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Expression of pluripotency-related genes in goat transgenic embryos having GFP gene targeted at Rosa 26 locus

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Abstract
Transcription activator-like effector nucleases (TALENs) are highly efficient genome-editing tools that facilitate precise site-directed modifications by inducing double-strand breaks in the host genome, enabling targeted gene knock-in or knock-out. The Rosa26 locus has been widely recognized as a safe harbor for gene integration across various species. This study aimed to assess the quantitative expression of pluripotency-related genes in transgenic goat embryos produced via hand-guided cloning, using goat fetal fibroblast cells with a GFP gene integrated at the Rosa26 locus via TALEN-mediated genome editing. Goat fetal fibroblast cells were co-transfected with a donor vector carrying the GFP gene and TALEN expression vectors. Transgenic goat embryos were generated using GFP-expressing transfected cells as nuclear donors for hand-guided cloning. The early-stage developmental rates of transgenic embryos were comparable to those of non-transgenic (control) embryos. qPCR analysis revealed no significant differences ($P<0.05$) in the relative expression of pluripotency markers OCT4 (0.996±0.298 vs. 1.00) and NANOG (0.744±0.354 vs. 1.00) between transgenic and non-transgenic cloned embryos. These findings indicate that TALEN-mediated transfection did not impair the regenerative capacity of donor cells, allowing for normal embryonic development comparable to non-transgenic embryos. Thus, TALENs represent a promising tool for targeted gene insertion in the production of site-specific transgenic embryos.

Keywords: TALEN, Rosa 26, Green Fluorescent Protein, Goat, transgenic embryo

Introduction
Transgenic animals are genetically modified organisms in which a foreign gene, derived from another species, is deliberately inserted into the genome (Li *et al.*, 2024) [10]. Several techniques have been developed for transgene integration; however, in recent years, transcription activator-like effector nucleases (TALENs) have emerged as one of the most versatile and widely used tools for generating transgenic animals (Wani, *et al.*, 2023) [22]. TALENs consist of transcription activator-like effectors (TALEs), a class of specialized effector proteins with a modular DNA-binding domain, flanked by N- and C-terminal regions essential for nuclear localization and transcriptional activation (Boch & Bonas, 2010) [2]. The central DNA-binding domain, which recognizes specific nucleotide sequences (Boch & Bonas, 2010; Bogdanove & Voytas, 2011; Miller *et al.*, 2011) [2, 3, 15], is fused to the non-specific endonuclease FokI, enabling the induction of targeted double-strand breaks (DSBs) in the genome. These DSBs are repaired by cellular mechanisms, facilitating gene knock-in, knock-out, correction, and point mutagenesis.

Not all genomic loci are suitable for transgene integration, as many are susceptible to silencing or can disrupt essential gene functions. However, the Rosa26 locus has been extensively used for site-specific transgene insertion, ensuring stable and consistent gene expression across various species, including mice, humans, rats, cattle, sheep and pigs (Irion *et al.*, 2007; Kasparek *et al.*, 2014; Kong *et al.*, 2014; Li *et al.*, 2014; Remy *et al.*, 2014; Ménoret *et al.*, 2015; Wu *et al.*, 2016; Wang *et al.*, 2018; Xie *et al.*, 2022) [6, 7, 8, 11, 14, 16, 21, 23, 24]. Due to its ability to support sustained gene expression without silencing effects, Rosa26 serves as a preferred locus for targeted genetic modifications. In our previous study, TALEN technology was employed to integrate the GFP gene at the Rosa26 locus in goat fetal fibroblast cells, which were subsequently used as nuclear donors for producing cloned transgenic goat embryos via hand-guided cloning (Vats *et al.*, 2021) [19].

It is well established that the efficiency of transgenic embryo production is generally lower than that of conventional cloned or *in vitro* fertilized (IVF) embryos. This reduction in efficiency is often attributed to the cytotoxic effects of transfection methods, which may impair the viability of donor cells, subsequently affecting embryonic development. Gene expression analysis of key developmental markers, particularly those involved in apoptosis, cellular differentiation, and pluripotency, provides valuable insights into the regeneration potential of transgenic embryos (Vining *et al.*, 2021) [20]. Among these, the pluripotency-associated genes OCT4 and NANOG play a critical role in maintaining embryonic stem cell identity and early embryo development (Kumar *et al.*, 2012; Shah *et al.*, 2015) [9, 17]. These genes are considered master regulators of the transcriptional network governing pluripotency, as they orchestrate key signalling pathways essential for embryonic cell fate determination (Singh *et al.*, 2013) [18]. Disruptions in the expression of OCT4 and NANOG can lead to impaired embryonic development, reduced viability, and early-stage arrest. Therefore, their expression analysis serves as a reliable indicator of the developmental competence and regenerative potential of transgenic embryos.

