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Title:

# Effects of voltage strength during electropolation on the development and quality of *in vitro*-produced porcine embryos

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*Running title:*

Electroporation conditions and embryonic development

**Contents**

This study was conducted to determine suitable conditions for an experimental method in which the CRISPR/Cas9 system is introduced into *in vitr*o-produced porcine zygotes by electroporation. In the first experiment, when putative zygotes derived from *in vitro* fertilization (IVF) were electroporated by either unipolar or bipolar pulses, keeping the voltage, pulse duration, and pulse number fixed at 30 V/mm, 1 msec, and five repeats, respectively, the rate of blastocyst formation from zygotes electroporated by bipolar pulses decreased compared to zygotes electroporated by unipolar pulses. In the second experiment, the putative zygotes were electroporated by electroporation voltages ranging from 20 V/mm – 40 V/mm with five 1-msec unipolar pulses. The rate of cleavage and blastocyst formation of zygotes electroporated at 40 V/mm was significantly lower ($p < 0.05$) than that of zygotes electroporated at less than 30 V/mm. Moreover, the apoptotic nuclei indices of blastocysts derived from zygotes electroporated by voltages greater than 30 V/mm significantly increased compared with those from zygotes electroporated by voltages less than 25 V/mm ($p < 0.05$). When zygotes were electroporated with Cas9 mRNA and single-guide RNA (sgRNA) targeting site in the *FGF10* exon 3, the proportions of blastocysts with targeted genomic sequences were 7.7% (2/26) and 3.6% (1/28) in the embryos derived from zygotes electroporated at 25 V/mm and 30 V/mm, respectively. Our results indicate that electroporation at 25 V/mm may be an acceptable condition for introducing *Cas9* mRNA and sgRNA into pig IVF zygotes under which the viability of the embryos is not significantly affected.

**Keywords:** CRISPR/Cas9, electroporation, genome editing, *in vitro* fertilization, pig

## 1 INTRODUCTION

The recently developed clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) 9 system has enabled high-efficiency genome modification in animal cells/embryos including site-specific modifications and gene knock-ins and knockouts (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013). Using CRISPR/Cas9, efficient gene targeting has been achieved in mice, rats, and monkeys via the co-injection of zygotes with Cas9 mRNA and single-guide RNA (Ma et al., 2014; Niu et al., 2014; Yasue et al., 2014), and this strategy has been applied to gene targeting in pigs (Wang et al., 2015). However, the microinjection of CRISPR/Cas9 system into zygotes requires a high level of skill, is time-consuming and may cause damage to embryos. Thus, the widespread production of gene-modified pigs may remain limited due to the use of micromanipulator systems for the microinjection of endonucleases into the cytoplasm of zygotes (Fan & Lai, 2013). Recently, we established the GEEP (gene editing by electroporation of Cas9 protein) method (Tanihara et al., 2016), a method in which the CRISPR/Cas9 system is introduced into porcine zygotes by electroporation, which leads to high-efficiency disruption of the targeted gene. Previous studies have also reported the generation of knockout animals (mice and rats) by introducing the CRISPR/Cas9 system into intact zygotes using a similar electroporation method (Hashimoto & Takemoto, 2015; Kaneko & Mashimo, 2015; Kaneko et al., 2014). Kaneko and Mashimo (2015) have suggested that the pulse polarity affects the success rate of transferring mRNA into intact zygotes. In a previous study, we demonstrated that when the presumptive zygotes were electroporated with Cas9 mRNA and single-guide RNA (sgRNA) targeting the *FGF10* gene, the frequency of base insertions or deletions (indels) in the targeted gene and blastocyst formation rates were influenced by electroporation conditions such as duration and number of pulses (Tanihara et al., 2016). However, information on the conditions

suitable for the introduction of the CRISPR/Cas9 system into intact embryos of pigs and other species by electroporation is limited.

To clarify suitable conditions for electroporation, we investigated the effects of pulse polarity and voltage on the development and quality of *in vitro*-produced porcine embryos. We then confirmed whether the selected conditions could be used to edit the *FGF10* gene in porcine embryos.

## 2 MATERIALS AND METHODS

There were no live animals used in this study, so no ethical approval was required.

