In utero morphological and functional properties of bovine trophoblastic vesicles

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Abstract

In many mammals, including ruminants, pregnancy requires pregnancy recognition signaling molecules secreted by the conceptus; however, the mechanism underlying embryo establishment in cattle remains unknown. Trophoblastic vesicles are artificially produced from the extraembryonic tissues of the elongating conceptus and may be useful tools for understanding conception. This study investigated the morphological and functional properties of trophoblastic vesicles in comparison to those of intact conceptuses. Trophoblastic vesicles were prepared from the extraembryonic tissues of conceptuses collected 14 days after artificial insemination, cryopreserved immediately after dissection, and cultured after thawing for subsequent transplantation into the uterus. The transferred trophoblastic vesicles were collected 7 days after transplantation and compared with extraembryonic tissue samples collected from conceptuses at 21 days post-artificial insemination. The recovered trophoblastic vesicles were 40 times longer than those of their pre-transplant counterparts. Microscopic evaluation revealed that their membrane structures consisted of trophoblast and hypoblast layers. The expression patterns of the cell differentiation markers, CDX2, SOX2, and GATA6, and interferon tau protein expression levels in the trophoblastic vesicles were similar to those in control extraembryonic tissue samples. These findings suggest that trophoblastic vesicles are capable of morphological elongation and maintain interferon tau production in a similar way as original trophoblasts.

Keywords: bovine, elongation, IFNT, peri-implantation development, trophectoderm

Introduction

In recent years, cattle have undergone significant genetic improvements to enhance their economic traits as a food resource; however, more efficient and environmentally friendly animal production methods are required (Lamb & DiLorenzo, 2014), as reproductive performance, including estrous signs and pregnancy rates, continue to decline (Dochi et al., 2010; Lucy, 2001; Reese et al., 2020; Thundathil et al., 2016; Walsh et al., 2011). One of the reasons for this decline in reproductive performance is early embryonic mortality within the first month of gestation (P. J. Hansen, 2011; Reese et al., 2020; Wiltbank et al., 2016). Addressing this limitation is a major challenge for the next-generation animal reproduction technologies.

During the first month of gestation in cattle, the embryo elongates, and the mother is exposed to the effects of multiple factors secreted by the embryo to establish pregnancy. The pregnancy recognition factor in ruminants is interferon tau (IFNT), which is specifically secreted by embryonic trophoblasts (Bai et al., 2022; Imakawa et al., 1987). IFNT derived from the conceptus has paracrine inhibitory effects on the upregulation of oxytocin receptors in the endometrial epithelium. As a result, IFNT inhibits the production of prostaglandin F2α that causes the regression of corpus luteum (T. R. Hansen et al., 2017). Consequently, in the mother, IFNT expression allows for the prolongation of the estrous cycle and the preparation of the endometrium for implantation (Spencer et al., 1995; Spencer & Bazer, 1996). In addition, during this period, the trophoblast cells of the embryo proliferate rapidly, resulting in trophectoderm elongation; this process increases the overall IFNT secretion rate and facilitates both conceptus attachment and uterine receptivity to implantation via some paracrine effects on the endometrial expression of interferon-stimulated genes (Betteridge & Fléchon, 1988; Chang, 1952; Ealy & Yang, 2009; Forde & Lonergan, 2017; T. R. Hansen et al., 2017). Thus, the first month of pregnancy is a critical period

in which several changes occur for pregnancy establishment. The molecular mechanisms underlying embryo–uterus interactions during implantation need to be better understood to prevent early embryonic mortality during the critical phase of pregnancy establishment.