Considering these factors, the present study aimed to compare the expression of OCT4 and NANOG between transgenic and non-transgenic goat embryos produced through hand-guided cloning. This comparison will help evaluate whether TALEN-mediated gene integration affects the regenerative capacity and developmental potential of cloned transgenic embryos.

Materials and Methods

The present study was conducted at the Embryo Biotechnology Laboratory, Animal Biotechnology Centre, ICAR-National Dairy Research Institute, Karnal, India. All experiments were approved and conducted following the guidelines and regulations set by the Institute Bio-Safety Committee (IBSC/NDRI/Dt.29.04.2014). Goat ovaries were collected from a local abattoir as per the guidelines of the Institute Animal Ethics Committee. All chemicals and media for cell and embryo culture were purchased from Sigma Chemical (St Louis, MO); unless otherwise specified. Molecular biology reagents were obtained from Thermo Fisher Scientific (Waltham, MA, USA), fetal bovine serum (FBS) from HyClone (South Logan, UT), and plastic ware from Nunc (Roskilde, Denmark).

Production of transgenic and non-transgenic embryos

TALEN and donor vectors generated in our previous study were used for generation of transgenic cells (Vats *et al.*, 2021) [19]. These transgenic cells were used as nuclear donor for production of transgenic embryos by hand-guided cloning. Non- transgenic embryos (control) were produced from non-transfected goat fetal fibroblast cells. Recipient oocytes were prepared via *in vitro* maturation, cumulus and zona removal, and manual enucleation following the method used in our previous study (Vats *et al.*, 2021) [19]. GFP-expressing embryos were randomly selected, lysed, and subjected to PCR to detect GFP expression. Single embryo PCR was also performed to screen for correctly targeted integrations in embryos using specific primers as done in our earlier study (Vats *et al.*, 2021) [19].

RNA extraction and complementary DNA (cDNA) preparation

RNA was isolated from a pool of 15 transgenic and non-transgenic cloned embryos using the RNAqueous Micro Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Prior to cDNA synthesis, the RNA was treated with DNase-I (1U) for 15 minutes at 37°C, followed by DNase inactivation. RNA was quantified using a Nanoquant spectrophotometer.

cDNA synthesis from the isolated RNA was performed using the Superscript III First Strand Synthesis Kit (Invitrogen, USA) according to the manufacturer's instructions. The reverse transcriptase (RT) reaction was set up on ice, including a negative RT control to rule out genomic DNA contamination. After reverse transcription, PCR was performed to amplify the desired gene cDNA, using the negative RT reaction product as a template (RT-ve control) and a PCR-negative control for quality assurance.

The RT reaction was prepared by mixing RNA (2 µl), oligo (dT) (1 µl), 10 mM dNTPs (1 µl), and DEPC-treated water (6 µl). This mixture was incubated at 65°C for 5 minutes, then chilled on ice. A second mixture containing 10x RT buffer (2.00 µl), 25 mM MgCl2 (4.00 µl), 0.1M DTT (2.00 µl), RNase OUT (1.00 µl), and Superscript III RT (1.00 µl) was prepared and combined with the first mixture. The combined mixture was incubated in a thermal cycler at 50°C for 50 minutes, then at 85°C for 5 minutes. After adding 1 µl of RNase H, the mixture was incubated at 37°C for 20 minutes. The resulting cDNA was stored at -20°C until further use.

Comparative gene expression analysis by Real-time PCR

Real-time PCR was performed on CFX96 system (Bio-Rad) using SYBR green (double-stranded DNA-specific fluorescent dye) master mix from Fermentas. The reaction was performed in 96-well tubes for real time in a final volume of 10 µL. Equal amount of transcribed RNA was used in each group study. The cDNA templates were added to SYBR green reaction mix in triplicate along with two NTC (No template control). The reaction was set in a final volume of 10 µL having 2X SYBR green mix (5.0 µl), 10 µM Primer mix (1.0 µl), Template (1.0 µl) and Nuclease free water (3.0 µl). The reaction mixture was transferred to 96-well tubes and placed on the real-time machine following the program- initial denaturing of 5 min at 95°C; followed by 39 PCR cycles (denaturing: 95°C for 15 sec; annealing temperature: 58/60°C for 30 sec; extension: 72°C for 30 sec). Melt curve was set at 65°C to 95°C; increment 0.5°C for 0.05s. At the end of the reaction, the dissociation cycle was run to check the specificity of the amplification reaction. The list of primer sequences used is given in Table 1.

