CRISPR-on for activation of endogenous SMARCA4 and TFAP2C expression in bovine embryos

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Abstract

CRISPR-mediated transcriptional activation, also known as CRISPR-on, has proven efficient for activation of individual or multiple endogenous gene expression in cultured cells from several species. However, the potential of CRISPR-on technology in preimplantation mammalian embryos remains to be explored. Here, we report for the first time the successful modulation of endogenous gene expression in bovine embryos by using the CRISPR-on system. As a proof of principle, we targeted the promoter region of either *SMARCA4* or *TFAP2C* genes, transcription factors implicated in trophoblast lineage commitment during embryo development. We demonstrate that CRISPR-on provides temporal control of endogenous gene expression in bovine embryos, by simple cytoplasmic injection of CRISPR RNA components into one cell embryos. dCas9VP160 activator was efficiently delivered and accurately translated into protein, being detected in the nucleus of all microinjected blastomeres. Our approach resulted in the activation of *SMARCA* expression shortly after microinjection, with a consequent effect on downstream differentiation promoting factors, such as *TFAP2C* and *CDX2*. Although targeting of *TFAP2C* gene did not result in a significant increase in *TFAP2C* expression, there was a profound induction in *CDX2* expression on day 2 of development. Finally, we demonstrate that CRISPR-on system is suitable for gene expression modulation during the preimplantation period, since no detrimental effect was observed on microinjected embryo development. This study constitutes a first step toward the application of the CRISPR-on system for the study of early embryo cell fate decisions in cattle and other mammalian embryos, as well as to design novel strategies that may lead to an improved trophectoderm development.

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Introduction

Over the past 10 years, engineered-programmable DNA nucleases have resulted in unprecedented opportunities for gene editing. The CRISPR/Cas9 system (from Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR-associated nuclease9) stands out among engineered-nucleases given its simple design, flexibility and species adaptability (Mojica *et al.* 2005, Jinek *et al.* 2012, Cong *et al.* 2013, Knott & Doudna 2018). This system consists of a single guide RNA (sgRNA), which directs the nuclease Cas9 to its target sequence in the genome by complementary base pairing, generating double-stranded breaks that, upon repair by either non-homologous end-joining or homologous recombination,

can potentially result in the knock-out of any gene of interest.

Soon after its introduction as gene editing tool, the CRISPR/Cas9 system was also re-purposed for multiple site-specific nuclease-independent modifications (Larson *et al.* 2013, Zalatan *et al.* 2015, Jusiak *et al.* 2016, Knott & Doudna 2018, Engreitz *et al.* 2019). To achieve this, the Cas9 nuclease catalytic domains were mutated, resulting in a DNA-binding protein still capable of site-specific target recognition but unable to cut the DNA, called dCas9 (for dead Cas9, Qi *et al.* 2013). The fusion of dCas9 to different effector domains, including transcriptional activation or repression domains, allowed specific modulation of gene expression *in vitro* (Gilbert *et al.* 2013, Mali *et al.* 2013, Maeder *et al.* 2013, Perez-

Pinera *et al.* 2013). The first version of CRISPR-based transcriptional activator or CRISPR-on, consisted on the fusion of the dCas9 protein to several tandem repeats of VP16 herpes virus activation domain to generate the dCas9VP64 programmable activator (Gilbert *et al.* 2013, Perez-Pinera *et al.* 2013). Subsequent 'second-generation' activators were developed in order to co-recruit multiple activators by using the dCas9 protein as a scaffold. CRISPR-on tools have been successfully used to turn on the expression of individual or multiple endogenous genes in cell culture from several species (Cheng *et al.* 2013, Gilbert *et al.* 2013, Maeder *et al.* 2013, Perez-Pinera *et al.* 2013, Konermann *et al.* 2015). However, up to date, CRISPR-on-mediated endogenous gene activation has not been applied to mammalian embryos.

The proper development of the early mammalian embryo involves both temporal and spatial regulation of gene expression. During early embryogenesis, the first cell fate decision results in segregation of the inner cell mass (ICM), which will ultimately contribute mainly to the fetus, and trophoblast (TE), which serves as the precursor of extraembryonic tissues. This early differentiation occurs before implantation and has been widely studied in mice. Particularly in this species, lineage segregation is directed by the antagonistic expression of *Oct4* and *Cdx2* followed by OCT4-specific localization to the pluripotent ICM and CDX2 confinement to the trophoblast epithelium at the late blastocyst stage (Rossant 2004). The pluripotent state of the murine ICM is maintained through the formation of a complex between OCT4, SOX2 and NANOG (Avilion *et al.* 2003, Masui *et al.* 2007). Conversely, CDX2 plays a key role in trophoblast specification through the repression of *Oct4* and *Nanog* expression (Strumpf *et al.* 2005, Chen *et al.* 2009).

Although early embryo development is highly conserved within mammalian species, there are several species-specific differences underlying lineage commitment. As reported for mouse, the OCT4/SOX2/NANOG protein complex is also present in the bovine ICM (Degrelle *et al.* 2005, Ozawa *et al.* 2012) and CDX2 is located exclusively in the TE at the late blastocysts stage. However, while CDX2 is essential for TE maintenance, it is not crucial for TE establishment as it is for mouse (Berg *et al.* 2011). Moreover, in bovine, as well as in humans and pigs, the mechanism of differentiation does not require the restriction of OCT4 to the blastocyst ICM. In fact, *OCT4* and *CDX2* transcripts are co-expressed in the TE of *in vitro*-produced bovine embryos on day 7 (Simmet *et al.* 2018). It is only by day 11 that OCT4 expression is repressed in the TE lineage (Berg *et al.* 2011) suggesting that, in cattle, TE commitment might be regulated upstream of CDX2 and that cell fate segregation mechanisms have diverged between cattle and mouse.

