prevents off-target effects caused by the prolonged activity of dCas9 (Shakirova, Ovchinnikova and Dashinimaev, 2020).

2.3.1 DNA repair pathways

An important point for the generation of GMOs is the maintenance of genomic integrity, which is essential for the survival and development of organisms. As already mentioned above, once DNA DSB occurs, two major DNA repair pathways operate in higher eukaryotes in an attempt to avoid cell death: the homologous recombination

(HR) and the nonhomologous end joining (NHEJ). Such pathways, along with the gene editing technology, became modern genetic engineering tools used to modify the livestock genome, either through the NHEJ or HR repairs pathway, depending on the purpose, as such pathways operate in distinct ways and under different circumstances.

The classical NHEJ (c-NHEJ) has the potential to ligate any kind of DSB ends without the requirement of a homologous sequence, as opposed to the HR, which leads NHEJ mechanism to be considered the most powerful and relatively simple DSB repair pathway (Pardo *et al.*, 2009). To perform the reactions necessary for the repair, the NHEJ machinery relies on many protein factors that carry structural stabilization functions, as well as DNA degradation, polymerization and ligation functions (Fig. 9). All NHEJ reactions require the core NHEJ machinery that is composed of three protein complexes (MR(X)N, the KU and the DNA ligases complexes), potentially occurring throughout the cell cycle, with a dominant effect during G0/G1 and G2 phases (Pardo *et al.*, 2009; Karanam *et al.*, 2012; Chiruvella *et al.*, 2013). The NHEJ has frequently been considered an error-prone DSB-repair pathway, since it usually causes insertions and deletions of few bases (indels), thus resulting in errors (Lieber, 2010). As error-prone, or illegitimate, such pathway is effective for strategies involving the introduction of small mutations or random indels, which is necessary, for example, for disruption of functional alleles to promote gene knock-out.

The homologous recombination (HR) is one of the main homology-directed repair (HDR) mechanisms that requires homologous DNA. The HR is a key DNA repair pathway of high fidelity necessary to maintain genomic integrity, being active during the S and G2 phases. The DNA damage is processed to form an extended region of ssDNA, which is bound by the single stranded DNA binding protein RPA. Binding of RPA eliminates secondary structures in ssDNA, which is needed for competent Rad51 filaments to assemble (Heyer, Ehmsen and Liu, 2010). The Rad51 filament performs homology search and DNA strand invasion, generating the D-loop where the invading strand primes DNA synthesis (Heyer *et al.*, 2006). D-loop extension is followed by branch migration to produce double-Holliday junctions, the resolution of which completes the repair cycle. This resolution step can be accomplished via formation of two Holiday junctions, which are subsequently resolved to give crossover or non-crossover products (Wu *et al.*, 2008, Brandsma and Gen, 2012; Fig. 9).
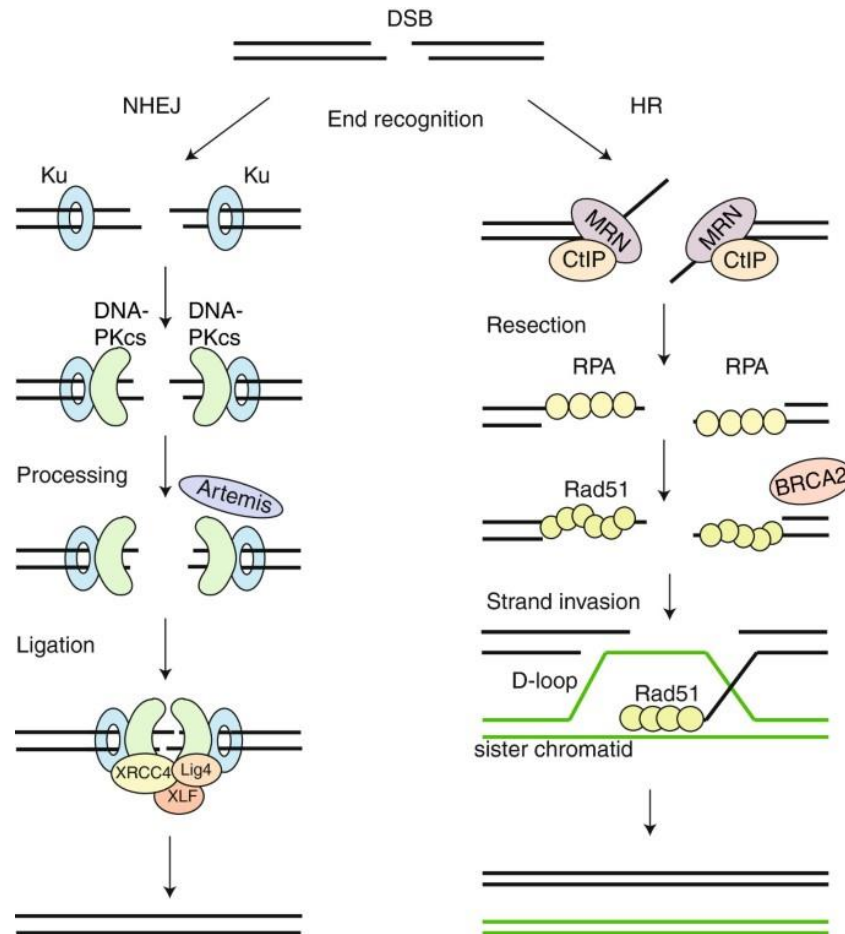
Figure 9. DNA repair pathways in eukaryotes upon a DNA double strand break (DSB). NHEJ: the nonhomologous end joining (NHEJ) pathway starts with recognition of the DNA ends by the Ku70/80 heterodimer, which recruits DNA-PKcs. If the ends are incompatible, nucleases such as Artemis can trim the ends. A DNA Ligase complex seals the break. HR: in the homologous recombination (HR) pathway, the MRN complex starts resection on the breaks to generate single stranded DNA (ssDNA). After resection, the break can no longer be repaired by NHEJ. The ssDNA is first coated by RPA, which is subsequently replaced by Rad51 with the help of BRCA2. These Rad51 nucleoprotein filaments mediate strand invasion on the homologous template. Extension of the D-loop and capture of the second end lead to repair. Source: adapted from Brandsma and Gen (2012).

The HDR mechanism allows the precise mutation of single or few nucleotides, which has been in use for the generation of animal models for human diseases. However, the NHEJ repair pathway is usually 1,000 to 10,000 more frequent than the HR in higher eukaryotes (Smith, 2001), which turns precise transgenesis process more difficult. Due to that, research groups around the world have been working on the development of new strategies to improve HDR recruitment and efficiency in many mammalian species.

2.3.2 Safe Harbor *Loci*

Traditionally, transgenic livestock animals have been produced by integrating a transgene into the genome in a random manner, as the exogenous DNA would be inserted wherever there would be a DSB. In such way, transgene integration and safety are limited by interaction between the newly integrated DNA and the host genome. An important factor for efficient transgene insertion and expression is the requirement of a genomic *locus* that leads to a safer and more efficient process, allowing gene integration and expression, without disrupting internal gene function (Sadelain, Papapetrou and Bushman, 2012; Ruan *et al.*, 2015). When randomly inserted, genes are subjected to position effects, generating unstable phenotypes and gene silencing, making their expression unreliable and unpredictable (Phelps *et al.*, 2003).

Although the question of where to introduce transgenes into the host genome to maximize safety and efficacy has not been completely elucidated, some predetermined genomic sites, known as safe harbor *loci* (SHL), appear to be an alternative to face such problems. Safe harbor *loci* are described as regions where an exogenous DNA can be targeted relatively easily by homologous recombination, supporting strong ubiquitous expression of inserted sequences while not being subjected to gene silencing (Irion *et al.,* 2007).

So far, the most targeted *locus* in mammals is the ROSA26, with extensively studies in mouse embryonic stem cells (Casola, 2010), rats (Kobayashi *et al.*, 2012) and humans (Irion *et al.,* 2007). The orthologous sequence of the mouse ROSA26 was also described in pigs (Kong *et al.*, 2014), cattle (Tan *et al.*, 2013) and goats (Tavares *et al.*, 2016). The ROSA26 *locus* is controlled by a promoter, which has a moderate strength that may in some instances result insufficient to achieve the desired levels of transgene expression (Casola, 2010). Recently, the transcriptionally active H11 *locus* has been described in mice (Tasic *et al.*, 2011), human stem cells (Zhu *et al.,* 2014) and pigs (Ruan *et al.*, 2015) as a safe *locus* that supports transgene insertion and expression, with an advantage to ROSA26, since the H11 *locus* does not contain any promoter, allowing the transgene to be expressed under its own promoter, as tissue-specific promoters (Ruan *et al.*, 2015).

2.3.3 Cellular Reprogramming

Considering the transgenic production by cloning by nuclear transfer (NT), no consensus has been reached on the influence of the cell type on transgene expression levels and post-reprogramming capacity after cloning procedures, and such factors appear to be related to the epigenetic profile of the cells. Initially, it was believed that pluripotent cells, such as embryonic stem cells (ESCs), would be more efficient for cloning due to a higher nuclear reprogramming success and proper embryo development (Prather *et al.*, 1987). The ESCs can self-renew, differentiate into all cell types, and undergo numerous cell divisions, giving rise to identical undifferentiated daughter cells. However, stem cell technology is still limited in livestock species as deriving and maintaining pluripotent cells *in vitro* are not yet fully characterized or reproducible in domestic animals (Brevini *et al.*, 2008; Kumar *et al.*, 2021). Conversely, somatic cells cannot divide indefinitely, having limiting proliferative potential (Banito and Gil, 2010). Thus, the ability to derive pluripotent cells from somatic cells by reversing the natural differentiation process that occurs during development has been long explored for cloning/transgenesis studies for applications in basic biology, drug development and regenerative medicine (Banito *et al.*, 2009).

Reprogramming somatic cells to pluripotency can be achieved by different approaches, including cloning by SCNT (Wilmut *et al.*, 1997), fusion between somatic and pluripotent cells (Ying *et al.*, 2002), and ectopic expression of specific transcription factors (TFs; Takahashi and Yamanaka, 2006). The use of genetically engineered pluripotent stem cells (i.e., embryonic stem cells, ESCs, or pluripotent stem cells, PSCs) as donor cells for cloning could simplify and improve efficiency for transgenic animal production, as already shown in mice (Zhou *et al.*, 2010). Reprogramming somatic cells into PSCs is the result of remodeling the somatic genome, epigenetically and transcriptionally, into an embryonic stem-like state, which includes the reactivation of pluripotency genes and the repression of lineage commitment genes (Maherali *et al.*, 2007; Sridharan *et al.*, 2009). Development of direct reprogramming technology offers an alternative approach for generation of pluripotent stem cells, applicable also in farm animals. Takahashi and Yamanaka (Takahashi and Yamanaka, 2006) first pinpointed key genetic factors that were able to reprogram committed cells into PSC. Such discovery allowed the development of a method to generate induced pluripotent stem cells (iPSCs) from mouse embryonic fibroblasts by induced expression of four transduced nuclear transcription factors (Takahashi and Yamanaka, 2006). Those authors first showed that mouse somatic cells could be reprogrammed to a pluripotent-

like state by expressing four transcription factors, so-called the 'Yamanaka factors' or OKSM (*Oct4, Klf4, Sox2*, and *c-Myc*). Since then, the iPSCs were established in many other animal species with different combinations of exogenous reprogramming factors, dependent on donor cell type and/or species, including human fibroblast cells, reprogramed using a combination of factors that included Nanog and Lin28, also effective to induce pluripotency (Takahashi *et al.*, 2007; Yu *et al.*, 2007; Yu *et al.*, 2009).

Reprogramming occurs as a gradual and usually inefficient process that results in only a small percentage of the cells becoming pluripotent, which indicate that TFs need to overcome a series of limiting events and epigenetic barriers to be able to operate (Takahashi and Yamanaka, 2006; Stadtfeld and Hochedlinger, 2010). Cell populations expressing OKSM pass through a sequence of distinct molecular and cellular events, where initially lineage-specific genes are gradually silenced, and embryonic markers become activated (Apostolou and Hochedlinger, 2013). Such events induce the expression of endogenous genes linked to pluripotency, acquiring a self-sustaining pluripotent state, which suggests an ordered process, accompanied by telomerase activation and telomere length extension (Takahashi *et al.*, 2007; Stadtfeld *et al.*, 2008).

In addition to the use of iPSCs to generate genetically modified animals, such elegant approach promises to further revolutionize genome reprogramming for numerous applications in medicine, agriculture, and biotechnology. As iPSCs can differentiate themselves into all cell types of an organism, such cells provide a powerful platform to study development, tissue regeneration, disease mechanisms, and gene therapeutic approaches (Colman and Dreesen, 2009; Rosselló *et al.*, 2009; Hwang *et al.*, 2011). In livestock species, iPSC have already been produced in the pig (Ezashi *et al.*, 2009; West *et al.*, 2010), horse (Nagy *et al.*, 2011; Breton *et al.*, 2013), cattle (Han *et al.*, 2011; Talluri *et al.*, 2015), sheep (Liu *et al.*, 2012; Sartori *et al.*, 2012) and goat (Song *et al.*, 2013; Sandmaier *et al.*, 2015). However, the pig has been the most intensively studied farm animal in genetic engineering, due to its organ size and physiology that best resembles the human organism, thus becoming a valuable model for testing new therapeutic approaches (Cibelli *et al.*, 2013). In that regard, the use of the CRISPRa system has emerged as a novel tool to direct cell reprogramming, promising to revolutionize cell biology and the applications into cell and gene therapy (Chavez *et al.*, 2015; Didovyk *et al.*, 2016; Weltner, *et al.*, 2018).

2.3.3.1 Cellular Senescence

The initial barrier for the success of reprogramming is the stress response triggered by the senescence pathway, that are related to DNA damage, oxidative stress and telomere loss, induced by replicative exhaustion in culture and also for the reprogramming process itself (Banito *et al.*, 2009; Fernández and Mallette, 2016). Senescence is the irreversible arrest during the G1 transition of the cell cycle that up-regulates genes into the apoptotic pathway, such as p53 and the cyclin-dependent kinase (CDK) inhibitors p16 and p21. Therefore, the higher the expression levels of such genes, the more difficult it is to reprogram the cells (Banito *et al.*, 2009). Several studies showed that knocking down p53 in human or mouse cells can significantly increase the efficiency of reprogramming (Zhao *et al.*, 2008; Banito *et al.*, 2009; Kawamura *et al.*, 2009). In mammary epithelial cells, suppression of p53 function induces cellular immortality, probably through the reactivation of telomerase (Kanaya *et al.*, 2000). The overlap between indirect telomerase regulation pathways and cell cycle checkpoint pathways, suggests that these genetic elements (p21, p53, and TERT) are also implicated in the process of senescence, caused in eukaryotic cell lines by telomere shortening (Lai *et al.*, 2005).

2.4 Applications

The gene editing technology for use in genetic engineering has brought numerous possibilities for DNA modifications aiming at the production of GMs and transgenic animals with different traits of interest, in a much faster way than the traditional process of crossbreeding in animal breeding programs. Most studies have been carried out on mice, but with the advances in this area, farm animals, such as sheep, cattle, pigs and goats, can be used for many purposes. The main applications of transgenic farm animals are to improve the performance of the animal itself, such as the generation of pigs with resistance to reproductive and respiratory syndrome (PRRS; Whitworth *et al.*, 2016); for the production of biopharmaceuticals or products, such as the production of milk without the presence of beta-lactoglobulin (BLG), a powerful known allergen in humans (Yu *et al.*, 2011); for xenotransplantation; and also in the generation of models that mimic human physiology for studies of diseases, such as cardiovascular problems with the production of knockout animals for the PPARγ

(peroxisome proliferator-activated Receptor Gamma) and LDL (low density lipoprotein) genes (Yang *et al.*, 2011; Carlson *et al.*, 2012). Gene editing, especially through the use of the CRISPR systems, to excise the DNA for any sort of purpose, or to activate genes through the CRISPRa system, is allowing a quick progress and spectacular advances in biology and in the way we manipulate the genome and the epigenome.

3. HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

a) The CRISPR activation (CRISPRa) system is a simple and effective tool to activate pluripotency genes to partially reprogram porcine somatic cells and overcome cell senescence.

b) The cytoplasmic microinjection into bovine IVP zygotes of the CRISPR/Cas9 system and donor repair templates targeted to bovine safe harbor *loci* (SHL) is innocuous to *in vitro* embryo development.

3.2 General Objective

a) To evaluate the strategies using the CRISPR system to promote partial cellular reprogramming in porcine cells in culture and to evaluate the effect of the CRISPR system when microinjected to promote homologous recombination on survival and development of bovine IVP embryos.

3.3 Specific Objectives

a) To evaluate the ability of the CRISPR activation approach to promote the upregulation of reprogramming genes in porcine somatic cells in culture;

b) To evaluate the ability of the CRISPR activation approach to promote the regression of cell senescence in porcine somatic cells in culture;

c) To evaluate the efficiency of nucleases dCpf1-VPR and dCas9-VPR to promote gene transcriptional activation in porcine somatic cells in culture;

d) To evaluate the effect of cytoplasmic microinjection of CRISPR/Cas9 system on survival and *in vitro* development of bovine IVP embryos;

e) To evaluate the effect of cytoplasmic microinjection of donor repair oligonucleotide templates under different concentrations on survival and *in vitro* development of bovine IVP embryos; and

f) To evaluate the effect of directing homologous recombination by CRISPR/Cas9 system into the ROSA26 and the H11 safe harbor *loci* on survival and *in vitro* development of bovine IVP embryos.

CHAPTER II: Efficiency of CRISPRa system to partially reprogram porcine fibroblast cells in culture

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Efficiency of CRISPRa system to partially reprogram porcine fibroblast cells in culture

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Abstract

Modified versions of the CRISPR system have been engineered to generate catalytically inactive nucleases, which can be fused to transcriptional activation domains to control gene expression. The CRISPR activation system serves as a tool for cellular reprogramming at the transcriptional level, leading to a transgene independent reprogrammed cell with transient expression of exogenous reprogramming factors. However, a lack of information exists regarding the use of the CRISPR activation system to induce expression of inactive reprogramming genes in porcine cells. In this study, we aimed to evaluate the efficiency of two nucleases fused to activation domains (dCas9-VPR and dCpf1-VPR) in enabling the transient upregulation of reprogramming target genes (*Oct4, Myc, Klf4, Sox2* and *Lin28a*), and the ability to alter transcription of downstream genes related to reprogramming of porcine somatic cells at advanced passages. When comparing nucleases, the dCas9-VPR more effectively upregulated single genes (overall fold change mean of 3.04) than dCpf1-VPR (overall fold change mean of 1.72), also using lower number of guide RNAs per gene, with highest results for *Myc* (fold change of 3.06) and *Lin28a* (fold change of 9.4). On the other hand, dCas9-VPR failed to upregulate multiple genes concomitantly, although we could observe downstream effects of the target genes in the expression of *p53* (fold change of 0.38) and *Dkc1* (fold change of 1.4). We suggest that the CRISPR activation system can promote partial cell reprogramming in pigs, first by expressing *Myc* and *Lin28a*, leading to transcriptionally activation of related genes, as *Dkc1*, and downregulation of *p53*, as a downstream effect. In addition to the efficiency of dCas9 for single gene activation, the advantages of multiplexing gRNAs with Cpf1 for multiple gene activation could be used in future studies to overcome the limitations of cellular senescence.

Keywords: Reprogramming; CRISPR activation system; Porcine; Somatic cells.

Introduction

The clustered regularly interspaced short palindromic repeats (CRISPR)-based system is a rather novel method for genome modification, containing an RNA sequence guide (gRNA) to specifically pair with the target DNA sequence, and a nuclease to cleave the DNA, creating double strand breaks (DSB) in specific sites of the genome (Tu *et al.*, 2015). The most common nuclease used in the CRISPR system is Cas9, and many studies have been made to increase its performance or to use it for other purposes. Modified versions of the Cas9 protein have been engineered by mutating two key amino acid residues within its nuclease domains, generating a deactivated Cas9 (or dead Cas9, dCas9), an RNA-programmable DNA-binding protein that lacks endonucleolytic activity, while retaining the capacity to interact with DNA (Gasiunas et al., 2012, Didovyk et al., 2016). Catalytically inactive Cas9 proteins fused to transcriptional activation domains (Ads) can be used to control gene expression (also known as CRISPR activation system, or CRISPRa). The CRISPRa enables Cas9 to serve as a tool for cellular reprogramming at the transcriptional level, leading to a fully transgene independent reprogrammed cell without persistent expression of exogenous reprogramming factors (Cheng *et al.*, 2013, Gilbert *et al.*, 2013, Maeder *et al.*, 2013, Chavez *et al.*, 2015). Several effector candidates with known transcriptional roles have been tested in mammalian cells, and three different Ads (VP64, p65, and Rta) showed the most meaningful induction when fused to form a so-called VPR activator (Chavez *et al.*, 2015, Didovyk *et al.*, 2016). Recently, new nucleases with better performances have been discovered to improve CRISPR procedures and to overcome limitations of the CRISPR-Cas9 system (Kim *et al.*, 2016). The Cpf1 is a new discovered smaller endonuclease, similar to Cas9 that also cleaves double-stranded DNA at the recognition site. However, it only requires a shorter (43 nucleotides) and simpler CRISPR RNA guide (crRNA or gRNA) that consists of a 5′-handle (20 nucleotides) and a guide segment (23 nucleotides; Li *et al.*, 2018). The simpler structure of CRISPR-Cpf1 allows it to encode two or more gRNAs in a multiplex single transcript (MST), which can be processed by the Cpf1 RNase activity, enabling the use of multiple gRNAs at the same time (Zetsche *et al.*, 2017). As dCas9, the deactivated form of Cpf1 (dCpf1) was also successfully used to control gene expression using VPR activation domains in mammalian cells (Tak *et al.*, 2017).