### 2.1 Oocyte collection, *in vitro* maturation and fertilization

Pig ovaries were obtained from prepubertal crossbred gilts (Landrace × Large White × Duroc breeds) at a local slaughterhouse. Cumulus-oocyte complexes (COCs) with a uniform ooplasm and compact cumulus cell mass were collected from follicles 2–6 mm in diameter; the COCs were cultured in maturation medium at 39°C in a humidified incubator containing 5% $CO_2$ as described previously, with minor modifications (Do et al., 2015). The maturation medium consisted of 25 mM HEPES tissue culture medium 199 with Earle's salts (TCM 199; Invitrogen Co., Carlsbad, CA, USA) supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma-Aldrich, St. Louis, MO, USA), 50 μM sodium pyruvate (Sigma-Aldrich), 2 mg/ml D-sorbitol (Wako Pure Chemical Industries Ltd., Osaka, Japan), 1 μg/ml 17 β-estradiol (Sigma-Aldrich), 10 IU/ml equine chorionic gonadotropin (Kyoritu Seiyaku, Tokyo, Japan), 10 IU/ml human chorionic gonadotropin (Kyoritu Seiyaku), and 50 μg/ml gentamicin (Sigma-Aldrich). After maturation for 20–22 h, the COCs were cultured for 24 h in maturation medium without hormones.

97 The matured oocytes were subjected to *in vitro* fertilization (IVF), as described
98 previously (Do et al., 2015). Briefly, frozen-thawed spermatozoa were transferred into 6
99 ml of porcine fertilization medium (PFM; Research Institute for the Functional Peptides
100 Co., Yamagata, Japan) and washed by centrifuging at 500 × *g* for 5 min. The pelleted
101 spermatozoa were resuspended in PFM and adjusted to $5 \times 10^6$ cells/ml. Next, COCs were
102 transferred to the sperm-containing PFM and co-incubated for 12 h at 39°C under 5%
103 $CO_2$ and 5% $O_2$. After co-incubation, the putative zygotes were denuded from the
104 cumulus cells and the attached spermatozoa by mechanical pipetting.
105
106 **2.2 Preparation of sgRNA targeting *FGF10*, and *Cas9* mRNA**
107 We introduced Cas9 mRNA and sgRNA targeting *Fgf10*, which was previously
108 transferred into eggs by the electropolation method (Tanihara et al., 2016) and elicited the
109 limbless phenotype (Hashimoto & Takemoto 2015). pDR274 plasmids carrying target
110 sequences were constructed by inserting annealed oligos into the BsaI site. The oligos (Fwd:
111 5′-TAGGAAAAGGAGCTCCCAGGAG-3′; and Rev: 5′-
112 AAACCTCCTGGGAGCTCCTTTT -3′) were purchased from Sigma-Aldrich. After DraI
113 digestion, sgRNAs were synthesized using the MEGAshortscript T7 Transcription Kit
114 (Ambion, Austin, TX, USA) and then purified by phenol-chloroform-isoamylalcohol
115 extraction and isopropanol precipitation. The precipitated RNA was dissolved in Opti-
116 MEM I (Life Technologies, Gaithersburg, MD, USA). The RNAs were quantified by
117 absorption spectroscopy and agarose gel electrophoresis and were stored at -30˚C until
118 use. *Cas9* mRNA was prepared as described previously (Hashimoto & Takemoto 2015).
119
120 **2.3 Electroporation and embryo culture**
121 Electroporation was performed 13 h after the initiation of IVF as described
122 previously (Tanihara et al., 2016). Briefly, an electrode (LF501PT1-20; BEX, Tokyo,

Japan) was connected to a CUY21EDIT II electroporator (BEX) and placed under a stereoscopic microscope. The putative zygotes (approximately 30 – 40 zygotes) were washed with Opti-MEM I solution and placed in a line in the electrode gap, in a chamber slide filled with 10 μL of Opti-MEM I solution with or without sgRNA and Cas9 mRNA. After electroporation, the zygotes were washed with pig zygote medium (PZM-5; Research Institute for the Functional Peptides Co.) and cultured for 3 days. Embryos cultured for 3 days were subsequently incubated in porcine blastocyst medium (PBM; Research Institute for the Functional Peptides Co.) for 4 days. As a control, some zygotes were cultured with PZM-5 and PBM for 7 days without performing electroporation.