Recently, two transcription factors (TF), TFAP2C and SMARCA4, were shown to have key roles in trophoblast lineage commitment through the modulation of *CDX2* and *NANOG* expression in murine embryos (Cao *et al.* 2015, Carey *et al.* 2015). TFAP2C is a TF with a high degree of sequence conservation present in a wide variety of species, playing an important role in the placenta development (Aston *et al.* 2009, Lee *et al.* 2015). In mice, *TFAP2C* mRNA abundance remains stable through fertilization to the blastocysts stage (Winger *et al.* 2006) and promotes differentiation toward the TE by regulating the establishment of cell polarity, repressing position-dependent HIPPO signaling, and activating *Cdx2* expression (Cao *et al.* 2015). SMARCA4 (also known as BRG1) is a key component of a remodeling chromatin protein complex that mediates crucial developmental events such as embryonic genome activation (EGA) and cell differentiation, through both activation and repression of gene expression. In mouse, it has been reported that *Oct4*, *Nanog* and *Sox2* are direct targets of SMARCA4 and *Smarca4* lack of expression impairs embryo implantation (Kidder *et al.* 2009, Choi *et al.* 2013, Carey *et al.* 2015). Since, in cattle, TE confinement seems to be regulated upstream of CDX2, it is likely that in this species SMARCA4 and TFAP2C have a more crucial role in the initial lineage segregation toward ICM and TE.

Based on the above, in this work we applied for the first time the CRISPR-on system to bovine preimplantation embryos for modulation of endogenous expression of *SMARCA4* and *TFAP2C*. Placentation failures are frequently reported for bovine *in vitro* embryo production (Hill *et al.* 2000, Chavatte-Palmer *et al.* 2012) and may be associated with aberrant trophoblast cell proliferation and differentiation during the pre-implantation stage (Knott & Paul 2014, Mainigi *et al.* 2016). Thus, we hypothesized that CRISPR-on may be suitable to induce an activation of *SMARCA4* and/or *TFAP2C* expression which ultimately would promote cell differentiation toward the TE lineage. To achieve this, in an initial experiment, we injected early zygotes with dCas9VP160 mRNA and evaluated dCas9VP160 mRNA stability and translation into protein during embryo development. Subsequently, we employed the CRISPR-on technology for activation of endogenous *SMARCA4* and *TFAP2C*, by simple cytoplasmic injection of dCas9VP160 together with the appropriate sgRNAs. Finally, we assessed the effect of transcriptional activation on preimplantation embryo development and TE proportion at the blastocyst stage. The possibility to precisely modulate the transcription of endogenous genes in a simple and transient fashion opens new and unprecedented possibilities to the study of cell differentiation in species other than mice and also to easily be applied for bovine *in vitro* embryo production programs improvement, since modulation of endogenous gene expression with CRISPR-on is independent of gene editing.

Materials and methods

Experimental design

The experiments described below were conducted to evaluate the potential of CRISPR-on system for modulation of endogenous gene expression in preimplantation bovine embryos:

In an initial experiment, dCas9VP160 mRNA decay and translation were assessed during embryo development. To do this, presumptive zygotes were cytoplasmically injected with dCas9VP160 mRNA and mRNA relative abundance was evaluated 2, 4 and 7 days after microinjection by RT-qPCR. The immunodetection of dCas9VP160 protein in bovine microinjected embryos was also assessed. In a second experiment, CRISPR-mediated activation of *SMARCA4* and *TFAP2C* expression was evaluated. The general scheme of the procedure is shown on Fig. 1A. First, four non-overlapping sgRNA were designed and synthesized targeting either bovine *SMARCA4* or *TFAP2C* proximal promoter region (Fig. 1B). Presumptive zygotes were microinjected with the dCas9VP160 mRNA and appropriated pool of sgRNAs, Table 1 summarizes the groups and microinjection components used. Embryos were *in vitro* cultured until day 2, 4 or 7 and stored for RT-qPCR analysis, as indicated in Fig. 1. RT-qPCR assay was performed to evaluate the expression of the targeted genes and downstream genes. Finally, in a third experiment, the effect of transcriptional modulation over embryo development was assessed by comparing cleavage, morulae and blastocysts rates within groups, at days 2, 5 and 7 of the *in vitro* culture.

Oocyte collection and in vitro maturation

Bovine ovaries were obtained from local slaughterhouses and transported to the laboratory at 25–30°C. Cumulus oocyte complexes (COCs) were aspirated from 2 to 8 mm follicles and collected into TALP-H medium. COCs were cultured for 24 h in 100 µL droplets of complete TCM medium, under mineral oil in 6.5% CO₂ in humidified air at 39°C, for *in vitro* maturation. Complete TCM medium consisted of M-199

(31100-035; Thermo Fisher Scientific), supplemented with 10% FCS (013/07; Internegocios, Mercedes, Buenos Aires, Argentina), 10 mg/mL follicle-stimulating hormone (NIH-FSH-P1, FolltropinR, Bioniche, Caulfield North, Victoria, Australia), 0.3 mM sodium pyruvate (P2256), 100 mM cysteamine (M9768), and 2% antibiotic-antimycotic (ATB, 15240-096; Thermo Fisher Scientific).