Melting peaks were determined using melting curve analysis in order to ensure the specific amplification. The relative quantification of gene expression was conducted using the method described by Livak and Schmittgen (2001) [12]. GAPDH mRNA was employed as an internal standard for the analysis of relative transcript levels of each gene and in negative controls, H_2O replaced cDNA in the reaction tubes. For comparison, the average expression level of each gene from control group of embryos was set as 1. Three separate experiments were performed with three replicates for each gene.

Statistical Analysis

Analysis was performed using Graphpad PRISM 7 software by one way analysis of variance (ANOVA) to compare the means of different groups followed by arcsine transformation. The differences were considered to be statistically significant at $p<0.05$. Data are presented as mean±SEM.

Results

Generation of transgenic cells

TALEN and donor vectors (Figure 1) were co-transfected into established female GFF cells. The transfected cells were observed under fluorescence microscope to detect GFP expression (Figure 2) and were enriched by antibiotic selection and low density dilution. GFP gene integration at the specified locus and its expression was analysed by 5' and 3' junction PCR.

Transgenic and Nontransgenic Embryo Production

The correctly targeted cells were used as nuclear donor for hand guided cloning and produced cloned goat embryos expressing GFP (Figure 3). The developmental rate of cloned embryos produced from transgenic and nontransgenic cells was compared and the percentage of reconstructs that developed to blastocyst was significantly lower ($p<0.05$) for cloned embryos produced using transgenic cells than those produced from nontransgenic cells (7.84±0.68% vs. 3.92±1.12%) while the cleavage rate was not significantly different in both the groups.

Gene expression in transgenic and nontransgenic embryos

The expression levels of key pluripotency-related genes were analyzed in both transgenic and non-transgenic embryos to assess whether TALEN-mediated gene integration affected early embryonic development. Quantitative PCR (qPCR) analysis revealed no statistically significant difference ($p>0.05$) in the relative expression levels of OCT4 (0.996±0.298) and NANOG (0.744±0.854) between transgenic and non-transgenic cloned embryos (Figure 4).

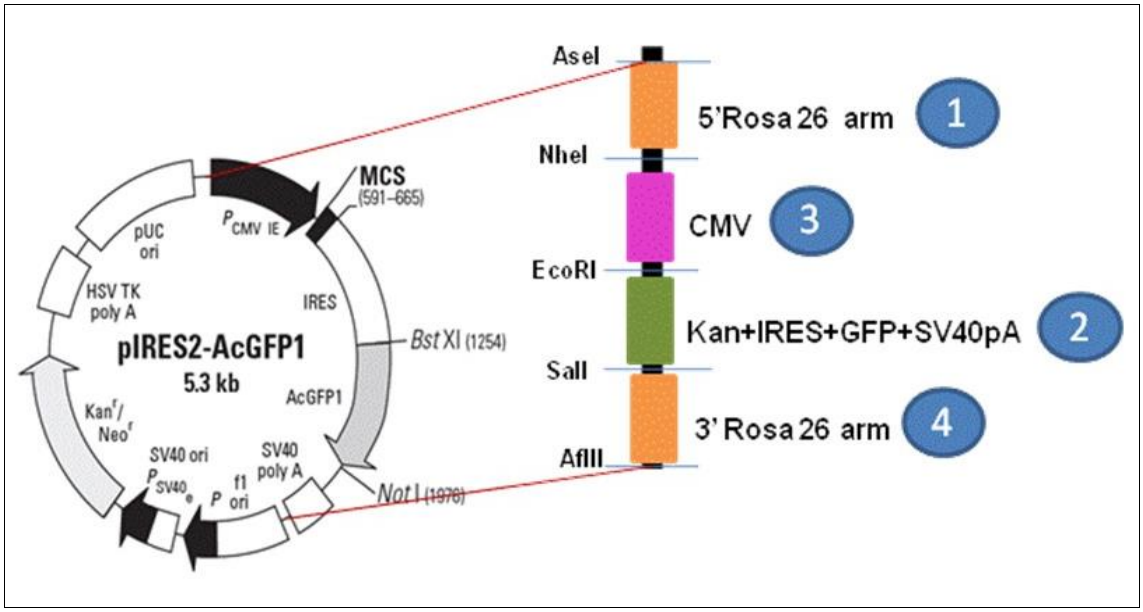

Fig 1. Donor vector P5'KC3'containing 5' Rosa 26 arm, KAN-IRES-GFP-SV40pA, CMV and 3' Rosa 26 arm into a pIRES2-AcGFP1 plasmid. The numbers indicate the sequential order in which the fragments were ligated into the plasmid.