**2.4 Assessment of blastocyst quality**

To evaluate the total cell number and existence of apoptosis in the blastocysts, the blastocysts were fixed on day 7 (day 0; insemination) and were analysed using a combined technique for simultaneous nuclear staining and terminal deoxynucleotidyl transferase nick-end labelling (TUNEL) modified from previously described procedures (Otoi et al., 1999). Briefly, blastocysts were fixed overnight at 4°C in 3.7% (w/v) paraformaldehyde diluted in PBS. After fixation, the blastocysts were permeabilized in PBS containing 0.1% (v/v) Triton-X100 for 40 min. The blastocysts were subsequently incubated overnight at 4°C in PBS containing 10 mg/ml bovine serum albumin (blocking solution) and then incubated in fluorescein-conjugated 2-deoxyuridine 5-triphosphate and terminal deoxynucleotidyl transferase (TUNEL reagent; Roche Diagnostics Co., Tokyo, Japan) for 1 h at 38.5°C. After TUNEL staining, the embryos were counterstained with 1 μg/ml DAPI (Invitrogen Co., Carlsbad, CA, USA) for 10 min and then treated with an anti-bleaching solution (Slow-Fade; Molecular Probes Inc., Eugene, OR, USA), mounted on glass slides and sealed with clear nail polish. Labelled blastocysts were examined using an epifluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan). Apoptotic

nuclei exhibited condensed and fragmented morphology (Brison & Schultz, 1997). The apoptotic index was calculated by dividing the number of cells containing apoptotic nuclei (labelled by TUNEL) by the total number of cells.

**2.5 Analysis of targeted genes after electroporation**

Genomic DNA was isolated by boiling individual blastocysts in 50 mM NaOH solution. After neutralization, the genomic regions flanking the sgRNA target sequences were PCR-amplified using specific primers (Fwd: 5′-CCATCCCATTTGATCTGCTT-3′; and Rev: 5′-CTTCAACTGGCAGCACAATG-3′). The PCR products were extracted using agarose-gel electrophoresis and the targeted genomic regions were sequenced. Sequencing was performed using a BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Thermo Fisher Scientific, Waltham, MA, USA) and an ABI 3500 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

**2.6 Experimental design**

In the first experiment, we examined the effect of pulse polarity with unipolar and bipolar pulses on the development of porcine embryos. Putative zygotes were placed in Opti-MEM I solution without sgRNA and Cas9 mRNA and were electroporated by either unipolar or bipolar pulses, keeping the voltage, pulse duration and pulse number fixed at 30 V/mm, 1 msec and five repeats, respectively.

In the second experiment, we tested the effect of electroporation voltages on the development and quality of porcine embryos. In the first experiment, the unipolar pulse was better than the bipolar pulse for the development of embryos. Thus, putative zygotes were electroporated in Opti-MEM I solution without sgRNA and Cas9 mRNA by electroporation voltages ranging from 20 V/mm – 40 V/mm with five 1-msec unipolar pulses.

175 The electroporation voltage found to be most suitable for the development and
176 quality of embryos in the second experiment was 25 V/mm, but the frequency of base
177 insertions or deletions (indels) in the target gene after introducing the CRISPR-Cas9
178 system into zygotes remained unclear. In the third experiment, we used two
179 electroporation voltages (25 V/mm and 30 V/mm) to compare the efficiency of genome
180 editing in porcine zygotes. The putative zygotes were electroporated with 400 ng/μl of
181 *Cas9* mRNA and 200 ng/μl of sgRNA targeting the *FGF10* gene (Sekine et al., 1999) by
182 electroporation at 25 V/mm and 30 V/mm with five 1-msec unipolar pulses. The
183 electroporated zygotes were cultured for 7 days until blastocyst formation. The
184 frequencies of base insertions or deletions (indels) in the *FGF10* gene of individual
185 blastocysts derived from zygotes electroporated at 25 V/mm (26 embryos) and 30 V/mm
186 (28 embryos) were analysed.
187
188 **2.7 Statistical analysis**
189 Statistical significance was inferred from analysis of variance (ANOVA) tests
190 followed by Fisher's protected least significant difference (PLSD) tests using STATVIEW
191 (Abacus Concepts, Inc., Berkeley, CA, USA). All percentage data were subjected to
192 arcsin transformation before statistical analysis. Differences with a probability value ($p$)
193 of 0.05 or less were regarded as significant.
194
195 **3 RESULTS**
196
197 As shown in Fig. 1, when putative zygotes were electroporated by either unipolar or
198 bipolar pulses, cleavage rates did not differ among the groups. However, the rate of
199 blastocyst formation from zygotes electroporated by bipolar pulses was significantly
200 lower ($p < 0.05$) than from zygotes electroporated by unipolar pulses. The rates of

blastocyst formation from electroporated zygotes decreased compared with control zygotes cultured without electroporation, irrespective of pulse polarity.