The CRISPRa system can be used as a new tool to reprogram somatic cells into pluripotent stem cells (PSCs) by a "non-viral" and "non-integrative" technique, with

a potential for application in several research fields. As induced PSCs (iPSCs) can potentially differentiate themselves into all cell types of an organism, such cells provide a powerful platform to study development, tissue regeneration, disease mechanisms, gene therapeutic approaches (Colman and Dreesen, 2009, Rosselló *et al.*, 2009, Hwang *et al.*, 2011). The iPSCs are also capable of generating cloned offspring through somatic cell nuclear transfer, as already shown in the production of transgenic mice (Zhou *et al.*, 2010). Several studies have reported reprogramming of mice and human cells using CRISPRa (Balboa *et al.*, 2015, Liu *et al.*, 2018, Weltner *et al.*, 2018, Yang *et al.*, 2019). However, a lack of information exists regarding the efficiency of CRISPRa in pig cells. The pig has been the most intensively studied farm animal in genetic engineering due to its organ size and physiology that best resembles the human organism, thus becoming a valuable model for testing new therapeutic approaches (Cibelli *et al.*, 2013). Thus, in the present study, we aimed to evaluate and compare the efficiency of the transcriptional activation approach between dCpf1-VPR and dCas9-VPR in enabling transient upregulation of reprogramming genes separately and in combination in porcine somatic cells in culture, and also the ability of CRISPRa approach to overcome limitations of cell reprogramming and promoting regression of cell senescence by analyzing expression of specific senescence genes in porcine somatic cells at advanced passages.

Materials and methods

Cell culture

Pig fetal fibroblast cells derived from fetuses aseptically collected at a local slaughterhouse were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, USA) containing 10% fetal bovine serum (FBS; Life Technologies), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Life Technologies). Medium was changed every other day and all cells were kept in an incubator at 37°C under 5% CO_2 and saturated humidity. Cells were cultured until passage number five to be used for the first experiment comparing dCas9 and dCpf1. Another group of cells were cultured until passage 22 for the second experiment involving the induction of cell reprogramming with dCas9 at late passages.

Guide RNA design and production

The guide RNAs (gRNAs) for the porcine target genes *Oct4, Myc, Klf4, Sox2* and *Lin28a* (OMKSL) were designed based on Weltner *et al.* (Weltner *et al.*, 2018). Guide RNAs were designed using Benchling (https://benchling.com/), targeting the

proximal promoters (-1 kb to +600 bp from transcription start site) of the targets OMKSL, and the control gRNAs were designed using a GenScript tool (https://www.genscript.com/tools/create-scrambled-sequence) to design scrambled sequences. Candidate gRNAs for each of the enzymes (Cas9 or Cpf1) were selected according to their off-target score and position and assembled as previously described (Fu *et al.*, 2013). Briefly, single guide RNA oligonucleotide duplexes, corresponding to space sequences with specific overhangs, were annealed and ligated into *BsmB*I-digested MLM3636 (SpCas9) or BPK3082 (LbCpf1) plasmids (a gift from Dr. K. Joung, Addgene numbers 43860 and 78742, respectively; http://www.jounglab.org), containing U6 promoter and gRNA scaffolds. Plasmids were cloned in competent *E. coli* under standard protocols for subsequent extraction and purification (Zyppy Plasmid Miniprep kit, Zymo Research, USA). Plasmids were digested with *BsmB*I and *Sal*I to confirm the insertion of the gRNAs, followed by electrophoresis in 1% agarose gel. Plasmids with correct band sizes were sequenced to confirm the insertion of the gRNA. Lists of guide RNA oligonucleotides for SpCas9 and LbCpf1 are provided in Supplemental Tables 1 and 2.

CRISPRa Vectors

Plasmids used to transfect dSpCas9 (*Streptococcus pyogenes*; SP-dCas9-VPR, a gift from G. Church, Addgene number 63798) and dLbCpf1 (*Lachnospiraceae bacterium*; JG1211, a gift from K. Joung, Addgene number 104567) contained VP64-p65-Rta (VPR) fused to C-terminus of the respective enzymes.

Cell transfection

Around 500.000 pig fetal fibroblast cells were transfected with a total amount of 1 µg DNA per transfection using Amaxa Nucleofector (Lonza, USA). In Experiment 1, treatment cells at passage 5 were transfected with a total of 400 ng of either dCas9-VPR or dCpf1-VPR, 200 ng pmaxGFP (GFP plasmid) and 400 ng gRNAs plasmids, with 3 to 5 gRNAs from each gene (*Oct4, Myc, Klf4, Sox2* or *Lin28a*; OMKSL) per transfection. In Experiment 2, treatment cells at passage 22 were transfected with a total of 400 ng dCas9-VPR, 40 ng pmaxGFP and 560 ng gRNAs plasmids (17 plasmids in total) for multiple target genes at the same transfection. Control cells were transfected with the same conditions of each experiment, but with the use of 200 ng of scrambled gRNAs per transfection. Afterwards, transfected cells in Experiment 1 were plated on 6-well tissue culture plates in culture medium and cultured for 2 days post-transfection. In Experiment 2, transfected cells were plated in a 6-well plate at different

cell concentrations in each well to obtain distinct patterns of cell growth rate, to allow the gene expression analyses on Days 2 and 17 of cell culture, under the same conditions as described above. Then, cells were collected for quantitative reverse transcription PCR (qRT-PCR) analyses for the OMKSL genes in both experiments, and for p21, p53, TERT and DKC1 genes in Experiment 2, as below.

Quantitative reverse transcription PCR

Total RNA was extracted from cells using the QuinckRNA Microprep kit (Zymo Research, USA). RNA quality and concentration were measured by spectrophotometry using Nanodrop ONE (Thermo Fisher Scientific, USA). One microgram of total RNA was used for cDNA synthesis using the AffinityScript Multiple Temperature cDNA Synthesis kit (Agilent Technologies, USA). For qRT-PCR reactions, a total of 100 ng of retrotranscribed RNA was amplified with 5 μL of forward and reverse primer mix at 10 μM each, using the iTaq Universal SYBR Green Supermix (Bio-Rad, USA) for a final volume of 10 μL. Samples were placed in a 96-well plate that was subsequently sealed, and the PCR was run in the Thermocycler qTOWER^{3}G (Analytik Jena AG, USA). The PCR cycles consisted of denaturation step of 30 s at 95°C, followed by 40 cycles of 15 s at 95°C, 64°C for 60 s, and the melting curve step. Relative quantification of gene expression was analyzed using the $^{\Delta\Delta}$Ct method, with β-actin as endogenous control, with the expression levels relative to control cells. A list of primers used is provided in the Supplemental Table 3.

Statistical analysis

The two experiments were run in duplicates in two independent replicates. The statistical analysis for comparison of gene expression data between treatment and control groups, on both experiments, was performed using one-tailed Student's test for $P<0.05$, and between treatment groups in Experiment 1 (dCas9-VPR and dCpf1-VPR) was performed using two-tailed Student's test, for $P<0.10$. Simple linear correlation and regression analyses were done between the gene expression data and the proportion of positively fluorescent cells (GFP+) 24 h after co-transfection, for both experiments.

Results

The sequences and position of the oligonucleotides from the Weltner *et al.* (Weltner *et al.*, 2018) study were compared within the pig genome (*Sus scrofa domesticus*, Assembly Sscrofa11.1) and were designed according to the possible regulatory region of each gene, as shown in Fig. 1. Different numbers of gRNAs were

transfected depending on the nuclease (dCas9 or dCpf1) to improve gene activation, according to previous tests performed by our group (unpublished data).
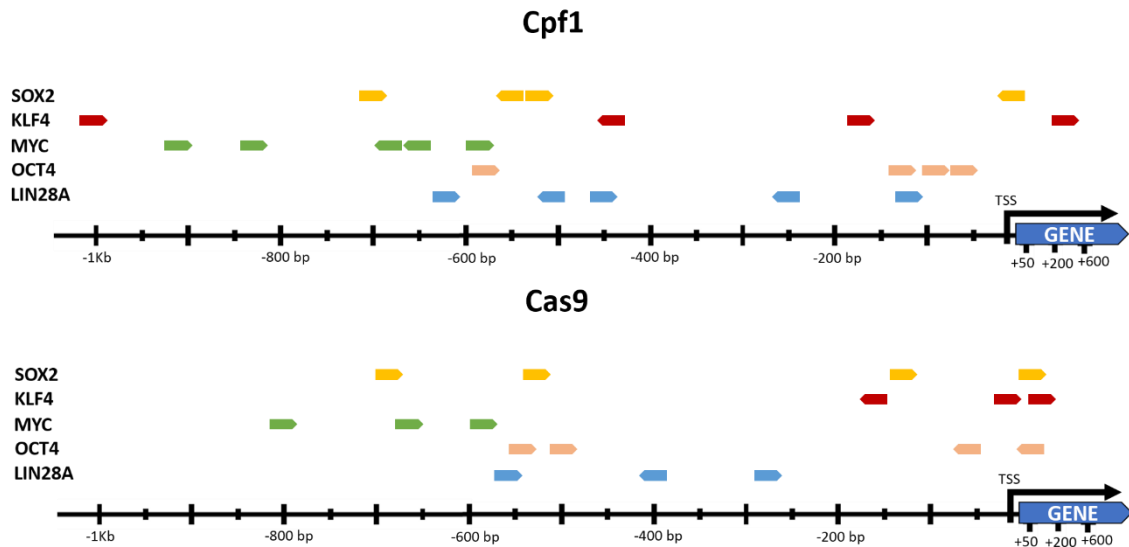
Figure 1. Guide RNA designs for Cpf1 and Cas9 enzymes and distances from the transcription start site (TSS) region of each porcine target gene *Oct4, Myc, Klf4, Sox2* and *Lin28a* (OMKSL).

Experiment 1

Fetal fibroblast cells at passage 5 were transfected to compare the effect of treatment with dCas9-VPR and dCpf1-VPR to induce expression of the target genes, related to pluripotency. Both enzymes were chosen based on the literature, with the Cas9 being a known enzyme commonly used for most CRISPRs experiments thus far, with favorable results even for transcription activation (Cheng *et al.*, 2013, Gilbert *et al.*, 2013, Maeder *et al.*, 2013, Chavez *et al.*, 2015), whereas the Cpf1 enzyme is known as a high fidelity enzyme with low off-target rates, being also easier to use with multiplex gRNAs (Zetsche *et al.*, 2017). The GFP plasmid was co-transfected with the gRNAs and each of the enzymes to evaluate the efficiency of the procedure and transfection rates. According with the analysis of GFP signal on each transfection, we observed that *Lin28a* had the higher transfection rates in both treatments (87% in the dCas9 group and 70% in the dCpf1 group), following by *Myc* (73% in the dCas9 group and 60% in the dCpf1 group) and *Sox2* (65% in the dCas9 and the dCpf1 groups). The *Oct4* had the lowest values in both treatments (30% in the dCas9 group and 35% in the dCpf1 group), following by *Klf4* (55% in the dCas9 group and 40% in the dCpf1 group), as presented in Supplemental Table 4.

Differences were observed regarding the expression of target genes between control and treatment in both groups (Fig. 2). The *Oct4* and *Myc* were significantly upregulated in the dCas9-VPR treatment group ($P < 0.05$), with *Lin28a* ($P = 0.08$) having a trend to be upregulated (Suppl. Table 5). On the other hand, *Myc* ($P = 0.1$) and *Klf4* ($P = 0.06$) showed only a tendency to be upregulated when transfected with dCpf1-VPR (Suppl. Table 5). Interestingly, after comparing differences between dCas9-VPR and dCpf1-VPR regarding the expression pattern of target genes, *Oct4* and *Klf4* showed opposite gene expression regulation depending on the treatment. While *Oct4* was upregulated in the dCas9-VPR group, the same gene in the dCpf1-VPR group was downregulated ($P < 0.05$), whereas *Klf4* showed a tendency for upregulation ($P = 0.06$) with dCpf1-VPR, and downregulation with dCas9-VPR ($P < 0.05$; Suppl. Table 5).
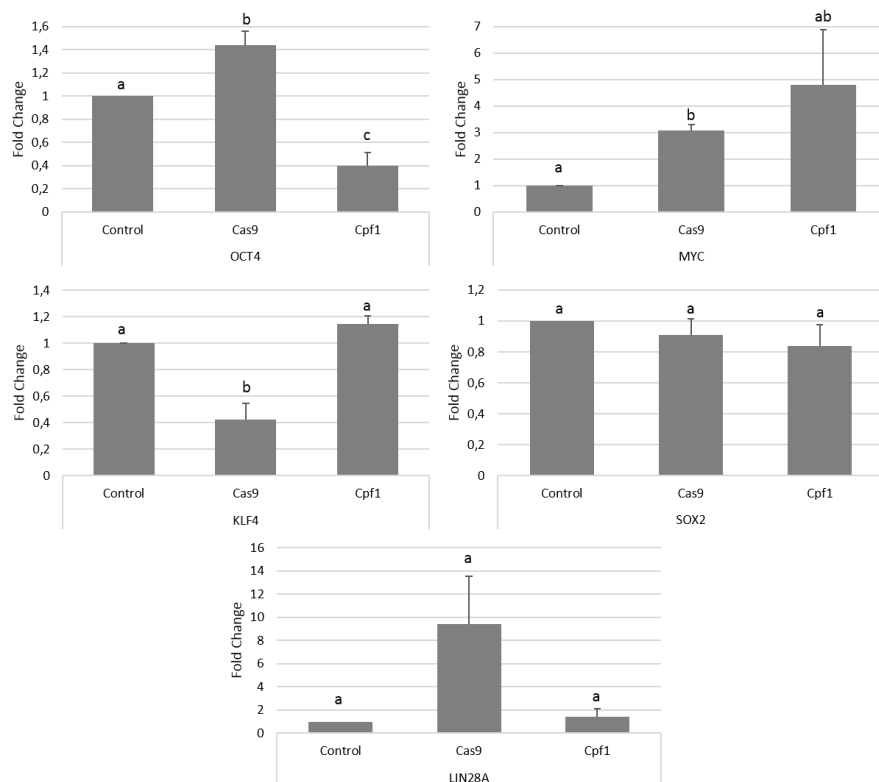
Figure 2. Relative expression pattern of target genes *Oct4, Myc, Klf4, Sox2* and *Lin28a* (OMKSL) in Experiment 1, shown as the fold change ($2^{-\Delta\Delta Ct}$) differences between treatment cells transfected with either the dCas9-VPR (Cas9) or the dCpf1-VPR (Cpf1) enzymes, and control cells transfected with control gRNAs, as reference. Data represent mean ± standard error of the mean (SEM) from two independent replicates. [a,b,c] $P < 0.05$.

The other genes had the same pattern of expression in both treatments when analyzed separately, but when all the genes were analyzed together for each

treatment, we could compare them with controls and identified that the overall gene expression in dCas9-VPR group were higher (fold change of 3.04, P = 0.06) in comparison with dCpf1-VPR group (fold change of 1.72, P = 0.129, Fig. 3).
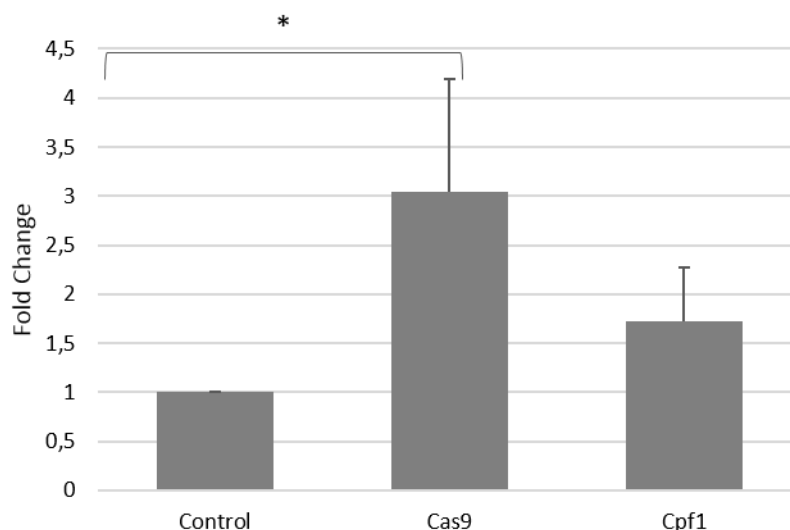
Figure 3. Target genes overall fold changes values for dCas9-VPR (Cas9) and dCpf1-VPR (Cpf1) treatment groups compared with control group in Experiment 1. Data represent mean ± standard error of the mean (SEM). *: P < 0.1.

As the expression of *Oct4, Myc, Klf4* and *Lin28a* genes showed to be activated after treatments, but with high sample variation, we evaluated whether the qPCR analysis could have been influenced by the transfection rates. A correlation analysis was performed with the log2 Fold Change and the transfection rate (GFP+) for the genes upregulated or with a tendency for upregulation in both treatments (Cas9 or Cpf1; Suppl. Table 4). A positive correlation was observed (r = 0.838, P = 0.002) between gene expression levels and transfection rate (proportion of GFP+ cells), with the regression analysis determining a dependence of the expression pattern on the transfection rate (adjusted R^2 of 0.665, Y = 0.045X - 1.15).

Experiment 2

Statistical differences in gene expression were observed between dCas9-VPR and dCpf1-VPR for *Oct4* and *Klf4* genes, but we had higher overall fold change values using the dCas9-VPR treatment than the dCpf1-VPR in Experiment 1 (Fig. 3). Such was the basis for the use of dCas9-VPR in Experiment 2, which aimed the activation of the expression of all OMKSL genes at the same time, to attempt to induce partial cell reprogramming. For that, porcine fetal fibroblasts at late passages were used to

evaluate the efficiency of the procedure and the transfection rate, which was 30% after our analysis of the GFP signal. To evaluate the effect of dCas9-VPR gene activation over time, transfected cells were cultured until Days 2 and 17.