Studies involving elongated embryos in ruminants and pigs require substantial effort due to the cost of breeding, the relatively long period required for sufficient embryonic development before retrieval, and the difficulty of embryo collection via uterine flushing. Furthermore, unlike for blastocysts, in vitro culture techniques for preparing elongating embryos have not yet been established (Akizawa et al., 2021; Ramos-Ibeas et al., 2020; Vejlsted et al., 2006). Therefore, attention has shifted to trophoblastic vesicles (TVs), which are vesicle-like tissues produced through in vitro culture of the extraembryonic tissue derived from elongating embryos (Heyman et al., 1984, 1987). TVs may prolong the estrous cycle when transferred into the uterus and improve fertility when co-transferred with embryos (Hashiyada et al., 2005; Heyman et al., 1984). Furthermore, because multiple TVs can be easily produced from a single elongated embryo, they represent potential experimental materials for studies on the mechanisms of implantation in cattle. However, the characteristics of TVs after transplantation into the uterus, particularly their morphology and functional properties, such as differentiation-associated gene expression and IFNT production, have not been investigated.

In this study, we aimed to determine the morphological and functional properties of TVs following their transfer into the uterus. First, we established an efficient method for TV production. Next, frozen-thawed TVs were transferred into cattle uteri in the luteal phase, and re-elongated TVs were collected and analyzed 7 days after transfer. The morphological features and IFNT-producing abilities of the retrieved TVs were compared with those of the trophectoderms of intact embryos matching the elapsed post-fertilization period.

Materials and methods

Animal care

All experimental protocols were approved by the Regulatory Committee for the Care and Use of Laboratory Animals of the Aomori Prefectural Industrial Technology Research Center.

Preparation of trophoblastic vesicles

Elongated embryos were collected by superovulation and uterine flushing using a modified version of a previously described method (Fujii et al., 2017; Hiraizumi et al., 2015). Briefly, Japanese black donor cows were treated using an intravaginal progesterone-releasing device (CIDR; InterAg, Hamilton, New Zealand) and 0.5 mg of cloprostenol (Zenoadin C 2 mL im; Nippon Zenyaku, Fukushima, Japan) at a random stage of the estrous cycle after the day of estrus (designated Day 0). Then, 125 μg of fertirelin acetate (Conceral 2.5 mL im; MSD Animal Health, Rahway, NJ, USA) was administrated to the cows on day 7. Next, a single injection of 20 A.U. pFSH (Antrin R·10; Kyoritsu Seiyaku, Tokyo, Japan) dissolved in 50 mL of normal saline was subcutaneously administered to the cows at the neck region on day 10. Then, the CIDR was removed and cloprostenol (0.5 mg) and fertirelin acetate (125 μg) were administered on days 12 and 13, respectively. Fixed-time artificial insemination (AI) was performed 24 h after cloprostenol administration using frozen-thawed semen. Uterine flushing was performed under caudal epidural anesthesia (4 mL of 2% [v/w] procaine hydrochloride) 14, 15, and 17 days after AI using an 18-Fr balloon catheter with enlarged holes at the tip to allow the conceptus to pass. The embryonic discs of the recovered elongating embryos were removed, and the remaining tubal extraembryonic parts were dissected into fragments of approximately 1 mm in diameter using a scalpel (#15; Feather Safety Razor, Osaka, Japan). Then, extraembryonic tissue fragments were cultured regardless of

the original section source in Medium199 HEPES (M199, Thermo Fisher Scientific, Waltham, MA) supplemented with 20% (v/v) newborn calf serum (NBCS; Thermo Fisher Scientific) at 38.5 °C in a 5% CO_2 atmosphere for 24 h using non-treated culture plates. After culturing, tissues that formed spherical shapes without degeneration were considered TVs.

Cryopreservation and thawing of extraembryonic tissue fragments and TVs

Cryopreservation of extraembryonic tissue fragments and TVs was performed following the direct transfer method for bovine embryos, with slight modifications (Dochi, 2019). Briefly, four to six extraembryonic tissue fragments or TVs were equilibrated for 15 min in M199 medium supplemented with 20% (v/v) NBCS, 10% (v/v) ethylene glycol, and 0.1 mol/L trehalose, and then loaded into a 0.25-mL cryopreservation straw. Then, the straws were placed in a pre-cooling programmable freezer (ET-1; Fujihira Industry Co., Ltd., Tokyo, Japan) at –7 °C, for seeding. The cooling cycle was programmed as follows; −7 °C for 10 min, −0.3 °C/min up to −30 °C. After cooling, the straws were immediately immersed in liquid nitrogen for storage. For thawing, the straws were held in air for 10 s and then immersed in a water bath at 30 °C for 10 s. Then, the contents of the straw were incubated in M199 medium supplemented 20% (v/v) NBCS at 38.5 °C in a 5% CO_2 atmosphere for 24 h, and survival rate was determined by calculating the rate of TV formation.