In vitro fertilization (IVF) procedure

Frozen sperm straws of bulls with proven fertility were thaw in a 37°C water bath and in Brackett-Oliphant (BO) medium (Brackett & Oliphant 1975). Spermatozoa were then washed twice by centrifugation at 490 g for 5 min to remove cryoprotectants and finally resuspended in BO supplemented with 5 mM caffeine (C4144, SIGMA) and 20 IU/mL heparin (H3149, SIGMA). Spermatozoa were adjusted to 16 × 10⁶/mL with BO containing fatty acid-free BSA (10 mg/mL, A6003 SIGMA). For IVF, 20 to 25 COCs were placed in 100 µL droplets of sperm suspension for 5 h under 5% CO₂ in humidified air. Then, cumulus cells were removed by vortexing the presumptive zygotes for 2 min in hyaluronidase (H-4272) (1 mg/mL in Dulbecco's PBS) and then washed 3 times in TALP-Hepes. Only presumptive zygotes were used for microinjection.

CRISPR-on system

The Zhang Lab CRISPR design tool (<http://crispr.mit.edu>) was used to design four non-overlapping sgRNAs, targeting the proximal promoter region of either *SMARCA4* or *TFAP2C*. For both genes, the regulatory region ranging from –200 to –50 bp upstream of the TSS was considered as the optimal activation window (Fig. 1B, Gilbert et al. 2013, Konermann et al. 2015). In all cases, the design parameters were set to minimize off-target binding sites in the genome. pUC57-sgRNA expression vector was a gift from Xingxu Huang (Addgene plasmid #51132; <http://n2t.net/addgene:51132>; Shen et al. 2014). All sgRNAs were cloned into pUC57-sgRNA

Figure 1 (A) Scheme of general procedure for CRISPR-mediated modulation of gene expression in bovine embryos. Gene expression was assessed by RT-qPCR from pools of 10 embryos at day 2 or day 4 of the *in vitro* culture or 5 blastocysts at day 7. Data sets were analyzed by Mann–Whitney *t*-test. Embryo development was assessed by cleavage, morulae and blastocysts rates at days 2, 5 and 7 of the *in vitro* culture, respectively. Differences were determined by Fisher's exact test. (B) Scheme of *B. taurus* *SMARCA4* and *TFAP2C* regulatory region. Green arrows indicate orientation and position of 4 non-overlapping sgRNAs designed for transcriptional activation of targeted genes. The optimal activation window was considered between –200 and –50 bp upstream of each TSS, indicated in the figure as '+1'.

Table 1 Experimental groups and components of microinjection mix.

	dCas9_SM	dCas9_TF	SHAM
dCas9VP160	+	+	+
SM1, SM2, SM3, SM4	+	–	–
TF1, TF2, TF3, TF4	–	+	–

SM (1, 2, 3 and 4) are sgRNAs targeting *SMARCA4* promoter region. TF (1, 2, 3 and 4) are sgRNAs targeting *TFAP2C* promoter region. Microinjection mix was prepared in PVP.

expression vector and the correct sequence was confirmed by capillary Sanger sequencing (Genomic Unit, Biotechnology Institute, INTA, Hurlingham, Argentina). dCas9 fused to 10 tandem repeats of VP16 domain (dCas9VP160) was used as a programmable transcriptional activator. Plasmid coding for dCas9VP160 was a gift from Rudolf Jaenisch (Addgene plasmid #48240; <http://n2t.net/addgene:48240>; Cheng *et al.* 2013). In order to generate dCas9VP160 mRNA, a pT3-dCas9VP160 PCR product was amplified using a Forward primer containing the T3 sequence (T3_dCas9VP160_f) and a BGH-Reverse primer (1 cycle at 95°C for 5 min, followed by 95°C for 30 s, 58°C for 30 s and 68°C for 5 min, during 35 cycles, and a final extension at 68°C for 5 min). The Q5 High fidelity DNA polymerase (M0491s, NEB) was employed for accurate amplification. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, 28104) and used as template for *in vitro* transcription (IVT) reaction. The sequences of all sgRNAs and primers used for dCas9VP160 amplification are listed in Table 2.

***In vitro* transcription (IVT) of RNAs and injection of bovine zygotes**

For sgRNAs IVT, the MEGAshortscript Kit (AM1354, Ambion) was used, with Dral-digested pUC57-sgRNA plasmids as templates. sgRNAs were purified using the MEGAclear Kit (AM1908, Ambion). For dCas9VP160 IVT, the pT3-dCas9VP160 PCR product was used as template. Capped mRNA was synthesized using mMESSAGE mMACHINE™ T3 Transcription Kit (Ambion, AM1348) and purified with the RNeasy Mini Kit (Qiagen). dCas9VP160 mRNA proper size was confirmed by denaturing gel electrophoresis. dCas9VP160 mRNA (100 ng/μL) and mix of 4 sgRNAs targeting either *SMARCA4* or *TFAP2C* regulatory region (25 ng/μL for each)

were mixed in PVP and microinjected into the cytoplasm of bovine presumptive zygotes.