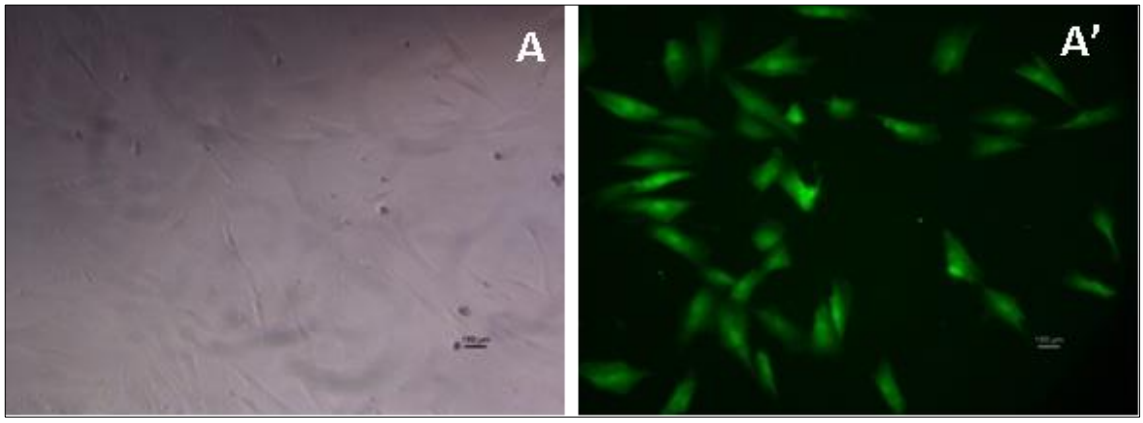

Fig 2: Co-transfection of TALEN and donor vector into GFF by nucleofection A) under bright light, A') under fluorescence, (200 X; 100 µm)

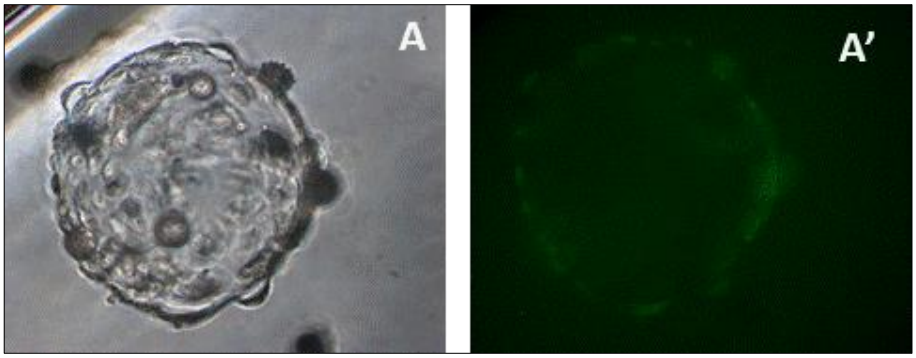

Fig 3: Transgenic cloned goat embryos expressing GFP gene under Bright light (A); Blue light (A') (Magnification: 200X)

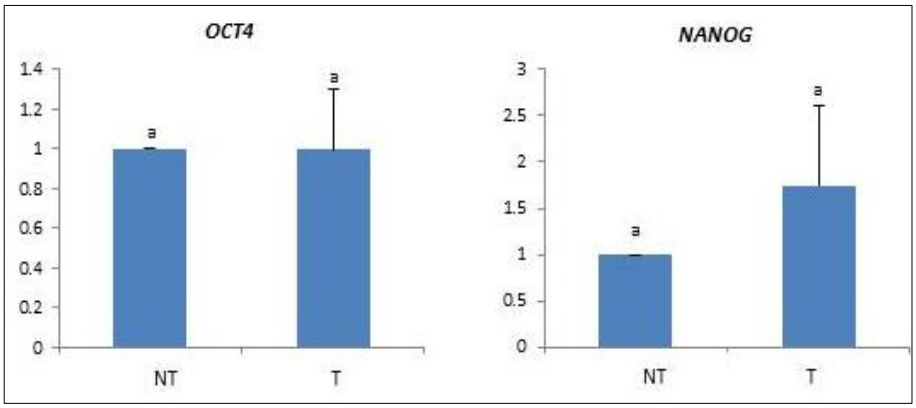

Fig 4: Relative gene expression study related to pluripotency in non-transgenic (NT) and transgenic (T) embryos by qPCR, (P<0.05)