　　　　After 2 or 17 days of cell culture, the transfected fibroblast cells were collected for RNA extraction to perform RT-qPCR. The experiment was run in two independent runs, in duplicates, but after Day 2, one of the cell culture duplicates did not grow sufficiently to render samples for the RT-qPCR analyses. Thus, the qPCR analyses on Day 17 were performed with only one transfection round duplicate (Figs. 4 and 5).
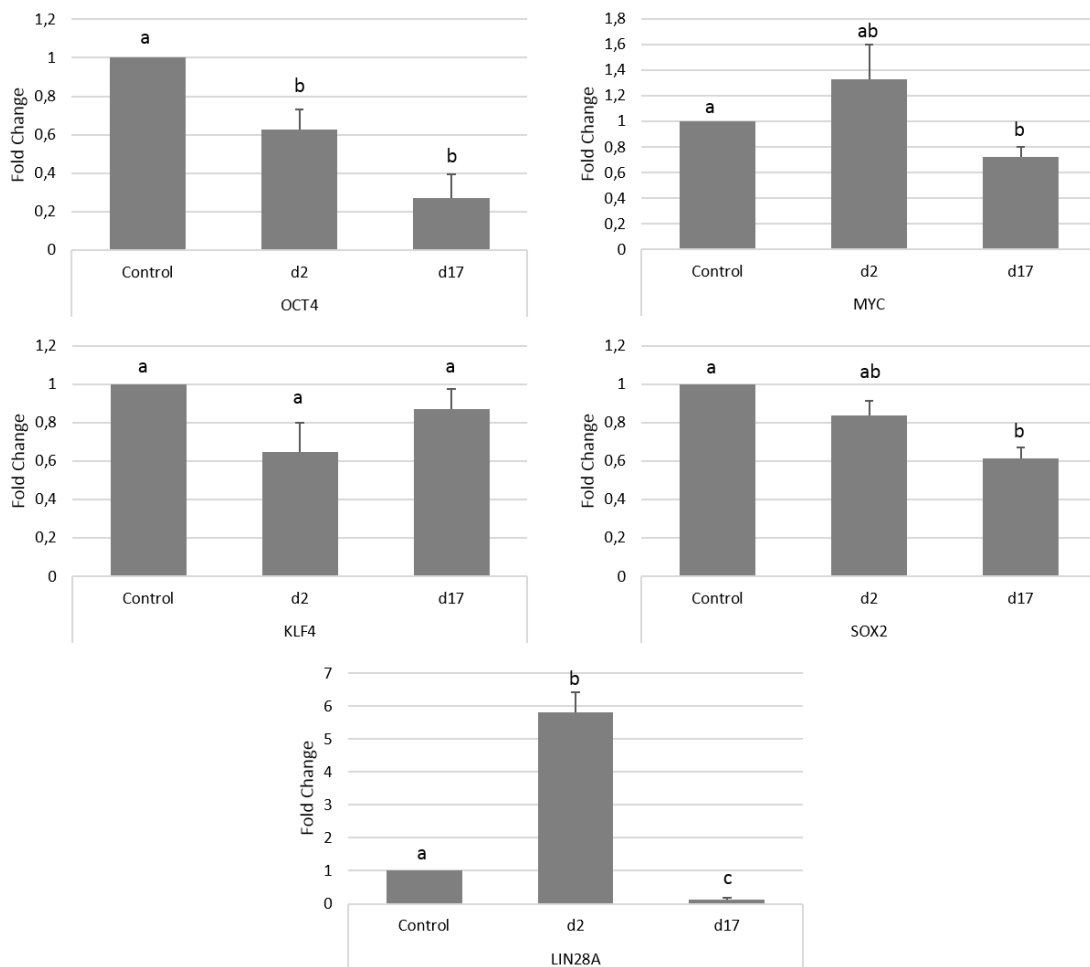
Figure 4. Relative expression of OMKSL target genes (mean ± SEM) in Experiment 2, shown as the fold change ($2^{-\Delta\Delta Ct}$) differences between treated cells co-transfected with the dCas9-VPR enzyme and gRNAs for all target genes, and control cells transfected with control gRNAs, as reference. Cells were analyzed on Days 2 (d2) and 17 (d17). [a,b] $P < 0.05$.
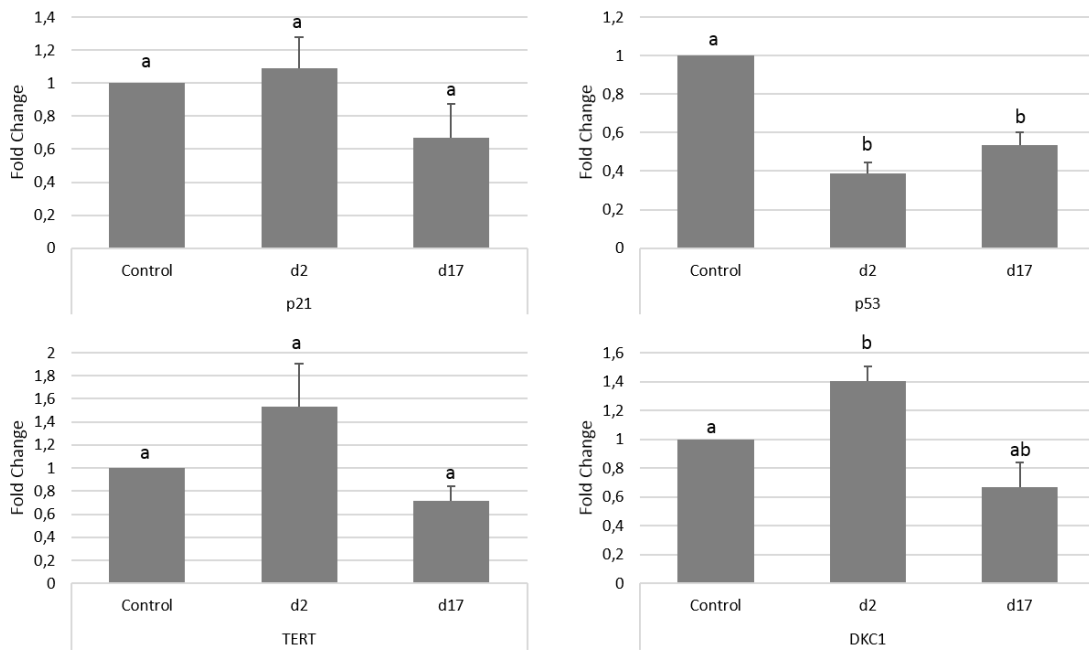
Figure 5. Relative expression of reprogramming-related genes (mean ± SEM) in Experiment 2, shown as the fold change ($2^{-\Delta\Delta Ct}$) differences between treated cells co-transfected with dCas9-VPR enzyme and gRNAs for all target genes, and control cells transfected with control gRNAs, as reference. Cells were analyzed on Days 2 (d2) and 17 (d17). [a,b:] $P < 0.05$.

Significant differences ($P < 0.05$) were observed in the dCas9-VPR treatment group compared with the control, as an upregulation of *Lin28a* and *Dkc1* and a downregulation of *p53* were observed (Figs. 4 and 5). As expected, better results for gene activation occurred mostly on Day 2, following a downregulation in almost all genes on Day 17. As in Experiment 1, the transfection rate in this experiment was also rather low (30%), which could have influenced the expression level measured by qPCR, according with the regression analysis made on Experiment 1.

Discussion

The ability to derive pluripotent cells from somatic cells (induced pluripotent somatic cells, or iPSCs) by reversing the natural differentiation process that occurs during development has been long explored (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007; Yu *et al.*, 2007). Genome-integrating methods using viral transduction (mostly lentiviruses and retroviruses) remain gold standard in iPSC generation, enabling cells to overcome the senescence barrier, but novel methods, the so-called "non-integrating techniques", are being extensively developed and evaluated (González *et al.*, 2011, Schlaeger *et al.*, 2015). The modified version of the CRISPR system, named CRISPR activation (CRISPRa), allows the use of non-viral vectors for

many purposes, including reprogramming cells (González *et al.*, 2011, Gilbert *et al.*, 2013, Chavez *et al.*, 2015, Konermann *et al.*, 2015, Schlaeger *et al.*, 2015). However, no study has been made testing the performance of such technology to transcriptionally activate reprogram-related genes in porcine cells, aiming to evaluate the best experimental conditions. Due to the lack of information in this field, we aimed to test and compare the efficiency of gene activation from dCas9-VPR and dCpf1-VPR and finally to test the performance of CRISPRa to alter transcription of downstream genes related to reprogramming of porcine cells in culture.

When comparing the efficiency of transcription activation between dCas9-VPR and dCpf1-VPR in the first experiment, we found that dCas9 was able to upregulate *Oct4* and *Myc* and had better overall results with higher fold change values compared with dCpf1 (dCas9 fold change mean of 3.04, dCpf1 fold change mean of 1.72; Fig. 3). The best performance observed with dCas9 was also related with the lower number of gRNAs (Fig. 1) that was necessary to obtain a higher fold change increase compared with the control group. With dCas9, only three gRNAs were needed to upregulate *Myc*, while five gRNAs were needed when using dCpf1 to observe a trend for upregulation. It was interesting to note that for *Oct4* and *Klf4* the effect of dCas9 and dCpf1 in gene regulation was the opposite (Fig. 2), and depending on treatment, the genes were downregulated. The unexpected event of gene repression using CRISPRa was also reported by other authors, who showed a potential of gRNAs to modulate gene expression depending on the position related to the promoter sequence (Farzadfard *et al.*, 2013, Deaner *et al.*, 2017). Those authors observed that when using dCas9-VPR with gRNAs targeting sites in close proximity with TATA box and TSS, the targeted genes were downregulated, likely due to interference of elements of such system with the transcriptional initiation complex (Kuras and Struhl, 1999, Deaner *et al.*, 2017, Jensen, 2018). Indeed, in our experiment, the gRNAs used with dCpf1-VPR to target *Oct4* and dCas9-VPR for *Klf4* were closer to the TSS site, which could have promoted the observed downregulation (Suppl. Table 1 and 2).

We also noticed a higher variation in gene expression between samples, which likely influenced the qPCR results by the distinct proportion of transfected cells per treatment. In such case, even if a particular gene was overexpressed after exposure to CRISPR activators, the gene expression pattern of the non-transfected cells likely masked the real results by lowering the overall gene expression. In spite of that, we still had statistical upregulation of genes using dCas9 in Experiment 1, as described

above, but only a trend of upregulation for *Myc* and *Klf4*, using dCpf1 and for *Lin28a* with dCas9, which could be confirmed statistically at higher transfection rates, also reducing the expression variability observed between samples. This inference is based on a closer analysis of the higher fold difference values between treatment and control groups observed for the *Myc* and *Lin28a* genes in dCas9-VPR group (Fold Change > 3; Fig. 2), which had higher co-transfection efficiency (GFP+ > 60%; Suppl. Table 4), confirmed by the correlation and regression analyses.

The fact that *Myc* was upregulated with high fold-change using dCas9-VPR shows the potential of this system to promote reprogramming in porcine cells, since *Myc* is a central hub gene involved in multiple mechanisms for maintenance of pluripotency, exerting its function to induce reprogramming since early stages of this process (Mikkelsen *et al.*, 2008, Sridharan *et al.*, 2009, Fagnocchi and Zippo, 2017). *Myc* binding has been associated with activation of its target genes and interaction with transcriptional co-activators, accomplished through the recruitment of chromatin modifying factors (such as histone acetyltransferases), mediating early global epigenetic changes (Cole and Nikiforov, 2006, Zippo *et al.*, 2007, Zippo *et al.*, 2009). One of the mechanisms of *Myc* to support pluripotency is by limiting the expression of microRNAs from let-7 family, which promotes cell differentiation (Fagnocchi and Zippo, 2017). The inhibition occurs through *Myc* directly binding the microRNAs or by transcriptionally inducing its target LIN28A, that is a known let-7 repressor (Chang *et al.*, 2008, Chang *et al.*, 2009, Dangi-Garimella *et al.*, 2009, Melton *et al.*, 2010, Zhong *et al.*, 2010, Fagnocchi and Zippo, 2017). Furthermore, in other studies, the overexpression of *Lin28a* combining with the Yamanaka factors (OMKS) was able to promote the reprogramming of human fibroblast cells into self-renewing iPSCs, suggesting that *Lin28a* is critical to pluripotent stem cell self-renewal (Hanna *et al.*, 2009, Shyh-Chang and Daley, 2013). In the first experiment, the higher fold value observed for *Lin28a* (Fold Change = 9.4; Fig. 2) could also have been influenced by *Myc* upregulation, and since both are important to maintain cell pluripotency, the dCas9-VPR system could be advantageous to induce reprogramming in porcine fibroblast cells.

In Experiment 2, we aimed to test whether the dCas9-VPR system could promote multiple gene activations, and perhaps, induce downstream molecular events leading to partial cell reprogramming, since we obtained favorable results after single gene activations in Experiment 1. For that, a combination of various plasmids was

transfected into cells, which could be related to the low transfection rate (Suppl. Table 4). In addition, low moiety amounts of each gRNA plasmid was transfected for all genes, as a higher concentration, as performed in Experiment 1, could be detrimental to cells. Consequently, the OMKSL gene expression levels were anticipated to be lower than in Experiment 1, as observed on Days 2 and 17 (Fig. 4). The interference of a combination of a large number of gRNAs was already described by Kurata *et al.* (Kurata *et al.*, 2018) to lower the efficiency of CRISPR system using Cas9 in HEK293T cells. Interestingly, and in spite of that, significant expression effects ($P < 0.05$) were observed for the target gene *LIN28A* and for *DKC1* and *p53*, reprogramming-related genes (Figs. 4 and 5).

The *p53-p21* gene pathway is important to trigger the expression of a network of downstream targets, leading to activation of several cellular responses that can suppress proliferation, promote differentiation, cell cycle arrest and the shortening of telomers, leading to cell senescence (Lin *et al.*, 2012). Such downstream events occur in part, by the effects of *p53* binding to target genes, as *Tert* and *p21*. In cells at late passages that already initiated a process of senescence, the expression of *p53* has been shown to be usually high, leading to the transactivation of *p21*, contributing to cellular senescence (Kanaya *et al.*, 2000, Lai *et al.*, 2005). In our study, a *p53* downregulation was observed in treated cell, but no significant differences were detected between the control and the treatment groups for neither *p21* nor *Tert*. The low *p53* expression levels in treated cells could be a consequence of an initial mild cell reprogramming response, which in fact would be the first target of the process, failing to trigger a *p21* downregulation later on, which could explain the fact that *p21* was not different from control on Day 2 (Fig. 5).

Reprogrammed cells have a reactivation of the telomerase activity, as an initial reprogramming event, by upregulation of telomerase-related genes to lengthen the telomers (Agarwal *et al.*, 2010). Although the *Tert* mean mRNA expression level was higher, no statistically significance was detected (Fig. 5). However, the *Dkc1*, another telomerase associated protein, responsible for the assembling and stabilization of telomerase (Ly, 2011, Marrone and Mason, 2003), was upregulated in treated cells on Day 2 (Suppl. Table 6). Other studies observed a correlation between *Tert* and *Dkc1* mRNA expression in iPSC derived from porcine and in human tumor cells (Ji *et al.*, 2013, Çalışkan Can *et al.*, 2017). We infer that the conditions of our experiment could have influenced and masked the expression results, since the fold change value for

Tert was higher in treat cells than in controls, being similar to values observed for the *Dkc1* gene. The same occurred with *Myc*, that had higher fold change values in treated cells, albeit not statistically upregulated, differently to what was observed in Experiment 1 when dCas9-VPR was used to induce single gene activation. It is important to mention that *Myc* is one of the genes described to promote reactivation of telomerase activity, promoting epigenetic modulations in the chromatin to induce the transcription of *Tert* and associated genes (Wu *et al*., 1999, Patel *et al.,* 2016), becoming an important gene to overexpress when the goal is to attain cellular reprogramming.

Conclusion

Generally, the CRISPR activation system used in this study was efficient to significantly induce the overexpression of single target genes in porcine fibroblast cells but failed to effectively activate multiple genes concomitantly under the conditions of our experiments. The results observed in Experiment 2 could have been attributed to either the rather low transfection efficiency and/or the low concentration of combined gRNAs used in the co-transfection of cells. Perhaps the use of polycistronic plasmids, with the combined sequences of gRNAs within the same plasmids, could result in more pronounced expression differences in the target genes, due to higher moieties of each gRNAs acting upon transfection. Thus, as expected, the reprogramming effects on porcine cells were not readily detected, even though downstream events, such as p53 downregulation and *Dkc1* upregulation, were observed. We suggest that the CRISPR activation system can promote partial cell reprogramming, first by overexpressing *Myc* and *Lin28a*, leading to transcriptionally activation of its related genes (*Tert* and *Dkc1*). At the same time, the downregulation of *p53* may promote the suppression of *p21*, as a downstream effect. Moreover, dCas9-VPR showed higher levels of transcriptional activation efficiency on target genes than dCpf1-VPR in porcine fibroblast cells, but the advantages of multiplexing gRNAs with Cpf1 for multiple gene activation still needs to be further investigated.

Acknowledgments

Statement of Interest

The authors declare that there is no conflict of interest in the research reported.

References

Agarwal, S., Loh, Y.-H., McLoughlin, E. M., Huang, J., Park, I.-H., Miller, J. D., Huo, H., Okuka, M., Dos Reis, R. M., Loewer, S., Ng, H.-H., Keefe, D. L., Goldman, F. D., Klingelhutz, A. J., Liu, L. & Daley, G. Q. 2010. Telomere elongation in induced pluripotent stem cells from dyskeratosis congenita patients. *Nature,* 464**,** 292-296.

Balboa, D., Weltner, J., Eurola, S., Trokovic, R., Wartiovaara, K. & Otonkoski, T. 2015. Conditionally Stabilized dCas9 Activator for Controlling Gene Expression in Human Cell Reprogramming and Differentiation. *Stem Cell Reports,* 5**,** 448-459.

Chang, T.-C., Yu, D., Lee, Y.-S., Wentzel, E. A., Arking, D. E., West, K. M., Dang, C. V., Thomas-Tikhonenko, A. & Mendell, J. T. 2008. Widespread microRNA repression by Myc contributes to tumorigenesis. *Nature Genetics,* 40**,** 43-50.

Chang, T.-C., Zeitels, L. R., Hwang, H.-W., Chivukula, R. R., Wentzel, E. A., Dews, M., Jung, J., Gao, P., Dang, C. V., Beer, M. A., Thomas-Tikhonenko, A. & Mendell, J. T. 2009. Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation. *Proceedings of the National Academy of Sciences,* 106**,** 3384.

Chavez, A., Scheiman, J., Vora, S., Pruitt, B. W., Tuttle, M., P R Iyer, E., Lin, S., Kiani, S., Guzman, C. D., Wiegand, D. J., Ter-Ovanesyan, D., Braff, J. L., Davidsohn, N., Housden, B. E., Perrimon, N., Weiss, R., Aach, J., Collins, J. J. & Church, G. M. 2015. Highly efficient Cas9-mediated transcriptional programming. *Nature Methods,* 12**,** 326.

Cheng, A. W., Wang, H., Yang, H., Shi, L., Katz, Y., Theunissen, T. W., Rangarajan, S., Shivalila, C. S., Dadon, D. B. & Jaenisch, R. 2013. Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Research,* 23**,** 1163-1171.

Cibelli, J., Emborg, M. E., Prockop, D. J., Roberts, M., Schatten, G., Rao, M., Harding, J. & Mirochnitchenko, O. 2013. Strategies for improving animal models for regenerative medicine. *Cell Stem Cell,* 12**,** 271-274.

Cole, M. D. & Nikiforov, M. A. 2006. Transcriptional Activation by the Myc Oncoprotein. In: Eisenman, R. N. (ed.) *The Myc/Max/Mad Transcription Factor Network.* Springer Berlin Heidelberg, Berlin, Heidelberg.

Colman, A. & Dreesen, O. 2009. Pluripotent Stem Cells and Disease Modeling. *Cell Stem Cell,* 5**,** 244-247.

Çalışkan Can, E., Atalay, M. C., Miser Salihoğlu, E., Yalçıntaş Arslan, Ü., Şimşek, H. B. & Yardım Akaydın, S. 2017. Normal and Tumour Tissue mRNA Expressions of

Telomerase Complex Genes in Several Types of Cancer. *Balkan Medical Journal,* 34**,** 269-274.

Dangi-Garimella, S., Yun, J., Eves, E. M., Newman, M., Erkeland, S. J., Hammond, S. M., Minn, A. J. & Rosner, M. R. 2009. Raf kinase inhibitory protein suppresses a metastasis signalling cascade involving LIN28 and let-7. *The EMBO Journal,* 28**,** 347-358.

Deaner, M., Mejia, J. & Alper, H. S. 2017. Enabling Graded and Large-Scale Multiplex of Desired Genes Using a Dual-Mode dCas9 Activator in *Saccharomyces cerevisiae*. *ACS Synthetic Biology,* 6**,** 1931-1943.

Didovyk, A., Borek, B., Tsimring, L. & Hasty, J. 2016. Transcriptional Regulation with CRISPR-Cas9: Principles, Advances, and Applications. *Current Opinion in Biotechnology,* 40**,** 177-184.

Fagnocchi, L. & Zippo, A. 2017. Multiple Roles of MYC in Integrating Regulatory Networks of Pluripotent Stem Cells. *Frontiers in Cell and Developmental Biology,* 5**,** 7-7.

Farzadfard, F., Perli, S. D. & Lu, T. K. 2013. Tunable and multifunctional eukaryotic transcription factors based on CRISPR/Cas. *ACS Synthetic Biology,* 2**,** 604-613.

Fu, Y., Foden, J. A., Khayter, C., Maeder, M. L., Reyon, D., Joung, J. K. & Sander, J. D. 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature Biotechnology,* 31**,** 822-826.

Gasiunas, G., Barrangou, R., Horvath, P. & Siksnys, V. 2012. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences of the United States of America,* 109**,** E2579-E2586.

Gilbert, Luke A., Larson, Matthew H., Morsut, L., Liu, Z., Brar, Gloria A., Torres, Sandra E., Stern-Ginossar, N., Brandman, O., Whitehead, Evan H., Doudna, Jennifer A., Lim, Wendell A., Weissman, Jonathan S. & Qi, Lei S. 2013. CRISPR-Mediated Modular RNA-Guided Regulation of Transcription in Eukaryotes. *Cell,* 154**,** 442-451.

González, F., Boué, S. & Belmonte, J. C. I. 2011. Methods for making induced pluripotent stem cells: reprogramming à la carte. *Nature Reviews Genetics,* 12**,** 231.

Hanna, J., Saha, K., Pando, B., van Zon, J., Lengner, C. J., Creyghton, M. P., van Oudenaarden, A. & Jaenisch, R. 2009. Direct cell reprogramming is a stochastic process amenable to acceleration. *Nature,* 462**,** 595-601.

Hwang, D. H., Jeong, S. R. & Kim, B. G. 2011. Gene transfer mediated by stem cell grafts to treat CNS injury. *Expert Opinion on Biological Therapy,* 11**,** 1599-1610.

Jensen, M. K. 2018. Design principles for nuclease-deficient CRISPR-based transcriptional regulators. *FEMS Yeast Research,* 18**,** foy039.

Ji, G., Ruan, W., Liu, K., Wang, F., Sakellariou, D., Chen, J., Yang, Y., Okuka, M., Han, J., Liu, Z., Lai, L., Gagos, S., Xiao, L., Deng, H., Li, N. & Liu, L. 2013. Telomere Reprogramming and Maintenance in Porcine iPS Cells. *Plos One,* 8**,** e74202.

Kanaya, T., Kyo, S., Hamada, K., Takakura, M., Kitagawa, Y., Harada, H. & Inoue, M. 2000. Adenoviral Expression of p53 Represses Telomerase Activity through Down-Regulation of Human Telomerase Reverse Transcriptase Transcription. *Clinical Cancer Research,* 6**,** 1239.

Kim, Y., Cheong, S.-A., Lee, J. G., Lee, S.-W., Lee, M. S., Baek, I.-J. & Sung, Y. H. 2016. Generation of knockout mice by Cpf1-mediated gene targeting. *Nature Biotechnology,* 34**,** 808.

Konermann, S., Brigham, M. D., Trevino, A. E., Joung, J., Abudayyeh, O. O., Barcena, C., Hsu, P. D., Habib, N., Gootenberg, J. S., Nishimasu, H., Nureki, O. & Zhang, F. 2015. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature,* 517**,** 583-588.