***In vitro* embryo culture**

Presumptive zygotes were cultured in synthetic oviductal fluid medium (SOF, Holm *et al.* 1999) supplemented with 2.5% FBS at 39°C in humidified gas mixture (5% CO₂, 5% O₂, 90% N₂). Culture medium was renewed on days 2 and 5 of culture. Cleavage was evaluated on day 2 and morulae and blastocyst rate were determined on day 5 and 7, respectively.

RNA isolation and RT-qPCR analysis

Pools of ten day 2 or day 4 embryos or pools of 5 blastocysts were stored at –20°C in RNA later (AM7020, Ambion) until RNA extraction. The Arcturus PicoPure RNA isolation kit (Applied Biosystems) was used for total RNA isolation, according to manufacturer's instruction and eluted in 11 μL. First-strand cDNA was synthesized using random primers and the SuperScript II reverse transcriptase (Life Technologies) with 10 μL of total RNA in a 20-μL final reaction. qPCR analysis was performed with gene-specific primers (Table 3), using SYBR green detection on a StepOne Plus thermocycler (Applied Biosystems). Primers targeting *SMARCA4* promoter were designed from mRNA and genomic sequences obtained from NCBI, using PerlPrimer® Software v1.1.21 (Marshall 2004). At least three biological replicates of IVF were evaluated for all time points assessed, each one including non-injected as well as injected groups. Samples were loaded as duplicates (technical replicates) in a 15 μL reaction, containing 8 μL Sybr green, 1 μL of each primer (20 mmol/μL), 1 μL cDNA template, and 4 μL MilliQ water. Data was analyzed by the $\Delta\Delta$ CT method using the geometric mean of *ACTIN* and *GAPDH* as internal standard. One biological.

Immunocytochemistry (ICC)

Preimplantation stage embryos were analyzed by immunofluorescence to determine dCas9VP160 protein presence 3 days after microinjection. Embryos were fixed in 4% Paraformaldehyde (F-1635, Sigma) in DPBS (14287-072; Gibco) for 20 min and permeabilized in 0.1% Triton-X

Table 2 Sequence of primers used to obtain the dCas9VP160 coding sequence under the T3 promoter and sgRNAs used to target *SMARCA4* and *TFAP2C* proximal promoter regions.

Abrev	Name	Sequence (5'–3')
SM1	sgRNA_SMARCA4_1Fw	TAGGGCGGGGCGCGGGCAGCGTG
SM2	sgRNA_SMARCA4_2Fw	TAGGGAAGCGAGAGAGGGAGTTCC
SM3	sgRNA_SMARCA4_3Fw	TAGGGCACGCGCGCTAGGAGCGGA
SM4	sgRNA_SMARCA4_4Fw	TAGGGTTGTCTGGGAGAGGTGGGT
TF1	sgRNA_TFAP2C_1Fw	TAGGGTCCTTCTCATTAAAGGCTCCC
TF2	sgRNA_TFAP2C_2Fw	TAGGGCTAGGCTGGGACTGGCTGG
TF3	sgRNA_TFAP2C_3Fw	TAGGGCGGAGACCCACGCTGCGTG
TF4	sgRNA_TFAP2C_4Fw	TAGGGTGCGTGGTGAGGGTGACTGA
	T3_dCas9VP160_F ^a	AATTAACCCTCACTAAAGGGAGAcaggtggaccgtgccaccA
	BGH-Rev	TAGAAGGCACAGTCGAGG

^aUppercase letters indicate the T3 minimum promoter sequence.

Table 3 Sequence of primers used for RT-qPCR analysis in bovine embryos.

Gene	Accession number	Primer Sequence (5'–3')		Amplicon (bp)	Reference
		Forward	Reverse		
TFAP2C	NM_001083748	ATTCGCAAAGGTCCTATTTC	TAGATGTAGAGCTGAGGAGGGA	139	Zhenhua <i>et al.</i> 2017
CDX2	AM293662	CGTCTGGAGCTGGAGAAGGA	CGGCCAGTTCGGCTTTC	70	Tripurani <i>et al.</i> 2011
NANOG	-	TCCAGCAAATGCAAGAACTTTC	TTACATTTTCATTCTCTGGTTCTGGAA	88	Wrenzycki <i>et al.</i> 2002
GAPDH	NM_001034034	TTCAACGGCACAGTCAAGG	ACATACTCAGCACCAGCATCAC	119	Khan <i>et al.</i> 2012
ACTB	NM_173979	GACATCCGCAAGGACCTCTA	ACATCTGCTGGAAGGTGGAC	205	Canel <i>et al.</i> 2017
SMARCA4	NM_001105614.1	CTGCAGGAACGGGAATACAG	CTGGAAGTTCAGCAGTCTGAG	140	This work

bp, basepair.

(T-9284) in DPBS for 15 min. Non-specific immunoreactions were blocked by incubation for 1 h in blocking solution (DPBS containing 4% BSA and 0.2% Tween® 20; H5152, Promega). Incubation with Rabbit Anti-HA tag antibody (9110 Abcam) diluted 1:300 in blocking solution, was performed overnight at 4°C. Incubation with the secondary antibody (Goat anti-Rabbit IgG, Invitrogen) was performed for 1 h in the dark, at room temperature. Then, embryos were mounted on glass slides, in 70% v/v glycerol under a cover slip using VECTASHIELD® Mounting Medium (H-1000, Vector Laboratories). Negative controls were generated using only the secondary antibody. For blastocyst staining, embryos were collected 7 days after IVF and ICC was performed as indicated before. To estimate TE proportion in blastocysts, two biological replicates were performed using a ready-to-use Anti-CDX2 antibody (AM392-5M, BioGenex). CDX2⁺ nuclei served as TE indicator, while DAPI staining was used for total nuclei count. The cell counter plugin in Fiji (ImageJ) was used for nuclei counting. Non-injected IVF blastocyst were included as control.