As shown in Table 1, when putative zygotes were electroporated by electroporation voltages ranging from 20 V/mm – 40 V/mm, the rate of cleavage and blastocyst formation of zygotes electroporated at 40 V/mm was significantly lower ($p < 0.05$) than that of zygotes electroporated at less than 30 V/mm. Moreover, the apoptotic nuclei indices of embryos derived from zygotes electroporated at voltages greater than 30 V/mm significantly increased compared with those from zygotes electroporated at voltages less than 25 V/mm ($p < 0.05$). The apoptotic nuclei indices of embryos from electroporated zygotes increased compared with embryos from control zygotes cultured without electroporation, irrespective of the voltage used.

When putative zygotes were electroporated with Cas9 mRNA and sgRNA targeting site in *FGF10* exon 3, the proportions of blastocysts with targeted genomic sequences were 7.7% (2/26) and 3.6% (1/28) in embryos derived from zygotes electroporated at 25 V/mm and 30 V/mm, respectively. All mutated blastocysts (3 embryos) carried wild-type sequences at variable ratios (Fig. 2).

## 4 DISCUSSION

Electroporation, which permeabilizes the plasma membrane with an electric pulse, can deliver exogenous molecules into cells (Mir, 2001). However, the method has been limited by low gene transfer efficiency compared with viruses and their transient gene expression (Nishikawa & Huang, 2001). As a function of the field strength and duration, the permeabilization of the cell membrane can be reversible or irreversible. Irreversible electroporation occurs if the cell cannot recover from the membrane disruption (Davalos et al., 2005). Therefore, the high levels of cell damage incurred by electroporation must

be considered to obtain successful transfection efficiency. Changing pulse polarity may increase permeabilized membrane area and, consequently, increase gene expression (Faurie et al., 2004). Tekle et al. (1991) reported that the efficiency of DNA transfection *in vitro* was significantly higher when using bipolar pulses than when using unipolar pulses. A bipolar pulse is a sequence of two consecutive, oppositely polarized unipolar pulses. If the survival rate of embryos electroporated by bipolar pulses is similar to the survival rate of embryos electroporated by unipolar pulses, permeabilization could be expected to achieve increased molecular uptake. In the present study, however, we found that the development of zygotes electroporated by bipolar pulses decreased compared with those electroporated by unipolar pulses. Kotnik et al. (2001) reported that pulse strengths were lower when using bipolar pulses. Under our conditions, the same voltage, pulse duration and pulse number were used for electroporation. Therefore, the decreased development of zygotes electroporated by bipolar pulses might result from damage induced by higher pulse strengths.

While high levels of gene expression are required following transfection by electroporation, it is also desirable to minimize damage to embryos. Therefore, optimizing electroporation conditions have become a key factor affecting the development and quality of embryos. In a previous study, we reported that the frequency of indels increased with increasing pulse duration and number, whereas blastocyst formation rates markedly decreased (Tanihara et al., 2016). Moreover, the optimal duration and number of pulses were 1-ms and five, respectively, for introducing *Cas9* mRNA and sgRNA into pig IVF zygotes. However, the optimal voltage to use for electroporation has remained unclear. In the present study, zygotes were electroporated using electroporation voltages ranging from 20 V/mm – 40 V/mm with five 1-msec unipolar pulses. We found that 25 V/mm was most suitable for embryo development and quality, although the apoptotic nuclei indices of electroporated embryos were higher than