Kuras, L. & Struhl, K. 1999. Binding of TBP to promoters in vivo is stimulated by activators and requires Pol II holoenzyme. *Nature,* 399**,** 609-613.

Kurata, M., Wolf, N. K., Lahr, W. S., Weg, M. T., Kluesner, M. G., Lee, S., Hui, K., Shiraiwa, M., Webber, B. R. & Moriarity, B. S. 2018. Highly multiplexed genome engineering using CRISPR/Cas9 gRNA arrays. *PloS One,* 13**,** e0198714-e0198714.

Lai, S. R., Phipps, S. M. O., Liu, L., Andrews, L. G. & Tollefsbol, T. O. 2005. Epigenetic control of telomerase and modes of telomere maintenance in aging and abnormal systems. *Frontiers in Bioscience,* 10**,** 1779-1796.

Li, B., Zeng, C. & Dong, Y. 2018. Design and assessment of engineered CRISPR–Cpf1 and its use for genome editing. *Nature Protocols,* 13**,** 899.

Lin, C.-P., Choi, Y. J., Hicks, G. G. & He, L. 2012. The emerging functions of the p53-miRNA network in stem cell biology. *Cell Cycle,* 11**,** 2063-2072.

Liu, P., Chen, M., Liu, Y., Qi, L. S. & Ding, S. 2018. CRISPR-Based Chromatin Remodeling of the Endogenous Oct4 or Sox2 Locus Enables Reprogramming to Pluripotency. *Cell Stem Cell,* 22**,** 252-261.e4.

Ly, H. 2011. Telomere dynamics in induced pluripotent stem cells: Potentials for human disease modeling. *World Journal of Stem Cells,* 3**,** 89-95.

Maeder, M. L., Linder, S. J., Cascio, V. M., Fu, Y., Ho, Q. H. & Joung, J. K. 2013. CRISPR RNA-guided activation of endogenous human genes. *Nature Methods,* 10**,** 977-979.

Marrone, A. & Mason, P. J. 2003. Human Genome and Diseases:¶Dyskeratosis congenita. *Cellular and Molecular Life Sciences CMLS,* 60**,** 507-517.

Melton, C., Judson, R. L. & Blelloch, R. 2010. Opposing microRNA families regulate self-renewal in mouse embryonic stem cells. *Nature,* 463**,** 621-626.

Mikkelsen, T. S., Hanna, J., Zhang, X., Ku, M., Wernig, M., Schorderet, P., Bernstein, B. E., Jaenisch, R., Lander, E. S. & Meissner, A. 2008. Dissecting direct reprogramming through integrative genomic analysis. *Nature,* 454**,** 49-55.

Patel, P. L., Suram, A., Mirani, N., Bischof, O. & Herbig, U. 2016. Derepression of hTERT gene expression promotes escape from oncogene-induced cellular senescence. *Proceedings of the National Academy of Sciences of the United States of America,* 113**,** E5024-E5033.

Rosselló, R. A., Wang, Z., Kizana, E., Krebsbach, P. H. & Kohn, D. H. 2009. Connexin 43 as a signaling platform for increasing the volume and spatial distribution of regenerated tissue. *Proceedings of the National Academy of Sciences,* 106**,** 13219.

Schlaeger, T. M., Daheron, L., Brickler, T. R., Entwisle, S., Chan, K., Cianci, A., DeVine, A., Ettenger, A., Fitzgerald, K., Godfrey, M., Gupta, D., McPherson, J., Malwadkar, P., Gupta, M., Bell, B., Doi, A., Jung, N., Li, X., Lynes, M. S., Brookes, E., Cherry, A. B. C., Demirbas, D., Tsankov, A. M., Zon, L. I., Rubin, L. L., Feinberg, A. P., Meissner, A., Cowan, C. A. & Daley, G. Q. 2015. A comparison of non-integrating reprogramming methods. *Nature Biotechnology,* 33**,** 58-63.

Shyh-Chang, N. & Daley, G. Q. 2013. Lin28: primal regulator of growth and metabolism in stem cells. *Cell Stem Cell,* 12**,** 395-406.

Sridharan, R., Tchieu, J., Mason, M. J., Yachechko, R., Kuoy, E., Horvath, S., Zhou, Q. & Plath, K. 2009. Role of the murine reprogramming factors in the induction of pluripotency. *Cell,* 136**,** 364-377.

Tak, Y. E., Kleinstiver, B. P., Nuñez, J. K., Hsu, J. Y., Horng, J. E., Gong, J., Weissman, J. S. & Joung, J. K. 2017. Inducible and multiplex gene regulation using CRISPR-Cpf1-based transcription factors. *Nature Methods,* 14**,** 1163-1166.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. & Yamanaka, S. 2007. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell,* 131**,** 861-872.

Takahashi, K. & Yamanaka, S. 2006. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell,* 126**,** 663-676.

Tu, Z., Yang, W., Yan, S., Guo, X. & Li, X.-J. 2015. CRISPR/Cas9: a powerful genetic engineering tool for establishing large animal models of neurodegenerative diseases. *Molecular Neurodegeneration,* 10**,** 35.

Weltner, J., Balboa, D., Katayama, S., Bespalov, M., Krjutškov, K., Jouhilahti, E.-M., Trokovic, R., Kere, J. & Otonkoski, T. 2018. Human pluripotent reprogramming with CRISPR activators. *Nature Communications,* 9**,** 2643.

Wu, K.-J., Grandori, C., Amacker, M., Simon-Vermot, N., Polack, A., Lingner, J. & Dalla-Favera, R. 1999. Direct activation of TERT transcription by c-MYC. *Nature Genetics,* 21**,** 220.

Yang, J., Rajan, S. S., Friedrich, M. J., Lan, G., Zou, X., Ponstingl, H., Garyfallos, D. A., Liu, P., Bradley, A. & Metzakopian, E. 2019. Genome-Scale CRISPRa Screen Identifies Novel Factors for Cellular Reprogramming. *Stem Cell Reports,* 12**,** 757-771.

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R., Slukvin, I. I. & Thomson, J. A. 2007. Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells. *Science,* 318**,** 1917.

Zetsche, B., Heidenreich, M., Mohanraju, P., Fedorova, I., Kneppers, J., DeGennaro, E. M., Winblad, N., Choudhury, S. R., Abudayyeh, O. O., Gootenberg, J. S., Wu, W. Y., Scott, D. A., Severinov, K., van der Oost, J. & Zhang, F. 2017. Multiplex gene editing by CRISPR-Cpf1 through autonomous processing of a single crRNA array. *Nature Biotechnology,* 35**,** 31-34.

Zhong, X., Li, N., Liang, S., Huang, Q., Coukos, G. & Zhang, L. 2010. Identification of MicroRNAs Regulating Reprogramming Factor LIN28 in Embryonic Stem Cells and Cancer Cells. *Journal of Biological Chemistry,* 285**,** 41961-41971.

Zhou, S., Ding, C., Zhao, X., Wang, E., Dai, X., Liu, L., Li, W., Liu, Z., Wan, H., Feng, C., Hai, T., Wang, L. & Zhou, Q. 2010. Successful generation of cloned mice using nuclear transfer from induced pluripotent stem cells. *Cell Research,* 20**,** 850.

Zippo, A., De Robertis, A., Serafini, R. & Oliviero, S. 2007. PIM1-dependent phosphorylation of histone H3 at serine 10 is required for MYC-dependent transcriptional activation and oncogenic transformation. *Nature Cell Biology,* 9**,** 932-944.

Zippo, A., Serafini, R., Rocchigiani, M., Pennacchini, S., Krepelova, A. & Oliviero, S. 2009. Histone Crosstalk between H3S10ph and H4K16ac Generates a Histone Code that Mediates Transcription Elongation. *Cell,* 138**,** 1122-1136.

Supplementary Files

Suppl. Table 1. Sequence of guide RNA oligonucleotides for SpCas9 enzyme and its position downstream or upstream (+) from transcription start site (TSS) of each target gene.

Primers gRNAs Cas9 5'-3'			
SOX2	**Primer**	**Sequence**	**TSS (bp)**
1	F	ACACCTCATGCAAAACCCGGCCGCGG	531
	R	AAAACCGCGGCCGGGTTTTGCATGAG	
2	F	ACACCACTTCCTTCGAAAAGGCGTGG	697
	R	AAAACCACGCCTTTTCGAAGGAAGTG	
3	F	ACACCCGGCCCGCAGCAAACTTCAGG	55+
	R	AAAACCTGAAGTTTGCTGCGGGCCGG	
4	F	ACACCCGGGAGCGCAGAGCTCCGCGG	130
	R	AAAACCGCGGAGCTCTGCGCTCCCGG	
KLF4			
1	F	ACACCGCTGCTATGGCAACGCGCGGG	166
	R	AAAACCCGCGCGTTGCCATAGCAGCG	
2	F	ACACCTATAAGTAAGGAGCGCGCGGG	40
	R	AAAACCCGCGCGCTCCTTACTTATAG	
3	F	ACACCGCGCTGATCTGCGGACTGGGG	190+
	R	AAAACCCCAGTCCGCAGATCAGCGCG	
MYC			
1	F	ACACCTTTATAGGCGAGGGTCTGCGG	593
	R	AAAACCGCAGACCCTCGCCTATAAAG	
2	F	ACACCTCCCGGGTTCCCAAAGCCGAG	670
	R	AAAACTCGGCTTTGGGAACCCGGGAG	
3	F	ACACCGCGCGCGCAGTTAATTCATGG	811
	R	AAAACCATGAATTAACTGCGCGCGCG	
OCT4			
1	F	ACACCGTGGGAGAAACTGAGGCGGAG	77
	R	AAAACTCCGCCTCAGTTTCTCCCACG	
2	F	ACACCGTACGGAATGGAAGCCCGTGG	544
	R	AAAACCACGGGCTTCCATTCCGTACG	
3	F	ACACCGTGGAATCTAATAGGCTGGGG	501
	R	AAAACCCCAGCCTATTAGATTCCACG	
4	F	ACACCCCGGGGGCCCAGTAAAACCAG	70+
	R	AAAACTGGTTTTACTGGGCCCCCGGG	
LIN28A			
1	F	ACACCCTAAGAAGTCTTGAGTACCCG	408
	R	AAAACGGGTACTCAAGACTTCTTAGG	
2	F	ACACCATGTATAATTATCTGCACGGG	562
	R	AAAACCCGTGCAGATAATTATACATG	
3	F	ACACCTGTCAGAGACTGCAGTGGTGG	278
	R	AAAACCACCACTGCAGTCTCTGACAG	

Primers control gRNAs Cas9 5'-3'		
SOX2_Ctr	**Primer**	**Sequence**
1	F	ACACCGTCCGTCGTAGTATACGCAAG
	R	AAAACTTGCGTATACTACGACGGACG
KLF4_Ctr		
1	F	ACACCGTGCGGTCGTACGGCGCACAG
	R	AAAACTGTGCGCCGTACGACCGCACG
MYC_Ctr		
1	F	ACACCGGCGCGTTAAGGTTGTAGTCG
	R	AAAACGACTACAACCTTAACGCGCCG
OCT4_Ctr		
1	F	ACACCAGGAGGCGCGTAAGGTAAGG
	R	AAAACCTTACCTTACGCGCCTCCTG
LIN28A_Ctr		
1	F	ACACCATTGCGTAATCGTCACCGAAG
	R	AAAACTTCGGTGACGATTACGCAATG

F-forward
R-reverse

Suppl. Table 2. Sequence guide RNA oligonucleotides for LbCpf1 enzyme and its position downstream or upstream (+) from transcription start site (TSS) of each target gene.

	Primers gRNAs Cpf1 5'-3'		
SOX2	**Primer**	**Sequence**	**TSS (bp)**
1	F	AGATATGCAAAACCCGGCCGCGAG	529
	R	AAAACTCGCGGCCGGGTTTTGCAT	
2	F	AGATCCCACTTCCTTCGAAAAGGC	700
	R	AAAAGCCTTTTCGAAGGAAGTGGG	
3	F	AGATCATGAAAGGGGGCGGGGCCT	546
	R	AAAAAGGCCCCGCCCCCTTTCATG	
4	F	AGATCTGCGGGCCGGGCGGCTTCA	25+
	R	AAAATGAAGCCGCCCGGCCCGCAG	
KLF4			
1	F	AGATGCTGCTATGGCAACGCGCGG	166
	R	AAAACCGCGCGTTGCCATAGCAGC	
2	F	AGATCGCCCTAGAGAAGAGCGCGA	490
	R	AAAATCGCGCTCTTCTCTAGGGCG	
3	F	AGATCAGCCAAGTCCCTTCGGTGG	1001
	R	AAAACCACCGAAGGGACTTGGCTG	
4	F	AGATCCCCCTCTTCGTTGACTGGG	520+
	R	AAAACCCAGTCAACGAAGAGGGGG	
MYC			
1	F	AGATTAGGCGAGGGTCTGCGCGGC	589
	R	AAAAGCCGCGCAGACCCTCGCCTA	
2	F	AGATGGAACCCGGGAGGGGCGCTT	679
	R	AAAAAAGCGCCCCTCCCGGGTTCC	
3	F	AGATAGCGGGAGCAAAAGAAAATG	835
	R	AAAACATTTTCTTTTGCTCCCGCT	
4	F	AGATTTTTTCCCCCCGCCCTCGGC	655
	R	AAAAGCCGAGGGCGGGGGGAAAAA	
5	F	AGATAGCACAAGGGACCAGTATGC	911
	R	AAAAGCATACTGGTCCCTTGTGCT	
OCT4			
1	F	AGATGCCCTCCAGACACCACCGCC	115
	R	AAAAGGCGGTGGTGTCTGGAGGGC	
2	F	AGATTCCCACCCCCACCGACCCCT	63
	R	AAAAAGGGGTCGGTGGGGGTGGGA	
3	F	AGATCGGGTTCCGGGGCCTCCCTT	571
	R	AAAAAAGGGAGGCCCCGGAACCCG	
4	F	AGATACTGGGCCCCGGCTTGGGG	77
	R	AAAACCCCAAGCCGGGGGCCCAGT	
LIN28A			
1	F	AGATCCTCAGGCTCCAGCTCTGGC	250

	Primer	Sequence	
	R	AAAAGCCAGAGCTGGAGCCTGAGG	
2	F	AGATAAGCCACGTGACTGCTCCCA	515
	R	AAAATGGGAGCAGTCACGTGGCTT	
3	F	AGATGGGACCCCCATTGAGTCCTT	451
	R	AAAAAAGGACTCAATGGGGGTCCC	
4	F	AGATTCCCTTGACAGGTGGTTTGT	637
	R	AAAAACAAACCACCTGTCAAGGGA	
5	F	AGATCCTCCGGACTTCTCTGGGGC	69
	R	AAAAGCCCCAGAGAAGTCCGGAGG	
Primers control gRNAs Cpf1 5'-3'			
SOX2_Ctr	**Primer**	**Sequence**	
1	F	AGATGAACGCGCTAGACAGCACGC	
	R	AAAAGCGTGCTGTCTAGCGCGTTC	
KLF4_Ctr			
1	F	AGATGGCCGCTAGAGCGTCGCGAT	
	R	AAAAATCGCGACGCTCTAGCGGCC	
MYC_Ctr			
1	F	AGATGTCGGCGCATAGGTGCGCGG	
	R	AAAACCGCGCACCTATGCGCCGAC	
OCT4_Ctr			
1	F	AGATACCGCCACCGTACCGCCACC	
	R	AAAAGGTGGCGGTACGGTGGCGGT	
LIN28A_Ctr			
1	F	AGATACCTTCCGACCGTTCGGCCG	
	R	AAAACGGCCGAACGGTCGGAAGGT	

F-forward
R-reverse

Suppl. Table 3. List of qPCR primer sequences for the target genes.

Gene	Primer	Sequence
SOX2	F	ACAGCTACGCGCACATGAAT
	R	CGAGCTGGTCATGGAGTTGT
KLF4	F	GCCAAACTACCCACCCTTCC
	R	TGGCATGAGCTCTTGGTAATGG
MYC	F	AGCGACTCTGAGGAGGAACA
	R	TTCCGACCTTTTGGCAGGGG
OCT4	F	CTCGGGCTAGAGAAGGATGTG
	R	CCTCTCGTTGCGAATAGTCACT
LIN28A	F	CCAAGGGAGACAGGTGCTAC
	R	CTTCCCGAAAGTAGGCTGGC
p21	F	ACCATGTGGACCTGTTGCTGT
	R	AGAAATCTGTCATGCTGGTCTGCC
p53	F	GGAACAGCTTTGAGGTGCGTGTTT
	R	AATACTCGCCATCCAGTGGCTTCT
DKC1	F	ACATGGTGACGATGCATGATGTGC
	R	ATGGCATTGACCGCACTGTCTTTC
TERT	F	GAAAGCCAGAAACGCAGGGAT
	R	CCCAGAAGACAGCTGTAGGTAACG
Actin	F	TTCTGCATCCTGTCGGCGAT
	R	TGCGGCATCCACGAAACTAC
GFP	F	AAGCTGACCCTGAAGTTCATCTGC
	R	CTTGTAGTTGCCGTCGTCCTTGAA

F-forward
R-reverse

Suppl. Table 4. Fold Change (FC), log2 Fold Change (Log2FC), and the % of GFP+ cells (GFP %) for each gene in both treatment groups in Experiment 1.

Treatment	Gene	FC (± SE)	Log2FC	GFP %
dCas9-VPR	LIN28A	13.524	3.757	87
		5.288	2.402	
	MYC	3.303	1.723	73
		2.828	1.500	
	OCT4	1.562	0.643	30
		1.311	0.391	
	SOX2	1.012	0.018	65
		0.805	-0.312	
	KLF4	0.543	-0.878	55
		0.302	-1.724	
dCpf1-VPR	MYC	6.880	2.782	60
		2.690	1.427	
	KLF4	1.206	0.270	40
		1.084	0.116	
	LIN28A	2.113	1.079	70
		0.763	-0.390	
	SOX2	0.976	-0.033	65
		0.695	-0.523	
	OCT4	0.510	-0.968	35
		0.282	-1.821	

SE – Standard Error

Suppl. Table 5. Relative expression patterns of target genes *Oct4, Myc, Klf4, Sox2* and *Lin28a* (OMKSL) in Experiment 1, shown as the fold change ($2^{-\Delta\Delta Ct}$) differences between pig fetal fibroblast cells transfected with either the dCas9-VPR (Cas9) or the dCpf1-VPR (Cpf1) enzymes, and control cells transfected with control gRNAs (Ctr), as reference.

Gene	Enzyme	Fold Change	SEM	Comparison	P value
OCT4	Cas9	1.436	0.125	Ctr-Cas9	0.036
	Cpf1	0.396	0.114	Ctr-Cpf1	0.016
				Cas9-Cpf1	0.025
MYC	Cas9	3.065	0.237	Ctr-Cas9	0.006
	Cpf1	4.785	2.095	Ctr-Cpf1	0.106
				Cas9-Cpf1	0.500
KLF4	Cas9	0.423	0.120	Ctr-Cas9	0.020
	Cpf1	1.145	0.060	Ctr-Cpf1	0.069
				Cas9-Cpf1	0.033
SOX2	Cas9	0.909	0.103	Ctr-Cas9	0.236
	Cpf1	0.836	0.140	Ctr-Cpf1	0.182
				Cas9-Cpf1	0.717
LIN28A	Cas9	9.406	4.118	Ctr-Cas9	0.088
	Cpf1	1.438	0.675	Ctr-Cpf1	0.291
				Cas9-Cpf1	0.196

SEM – Standard Error of the Mean
Ctr – Control group (Fold Change = 1)

Suppl. Table 6. Relative expression patterns of target genes *Oct4, Myc, Klf4, Sox2* and *Lin28a* (OMKSL) in Experiment 2, shown as the fold change ($2^{-\Delta\Delta Ct}$) differences between pig fetal fibroblast cells transfected with dCas9-VPR, and control cells transfected with control gRNAs (Ctr), as reference, on Days 2 (d2) and 17 (17) post-co-transfection.