Statistical analysis

Differences on rates of *in vitro* embryo development were determined by Fisher's exact test with a CI of 95%, using the GraphPad Prism, version 5.01 program. Differences were considered statistically significant with a value of $P \leq 0.05$. To avoid misinterpretation due to culture procedure data was also analyzed by ANOVA, considering each biological replicate as a variable. No differences were found between replicates. For qPCR experiments, the statistical analysis was performed using GraphPad prism software version 5.01, using the Mann–Whitney test, with a CI of 95%. For each time point assessed, differences between non-injected control and injected groups were considered statistically significant with a value of $P \leq 0.05$. For mRNA decay, one biological replicate at day 7 was used as reference, to highlight the high variability within the group. For TE nuclei proportion determination one way ANOVA was used and the level of significance was $P < 0.05$.

Results

dCas9VP160 mRNA decay and translation during bovine preimplantation embryo development

First, the dCas9VP160 mRNA stability and its translation into protein were evaluated during *in vitro* embryo

development. With this aim, *in vitro* produced zygotes were cytoplasmically microinjected with dCas9VP160 mRNA, and thereafter, mRNA abundance was assessed by RT-qPCR during embryo development. In early embryos, transcriptional activity is limited until major embryonic genome activation, which occurs at the 8-cell stage in bovine embryos, around day 4 of *in vitro* culture (Barnes & First 1991). For this reason, to ensure CRISPR-on components availability immediately after microinjection, sgRNAs and mRNA coding for dCas9VP160 were *in vitro* synthesized and used for embryo microinjection.

Microinjection of mRNA had no detrimental effect on embryo development and dCas9VP160 transcripts were detected at all preimplantation stages evaluated after microinjection (Fig. 2A). As expected, dCas9VP160 mRNA abundance was significantly lower at the blastocyst stage relative to days 2 and 4 ($p < 0.05$, Fig. 2A). Furthermore, the correct translation and localization of dCas9VP160 protein was confirmed by immunostaining. dCas9VP160 protein showed nuclear localization (Fig. 2B) due to the presence of a NLS at the C-terminal of its amino acid sequence. Consistent with results for mRNA decay, dCas9VP160 protein was not detected at the blastocyst stage (Supplementary Fig. 1, see section on [supplementary materials](#) given at the end of this article). Our results show that dCas9VP160 mRNA abundance is maintained through day 2 and 4 after injection but not at day 7, and mRNA is efficiently translated to protein.

Evaluation of the CRISPR/dCas9VP160 system for SMARCA4 and TFAP2C expression modulation in bovine embryos

Once we verified that the components of the CRISPR-on system were efficiently delivered and dCas9VP160 was effectively translated into protein in early embryos, we next evaluated the suitability of the system for modulation of endogenous expression of SMARCA4 or TFAP2C in bovine preimplantation embryos. Figure 1A illustrates the experimental design.

Presumptive zygotes were cytoplasmically injected with a mix containing dCas9VP160 mRNA and a pool

Figure 2 (A) Decay of the dCas9VP160 mRNA during *in vitro* development. Data is presented as the Ln fold-change value in mRNA abundance for each time point, relative to the amount present at blastocyst stage and normalized to the geometrical mean of Actin and GAPDH. (B) Immunodetection of dCas9VP160 protein in bovine D2 and D4 microinjected embryos. dCas9VP160 protein was detected in all blastomeric nuclei from microinjected embryos at day 2 and 4 of the *in vitro* culture using an anti-HA tag antibody.

of 4 sgRNAs targeting either *SMARCA4* (dCas9_SM group) or *TFAP2C* (dCas9_TF group). The induction and kinetics of CRISPR-on endogenous gene activation were measured by RT-qPCR on days 2, 4 and 7 in pools of embryos. The expression levels of the endogenous target genes were compared to the non-injected control group.

SMARCA4 and *TFAP2C* transcripts were detected at all developmental stages for injected and non-injected groups. Microinjection of CRISPR-on components into one-cell stage embryos triggered a significant increase in *SMARCA4* relative expression at day 2, for the dCas9_SM group. Moreover, *SMARCA4* induction resulted in increased gene expression of *TFAP2C* and other downstream effectors of TE differentiation, such as *CDX2*. This result demonstrates that CRISPR-on can efficiently induce endogenous gene expression in early bovine embryos. However, no significant changes were observed in the endogenous level of *NANOG*. In all cases, gene expression was activated in a transient

fashion, because by day 4 *SMARCA4* expression levels were no longer significantly different relative to the control group (Fig. 3). Regarding dCas9_TF group, CRISPR-on microinjection did not result in statistically significant induction of *TFAP2C*. Nonetheless, the increase in *TFAP2C* was biologically relevant as a significant increase in *CDX2* mRNA abundance was detected on day 2 after injection, similar to that observed for dCas9_SM group. On day 4 after injection *CDX2* expression levels were no longer statistically significant. Altogether, these results demonstrate that CRISPR-on is suitable for transient induction of *SMARCA4* as well as activation of downstream transcriptional targets such as *CDX2* and *TFAP2C*.