253 those of control embryos. Moreover, when zygotes were electroporated with Cas9 mRNA
254 and sgRNA targeting site in *FGF10* exon 3 at 25 V/mm and 30 V/mm, 25 V/mm resulted
255 in a high genome editing efficiency in the resulting blastocysts. Although we succeeded
256 in introducing indels into the porcine embryos, the mutation frequency was lower than
257 that observed in mice (Hashimoto & Takemoto 2015). Our results showed that all mutated
258 blastocysts carried wild-type sequences. These results indicate that electroporation at 25
259 V/mm may be an acceptable condition for introducing *Cas9* mRNA and sgRNA into pig
260 IVF zygotes, but further studies are necessary to achieve higher genome editing in porcine
261 embryos using the electroporation.
262 In conclusion, our results demonstrate that bipolar pulses have a detrimental effect
263 on the development of zygotes electroporated under our study conditions. Moreover,
264 when using five 1-msec unipolar pulses, electroporation at 25 V/mm is suitable for
265 introducing the CRISPR/Cas9 system into pig IVF zygotes.
266

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## CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

## AUTHOR CONTRIBUTIONS

All authors contributed to the work described in the paper and all take responsibility for it. Drs. K. Nishio, F. Tanihara, T-V. Nguyen, T. Kunihara、M. Nii, T. Takemoto, and M. Hirata, as co-authors, made significant contributions to the conception and design of experiments, and the analysis and interpretation of data. Dr. T. Otoi, as a corresponding author, participated in drafting the article or reviewing and/or revising its contents.

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FIGURE LEGENDS

**Fig. 1.** Efficiency of unipolar and bipolar rectangular electroporation pulses on the development of porcine embryos. Zygotes were electroporated using unipolar (549 zygotes) and bipolar (520 zygotes) pulses, using five 1-msec pulses at 30 V/mm. As a control, a set of zygotes (531 zygotes) was cultured without electroporation. Eleven replicates were analysed per treatment group. $^{a\text{-}c}$Bars with different letters differ significantly ($p < 0.05$).

**Fig. 2.** Representative genomic sequences of porcine blastocysts formed after zygote electroporation with Cas9 mRNA and *FGF10* sgRNA at 25 V/mm and 30 V/mm showing wild type (WT) and mutated type (25V and 30V). There were more than two peaks in the mutated embryos, indicating that *FGF10* mutation (deletion or/and insertion) had occurred. The arrowhead indicates the Cas9 cleavage site.

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Table 1. Effects of voltage strength on the development and quality of embryos electroporated after in vitro fertilization*

| Voltage strength (V/mm) ** | No. of oocytes examined | No. (%) of embryos | | Total cell number in blastocyst | Apoptotic nucleus index*** |
|---|---|---|---|---|---|
| | | cleaved | developed to blastocysts | | |
| Control | 125 | 108 (86.4 ± 1.6)[a,b] | 33 (26.4 ± 2.8)[a] | 51.1 ± 2.7[a] | 3.0 ± 0.4[a] |
| 20 | 115 | 105 (91.4 ± 2.1)[a] | 24 (21.0 ± 5.4)[a,b] | 47.5 ± 4.6[a] | 6.6 ± 0.6[b] |
| 25 | 121 | 108 (89.4 ± 2.3)[a] | 31 (25.8 ± 6.3)[a,b] | 49.4 ± 4.5[a] | 6.4 ± 0.6[b] |
| 30 | 127 | 103 (82.4 ± 7.2)[a,b] | 21 (17.0 ± 3.8)[a,b] | 46.7 ± 3.7[a] | 10.6 ± 1.1[c] |
| 35 | 118 | 83 (70.2 ± 6.0)[b,c] | 17 (14.3 ± 2.2)[b,c] | 33.4 ± 2.5[b] | 16.1 ± 1.8[d] |
| 40 | 121 | 74 (61.1 ± 9.3)[c] | 5 (4.1 ± 1.6)[c] | 36.4 ± 3.4[a,b] | 16.9 ± 1.3[d] |