Gene	Day	Fold Change	SEM	Comparison	P value
OCT4	2	0.627	0.104	Ctr-d2	0.035
	17	0.268	0.123	Ctr-d17	0.013
MYC	2	1.329	0.268	Ctr-d2	0.171
	17	0.721	0.081	Ctr-d17	0.038
KLF4	2	0.648	0.149	Ctr-d2	0.071
	17	0.868	0.106	Ctr-d17	0.171
SOX2	2	0.836	0.078	Ctr-d2	0.086
	17	0.614	0.054	Ctr-d17	0.009
LIN28A	2	5.817	0.597	Ctr-d2	0.007
	17	0.136	0.049	Ctr-d17	0.001
p21	2	1.090	0.190	Ctr-d2	0.341
	17	0.670	0.204	Ctr-d17	0.124
p53	2	0.387	0.057	Ctr-d2	0.004
	17	0.533	0.068	Ctr-d17	0.010
TERT	2	1.533	0.368	Ctr-d2	0.142
	17	0.714	0.125	Ctr-d17	0.075
DKC1	2	1.402	0.101	Ctr-d2	0.029
	17	0.667	0.171	Ctr-d17	0.096

SEM – Standard Error of the Mean
Ctr – Control group (Fold Change = 1)

CHAPTER III: Survival and developmental outcome of bovine IVP embryos following cytoplasmic microinjection of CRISPR/Cas9 system for homologous recombination (HR) into the H11 and Rosa26 safe harbor *loci*

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Survival and developmental outcome of bovine IVP embryos following cytoplasmic microinjection of CRISPR/Cas9 system for homologous recombination (HR) into the H11 and Rosa26 safe harbor *loci*

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Abstract

This study aimed to evaluate *in vitro* survival and developmental outcome of IVP bovine embryos after cytoplasmic microinjection (MI) of CRISPR/Cas9 system and DNA templates at the 1-cell stage embryo, targeting the safe harbor *loci* (SHL) H11 and Rosa26. Bovine COCs from slaughterhouse ovaries were *in vitro* matured for 20 h and fertilized for either 8 h (treatment groups) or 18 h (Intact Group). Groups of presumptive zygotes were partially denuded by pipetting 8 h post-fertilization (hpf), and then segregated into treatment groups: Semi-denuded (Semi), non-MI control; MI with CRISPR/Cas9; and SHL groups, targeting either the H11 or the Rosa26 *loci*, MI with CRISPR/Cas9, gRNA for each SHL, and one of two doses of repair oligonucleotide templates (5 ng/µL or 20 ng/µL). Embryos were *in vitro* cultured up to the blastocyst stage. Post-MI survival rates (D1), cleavage (D2) and blastocyst (D7) rates were compared by the Chi-square test (P<0.05). Survival was not affected by the injection of the CRISPR/Cas9 system, the doses, or the target *loci*, although the partial *cumulus* cells removal at 8 hpf, or the microinjection of donor oligonucleotides and the CRISPR/Cas9 system reduced development to the blastocyst stage in comparison to controls, being lower than 20% in most groups (Intact, 31.6%; Semi, 22.8%; CRISPR/Cas9, 23.9%; Oligo templates, 15.7%), irrespective of the injected dose or the targeted *locus*. In conclusion, the microinjection with repair templates and CRISPR/Cas9 system is feasible for homologous recombination experiments in bovine preimplantation IVP embryos, despite the reduction in embryo development.

Keywords: Homologous recombination; CRISPR system; bovine embryo

Introduction

Since the development of recombinant DNA technology in the last century, major steps have been taken in the areas of biotechnology and biomedicine. The animal platform, based on the use of transgenic animals as bioreactors for production of recombinant proteins for therapeutic purposes, is considered one of the greatest innovations in the biotechnology and pharmaceutical industry (Houdebine, 2009). In this context, the development of genetically modified organisms (GMOs) emerges as a key component in the search for improvements in the production process, offering attractive possibilities, such as low production cost and high productivity and quality of recombinant proteins (Bertolini *et al.*, 2016).

Despite the success of transgenics in large animal species, we are still far from an ideal situation, since many events during this process cannot yet be completely controlled. Normally, transgenes are integrated at random sites in the genome so the expression may vary and be altered due to position effects, such as transgene silencing (Chi *et al.*, 2019). One option to assist in solving part of the problems related to development of transgenic animal founders is to direct the insertion of the transgene (known as knock-in, KI) to specific sites into the genome less prone to silencing, known as safe harbor *loci* (SHL). Transgene KI into SHL can ensure good gene expression and secretion of recombinant proteins, as already demonstrated in mice and pigs (Maruyama *et al.*, 2015; Ruan *et al.*, 2015). Recent advances in the development of tools for genome editing, such as the clustered regularly interspaced short palindromic repeat/associated protein 9 nuclease system (or CRISPR/Cas9 system; Jinek *et al.*, 2012), can be used to genetically modify cells in culture and even allows direct embryo editing (Cong *et al.*, 2013; Hai *et al.*, 2014; Navarro-Serna *et al.*, 2020; Yoshimi e*t al.,* 2021). The CRISPR/Cas9 system have led to a revolution in genetic engineering in large animals, allowing site-directed changes in the genome to be made relatively easily, by homology-directed repair (HDR) through homologous recombination (HR), minimizing the possibility of undesirable effects, such as gene silencing (Sander and Joung, 2014; Navarro-Serna *et al.*, 2020). However, as the efficiency of transgene KI by HR in embryos is still low (Ran *et al.*, 2013), several methods have been under investigation to improve it. Recently, studies have focused on different designs and optimal lengths of the repair donor oligonucleotides templates to increase HDR rates in early embryos, such as the use of double-stranded donor templates with 3' overhangs and asymmetric single-stranded donor templates, that improved the

integration of long DNA sequences and a single nucleotide substitution in human cells, respectively (Liang *et al.*, 2017; Richardson *et al.*, 2016). Nevertheless, the impact of the use of the CRISPR/Cas9 system for the KI by HDR of such different oligonucleotide donor templates in early bovine embryos on subsequent *in vitro* embryo development and KI efficiency is still ill defined. Therefore, the aim of this study was to evaluate the effect of the cytoplasmic microinjection of 1-cell bovine IVP embryos with the CRISPR/Cas9 system and donor repair oligonucleotides for the KI by HR into bovine SHL on embryo viability and on *in vitro* embryo developmental outcome.

Materials and Methods

Chemicals and reagents were from Sigma Chemical Company (Sigma-Aldrich, St. Louis, MO, USA), unless stated otherwise.

Orthologous sequence identification and guide RNA (gRNAs) design

The orthologous sequence of bovine H11 (bH11) *locus* was identified based in the alignment of the bovine genome with the previous described sequences from mice (Tasic *et al.*, 2011), humans (Zhu *et al.*, 2014) and pigs (Ruan *et al.*, 2015), using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The bovine ROSA26 sequence (bRosa26) was previously described by Tan *et al.* (2013) and confirmed by sequence alignment with the mouse (Casola, 2010), rat (Kobayashi *et al.*, 2012) and human (Irion *et al.*, 2007) genomes. Then, sgRNAs were designed according to the bROSA26 (sgRosa26, 5'-CACCGTATTATTTCTTAAACTCCT-3') or bH11 (sgH11, 5'-CACCGTAGCCATAAGACTACCTAT-3') *locus* sequences, using the ZiFiT online software at http://zifit.partners.org/ZiFiT/ (Suppl. Table 1). Oligonucleotides were annealed and cloned into the pX458 vector (Addgene, #48138, USA) at the *Bbs*I restriction site, then *in vitro* transcribed using the MEGAshortscript™ T7 Transcription Kit (Invitrogen, USA) and purified by ethanol precipitation, following the manufacturer's recommendation.

Donor Repair Oligonucleotides Design

Four distinct donor repair oligonucleotide templates (named SST, SSNP, SSP, and DS; Suppl. Table 2) were designed according to Liang *et al.* (2017), with homology to either the bROSA26 or the bH11 *locus*. One symmetric sense single-stranded oligonucleotide (SST) donor template for HR was designed with a restriction enzyme site (*Kpn*I) positioned at the center of the oligo, flanked by 47 nucleotides on each side (both HR arms). Two asymmetric single-stranded oligonucleotide donor templates were designed with 30 nucleotides for HR placed on the left arm and 67 nucleotides

on the right arm, both flanking the *Kpn*I restriction enzyme site. The asymmetric oligonucleotide templates were differentiated by the target strand, with homology to the sense (corresponding to the strand having the PAM sequence, SSP) or the antisense (corresponding to the non-PAM strand, SSNP) strand, considering the Cas9 specific SHL targeting sequences. One double-stranded donor oligonucleotide (DS) template for HR was designed with single-stranded overhangs and with an insertion element, such as a FLAG tag at the 3' end (with 31 nucleotides), corresponding to the sites for five distinct restriction enzymes (*Bgl*II, *Blp*I, *Kpn*I, *Xba*I and *Eco*RI), and the respective homology arms at the opposite ends (with 30 nucleotides). Once annealed, at 95ºC for 3 min, the tag was within the dsDNA region and the homology arms were single-stranded.

In vitro production (IVP) of bovine embryos

Twenty-two independent replicates for the *in vitro* production (IVP) of bovine embryos were performed by *in vitro* fertilization, following our established procedures (Ribeiro *et al.*, 2009; Gerger *et al.*, 2017; Campagnolo *et al.*, 2020), in seven replicates for Experiment I (H11, 5 ng/µL), five for Experiment II (Rosa26, 5 ng/µL), five for Experiment III (H11, 20 ng/µL) and five for Experiment IV (Rosa26, 20 ng/µL).

In vitro maturation (IVM)

Bovine ovaries were obtained from a regional slaughterhouse and transported to the laboratory in saline solution (0.9% NaCl; 30ºC). *Cumulus*–oocyte complexes (COCs) were aspirated from 3- to 8-mm follicles using a 5-mL syringe coupled to an 18 G needle. A total of 5,389 grades 1 and 2 bovine COCs were selected based on Stojkovic *et al.* (2001), and groups of 15 to 20 COCs were *in vitro*-matured (IVM) for 20 h into 100 µL microdrops of IVM medium under mineral oil at 38.5ºC, 5% CO_2 in air and saturated humidity. The IVM medium was composed of TCM-199 with Earle's salts, L-glutamine and HEPES, 0.2 mM sodium pyruvate, 26.1 mM sodium bicarbonate, 10% heat-inactivated fetal bovine serum (FBS), supplemented with 5 IU/mL FSH-p (Folltropin, Bioniche, USA), 10 IU/mL hCG (Chorulon, Intervet, Inc., USA), and 1 mg/mL 17-β estradiol. A solution containing 10^5 IU/mL sodium penicillin, 10 mg/mL streptomycin sulfate, and 25 mg/mL amphotericin B (GIBCO-BRL, Life Technologies, Grand Island, NY, USA) was added to the medium (1:100).

In vitro fertilization (IVF)

Procedures for *in vitro* sperm capacitation and IVF were based on Parrish *et al.* (1986), modified by Ribeiro *et al.* (2009), Gerger *et al.* (2017) and Campagnolo *et al.*

(2020). Frozen–thawed bovine sperm cells were segregated by Percoll® gradient with Sperm-TALP medium. Following IVM, groups of 15 to 20 COCs were co-cultured with capacitated sperm cells, in 50 µL microdrops of IVF-TALP medium, under mineral oil, at an insemination dose of 5.000 viable sperm cells/COC, at 38.5°C, 5% CO_2 and saturated humidity. Manipulated/microinjected presumptive zygotes were partially denuded (semi-denuded) by pipetting 8 h post-fertilization (hpf), whereas Control (intact, non-manipulated, non-microinjected) presumptive zygotes were completely denuded 18 hpf.

Cytoplasmic Microinjection (MI)

Zygote cytoplasmic microinjection followed procedures according to Campagnolo *et al.* (2020). Semi-denuded 1-cell stage bovine IVP embryos were allocated to microdrops with HEPES-buffered M-199 and 10% FBS supplemented with 5 µg/mL cytochalasin B, under mineral oil. Microinjection was performed using a microinjector apparatus (Femtojet 4i, Eppendorf, Germany) coupled to a micromanipulator. The injected volume into each zygote per group (microinjection mixes described below) was approximately 15 pL (1.5% of the total volume of the zygote). Embryo survival rate was assessed 24 h after microinjection, with the removal of lysed structures from each group.

Embryo in vitro culture (IVC)

After cytoplasmic microinjection, structures from all five microinjected groups (below) and the non-injected groups (Intact Control group and Semi-denuded non-injected Control group) were *in vitro*-cultured (IVC) into four-well dishes containing 450 µL of modified SOF culture medium (Holm *et al.*, 1999), supplemented with 1.5 mM D-glucose and 5% FBS. Structures were cultured in the foil bag system, at 38.5°C, saturated humidity and 5% CO_2, 5% O_2 and 90% N_2 up to the blastocyst stage on Day 7 of development. Cleavage and blastocyst rates were determined on Days 2 and 7 of development (IVF=Day 0), respectively.

Experimental Design

Zygotes were segregated into two control groups and five treatment groups for KI experiments by HR. The IVP control groups were as follows: (a) Intact (non-manipulated, non-microinjected) control group, composed of COCs subjected to IVF for a period of 18 h prior to total *cumulus* cell removal and IVC; and (b) Semi-denuded (manipulated, non-microinjected) control group, which was composed of presumptive zygotes that were partially denuded 8 hpf, followed by IVC. The treatment groups were

comprised of presumptive zygotes that were partially denuded 8 hpf, followed by cytoplasmic microinjection and IVC (Fig. 1A). Treatment groups were microinjected with different combinations of the ribonucleoprotein (RNP) of the CRISPR/Cas9 system and/or one of the four oligonucleotide donor templates for either the bH11 or the bROSA26 *locus*, as follows: (c) CRISPR/Cas9 group, a microinjection RNP control group containing zygotes microinjected with 30 ng/µL of Cas9 protein (GeneArt™ Platinum™ Cas9 Nuclease; Invitrogen, USA) and a guide RNA (20 ng/µL) in Tris-EDTA (TE) solution for one of the SHL, with no oligonucleotide templates; and zygotes microinjected with RNP (30 ng/µL of Cas9 protein and 20 ng/µL of guide RNA) in TE solution for one of the SHL and either 5 ng/µL or 20 ng/µL of the (d) DS; (e) SSNP; (f) SSP or (g) SST oligonucleotide donor templates targeting either the bH11 or the bRosa26.
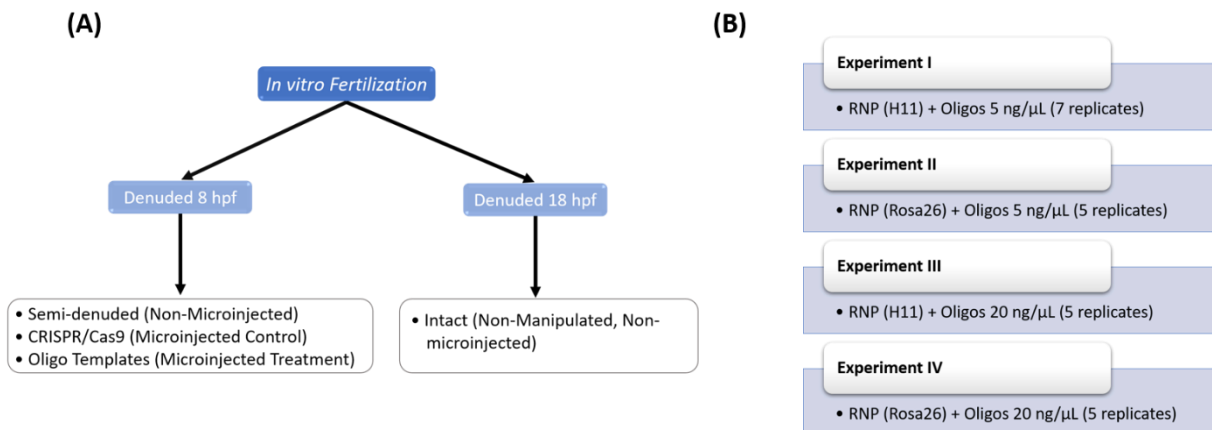
Figure 1. Scheme of Experimental Design. A: Zygote groups based on denuding time after FIV. B: Treatment groups of microinjected zygotes segregated into experiments 1 to 4.

Four experiments were performed, according to the injected dose of the oligonucleotides and the target *loci* (Fig. 1B). In Experiments I and III (bH11), zygotes were microinjected with RNP and 5 ng/µL or 20 ng/µL, respectively, of donor repair oligonucleotides (DS, SSNP, SSP, and SST) for the bH11 *locus*. In Experiments II and IV (bRosa26), zygotes were microinjected with RNP and 5 ng/µL or 20 ng/µL, respectively, of donor repair oligonucleotides (DS, SSNP, SSP, and SST) for the bRosa26 *locus*. In all experiments, the IVP control groups were used to compare the overall efficiency of the IVP procedures (Intact group) and the effect of the partial

cumulus cell removal at 8 hpf on embryo development (Semi-denuded group). In Experiments I and II (5 ng/µL), the CRISPR/Cas9 MI control group was used to compare the effect of MI with RNP (gRNA and Cas9 protein) on subsequent embryo development. In Experiments III and IV (20 ng/µL), the CRISPR/Cas9 MI control group was not included due to the limited number of structures available in each replication.

Data analyses

Post-MI survival rates (D1), cleavage (D2) and blastocyst (D7) rates, and comparative overall efficiency were compared between groups by the Chi-square test (P < 0.05). Blastocysts from each group were individually collected for genomic studies on the KI efficiency (pending analyses).

Results

Survival Rates after Cytoplasmic Microinjection

Survival rates after the cytoplasmic microinjection of 1-cell stage IVP embryos between groups is presented in Table 1. Survival rates were similar between the microinjected control groups in all experiments, also not differing from most of the treatment groups, except for the SSP group in Experiment I (H11, 5 ng/µL, 81.9%), which had a significant lower survival rate (75.8%). Such information demonstrated that the target *locus* and the oligonucleotide design and doses did not affect survival rates following cytoplasmic microinjection.

Cleavage and Blastocyst Rates

Table 2 displays cleavage rates of IVP bovine embryos between control and treatment groups. Mean cleavage rates for the Intact control group was 67% and for the Semi-denuded and MI control groups were 60%. Treatment groups in Experiments I, II, III and IV attained mean cleavage rates of 60.0%, 58.6%, 51.8% and 61.5%, respectively. Only Experiment III was significantly different from the others (P<0.05). Cleavage rates in treatment groups were not statistically different from the Semi-denuded or the MI control groups in each experiment (P>0.05), demonstrating that, in general, cleavage remained similar among groups, even after the injection of repair donor templates, regardless the target *loci* and injected dose. Cleavage rates in Experiment III were the lowest in almost all groups, with rates below 50%, except for the Intact control group (60.4%) and the SSP treatment group (68.3%).

Blastocyst rates between control and treatment groups are presented in Table 3. Control groups (IVP and MI) had blastocyst rates above 20% in all experiments, reaching 31.6%, 22.8% and 23.9% in the Intact, Semi-denuded and CRISPR/Cas9

control groups, respectively. The manipulation of structures to remove *cumulus* cells in a short time after the onset of IVF (8 h) and the cytoplasmic microinjection of 1-cell stage IVP embryos with the CRISPR/Cas9 system, irrespective of the target *locus*, decreased blastocyst yields, but in general, did not significantly affect embryo development to the blastocyst stage.

Table 1. Survival rates on Day 1 after cytoplasmic microinjection (MI) of bovine 1-cell stage IVP embryos with CRISPR/Cas9 system or different doses of donor repair oligonucleotide templates.

Survival after MI*		H11 (Experiment I)			Rosa26 (Experiment II)			H11 (Experiment III)			Rosa26 (Experiment IV)		
IVP embryos	Experimental groups**	Total	Dose 5 ng/µL		Total	Dose 5 ng/µL		Total	Dose 20 ng/µL		Total	Dose 20 ng/µL	
		n	n	%	n	n	%	n	n	%	n	n	%
Microinjected	CRISPR/Cas9	209	170	81.3[abA]	62	56	90.3[aA]	-	-	-	-	-	-
	DS	204	176	86.3[aA]	188	158	84.0[aA]	98	89	90.8[aA]	229	195	85.1[aA]
	SSNP	194	164	84.5[aA]	251	208	82.9[aA]	131	112	85.5[aA]	153	135	88.2[aA]
	SSP	211	160	75.8[bB]	234	208	88.9[aA]	135	120	88.8[aA]	152	128	84.2[aAB]
	SST	198	161	81.3[abB]	131	117	89.3[aA]	158	136	86.0[aAB]	113	98	86.7[aAB]
	Mean[†]	807	661	81.9[B]	804	691	85.9[A]	522	457	87.5[A]	647	556	85.9[A]

*DS: Double-Stranded; SSNP: Asymmetric Single-Stranded non-PAM; SSP: Asymmetric Single-Stranded PAM; SST: Symmetric Single-Stranded.

**Survival rates are based on the total number of COCs used for IVF in each group.

[†]Mean values for the DS, SSNP, SSP, and SST groups in each experiment.

[a,b:] Different superscripts in the same column differ, for $P<0.05$.

[A,B:] Different superscripts in the same row differ, for $P<0.05$.

Table 2. Cleavage rates on Day 2 of development of non-microinjected control groups and microinjected groups, after cytoplasmic microinjection (MI) of bovine 1-cell stage IVP embryos with CRISPR/Cas9 system or different doses of donor repair oligonucleotide templates.

Cleavage Rate*		H11 (Experiment I)			Rosa26 (Experiment II)			H11 (Experiment III)			Rosa26 (Experiment IV)		
IVP embryos	Experimental Groups**	Survival after MI	Dose 5 ng/µL		Survival after MI	Dose 5 ng/µL		Survival after MI	Dose 20 ng/µL		Survival after MI	Dose 20 ng/µL	
		n	n	%	n	n	%	n	n	%	n	n	%
Non-microinjected Control	**Intact**	403	288	71.5[aA]	217	147	67.7[aAB]	210	127	60.4[aB]	241	164	68.0[aAB]
	Semi-Denuded	395	248	62.8[bA]	256	158	61.7[abA]	129	60	46.5[bB]	259	177	68.3[aA]
Microinjected	**CRISPR/Cas9**	170	101	59.4[bcA]	56	34	60.7[abA]	-	-	-	-	-	-
	DS	176	106	60.2[bcAB]	158	89	56.3[bAB]	89	44	49.4[bB]	195	126	64.6[abA]
	SSNP	164	104	63.6[abA]	208	133	63.9[abA]	112	52	46.4[bB]	135	87	64.4[abA]
	SSP	160	84	52.5[cB]	208	118	56.7[bB]	120	82	68.3[aA]	128	75	58.5[abAB]
	SST	161	103	64.0[abA]	117	65	55.6[bAB]	136	59	43.3[bB]	98	54	55.1[bAB]
	Mean†	661	397	60.0[A]	691	405	58.6[A]	457	237	51.8[B]	556	342	61.5[A]

*Rates are based on the number of COCs that survived after MI in each group.