Effect of CRISPR/dCas9VP160 mediated *SMARCA4* and *TFAP2C* expression modulation on bovine embryo development

Next, we evaluated the effect of CRISPR-mediated temporal induction of *SMARCA4* on *in vitro* embryo development. Although *TFAP2C* expression was not significantly induced, it did result in a significant induction of *CDX2*; therefore, we also decided to assess *in vitro* embryo development of this group. As before, zygotes were cytoplasmically injected with a mix containing dCas9VP160 mRNA and 4 sgRNAs targeting the regulatory region of either *SMARCA4* or *TFAP2C* and *in vitro* cultured until blastocyst stage. As control (SHAM), a group injected only with dCas9VP160 was included. Results of embryo development are summarized in Table 4. Interestingly, while *SMARCA4* mRNA levels were increased by day 2, embryo cleavage rates were not statistically different from the control group. Instead, an effect of dCas9VP160-mediated induction of *SMARCA4* expression was evident by the morulae stage, where a significantly higher rate was detected for dCas9_SM group respect to both, dCas9_TF group and dCas9VP160 control group. However, this effect was no longer noticeable by the blastocyst stage, where no significant differences were observed between microinjected groups. On the other hand, CRISPR-mediated induction of *TFAP2C*, under the conditions tested, did not affect *in vitro* embryo development.

Furthermore, we evaluated whether the modulation of gene expression affected the proportion of TE cells in blastocyst at day 7 after IVF. TE cells were differentially immunostained using Anti-*CDX2* antibody, while total cell nuclei were labeled using DAPI. Representative examples of differential cell staining are shown in Supplementary Fig. 2A. There was no effect of gene expression modulation on the proportion of *CDX2*⁺ blastomeres in the microinjected embryos, relative to the non-injected control (Supplementary Fig. 2B).

Together, our results suggest that CRISPR-on system had no detrimental effect on embryo development. *SMARCA4* induction of expression early in the

Figure 3 Relative abundance of transcripts in microinjected and control bovine embryos. The relative abundance of *SMARCA4*, *TFAP2C*, *NANOG* and *CDX2* in embryos of the dCas9_SM group (left, striped bars) and dCas9_TF (right, white bars) was evaluated on days 2, 4 and 7 of the *in vitro* culture. The results are expressed as fold change over IVF control embryos (dotted line). *Significant differences with respect to the IVF control, Mann Whitney *t*-test $P < 0.05$.

development improved morulae rates, nonetheless blastocyst rate was not altered.

Discussion

While the CRISPR/Cas9 nuclease system has been shown to be highly efficient for genome editing in embryos of several mammalian species (Mashiko *et al.* 2014, Shao *et al.* 2014, Chen *et al.* 2015, Crispo *et al.* 2015, Wang *et al.* 2015a,b, Bevacqua *et al.* 2016), the application of the CRISPR-on system has thus far been limited to *in vitro* somatic cell cultures. The present work demonstrates for the first time the effectiveness of the CRISPR-on system for activation of endogenous gene expression in preimplantation bovine embryos, by simple cytoplasmic injection of RNA CRISPR components. In particular, we showed that our strategy leads to efficient delivery and

Table 4 *In vitro* development of bovine embryos microinjected with the CRISPR/dCas9VP160 system.

Groups	<i>n</i>	Cleavage	Morulae	Blastocyst (%)
dCas9_SM	186	172 (92) ^a	115 (62) ^a	94 (50.5) ^a
dCas9_TF	172	155 (90) ^a	93 (54) ^b	70 (40.7) ^a
SHAM	62	58 (97) ^a	36 (58) ^b	33 (53.2) ^a

n: number of microinjected presumptive embryos that were *in vitro* cultured. Data from at least three independent replicates.

^{a,b}Different superscripts indicate significant differences (Fisher test; $P < 0.05$).

proper translation of dCas9VP160 mRNA into protein in bovine zygotes. Moreover, dCas9VP160 triggered not only activation of the target gene shortly after microinjection, but also downstream genes. Finally, we confirmed that the CRISPR-on system is suitable for modulation of expression in early preimplantation embryos, while not causing a detrimental effect on development.

In bovine, major EGA takes place at the eight-cell stage (around day four of the IVC, Barnes & First 1991) and therefore, microinjection of CRISPR-on RNA components is required to ensure prompt availability of dCas9VP160 protein immediately after injection. In this regard, in the first experiment we assessed stability of dCas9VP160 mRNA and confirmed that it was correctly processed by the embryo translation machinery. We found that while high levels of mRNA and protein were present both at days 2 and 4; they were dramatically reduced by day 7, probably due to mRNA and protein degradation and dilution due to cell growth and division by the blastocyst stage (Momose *et al.* 1999). It is interesting to note that delivery of CRISPR-on RNA components did not impair embryo development. Therefore, CRISPR-on mediated by mRNA injection constitutes an excellent system for transient activation of endogenous gene expression soon after microinjection of CRISPR-on components.