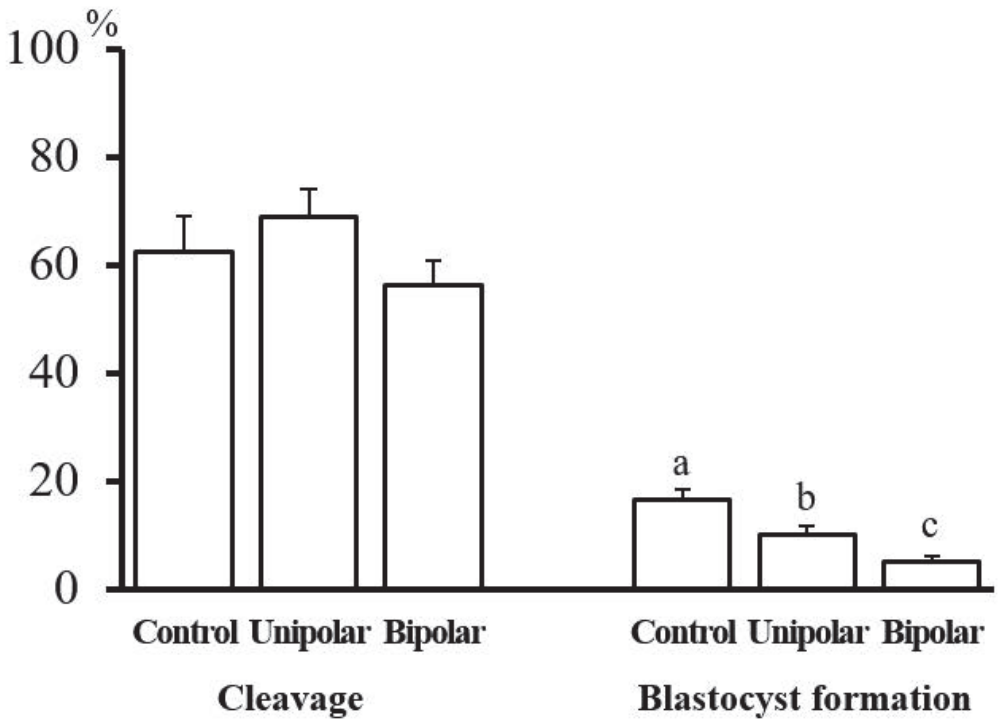
*Four replicate trials were carried out. Data are expressed as mean ± SEM.

**Electroporation was performed by five 1-ms pulses at various voltages. As control, the zygotes were cultured without performing electroporation.

***The apoptotic index was defined as the ratio of the number of cells containing apoptotic nucleus and the total number of cells in a blastocyst.

[a-d] Values with different superscripts in the same column are significantly different ($P < 0.01$).

Figure 1


100 %
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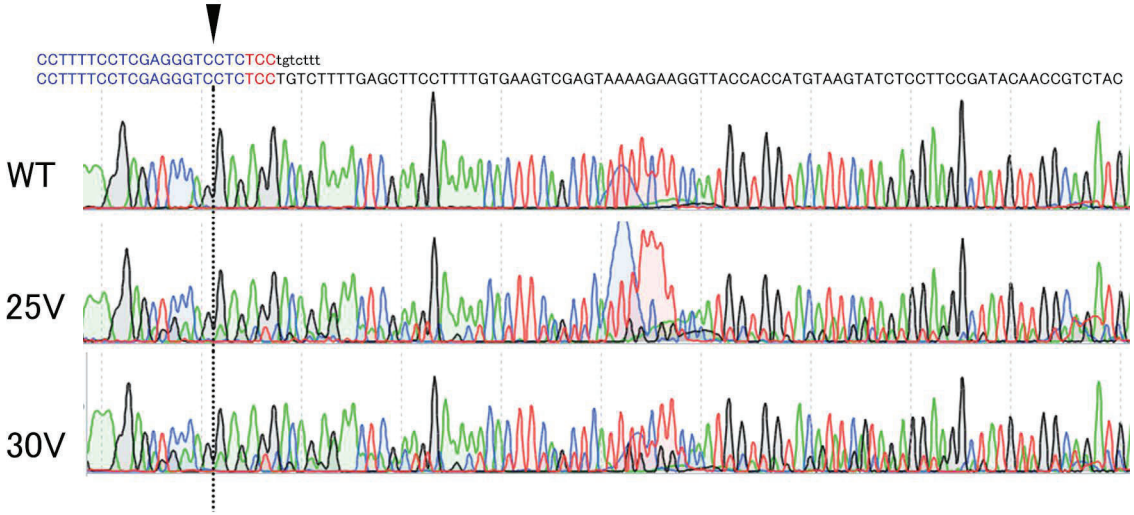
Blastocyst formation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CCTTTTCCTCGAGGGTCCTCTCCTGTCTTTTGAGCTTCCTTTTGTGAAGTCGAGTAAAAGAAGGTTACCACCATGTAAGTATCTCCTTCCGATACAACCGTCTAC
WT
25V
30V