**DS: Double-Stranded; SSNP: Asymmetric Single-Stranded non-PAM; SSP: Asymmetric Single-Stranded PAM; SST: Symmetric Single-Stranded.

†Mean values for the DS, SSNP, SSP, and SST groups in each experiment.

[a,b]: Different superscripts in the same column differ, for $P<0.05$.

[A,B]: Different superscripts in the same row differ, for $P<0.05$.

In contrast, the microinjection of oligonucleotide templates reduced blastocyst development in most experimental groups, compared with controls, being lower than 20% in most groups. Therefore, even if the injection of oligonucleotides did not affect the survival of structures, it was detrimental to embryo development, significantly decreasing blastocyst yields. Nevertheless, no significant differences in blastocyst rates were observed between treatment groups and between experiments, although the groups microinjected with 20 ng/µL of oligonucleotides had a trend for slightly higher blastocyst rates than the groups injected with 5 ng/µL, regardless of the target *locus* (Experiment I, 13.9%; Experiment II, 14.1%; Experiment III, 17.0%; and Experiment IV, 17.9%).

Relative Efficiency

To determine the overall relative efficiency of each experimental group, the probability for survival after cytoplasmic microinjection and for development to the blastocyst stage from the total number of COCs used for IVF was calculated based on data from each group and experiment, with combined data shown in Table 4. As expected, the relative efficiency of the Intact control group was higher than the other groups, where one blastocyst was produced out of three COCs (1:3.2), followed by the Semi-denuded and the CRISPR/Cas9 control groups (1:4.3 and 1:5.7, respectively), which were similar with one another. Nevertheless, all treatment groups had lower relative efficiencies to generate blastocysts in comparison with controls. However, the groups of zygotes microinjected with 20 ng/µL of oligonucleotides had similar efficiency (1:7.6) to the CRISPR/Cas9 control group, regardless the target *locus* and the oligonucleotide design.

Table 3. Blastocyst rates on Day 7 of development of non-microinjected control groups and microinjected groups, after cytoplasmic microinjection (MI) of bovine 1-cell stage IVP embryos with CRISPR/Cas9 system or different doses of donor repair oligonucleotide templates.

Blastocyst Rate*		H11 (Experiment I)			Rosa26 (Experiment II)			H11 (Experiment III)			Rosa26 (Experiment IV)		
IVP embryos	Experimental Groups**	Survival after MI	Dose 5 ng/µL		Survival after MI	Dose 5 ng/µL		Survival after MI	Dose 20 ng/µL		Survival after MI	Dose 20 ng/µL	
		n	n	%	n	n	%	n	n	%	n	n	%
Non-microinjected Control	Intact	403	117	29.0ᵃᴬ	217	75	34.6ᵃᴬ	210	62	29.5ᵃᴬ	241	81	33.6ᵃᴬ
	Semi-Denuded	395	92	23.3ᵃᵇᴬ	256	54	21.1ᵇᴬ	129	27	20.9ᵃᵇᴬ	259	68	26.2ᵃᵇᴬ
Microinjected	CRISPR/Cas9	170	45	26.5ᵃᵇᴬ	56	12	21.4ᵇᴬ	-	-	-	-	-	-
	DS	176	37	21.0ᵇᶜᴬ	158	15	9.5ᵈᴮ	89	15	16.8ᵇᶜᴬᴮ	195	27	13.8ᵈᴬᴮ
	SSNP	164	17	10.4ᵈᴮ	208	26	12.5ᶜᵈᴮ	112	15	13.3ᶜᴮ	135	31	22.9ᵇᶜᴬ
	SSP	160	23	14.4ᶜᵈᴬ	208	36	17.3ᵇᶜᴬ	120	23	19.1ᵃᵇᴬ	128	22	17.1ᶜᵈᴬ
	SST	161	15	9.3ᵈᴮ	117	21	17.9ᵇᶜᴬ	136	25	18.3ᵇᶜᴬ	98	20	20.4ᵇᶜᵈᴬ
	Mean†	661	92	13.9ᴬ	691	98	14.1ᴬ	457	78	17.0ᴬ	556	100	17.9ᴬ

*Rates are based on the number of COCs that survived after MI in each group.

**DS: Double-Stranded; SSNP: Asymmetric Single-Stranded non-PAM; SSP: Asymmetric Single-Stranded PAM; SST: Symmetric Single-Stranded.

†Mean values for the DS, SSNP, SSP, and SST groups in each experiment.

[a,b]: Different superscripts in the same column differ, for $P<0.05$.

[A,B]: Different superscripts in the same row differ, for $P<0.05$.

Table 4. Probability outcomes and relative efficiency for non-microinjected control groups and microinjected groups, after cytoplasmic microinjection (MI) of bovine 1-cell stage IVP embryos with CRISPR/Cas9 system or different doses of donor repair oligonucleotide templates and development to the blastocyst stage, from the total number of COCs used for IVF in each group.

IVP embryos	Experimental groups**	Safe harbor *locus*	Dose (ng/µL)	COCs (IVF) n	Probability		Relative efficiency (ratio)*
					Survival after IM (IVC)	Blastocyst	
Non-microinjected	Intact	H11 + Rosa26	5+20	1071	-	0.318	0.318[a] (1:3.2)
Control	Sem-denuded	H11 + Rosa26	5+20	1039	-	0.232	0.232[b] (1:4.3)
Microinjected	Total microinjected			3051	0.849	0.139	0.118[d] (1:8.5)
	CRISPR/Cas9	H11 + Rosa26	5	271	0.834	0.210	0.175[bc] (1:5.7)
	DS	H11 + Rosa26	5 + 20	719	0.860	0.181	0.131[cd] (1:7.6)
	SSNP	H11 + Rosa26	5 + 20	729	0.849	0.122	0.104[d] (1:9.6)
	SSP	H11 + Rosa26	5 + 20	732	0.842	0.142	0.120[d] (1:8.3)
	SST	H11 + Rosa26	5 + 20	600	0.853	0.135	0.115[d] (1:8.7)
	All oligos	H11 + Rosa26	5 + 20	2780	0.851	0.132	0.112[d] (1:8.9)
	All oligos	H11	5 + 20	1329	0.841	0.128	0.108[d] (1:9.3)
		Rosa26	5 + 20	1451	0.859	0.136	0.117[d] (1:8.6)
	All oligos	H11 + Rosa26	5	1611	0.839	0.118	0.100[d] (1:10)
			20	1169	0.867	0.152	0.132[cd] (1:7.6)

*Relative efficiency to obtain a blastocyst on Day 7 of development. Ratio refers to the number of COCs necessary to obtain a blastocyst on Day 7 of development. Determination of relative efficiency and ratio did not include data on survival after MI for the nonmicroinjected control groups.

**DS: Double-Stranded; SSNP: Asymmetric Single-Stranded non-PAM; SSP: Asymmetric Single-Stranded PAM; SST: Symmetric Single-Stranded.

[a,b]: Different superscripts in the same column differ, for $P<0.05$.

Discussion

The recent advances in CRISPR-mediated genome engineering have allowed researchers to efficiently induce double-strand breaks (DSBs) in genomic DNA using Cas9 and an appropriate single-guide RNA (sgRNA; Cho *et al.*, 2013; Jiang *et al.*, 2013; Wang *et al.*, 2013; Mali *et al.*, 2013), enabling the introduction of a DNA fragments to generate a KI model (Navarro-Serna *et al.*, 2020). In mammalian cells, most DSBs are repaired by the nonhomologous end joining (NHEJ) pathway, which is error-prone resulting in disruptive insertions or deletions (indels) at targeted *loci*, possibly creating gene knockouts. Alternative repair pathways include the use of sister chromatids or an exogenous repair donor DNA template via components of the homology-directed repair (HDR) pathway. Such event is desired for a correct genome editing, with the most common form of HDR being HR (Navarro-Serna *et al.*, 2020). However, the efficiency of DNA repair by HDR via HR is relatively low (Navarro-Serna *et al.*, 2020; Ran *et al.*, 2013), and several methods have been explored to improve the utility of such approach for genome editing (Liang *et al.*, 2017).

The standard practice for CRISPR editing relies on microinjection of CRISPR/Cas9 system into one-cell zygotes, a process that is technically demanding, laborious and costly (Wang *et al.*, 2013; Yang *et al.*, 2013). Moreover, physical damage caused by microinjection significantly reduces embryo viability, further decreasing efficiency in genetic modifications (Brinster *et al.*, 1985; Chen *et al.*, 2016). The parameters controlling the efficacy of CRISPR/Cas9 microinjection to mediate targeted insertion are not fully established, thus this study aimed to evaluate bovine embryo development after CRISPR/Cas9 system cytoplasmic microinjection to produce KI by HDR into Rosa26 and H11 *loci*.

The timing after fertilization, the type of components of the CRISPR/Cas9 system (expressing vector, RNA, protein) and the concentration doses used for the cytoplasmic microinjection of presumptive zygotes after IVF may have significant effects on the mutation efficiency, level of mosaicism, and potential off-target mutations (Tanihara *et al.*, 2019; Hennig *et al.*, 2020; Navarro-Serna *et al.*, 2020; Le *et al.,* 2021). The standard times usually used for the microinjection of bovine IVP embryos for gene editing (16-20 hpf), often adopted for practical reasons, as such time coincides with the end of the IVF period and the completion of DNA replication into the pronuclei, are often associated with high rates of mosaicism (Lamas-Toranzo *et al.*, 2019; Mehravar *et al.*, 2019). The cytoplasmic microinjection of RNP or RNA into MII bovine oocytes

prior to IVF or into 1-cell stage bovine IVP embryos at 10 hpf significantly reduced mosaicism in developing embryos when compared with injection into zygotes at 20 hpf (10 to 30% *vs.* 100%, respectively; Lamas-Toranzo *et al.*, 2019). Moreover, Meng *et al.* (2015) demonstrated that the expression of the eGFP reporter gene in buffalo zygotes was higher after cytoplasmic microinjection at an earlier time (7-8 hpf), which corresponds to an early pronuclear stage in cattle (Xu and Greve, 1988; Mezzalira *et al.*, 2011), than later (18-19 hpf), with no effects on blastocyst yield. Thus, as an attempt to reduce or minimize mosaicism in edited embryos, *cumulus* cell partial removal was performed at 8 hpf in this study for cytoplasmic microinjection to be completed prior to pronuclear formation, or prior to 10 hpf, despite the anticipated potential decrease in subsequent embryo development, as discussed below.

It was apparent, though, that partially removing *cumulus* cells at 8 hpf, a useful procedure for effective control of the cytoplasmic microinjection, slightly compromised development when compared with the Intact group. Therefore, the manipulation of structures prior to microinjection, at 8 hpf, and not the cytoplasmic microinjection *per se*, had a significant impact on embryo development. Likely, manipulation of the structures affected cleavage rates in Experiment III, since the Semi-denuded group had a lower cleavage rate (46.5%) compared to the other experiments and to the Intact control group (Table 2). Such technical factor probably caused a delay in embryo development on Day 2 of development, with no impact on blastocyst rates on Day 7, as shown on Table 3. Moreover, Ward *et al.* (2002) demonstrated that *cumulus* cell removal at 1, 5 and 10 hpf after the onset of bovine IVF reduced cleavage rates (1, 5 and 10 hpf) and blastocyst yields up to Day 8 of development (1 and 5 hpf), as compared with *cumulus* cell removal at 15, 20 or 24 hpf. In human IVF, removing *cumulus* cells at 4-6 hpf as oppose to 18-20 hpf either reduced the percentage of fertilized oocytes and available embryos on Day 3 of development (Liu *et al.*, 2020) or increased polyspermy and decreased blastocyst rates and the proportion of high-quality blastocysts (Liu *et al.*, 2015). Consequently, as such potential effect on embryo development by partial *cumulus* cell removal at 8 hpf was anticipated, the Semi-denuded group was included in the study as control for such effect.

The genomic site where a target KI gene is integrated is an important factor in gene editing and in genetic engineering experiments. Depending on the site, the transgene may not be expressed due to gene silencing or may even cause gene knockout and potential detrimental effects on cell viability. The Rosa26 *locus* is a well-

established genomic SHL for the stable expression of a variety of target genes (Soriano, 1999). Recently, the H11 *locus* was also identified as an efficient SHL (Chi *et al.*, 2019). Thus, we tested whether the microinjection of oligonucleotides directed to such *loci* could interfere in embryo development. We observed no differences in embryo survival and development by targeting the CRISPR/Cas9 system to either the Rosa26 or the H11 bovine *loci*. Similarly, Hai *et al.* (2014) observed that CRISPR system cytoplasmic microinjection into pig zygotes had no significant negative effects on embryo development.

The combined injection of donor repair oligonucleotides had no effect on survival, regardless the SHL *locus*, the template design, or the injected dose, but was detrimental to embryo development, causing a significant fall in blastocyst yields in all treatment groups when compared to controls (Tables 3 and 4). It is known that the injection of large amounts of DNA into mouse zygotes may be toxic and may impair embryo development, even if such doses resulted in higher efficiency of transgene KI by HR (Brinster *et al.*, 1985; Raveux *et al.*, 2017). Therefore, to test the effect of the amount of the donor oligonucleotide templates microinjected into embryos on development and HDR efficiency (pending analysis), two oligo template doses were used, being one at a lower (5 ng/μL), and the other at a higher (20 ng/μL) dose. Such concentration doses were chosen based on previous studies that tested the efficiency of the cytoplasmic microinjection of repair oligonucleotides in embryo development, with ranging doses from 2 to 40 ng/μL (Meng *et al.*, 2015; Miura *et al.*, 2015; Raveux *et al.*, 2017). In our study, survival of structures assessed 24 h after microinjection was not affected by the oligo template doses, being similar to the control groups (Table 1). Interestingly, and even though the microinjection of oligos affected blastocyst development (Table 3). Despite the relatively small size of the oligo templates (61 bp to 103 bp), the amount of DNA injected into the zygotes, along with the CRISPR/Cas9 system, could have affected embryo development. This is concluded since the microinjected control group (with no oligo injection), reached better developmental rates than the experimental groups, being similar to the Semi-denuded control group. Nevertheless, the use of the higher oligo dose appeared to show slightly better blastocyst rates than the lower dose, which had an impact on the overall efficiency of the procedure (Table 4). Raveux *et al.* (2017) observed that the cytoplasmic microinjection with 20 ng/μL of repair oligonucleotides increased KI efficiency in 10% when compared to a lower dose of 2 ng/μL. This is an important result, since the

microinjection of 20 ng/μL provided similar survival rates as controls (Table 1). However, blastocyst yields on Day 7 of development reached rates above 20% in some groups (Table 3).

In summary, embryo cytoplasmic microinjection with any type of repair donor oligonucleotide templates in combination with the CRISPR/Cas9 system significantly reduced development to the blastocyst stage up to Day 7 of IVC, irrespective of the injected dose or the targeted *locus*. In addition, the microinjection procedure using the CRISPR/Cas9 system with Cas9-gRNAs RNP did not compromise survival or embryo development, as compared to the Semi-denuded control group. However, *cumulus* cell partial removal 8 h after the onset of the IVF affected embryo development when compared with the Intact control group. In general, the groups microinjected with oligonucleotides required more COCs to result in blastocysts on Day 7 of development, varying from 1 in 8 to 1 in 10. Despite the decrease in the overall efficiency in terms of embryo development, the microinjection with an oligonucleotide template dose of 20 ng/μL, along with the CRISPR/Cas9 system, is feasible for homologous recombination experiments in bovine preimplantation IVP embryos, as embryo development appeared to be higher than the 5 ng/μL dose, for acceptable blastocyst rates to support further studies. The genomic analyses of the integration rates of the oligonucleotides by HR into the SHLs still need to be performed, and once completed, it may elucidate which protocol was more efficient at inducing precise KI mutations in early bovine embryos.

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Statement of Interest

The authors declare that there is no conflict of interest in the research reported.

References

Bertolini, L.R., Meade, H., Lazzarotto, C.R., Martins, L.T., Tavares, K.C., Bertolini, M. and Murray, J.D. (2016) The transgenic animal platform for biopharmaceutical production. Transgenic Res, 25, 329-343.

Brinster, R.L., Chen, H.Y., Trumbauer, M.E., Yagle, M.K. and Palmiter, R.D. (1985) Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. PNAS, 82, 4438-4442.

Campagnolo, K., Ongaratto, F.L., Rodrigues de Freitas, C., Peña-Bello, C.A., Rodrigues, B.W., Mattos, K., Rodrigues, J.L. and Bertolini, M. (2020) In vitro development of IVF-derived bovine embryos following cytoplasmic microinjection for the episomal expression of the IGF2 gene. Reprod Dom Anim, 55, 574-583.

Casola, S. (2010) Mouse Models for miRNA Expression: The ROSA26 Locus. Vol. 667 (Ed, System., M. S. e. M. a. t. I.) Humana Press, Totowa, NJ, pp. 145-163.

Chen, S., Lee, B., Lee, A. Y.-F., Modzelewski, A.J. and He, L. (2016) Highly efficient mouse genome editing by CRISPR ribonucleoprotein electroporation of zygotes. J of Biol Chem, 291, 14457-14467.

Chi, X., Zheng, Q., Jiang, R., Chen-Tsai, R.Y. and Kong, L.-J. (2019) A system for site-specific integration of transgenes in mammalian cells. PLOS ONE, 14, e0219842.

Cho, S.W., Kim, S., Kim, J.M. and Kim, J.-S. (2013) Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat Biotechnol, 31, 230-232.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A. and Zhang, F. (2013) Multiplex genome engineering using CRISPR/Cas systems. Science, 339, 819-823.

Gerger, R.P.C., Zago, F.C., Ribeiro, E.S., Gaudencio Neto, S., Martins, L.T., Aguiar, L.H., Rodrigues, V.H.V., Furlan, F.H., Ortigari, I., Sainz, R.D., Ferrell, C.L., Miglino, M.A., Ambrósio, C.E., Rodrigues, J.L., Rossetto, R., Forell, F., Bertolini, L.R. and Bertolini, M. (2017) Morphometric developmental pattern of bovine handmade cloned concepti in late pregnancy. Reprod Fertil Dev, 29, 950-967.

Hai, T., Teng, F., Guo, R., Li, W. and Zhou, Q. (2014) One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. Cell Res, 24, 372-375.

Hennig, S.L., Owen, J.R., Lin, J.C., Young, A.E., Ross, P.J., Van Eenennaam, A.L. and Murray, J. D. (2020) Evaluation of Mosaicism and Off Target Mutations in CRISPR-Mediated Genome Edited Bovine Embryos. bioRxiv, 2020.2006.2004.134759.

Holm, P., Booth, P.J., Schmidt, M.H., Greve, T. and Callesen, H. (1999) High bovine blastocyst development in a static in vitro production system using sofaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology, 52, 683-700.

Houdebine, L.-M. (2009) Production of pharmaceutical proteins by transgenic animals. Comp Immunol Microbiol Infect Dis, 32, 107-121.

Irion, S., Luche, H., Gadue, P., Fehling, H.J., Kennedy, M. and Keller, G. (2007) Identification and targeting of the ROSA26 locus in human embryonic stem cells. Nature Biotechnology, 25, 1477-1482.

Jiang, W., Bikard, D., Cox, D., Zhang, F. and Marraffini, L.A. (2013) RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol, 31, 233-239.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A. and Charpentier, E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science, 337, 816-821.

Kobayashi, T., Kato-Itoh, M., Yamaguchi, T., Tamura, C., Sanbo, M., Hirabayashi, M. and Nakauchi, H. (2012) Identification of rat Rosa26 locus enables generation of knock-in rat lines ubiquitously expressing tdTomato. Stem Cells Dev, 21, 2981-2986.

Lamas-Toranzo I., Galiano-Cogolludo B., Cornudella-Ardiaca F., Cobos-Figueroa J., Ousinde O., Bermejo-Álvarez P. (2019) Strategies to reduce genetic mosaicism following CRISPR-mediated genome edition in bovine embryos. Sci Rep, 9(1):14900. doi: 10.1038/s41598-019-51366-8.

Le, Q. A., Tanihara, F., Wittayarat, M., Namula, Z., Sato, Y., Lin, Q., Takebayashi, K., Hirata, M. and Otoi, T. (2021) Comparison of the effects of introducing the CRISPR/Cas9 system by microinjection and electroporation into porcine embryos at different stages. BMC Rese Notes, 14, 7.

Liang, X., Potter, J., Kumar, S., Ravinder, N. and Chesnut, J.D. (2017) Enhanced CRISPR/Cas9-mediated precise genome editing by improved design and delivery of gRNA, Cas9 nuclease, and donor DNA. J Biotechnol, 241, 136-146.

Liu, J., Chen, M., Lin, C., Weng, X., Meng, Z. and Tang, W. (2015) Effect of Early Cumulus Cell Removal on the Fertilization and Clinical Outcome in Human in Vitro Fertilization. ARSci, 3, 50-56. doi: 10.4236/arsci.2015.33006.