Table 1: Primers for pluripotency related genes

Gene	Sequence	Annealing temp (°C)	Product size	Accession ID
NANOG	F-GGAATCTTCACCCATGCCTG R-GACCTTCTCTTCCTTCTCTGTG	58	198bp	JF923884
OCT4	F-TACTCTTCGGTCCCATTCCC R-CCAAAGCCCTAGTACAAACCC	60	165 bp	NM_001285590.1
GAPDH	F- CTCCCAACGTGTCTGTTGTG R- CCCAGCATCGAAGGTAGAAG	60	185bp	GU324291.1

Discussion

Transgene integration at a safe locus using site-specific nucleases is crucial for generating genetically modified animals for agricultural and biomedical applications (Yamashita *et al.*, 2023) [25]. While this strategy is well established in mice, rats, humans, pigs, and cattle (Irion *et al.*, 2007; Kasparek *et al.*, 2014; Li *et al.*, 2014; Remy *et al.*, 2014; Ménoret *et al.*, 2015; Xie *et al.*, 2022) [6, 7, 11, 14, 16, 24], its potential for stable transgenesis in goats remains underexplored. In this study, we successfully produced transgenic goat embryos with GFP integrated at the Rosa26 locus using TALENs and hand-guided cloning and conducted a comparative analysis of pluripotency gene expression between transgenic and non-transgenic embryos.

TALENs have demonstrated significant efficacy in transgenesis due to their high targeting specificity, reduced cytotoxicity, and minimal off-target effects (González *et al.*, 2021) [5]. Our results indicate no significant differences in developmental competence and the expression of pluripotency-related genes OCT4 and NANOG between transgenic and non-transgenic cloned embryos. This suggests that TALEN mediated GFP integration at the Rosa26 locus did not alter pluripotency marker expression. This observation is consistent with prior studies which have demonstrated that the maintenance of pluripotency is not significantly impacted by the integration of exogenous genes, as long as key regulatory pathways remain intact (Avilion *et al.*, 2003; Boyer *et al.*, 2005; Yin *et al.*, 2020) [1, 4, 26].

A previous study has showed that OCT4 and NANOG expression levels were lower in cloned embryos (both transgenic and non-transgenic) compared to IVF-derived embryos, consistent with the expected effects of nuclear reprogramming in SCNT embryos. OCT4 levels remained unchanged and NANOG expression was slightly lower in transgenic embryos as compared to non-transgenic embryos (Mehta *et al.*, 2019) [13]. Additionally, they have also reported, reduced developmental potential, increased apoptosis, and altered gene expression in transgenic SCNT embryos, which could be due to random transgene insertion and prolonged G-418 selection during donor cell culture (Mehta *et al.*, 2019) [13]. These variations may be attributed to differences between site-directed and random transgene integration.

As previously established, OCT4 and NANOG play essential roles in sustaining early embryonic development (Kumar *et al.*, 2012; Singh *et al.*, 2013; Shah *et al.*, 2015) [9, 17, 18], and the stable expression of these genes in our study suggests that transgenic embryos retain their developmental potential, which is crucial for successful transgenic animal production. The comparable developmental competence and pluripotency gene expression observed in our study underscore the benefits of precise site-directed transgenesis in maintaining normal gene expression and embryo quality. However, further analysis of additional developmental and

apoptotic markers is necessary to fully assess the impact of TALEN-mediated transgenesis on embryo viability and long-term developmental potential.

Overall, our findings support the use of TALENs as a reliable and efficient genome-editing tool for targeted transgene insertion in livestock species, particularly at safe harbor loci such as Rosa26. These results contribute to the advancement of precise genetic modifications in goats, with promising implications for biopharmaceutical production, disease modeling, and genetic improvement in livestock breeding.

Conclusion

We successfully produced transgenic cloned goat embryos with the GFP gene integrated at the Rosa 26 locus and conducted a comparative analysis of their developmental competence and gene expression relative to non-transgenic embryos. Maintenance of pluripotency, as indicated by stable OCT4 and NANOG expression levels, helped in sustaining embryonic development. The present study reinforces the suitability of TALEN for precise and efficient genome modifications without adversely affecting early embryogenesis.

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