Once we confirmed that our microinjection strategy ensured proper delivery of CRISPR components and stable translation of dCas9VP160 protein, we evaluated the feasibility of employing this technology for upregulation of endogenous gene expression in embryos. While there is one report on the use of CRISPR-on system in mouse embryos (Cheng *et al.* 2013), it describes the transcriptional activation of a naked episomal reporter gene, which is facilitated by the presence of high number of copies of the reporter gene and avoids the complex natural genomic context of the cell. Williams *et al.* (2018) have reported the successful induction of endogenous gene expression in chick embryos by employing constitutively expression of CRISPR-on components. In this work, we evaluated whether endogenous gene-specific transcriptional activation can be achieved in preimplantation bovine embryos by using RNA CRISPR-on tools. As this approach is independent of gene edition (i.e., transgenesis), this

strategy could be easily translated to bovine *in vitro* embryo production programs.

As a proof of principle, we employed the CRISPR-on system for upregulation of endogenous *SMARCA4* and *TFAP2C* expression, key transcriptional regulators implicated in trophoblast lineage commitment. Previously, it was demonstrated that *SMARCA4*-deficient blastomeres exhibit a preferential commitment to the ICM lineage (Carey *et al.* 2015), while *TFAP2C* overexpression promotes trophoblast differentiation in mouse ESCs (Kuckenberg *et al.* 2010). Thus, CRISPR-mediated transcriptional activation of these or other TF may provide unique opportunities for understanding cell fate differentiation, with implications for *in vitro* embryo production improvement. Furthermore, the temporal expression pattern of *SMARCA4* has not previously been studied in preimplantation bovine embryos, and there is only one report describing the expression profile of *TFAP2C* in bovine embryos produced by IVF (Aston *et al.* 2009). While not the main objective of the current study, future experiments will address whether CRISPR-based gene activation results in the modulation of cell-fate decisions toward ICM or TE.

Consistent with its presumptive role in EGA, and in accordance with the mouse model, *SMARCA4* transcripts were detected at all preimplantation stages in bovine embryos (Bultman *et al.* 2006). Notably, *TFAP2C* transcripts were also detected in embryos at days 2, 4 and 7 of IVC. These results are consistent with *TFAP2C* expression pattern in mouse embryos; however, they differ from those reported by Aston *et al.* for bovine embryos (2009). We confirmed that the CRISPR-on system is efficient to induce *SMARCA4* expression only 2 days after microinjection of dCas9VP160 mRNA and the four sgRNAs targeting *SMARCA4* promoter region (dCas9_SM group). This prompt activation of gene expression suggests that CRISPR-on is capable of overriding the normal timing of genome activation. The CRISPR-on system targeting *TFAP2C* showed only a mild trend to increase *TFAP2C* mRNA levels (dCas9_TF group). This difference may be due to the targeted sequence itself or to inherent properties of the sgRNAs used, as it is well known that both traits critically affect CRISPR efficiency (Chakraborty *et al.* 2014). While achieving a mild induction of expression from the endogenous promoter is desirable for many applications, some purposes might require longer term activation of gene expression than the one reported here. In somatic cells, it was possible to induce the activation of endogenous gene expression for up to 21 days by using covalently close circle DNA (Giménez *et al.* 2016). Future studies will need to address whether this system can be efficiently adapted to embryos.

Importantly, the CRISPR-on system not only resulted in the modulation of its target genes, but also activation of downstream effectors known to play a role in TE maintenance. The observed overexpression of *TFAP2C*

as consequence of *SMARCA4* induction is worth mentioning, since it suggests that both of these TFs act synergistically not only in mice, but also in bovine. The interaction between *SMARCA4* and *TFAP2C* has been reported previously in murine trophoblastic stem cells culture. The authors showed that *TFAP2C* and *SMARCA4* bind simultaneously to the promoter region of more than 600 genes, which suggests a coordinated regulation between these transcription factors (Kidder & Palmer 2010). In addition, *CDX2*, hallmark modulator of TE specification (Aston *et al.* 2010, Choi *et al.* 2012, Goisis & Cibelli 2014), showed increased expression levels on day 2 for both, dCas9_SM and dCas9_TF groups ($P < 0.05$). Jedrusik *et al.* (2008) reported that *CDX2* overexpression in one blastomere from a 2-cell mouse embryo triggers its commitment to the TE lineage. Therefore, the CRISPR-on system may be useful not only for *in vitro* reprogramming of mouse somatic cells toward iPSC (Liu *et al.* 2018) or trophoblast stem cells (Wei *et al.* 2016), but also to arbitrarily manage blastomere commitment in preimplantation embryos toward TE lineage. Further work is needed to optimize the efficiency of CRISPR-on for blastomere reprogramming.

Our data differs from expectations based on mouse reports, since *NANOG* expression was not repressed by *SMARCA4* overexpression. Possibly, increased levels of *NANOG* and other TFs acting opposite to *SMARCA4* and *TFAP2C* were triggered secondarily to *SMARCA4* and *CDX2* overexpression, which could also account for the lack of CRISPR-on effect observed by day 4. However, it is worth noting that our results are consistent with previous findings in bovine where *NANOG* expression is independent from *CDX2*. Several studies reported that bovine *CDX2*-deficient embryos exhibit normal *NANOG* expression during preimplantation development (Berg *et al.* 2011, Goisis & Cibelli 2014, Wu *et al.* 2016), whereas in mice *NANOG* and *CDX2* cross-regulate each other in the developing embryo (Chen *et al.* 2009). Our results, indeed, emphasize the species-specific differences between cattle and mouse during preimplantation development. Thus, the CRISPR-on system could help to better understand the bovine-specific early preimplantation embryonic development.