Liu, M.H., Sun, L.J., Pan, J.P., Liang, S.S., Huang, M.Y. and WU, H.X. (2020) Early cumulus cell removal reduces available embryo rate while having no negative effect on live-birth and malformation rate in IVF: a propensity score-matched cohort study. Res Square, 1. doi: 10.21203/rs.2.20980/v1

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E. and Church, G. M. (2013) RNA-guided human genome engineering via Cas9. Science, 339, 823-826.

Maruyama, T., Dougan, S.K., Truttmann, M. C., Bilate, A.M., Ingram, J.R. and Ploegh, H.L. (2015) Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. Nat Biotechnol, 33, 538-542.

Mehravar, M., Shirazi, A., Nazari, M. and Banan, M. (2019) Mosaicism in CRISPR/Cas9-mediated genome editing. Dev Biol, 445, 156-162.

Meng, F., Li, H., Wang, X., Qin, G., Oback, B. and Shi, D. (2015) Optimized production of transgenic buffalo embryos and offspring by cytoplasmic zygote injection. J Anim Sci Biotechnol, 6, 44.

Mezzalira, J.C., Ohlweiler, L.U., da Costa Gerger, R.P., Casali, R., Vieira, F.K., Ambrósio, C.E., Miglino, M.A., Rodrigues, J.L., Mezzalira, A. and Bertolini, M. (2011) Production of Bovine Hand-Made Cloned Embryos by Zygote–Oocyte Cytoplasmic Hemi-complementation. Cell Reprogram, 13, 65-76.

Miura, H., Gurumurthy, C.B., Sato, T., Sato, M. and Ohtsuka, M. (2015) CRISPR/Cas9-based generation of knockdown mice by intronic insertion of artificial microRNA using longer single-stranded DNA. Sci Rep, 5, 12799-12799.

Navarro-Serna, S., Vilarino, M., Park, I., Gadea, J. and Ross, P.J. (2020) Livestock gene editing by one-step embryo manipulation. J Equine Vet Sci, 89, 103025.

Parrish, J.J., Susko-Parrish, J.L., Leibfried-Rutledge, M.L., Critser, E.S., Eyestone, W. H. and First, N.L. (1986) Bovine in vitro fertilization with frozen-thawed semen. Theriogenology, 25, 591-600.

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A. and Zhang, F. (2013) Genome engineering using the CRISPR-Cas9 system. Nat Protoc, 8, 2281-2308.

Raveux, A., Vandormael-Pournin, S. and Cohen-Tannoudji, M. (2017) Optimization of the production of knock-in alleles by CRISPR/Cas9 microinjection into the mouse zygote. Sci Rep, 7, 42661.

Ribeiro, E.S., Gerger, R.P.C., Ohlweiler, L.U., Ortigari, I., Mezzalira, J.C., Forell, F., Bertolini, L.R., Rodrigues, J.L., Ambrósio, C.E., Miglino, M.A., Mezzalira, A. and Bertolini, M. (2009) Developmental potential of bovine hand-made clone embryos reconstructed by aggregation or fusion with distinct cytoplasmic volumes. Cloning Stem Cells, 11, 377-386.

Richardson, C.D., Ray, G.J., DeWitt, M.A., Curie, G.L. and Corn, J.E. (2016) Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. Nat Biotechnol, 34, 339-344.

Ruan, J., Li, H., Xu, K., Wu, T., Wei, J., Zhou, R., Liu, Z., Mu, Y., Yang, S., Ouyang, H., Yanru Chen-Tsai, R. and Li, K. (2015) Highly efficient CRISPR/Cas9-mediated transgene knockin at the H11 locus in pigs. Sci Rep, 5, 14253.

Sander, J.D. and Joung, J.K. (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotech, 32, 347-355.

Soriano, P. (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet, 21, 70-71.

Stojkovic, M., Machado, S. A., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Gonçalves, P.B. and Wolf, E. (2001) Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. Biol Reprod, 64, 904-909.

Tan, W., Carlson, D.F., Lancto, C.A., Garbe, J.R., Webster, D.A., Hackett, P.B. and Fahrenkrug, S.C. (2013) Efficient nonmeiotic allele introgression in livestock using custom endonucleases. PNAS, 110, 16526-16531.

Tanihara, F., Hirata, M., Nguyen, N.T., Le, Q.A., Hirano, T. and Otoi, T. (2019) Effects of concentration of CRISPR/Cas9 components on genetic mosaicism in cytoplasmic microinjected porcine embryos. J Rep Dev, 65, 209-214.

Tasic, B., Hippenmeyer, S., Wang, C., Gamboa, M., Zong, H., Chen-Tsai, Y. and Luo, L. (2011) Site-specific integrase-mediated transgenesis in mice via pronuclear injection. PNAS, 108, 7902.

Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F. and Jaenisch, R. (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell, 153, 910-918.

Ward, F., Enright, B., Rizos, D., Boland, M. and Lonergan, P. (2002) Optimization of in vitro bovine embryo production: effect of duration of maturation, length of gamete co-incubation, sperm concentration and sire. Theriogenology, 57, 2105-2117.

Xu, K. and Greve, T. (1988). A detailed analysis of early events during in-vitro fertilization of bovine follicular oocytes. J Reprod Fertil, 82(1), 127–34.

Yang, H., Wang, H., Shivalila, C.S., Cheng, A.W., Shi, L. and Jaenisch, R. (2013) One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell, 154, 1370-1379.

Yoshimi, K., Oka, Y., Miyasaka, Y., Kotani, Y., Yasumura, M., Uno, Y., Hattori, K., Tanigawa, A., Sato, M., Oya, M., Nakamura, K., Matsushita, N., Kobayashi, K., Mashimo, T. (2021) Combi-CRISPR: combination of NHEJ and HDR provides efficient and precise plasmid-based knock-ins in mice and rats. Hum Genet, 140, 277–287.

Zhu, F., Gamboa, M., Farruggio, A.P., Hippenmeyer, S., Tasic, B., Schüle, B., Chen-Tsai, Y. and Calos, M. P. (2014) DICE, an efficient system for iterative genomic editing in human   pluripotent stem cells. Nucleic Acids Res, 42, e34.

Supplementary Tables

Suppl. Table 1: Single guide RNA (sgRNA) sequences for the CRISPR/Cas9 system
targeted to the bovine H11 (bH11) or bovine ROSA26 (bRosa26) *loci.*

sgRNA	Sequence 5' - 3'
sg*H11*	CACCGTAGCCATAAGACTACCTAT
sg*Rosa26*	CACCGTATTATTTCTTAAACTCCT

Suppl. Table 2. Repair donor oligonucleotide template sequences targeted to the bovine H11 (bH11) or bovine ROSA26 (bRosa26) *loci.*

Rosa26 *locus*	
Repair Templates*	**Sequence 5' - 3'**
SST	CCACTACTTAGCTCCTTTTGAAGTAGAGCCATATTATTTCTTAAACT<u>GGTACC</u>CCTAGGACAAAAAATGAGTAGAATGAAAACATACTTGCATGAGAGAA
SSNP	AGCACTTACAAAACCTTCAATTCTCTCATGCAAGTATGTTTTCATTCTACTCATTTTTTGTCCTAGG<u>GGTACC</u>AGTTTAAGAAATAATATGGCTCTACTTCAA
SSP	TTGAAGTAGAGCCATATTATTTCTTAAACT<u>GGTACC</u>CCTAGGACAAAAAATGAGTAGAATGAAAACATACTTGCATGAGAGAATTGAAGGTTTTGTAAGTGCT
DS Sense	<u>AGATCTGCTAAGCGGTACCTCTAGAGAATTC</u>CCTAGGACAAAAAATGAGTAGAATGAAAAC
DS Antisense	<u>GAATTCTCTAGAGGTACCGCTTAGCAGATCT</u>AGTTTAAGAAATAATATGGCTCTACTTCAA

H11 *locus*	
Repair Templates*	**Sequence 5' - 3'**
SST	ATTTTAGAAATTACACATTATCATCTGATATTAGCCATAAGACTACC<u>GGTACC</u>TATAGGGTCAGCTCAGTCTAAACTCACCCATTGGAGTCATTAGGCTC
SSNP	AAGCCATGGCCCTCTTTCTTGAGCCTAATGACTCCAATGGGTGAGTTTAGACTGAGCTGACCCTATA<u>GGTACC</u>GGTAGTCTTATGGCTAATATCAGATGATAA
SSP	TTATCATCTGATATTAGCCATAAGACTACC<u>GGTACC</u>TATAGGGTCAGCTCAGTCTAAACTCACCCATTGGAGTCATTAGGCTCAAGAAAGAGGGCCATGGCTT
DS Sense	<u>AGATCTGCTAAGCGGTACCTCTAGAGAATTC</u>TATAGGGTCAGCTCAGTCTAAACTCACCCA
DS Antisense	<u>GAATTCTCTAGAGGTACCGCTTAGCAGATCT</u>GGTAGTCTTATGGCTAATATCAGATGATAA

[1]Underlined sequence: Restriction enzyme site sequences (*Bgl*II, *Blp*I, *Kpn*I, *Xba*I *Eco*RI)

*DS: Double-Stranded; SSNP: Asymmetric Single-Stranded non-PAM; SSP: Asymmetric Single-Stranded PAM; SST: Symmetric Single-Stranded.

4. CONCLUSIONS

In Chapter II, we concluded that the CRISPR activation system promoted partial pig cell reprogramming, leading to transcriptionally activation of pluripotency genes, but with no effect on cell senescence. Moreover, the dCas9-VPR system showed higher levels of transcriptional activation efficiency on target genes than the dCpf1/Cas12a-VPR system in porcine fibroblast cells. However, the advantages of multiplexing gRNAs with Cpf1 for multiple gene activation still needs to be further investigated.

In Chapter III, we concluded that the cytoplasmic microinjection of the CRISPR/Cas9 system along with donor repair oligonucleotide templates into 1-cell stage *in vitro*-produced bovine embryos decreased the overall efficiency in terms of embryo development, irrespective of the template dose and targeted safe harbor *locus*. However, the microinjection of the CRISPR/Cas9 system (with only Cas9 protein and gRNAs) proved to be innocuous to *in vitro* embryo development, with no negative effects on cleavage and blastocyst rates. The genomic analyses of the integration rates of the oligonucleotides by HR into the SHLs into developed embryos still need to be performed.

5. PERSPECTIVES

Gene editing in farm animals may assist breeding programs by allowing changes in traits of interest in a more effective way, reducing the time for animal breeding cycles, consequently reducing costs. Many studies have been carried out to overcome the limitations and to increase the efficiency of procedures for the production of genetically modified animals. Using gene editing tools, many animals have already been generated, presenting advantages not only for the livestock sector with the increase in production and disease resistance, for instance, but also for human health. Through advances in the discovery of candidate genes related to production traits and even diseases, in a short time we will be able to use such information from gene editing to better understand human and animal physiology, also using it for biopharming, xenotranplantation and cell and gene therapy.

In general, and in this study, the strategies using CRISPR approaches to assist in gene editing were feasible, but further studies still need to be performed to improve and to simplify procedures and technologies for the development of new and effective research tools for genome and epigenome modifications. Our next steps are related to the improvement of the CRISPRa approach, through multiplexing gRNAs in a simple design to use with Cpf1/Cas12a, thus minimising the negative effect of transfecting high plasmid concentration into cells. If more reprogrammed cells are produced, such cells can be used for cloning by SCNT to determine whether induced reprogramming through CRISPRa in fact improves epigenetic plasticity after cloning demonstrated by higher rates of development and outcome after cloning. Moreover, the genomic analyses of the microinjected bovine IVP embryos will determine the efficiency of the integration of the DNA repair donor templates into the targeted safe harbor *loci*, and whether differences existed between template designs under distinct doses and between targeted SHL. Once defined, other strategies, such as the manipulation of the DNA repair machinery, may be included in future studies aimed to precisely modify cell and embryos at the genomic and epigenomic levels.

REFERENCES

APOSTOLOU, E., HOCHEDLINGER, K. Chromatin dynamics during cellular reprogramming. **Nature**, London, v. 502, p. 462-471, 2013.

BAILEY, J. Genetic modification of animals: scientific and ethical issues. *In:* HERMANN, K.; JAYNE, K. **Animal Experimentation**: working towards a paradigm change. Leiden: Brill, 2019. cap. 19, p. 443-479 DOI: 10.1163/9789004391192_020.

BALDASSARRE, H. *et al.* Production of transgenic goats by pronuclear microinjection of in vitro produced zygotes derived from oocytes recovered by laparoscopy. **Theriogenology**, New York, v. 59, n. 3, p. 831-839, 2003. DOI: 10.1016/S0093-691X(02)01128-7.

BANITO, A.; GIL, J. Induced pluripotent stem cells and senescence: learning the biology to improve the technology. **EMBO Reports**, London, v. 11, p. 353-359, 2010.

BANITO, A. *et al.* Senescence impairs successful reprogramming to pluripotent stem cells. **Genes & Development**, New York, v. 23, p. 2134-2139, 2009.

BARRANGOU, R.; DOUDNA, J. A. Applications of CRISPR technologies in research and beyond. **Nature Biotechnology**, New York, v. 34, p. 933-941, 2016.

BERTOLINI, L. R. *et al.* The transgenic animal platform for biopharmaceutical production. **Transgenic Research**, United Kingdom, v. 25, p. 329–343. DOI: 10.1007/s11248-016-9933-9, 2016.

BOCH, J., *et al.* Breaking the code of DNA binding specificity of TAL-type III effectors. **Science**, Washington, v. 326, p. 1509-1512, 2009.

BRANDSMA, I.; VAN GENT, D.C. Pathway choice in DNA double strand break repair: observations of a balancing act. **Genome Integrity**, London, v. 3, n. 9, p. 1-10, 2012.

BRESSAN, F. *et al.* Production of transgenic animals by nuclear transfer: model for biological studies. **Revista Brasileira de Reprodução Animal**, Belo Horizonte, v. 32, p. 240-250, 2008.

BRETON, A. *et al.* Derivation and characterization of induced pluripotent stem cells from equine fibroblasts. **Stem Cells and Development**, United States of America, v. 22, p. 611-621, 2013.

BREVINI, T. A. *et al.* Recent progress inembryonic stem cell research and its application in domestic species. **Reproduction in Domestic Animals**, Germany, v. 43, p. 193–199, 2008.

BRINSTER, R. L. *et al.* Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. **Proceedings of the National Academy of Sciences of the United States of America**, United States of America, v. 82, p. 4438-4442, 1985.

CARLSON, D. F. *et al.* Efficient TALEN-mediated gene knockout in livestock. **Proceedings of the National Academy of Sciences of the United States of America**, United States of America, v. 109, p. 17382-17387, 2012.

CARROLL, D. Genome engineering with Zinc-Finger nucleases. **Genetics**, Baltimore, v. 188, p. 773-782, 2011.

CASOLA, S. Mouse models for miRNA expression: the ROSA26 locus. **Methods in Molecular Biology**, Clifton, v. 667, p. 145-163, 2010. DOI: 10.1007/978-1-60761-811-9_10.

CHANDRASEKARAN, A. P.; SONG, M.; RAMAKRISHNA, S. Genome editing: a robust technology for human stem cells. **Cellular and Molecular Life Sciences**, Switzerland, v. 74, n.18, p. 3335-3346, 2017. DOI:10.1007/s00018-017-2522-0.

CHAVATTE-PALMER, P*. et al.* Review: placental perturbations induce the developmental abnormalities often observed in bovine somatic cell nuclear transfer. **Placenta**, United Kingdom, v. 33, p. 99-104, 2012.

CHAVEZ, A. *et al.* Highly efficient Cas9-mediated transcriptional programming. **Nature Methods**, New York, v. 12, p. 326-328, 2015.

CHENG, A. *et al.* Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. **Cell Research**, China, v. 23, p. 1163-1171, 2013.

CHI, X. *et al.* A system for site-specific integration of transgenes in mammalian cells. **PLos One**, San Franscisco, v. 14, n. 7, p. 1-14, 2019.

CHIRUVELLA, K. K.; LIANG, Z.; WILSON, T. E. Repair of double-strand breaks by end joining. **Cold Spring Harbor Perspectives in Biology**, United States of America, v. 5, n. 5, p. 1-21, 2013.

CIBELLI, J. B. *et al.* Cloned transgenic calves produced from nonquiescent fetal fibroblasts. **Science**, Washington, DC, v. 280, p. 1256-1258, 1998.

CIBELLI, J. *et al.* Strategies for improving animal models for regenerative medicine. **Cell Stem Cell**, United States of America, v. 12, p. 271-274, 2013.

CLARK, A. J. *et al.* Gene targeting in livestock: a preview. **Transgenic Research**, United Kingdom, v. 9, p. 263-275, 2000.

CONG, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. **Science**, Washington, DC, v. 339, p. 819-823, 2013.

DEMAYO, J. L. *et al.* Genetically engineered mice by pronuclear DNA microinjection. **Current Protocols in Mouse Biology**, United States of America, v. 2, p. 245-262, 2012.

DIDOVYK, A. *et al.* Transcriptional regulation with CRISPR-Cas9: principles, advances, and applications. **Current Opinion in Biotechnology**, United Kingdom, v. 40, p. 177-184, 2016.

EYESTONE, W. H. Production and breeding of transgenic cattle using in vitro embryo production technology. **Theriogenology**, New York, v. 51, p. 509-517, 1999.

EZASHI, T. *et al.* Derivation of induced pluripotent stem cells from pig somatic cells. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, DC, v. 106, p. 10993-10998, 2009.

FERNÁNDEZ, E.; MALLETTE, F. A. The rise of FXR1: escaping cellular senescence in head and neck squamous cell carcinoma. **PLos Genetics**, San Franscisco, v. 12, n. 11, p. 1-4, 2016.

FLETCHER, C. J. *et al.* Somatic cell nuclear transfer in the sheep induces placental defects that likely precede fetal demise. **Reproduction**, Cambridge, v. 133, p. 243-255, 2007.

FU, Y. *et al.* High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. **Nature Biotechnology**, New York, v. 31, p. 822–826, 2013. DOI:10.1038/nbt.2623.

GAJ, T.; GERSBACH, C. A.; BARBAS, C. F. ZFN, TALEN and CRISPR/Cas-based methods for genome engineering. **Trends in Biotechnology**, Amsterdam, v. 31, p. 397-405, 2013.

GALLI, C. *et al.* Somatic cell nuclear transfer and transgenesis in large animals: current and future insights. **Reproduction in Domestic Animals**, Berlin, v. 47, p. 2-11, 2012.

GASIUNAS, G. *et al.* Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, DC, v. 109, p. 2579-2586, 2012.

GILBERT, L. A. *et al.* CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. **Cell**, Cambridge, v. 154, p. 442-451, 2013.

GONZÁLEZ, F.; BOUÉ, S.; BELMONTE, J. C. I. Methods for making induced pluripotent stem cells: reprogramming à la carte. **Nature Reviews Genetics**, London, v. 12, p. 231-242, 2011.

GORDON, J. W. *et al.* Genetic transformation of mouse embryos by microinjection of purified DNA. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, DC, v. 77, n. 12, p. 7380–7384, 1980. DOI: 10.1073/pnas.77.12.7380.

GORDON, J. W.; RUDDLE, F. H. Integration and stable germ line transmission of genes injected into mouse pronuclei. **Science**, New York, v. 11; n. 214, p. 244-246, 1981. DOI:10.1126/science.6272397.

HAMMER, R. E. *et al.* Production of transgenic rabbits, sheep and pigs by microinjection. **Nature**, London, v. 315, p. 680-683, 1985.

HAN, X. *et al.* Generation of induced pluripotent stem cells from bovine embryonic fibroblast cells. **Cell Research**, United Kingdom, v. 21, p. 1509-1512, 2011.

HEYER, W. D.; EHMSEN, K. T.; LIU, J. Regulation of homologous recombination in eukaryotes. **Annual Review of Genetics**, United States of America, v. 44, p. 113-139, 2010.

HEYER, W. D. *et al.* Rad54: the swiss army knife of homologous recombination? **Nucleic Acids Research**, Oxford, v. 34, n. 15, p. 4115-4125, 2006.

HODGES, C. A.; STICE, S. L. Generation of bovine transgenics using somatic cell nuclear transfer. **Reproductive Biology and Endocrinology**, London, v. 1, n. 81, p. 1-7, 2003.

HSU, P. D.; LANDER, E. S.; ZHANG, F. Development and applications of CRISPR-Cas9 for genome engineering. **Cell**, Cambridge, v. 157, n. 6, p. 1262-1278, 2014. DOI:10.1016/j.cell.2014.05.010.

HSU, P. D. *et al.* DNA targeting specificity of RNA-guided Cas9 nucleases. **Nature Biotechnology**, New York, v. 31, n. 9, p. 827-832, 2013. DOI: 10.1038/nbt.2647.

IRION, S. *et al.* Identification and targeting of the ROSA26 locus in human embryonic stem cells. **Nature Biotechnology,** New York, v. 25, p. 1477–1482, 2007.

ISHII, T. Genome-edited livestock: ethics and social acceptance. **Animal Frontiers**, London, v. 7, p. 24-32, 2017.

JAENISCH, R.; MINTZ, B. Simian Virus 40 DNA Sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, DC, v. 71, n. 4, p. 1250–1254, 1974.

JINEK, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. **Science**, New York, v. 337, p. 816-821, 2012.

KANAYA, T. *et al.* Adenoviral expression of p53 represses telomerase activity through down-regulation of human telomerase reverse transcriptase transcription. **Clinical Cancer Research**, United States of America, v. 6, n. 4, p. 1239-1247, 2000.

KARANAM, K. *et al.* Quantitative live cell imaging reveals a gradual shift between DNA repair mechanisms and a maximal use of HR in mid S phase. **Molecular Cell**, United States of America, v. 47, n. 2, p. 320-329, 2012.

KAWAMURA, T. *et al.* Linking the p53 tumour suppressor pathway to somatic cell reprogramming. **Nature**, London, v. 460, p. 1140-1144, 2009.

KIM, Y. *et al.* Generation of knockout mice by Cpf1-mediated gene targeting. **Nature Biotechnology**, New York, v. 34, p. 808-810, 2016.

KLEINSTIVER, B. P. *et al.* High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. **Nature**, London, v. 529, p. 490–495, 2016. DOI:10.1038/nature16526.

KOBAYASHI, T. *et al.* Identification of rat Rosa26 locus enables generation of knock-in rat lines ubiquitously expressing tdTomato. **Stem Cells and Development**, United States of America, v. 21, p. 2981-2986, 2012.

KONG, Q. *et al.* ROSA26 locus supports tissue-specific promoter driving transgene expression specifically in pig. **PLos One**, San Franscisco, v. 9, n. 9, p. 1-9, 2014.

KUBISCH, H. M. *et al.* Expression of two transgenes in in vitro matured and fertilized bovine zygotes after DNA microinjection. **Journal of Reproduction and Fertility**, Colchester, v. 104, p. 133-139, 1995.

KUES, W.A.; NIEMANN, H. Advances in farm animal transgenesis. **Preventive Veterinary Medicine**, Amsterdam, v. 102, p. 146-156, 2011.

KUMAR, D. *et al.* Perspectives of pluripotent stem cells in livestock. **World Journal of Stem Cells**, United States of America, v. 13, n. 1, p. 1-29, 2021. DOI:10.4252/wjsc.v13.i1.1.

LAI, S. R. *et al.* Epigenetic control of telomerase and modes of telomere maintenance in aging and abnormal systems. **Frontiers in Bioscience**, United States of America, v. 10, p. 1779-1796, 2005.

LAIBLE, G.; ALONSO-GONZÁLEZ, L. Gene targeting from laboratory to livestock: current status and emerging concepts. **Biotechnology Journal**, Germany, v. 4, p. 1278–1292, 2009.

LAMAS-TORANZO, I. *et al.* Strategies to reduce genetic mosaicism following CRISPR-mediated genome edition in bovine embryos. **Scientific Reports**, London, v. 9, n. 1, p. 1-8, 2019. DOI: 10.1038/s41598-019-51366-8.

LI, B.; ZENG, C.; DONG, Y. Design and assessment of engineered CRISPR–Cpf1 and its use for genome editing. **Nature Protocols**, United States of America, v. 13, p. 899-914, 2018.

LI, T. *et al.* TAL nucleases (TALNs): hybrid proteins composed of TAL effectors and FokI DNA-cleavage domain. **Nucleic Acids Research**, Oxford, v. 39, p. 359-372, 2011.

LIEBER, M. R. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. **Annual Review of Biochemistry**, Palo Alto, v. 79, p. 181-211, 2010.

LIN, S. *et al.* Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. **Elife**, Cambridge, v. 3, p. 1-13, 2014.

LIU, J. *et al.* Generation and characterization of reprogrammed sheep induced pluripotent stem cells. **Theriogenology**, New York, v. 77, p. 338-346, 2012.

MAEDER, M. L. *et al.* CRISPR RNA-guided activation of endogenous human genes. **Nature Methods**, New York, v. 10, p. 977-979, 2013.

MAHERALI, N. *et al.* Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. **Cell Stem Cell**, United States of America, v. 1, p. 55-70, 2007.

MENG, L. *et al.* Generation of five human lactoferrin transgenic cloned goats using fibroblast cells and their methylation status of putative differential methylation regions of IGF2R and H19 imprinted genes. **PLos One**, San Franscisco, v. 8, n. 10, p. 1-10, 2013. DOI:10.1371/journal.pone.0077798.

MENG, F. *et al.* Optimized production of transgenic buffalo embryos and offspring by cytoplasmic zygote injection. **Journal of Animal Science and Biotechnology**, London, v. 6, n. 44, p.1-7, 2015.

MILLER, J.; MCLACHLAN, A. D.; KLUG, A. Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. **The EMBO Journal**, Eynsham, v. 4, p. 1609-1614, 1985.

MOSCOW, M. J.; BOGDANOVE, A. J. A simple cipher governs DNA recognition by TAL Effectors. **Science**, Washingtom, DC, v. 326, p. 1501, 2009.

MURRAY, J. D.; MAGA, E.A. Genetically engineered livestock for agriculture: a generation after the first transgenic animal research conference. **Transgenic Research**, United Kingdom, v. 3, p. 321-327, 2016. DOI: 10.1007/s11248-016-9927-7.

MURRAY, J. D. *et al.* Production of transgenic merino sheep by microinjection of ovine metallothionein-ovine growth hormone fusion genes. **Reproduction, Fertility and Development**, East Melbourne, v. 1, n. 2, p. 147-155, 1989. DOI: 10.1071/RD9890147.

NAGY, K. *et al.* Induced pluripotent stem cell lines derived from equine fibroblasts. **Stem Cell Reviews**, United States of America, v. 7, p. 693-702, 2011.

NAVARRO-SERNA, S. *et al.* Livestock gene editing by one-step embryo manipulation. **Journal of Equine Veterinary Science**, United States of America, v. 89, p. 1-9, 2020.

NIEMANN H; KUES, W.A. Application of transgenesis in livestock for agriculture and biomedicine. **Animal Reproduction Science**, Amsterdam, v. 79, n. 3, p. 291-317, 2003. DOI: 10.1016/S0378-4320(03)00169-6.

NIEMANN, H.; LUCAS-HAHN, A. Somatic cell nuclear transfer cloning: practical applications and current legislation. **Reproduction in Domestic Animals**, Berlin, v. 47, p. 2-10, 2012.

OBACK, B. Climbing mount efficiency: small steps, not giant leaps towards higher cloning success in farm animals. **Reproduction in Domestic Animals**, Berlin, v. 43, p. 407-416, 2008. DOI:10.1111/j.1439-0531.2008.01192.x.

PARDO, B.; GÓMEZ-GONZÁLEZ, B.; AGUILERA, A. DNA repair in mammalian cells. **Cellular and Molecular Life Sciences**, Basel, v. 66, n. 6, p. 1039-1056, 2009.

PATTANAYAK, V. *et al.* High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. **Nature Biotechnology**, New York, v. 31, p. 839–843, 2013. DOI:10.1038/nbt.2673.

PETERSEN, B. Basics of genome editing technology and its application in livestock species. **Reproduction in Domestic Animals**, Berlin, v. 52, p. 4-13, 2017.

PHELPS, C. J. *et al.* Production of α1, 3-galactosyltransferase-deficient pigs. **Science**, Washington, DC, v. 299, n. 5605, p. 411-414, 2003. DOI:10.1126/science.1078942.

PRATHER, R. S. *et al.* Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyt. **Biology of Reproduction**, Champaig, v. 37, n. 4, p. 859-866, 1987.

RUAN, J. *et al.* Highly efficient CRISPR/Cas9-mediated transgene knockin at the H11 locus in pigs. **Scientific Reports**, London, v. 5, p. 1-10, 2015. DOI:10.1038/srep14253.

SADELAIN, M.; PAPAPETROU, E. P.; BUSHMAN, F. D. Safe harbours for the integration of new DNA in the human genome. **Nature Reviews Cancer**, United Kingdom, v. 12, n. 1, p. 51-58, 2012. DOI:10.1038/nrc3179.

SANDER, J. D.; JOUNG, J. K. CRISPR-Cas systems for editing, regulating and targeting genomes. **Nature Biotechnology**, New York, v. 32, p. 347-355, 2014.

SANDMAIER, S. E. S. *et al.* Generation of induced pluripotent stem cells from domestic goats. **Molecular Reproduction and Development**, United States of America, v. 82, p. 709-721, 2015.

SARTORI, C. *et al.* Ovine-induced pluripotent stem cells can contribute to chimeric lambs. **Cellular Reprogramming**, United States of America, v. 14, p. 8-19, 2012.

SCHLAEGER, T. M. *et al.* A comparison of non-integrating reprogramming methods. **Nature Biotechnology**, New York, v. 33, p. 58-63, 2015.

SCHNIEKE, A. E. *et al.* Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. **Science**, Washington, DC, v. 278, p. 2130-2133, 1997.

SHAKIROVA, K. M.; OVCHINNIKOVA, V. Y.; DASHINIMAEV, E. B. Cell reprogramming with CRISPR/Cas9 based transcriptional regulation systems. **Frontiers in Bioengineering and Biotechnology**, Lausanne, v. 8, n. 882, p. 1-16, 2020. DOI: 10.3389/fbioe.2020.00882.

SIMMET, K.; WOLF, E.; ZAKHARTCHENKO, V. Manipulating the Epigenome in Nuclear Transfer Cloning: Where, When and How. **International Journal of Molecular Sciences**, Basel, v. 22, n. 236, 1-17, 2021. DOI: 10.3390/ijms22010236.

SMITH, J. *et al.* Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. **Nucleic Acids Research**, Oxford, v. 28, p. 3361-3369, 2000.

SMITH, K. Theoretical mechanisms in targeted and random integration of transgene DNA. **Reproduction, Nutrition, Development**, France, v. 51, p. 465–485, 2001. DOI:10.1051/rnd:2001102.

SONG, H. *et al.* Induced pluripotent stem cells from goat fibroblasts. **Molecular Reproduction and Development**, United States of America, v. 80, p. 1009-1017, 2013.

SRIDHARAN, R. *et al.* Role of the murine reprogramming factors in the induction of pluripotency. **Cell,** Cambridge, v. 136, p. 364-377, 2009.

STADTFELD, M.; HOCHEDLINGER, K. Induced pluripotency: history, mechanisms, and applications. **Genes & Development**, United States of America, v. 24, p. 2239-2263, 2010.

STADTFELD, M. *et al.* Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. **Cell Stem Cell**, United States of America, v. 2, p. 230-240, 2008.

SUMIYAMA, K.; KAWAKAMI, K.; YAGITA, K. A simple and highly efficient transgenesis method in mice with the Tol2 transposon system and cytoplasmic microinjection. **Genomics**, San Diego, v. 95, p. 306-311, 2010.

TAKAHASHI, K. ; YAMANAKA, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. **Cell,** Cambridge, v. 126, p. 663-676, 2006.

TAKAHASHI, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. **Cell**, Cambridge, v. 131, p. 861-872, 2007.

TALLURI, T. R. *et al.* Derivation and characterization of bovine induced pluripotent stem cells by transposon-mediated reprogramming. **Cellular Reprogramming**, United States of America, v. 17, p. 131-140, 2015.

TAN, W. *et al.* Efficient nonmeiotic allele introgression in livestock using custom endonucleases. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, DC, v. 110, n. 41, p. 16526–16531, 2013.

TASIC, B. *et al.* Site-specific integrase-mediated transgenesis in mice via pronuclear injection. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, DC, v. 108, n. 19, p. 7902-7907, 2011.

TAVARES, K. C. S. *et al.* Transient expression of functional glucocerebrosidase for treatment of gaucher's disease in the goat mammary gland. **Molecular Biotechnology**, United States of America, v. 58, p. 47–55, 2016. DOI:10.1007/s12033-015-9902-1.

TERNS, M. P.; TERNS, R. M. CRISPR-Based adaptive immune systems. **Current Opinion in Microbiology**, Oxford, v. 14, p. 321-327, 2011.

TU, Z. *et al.* CRISPR/Cas9: a powerful genetic engineering tool for establishing large animal models of neurodegenerative diseases. **Molecular Neurodegeneration**, London, v. 10, n. 35, p. 1-8, 2015.

UCHIDA, M. *et al.* Production of transgenic miniature pigs by pronuclear microinjection. **Transgenic Research**, United Kingdom, v. 10, n. 6, p. 577-582, 2001. DOI:10.1023/A:1013059917280.

URNOV, F. D. *et al.* Genome editing with engineered zinc finger nucleases. **Nature Reviews Genetics**, London, v. 11, p. 636-646, 2010.

WEST, F. D. *et al.* Porcine induced pluripotent stem cells produce chimeric offspring. **Stem Cells and Development**, United States of America, v. 19, n. 8, p. 1211-20, 2010. DOI: 10.1089/scd.2009.0458.

WHEELER, M. B. Production of transgenic livestock: promise fulfilled1. **Journal of Animal Science**, London, v. 81, p. 32-37, 2003.

WHITWORTH, K. M. *et al.* Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. **Nature Biotechnology**, New York, v. 34, p. 20-22, 2016.

WILMUT, I. *et al.* Viable offspring derived from fetal and adult mammalian cells. **Nature**, London, v. 385, p. 810-813, 1997.

WU, Y. *et al.* Rad51 protein controls Rad52-mediated DNA annealing. **Journal of Biological Chemistry**, United States of America, v. 283, n. 21, p. 14883-14892, 2008.

WYMAN, C.; KANAAR, R. DNA double-strand break repair: all's well that ends well. **Annual Review of Genetics**, United States of America, v. 40, p. 363-383, 2006.

YANG, D. *et al.* Generation of PPARγ mono-allelic knockout pigs via zinc-finger nucleases and nuclear transfer cloning. **Cell Research**, United Kingdom, v. 21, p. 979-982, 2011.

YING, Q. L. *et al.* Changing potency by spontaneous fusion. **Nature**, London, v. 416, p. 545-548, 2002.

YU, J. *et al.* Human induced pluripotent stem cells free of vector and transgene sequences. **Science**, London, v. 324, p. 797-801, 2009.

YU, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. **Science**, London, v. 318, p. 1917-1920, 2007.

YU, S. *et al.* Highly efficient modification of beta-lactoglobulin (BLG) gene via zinc-finger nucleases in cattle. **Cell Research**, United Kingdom, v. 21, p. 1638-1640, 2011.

ZETSCHE, B. *et al.* Multiplex gene editing by CRISPR-Cpf1 through autonomous processing of a single crRNA array. **Nature Biotechnology**, New York, v. 35, p. 31-34, 2017.

ZHANG, S.; VOIGT, C. A. Engineered dCas9 with reduced toxicity in bacteria: implications for genetic circuit design. **Nucleic Acids Research**, Oxford, v. 46, n. 20, p. 1115-11125, 2018. DOI: 10.1093/nar/gky884.

ZHAO, Y. *et al*. Two supporting factors greatly improve the efficiency of human iPSC generation. **Cell Stem Cell**, United States of America, v. 3, p. 475-479, 2008.

ZHOU, S. *et al*. Successful generation of cloned mice using nuclear transfer from induced pluripotent stem cells. **Cell Research**, United Kingdom, v. 20, p. 850-853, 2010.

ZHU, F. *et al.* DICE, an efficient system for iterative genomic editing in human pluripotent stem cells. **Nucleic Acids Research,** Oxford, v. 42, n. 5, p. 1-13, 2014.

APPENDICES

Appendix 1: Guidelines for the preparation of Chapter II

Guide for authors - Zygote Journal

Scope

Zygote is an international journal dedicated to the rapid publication of original research in early embryology. It covers interdisciplinary studies in animals and humans, from gametogenesis through fertilization to gastrulation. The scope includes gametogenesis, sperm–oocyte interaction, gamete and embryo physiology, cell polarity, cell–cell interactions, nuclear transfer, haploidization, molecular genetics, developmental genetics, *in-vitro* fertilization, and stem-cell and cryoconservation technologies. **Please note:** papers of a technical nature or which involve industrial-scale IVF are not suitable for Zygote and should be submitted elsewhere.

The editors favour work describing fundamental processes in the cellular and molecular mechanisms of animal development, and, in particular, the identification of unifying principles in biology. New technologies, clinical papers, review articles, debates and letters will become prominent features.

Submissions

All manuscripts must be submitted online at:

http://mc.manuscriptcentral.com/zygote

Submission of a paper will be taken to imply that it is unpublished and it is not being considered for publication elsewhere.

There is no formal restriction on length; however, *Original Articles* and *Reviews* of less than 15000 words are likely to appear sooner than longer ones. *Short Communications* should not exceed 1500 words and *News and Views Commentaries* should not exceed 500 words.

Preparation of manuscripts

Manuscripts must contain continuous line numbering throughout and should be organised as follows:

The title page should include:

- The **title** of the article, which **should be short** (preferably up to 12 words) but informative and accurately reflect the content.
- Authors' names and contact details: please list a brief affiliation for each author including country (assigned with superscript numbers) below the author names, and in addition, indicate the corresponding author with an asterisk and in this case provide an email address

- Word count, including all text but excluding tables, figures and references.

An Abstract of not more than 250 words followed by 5 Keywords, Introduction, Materials and Methods, Results, Discussion (combined Results and Discussion may be used for short papers), Acknowledgements, References, Endnotes, Tables and Figure Legends.

Manuscripts should be prepared using SI units.

Figures

Figures should be numbered consecutively as they appear in the text. Any indication of features of special interest should also be included. Figures must be supplied electronically. They must be saved at final publication size and ideally supplied in the following file formats: halftone figures (black & white, and colour) as TIF files at 300 dpi; black & white line figures as TIF or EPS files at 1000–1200 dpi. PDF format is also accepted. When relevant, photographs should be submitted with proposed reduction or magnification indicated by a scale line on or beside, the illustration. The places for insertion into the text should be indicated in the text as 'Fig. 1' etc. Legends for all illustrations should be typed together, separately from the main text. There is no charge for online publication of colour photographs or figures. More detailed information is available at: www.cambridge.org/core/services/authors/journals/journals-artwork-guide.

Tables

Tables with concise headings should be placed at the end of the paper. Each table must have a text reference, in the form 'Table 1' etc.

References

References should be cited in the text 'as Conklin (1905) showed' or 'as shown (Conklin, 1905)'. For papers with three or more authors, use et al. A full list of references in alphabetical order should be given at the end of the text: surname of author and initials; year of publication (in parentheses); title of paper; journal or book name (the former being abbreviated in accordance with the World List of Scientific Periodicals); volume number; first and last page of the reference. For books and conference proceedings, the place of publication and publisher (and editor(s) if appropriate) should be included.

Acknowledgements

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Ethics Statements

Financial Support

Please provide details of the sources of financial support for all authors, including grant numbers. For example, "This work was supported by the Medical research Council (grant number XXXXXXX)". Multiple grant numbers should be separated by a comma and space, and where research was funded by more than one agency the different agencies should be separated by a semi-colon, with "and" before the final funder. Grants held by different authors should be identified as belonging to individual authors by the authors' initials. For example, "This work was supported by the Wellcome Trust (A.B., grant numbers XXXX, YYYY), (C.D., grant number ZZZZ); the Natural Environment Research Council (E.F., grant number FFFF); and the National Institutes of Health (A.B., grant number GGGG), (E.F., grant number HHHH)". Where no specific funding has been provided for research, please provide the following statement: "This research received no specific grant from any funding agency, commercial or not-for-profit sectors."

Conflict of Interest declaration

All authors must include a conflict of interest declaration in their manuscript. This declaration will be subject to editorial review and may be published in the article.

Conflicts of interest are situations that could be perceived to exert an undue influence on the content or publication of an author's work. They may include, but are not limited to, financial, professional, contractual or personal relationships or situations.

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Ethical Standards

Where research involves human and/or animal experimentation, the following statements should be included (as applicable): "The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008." and "The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals."

Publication Ethics

Please visit here for information on our ethical guidelines.

Appendix 2: Guidelines for the preparation of Chapter III

Guide for authors - Research in Veterinary Science

Research in Veterinary Science publishes original contributions and review articles on research concerning the health and disease of animals, including studies in comparative medicine.

Peer review

This journal operates a single anonymized review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. Editors are not involved in decisions about papers which they have written themselves or have been written by family members or colleagues or which relate to products or services in which the editor has an interest. Any such submission is subject to all of the journal's usual procedures, with peer review handled independently of the relevant editor and their research groups. More information on types of peer review.

Use of Word Processing Software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: https://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required separate file submissions. See also the section on Electronic artwork.To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Form of papers

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation

marks and also cite the source. Any modifications to existing methods should also be described.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• *Title.* Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• *Author names and affiliations.* Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

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• *Present/permanent address.* If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which

the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Highlights

Highlights are mandatory for this journal as they help increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the novel results of your research as well as new methods that were used during the study (if any). Please have a look at the examples here: example Highlights.

Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Abstract, self-contained and embodying the main conclusions. It should note the relevance to veterinary science as well as the aims and objectives of the work. Sentences such as 'the results are discussed', which merely describe the paper, are not allowed.

Graphical abstract

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VITA

Gabriella Borba de Oliveira, daughter of Cláudio Costa de Oliveira and Ana Beatriz Marques de Borba, was born in September 3, 1992, in Bagé, in the State of Rio Grande do Sul. She attended the initial grades of elementary school at "Colégio Nossa Senhora Auxiliadora", finishing high school at "Escola Estadual de Ensino Médio Dr. Carlos Antônio Kluwe". In 2010, she was admitted to the undergraduate course in Biotechnology at the Federal University of Pelotas - UFPel, and in 2014 joined the master's degree in Animal Science at the University of São Paulo - USP, under the supervision of Professor Dr. Luiz Lehman Coutinho, with an internship period abroad at the Iowa State University in the USA, under the supervision of Dr. James Reecy.

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