In our approach, dCas9VP160 was employed as gene activator for precise targeting of the endogenous regulatory regions of *SMARCA4* and *TFAP2C* genes. The first version of CRISPR-based transcriptional activator or CRISPR-on, consisted on the fusion of the dCas9 protein to 4 tandem repeats of VP16 herpes virus activation domain to generate the dCas9VP64 programmable activator (Gilbert *et al.* 2013, Perez-Pinera *et al.* 2013). In mammals, VP64 recruits basal transcription machinery elements to the target promoter thus inducing gene expression (Mittler *et al.* 2003). However, dCas9VP64 system could only achieve moderate transcriptional activation unless multiple non-overlapping guides were

used to synergistically target the same promoter (Cheng *et al.* 2013, Maeder *et al.* 2013, Mali *et al.* 2013, Perez-Pinera *et al.* 2013, Hu *et al.* 2014). With the fusion of 10 or 12 VP16 tandem repeats to dCas9, resulting in dCas9VP160 and dCas9VP192 respectively, the number of sgRNAs needed to induce gene expression was significantly reduced (Cheng *et al.* 2013, Balboa *et al.* 2015, reviewed by Vora *et al.* 2016). In its different versions, CRISPR-on technology provides a feasible approach to induce the expression of coding, non-coding, but also of extremely large genes as well as CG-rich genes for which ORF overexpression is troublesome and time consuming. Moreover, because endogenous expression is induced, natural ratios of different protein isoforms are maintained, opposite as ectopic overexpression assays (Liu *et al.* 2001, reviewed by La Russa & Qi 2015). A main advantage of CRISPR-on technology is that simultaneous activation of multiple genes can be successfully achieved by using the same dCas9-based transcription factor and, furthermore, these tools are easily transferable to different species (Cheng *et al.* 2013).

New versions of CRISPR-on based their design on the combined use of different activation domains and also trans-recruitment strategies (reviewed by Vora *et al.* 2016), achieving an induction up to 300 times stronger than dCas9VP64, known as the standard activator (Tanenbaum *et al.* 2014, Chavez *et al.* 2016; reviewed by Vora *et al.* 2016). However, considering that in cattle blastocyst, CDX2 mRNA is enriched in TE cells by less than 2.4-fold relative to the ICM cells, we presume that dCas9VP160 mediated induction of expression would allow as to more similarly resembling endogenous gene expression as opposed to other CRISPR-on technologies, thus preventing the overload of translational resources or a toxic effect due to stoichiometric imbalance in the embryo (Moriya 2015). Lastly, the need of multiple sgRNA to target a small activation window in a specific locus, together with the mild increase in gene expression induced by dCas9VP160 activator reduces significantly the risk of undesired off target activation (Vora *et al.* 2016).

Finally, we evaluated the effect of the CRISPR-on system on *in vitro* embryo development. Interestingly, while neither cleavage nor blastocyst rates were affected, there was an improvement in development to the morula stage in the CRISPR-on SMARCA4 group. This effect might be a consequence of increased expression of *SMARCA4*, *TFAP2C* and *CDX2* on day 2 of development. Because the CRISPR-on machinery was no longer active by day 7 (experiment 1), and gene expression was already compensated by that stage (experiment 2), it is not surprising that blastocysts rates or TE nuclei proportion were not affected. Although the *in vitro* production of blastocysts was not increased in this work, the ability of microinjected embryos to implant and survive after embryo transfer may be improved,

as it was reported for other strategies including DKK1 treatment (an antagonist of the WNT signaling pathway, shown to direct lineage commitment, Denicol *et al.* 2014) or embryo aggregation (Misica-Turner *et al.* 2007).

Overall, the application of the CRISPR-on system for endogenous gene modulation of early preimplantation embryos is, to our knowledge, unprecedented and could be adapted not only to study mechanisms regulating early cell fate differentiation, but also to improve the outcome of *in vitro* derived embryos, by means of time-restricted overexpression of desired transcription factors.

Concluding remarks

In summary, the present study reports the use of the CRISPR-on system to induce endogenous gene expression in preimplantation bovine embryos, by simple cytoplasmic injection of RNA CRISPR components. dCas9VP160 is efficiently delivered to bovine zygotes and accurately translated into protein, triggering not only target gene expression activation shortly after microinjection, but also other TE-related genes. CRISPR-on system is suitable for its use in embryo preimplantation stages, as it does not cause a detrimental effect on development.

In conclusion, our results demonstrate that the CRISPR-on system can be used to modulate endogenous gene expression in cattle preimplantation embryos. This system may serve as a potential strategy to study early cell-fate decisions and improve TE development in preimplantation embryos.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0517>.

Declaration of interest

Jason Knott is on the editorial board of *Reproduction*. Jason Knott was not involved in the review or editorial process for this paper, on which he is listed as an author. The other authors have nothing to disclose.

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Author contribution statement

V S and R J B took part in conceptualization and study design, performed experiments, data analysis and manuscript writing and editing. V A, N C and L R performed experiments. M I G was involved in RNA synthesis and critical discussions. R F M and D F S took part in conceptualization and study design,

data analysis, critical discussion and manuscript drafting and critical revision. D F S and S F were in charge of financial support and resources provision. J G K was involved in study design and manuscript revision. D F S was in charge of research coordination, supervision and manuscript submission. All the authors revised and accepted the draft version of the manuscript. R J B and D F S are equal authors.

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