Transfer and recovery of trophoblastic vesicles

For estrus synchronization, recipient cows were treated with CIDR devices for 7 days and with 0.5 mg of cloprostenol at the time of insertion. After withdrawal of the CIDR, ovulation was confirmed by ultrasonography. Based on data from a previous study (Heyman et al., 1984), TV transfer was performed 12 days after ovulation.

The TVs used for transfer were prepared from the extraembryonic fragments, which had been derived from day-14 conceptus. These fragments were frozen before TV formation, and the vesicle formation was confirmed after thawing and culture. The day before TV transfer, extraembryonic tissue fragments were thawed and cultured in M199 medium supplemented with 20% (v/v) NBCS at 38.5 °C in a 5% CO_2 atmosphere. Four TVs which had formed spherical shapes after 24 h of incubation were non-surgically transferred into the uterine horn, ipsilaterally to the corpus luteum of the recipient. TVs were recovered 7 days after transfer in the same way the elongating embryos were initially recovered.

Immunofluorescence assay

The primary antibodies used were rabbit anti-CDX2 (1:400, RRID: AB_1523334, ab76541, Abcam, Cambridge, UK), rabbit anti-SOX2 (1:500, RRID: AB_10585428, ab92494, Abcam), and rabbit anti-GATA6 (1:1000, RRID: AB_10705521, #5851, Cell Signaling Technology, Danvers, MA, USA) antibodies. Alexa Fluor 555 goat anti-rabbit IgG polyclonal antibodies (RRID: AB_2535849, A-21428, Thermo Fisher Scientific) were used as secondary antibodies. After transfer and retrieval, extraembryonic tissue fragments were fixed with 4% paraformaldehyde phosphate buffer solution (Nacalai Tesque, Kyoto, Japan) for 60 min at room temperature. Then, the fragments were dehydrated with graded ethanol (70, 80, 90, 95, 100%, v/v) and ethanol was exchanged with xylene. The tissues were embedded in paraffin and cut into 7-µm-thick sections. After being deparaffinized with xylene and rehydrated with graded ethanol (100, 95, 70%, v/v), the samples were microwaved for 10 min (500 W) with 0.01 mol/L citric acid (pH 6.0) containing 0.05% (v/v) Tween 20, and then cooled for 40 min. The samples were blocked using a blocking buffer (Blocking One [Nacalai Tesque] diluted 1:5 in 0.05% [v/v] Tween 20 in Tris-buffered saline [TBS]) for 60 min at room temperature. Thereafter, the sections were incubated overnight in

blocking buffer containing primary antibodies at 4 ℃; this was followed by incubation with secondary antibodies diluted at a 1:400 ratio with blocking buffer for 3 h at room temperature. After nuclear staining with 25 mg/mL Hoechst 33343 (Sigma–Aldrich, St. Louis, Mo, USA), the sections were observed under a TCS SP5 confocal laser-scanning microscope (Leica, Wetzlar, Germany).

Semi-ultrathin section preparation for optical microscopy

Sections were prepared as previously described (Akizawa et al., 2021; Hayashi et al., 2021). Briefly, after transfer and retrieval, extraembryonic tissue fragments were pre-fixed in 0.1 mol/L phosphate buffer (PB) (pH 7.4) containing 2.5% (v/v) glutaraldehyde for 2 h at 4 °C. After washing thrice in PB, the samples were fixed in 0.1 mol/L PB containing 1% (w/v) OsO_4 for 2 h at 4 °C and then washed again thrice in PB. Next, the samples were dehydrated by immersion in graded ethanol. Then, ethanol was removed using methyl oxirane and the samples were embedded in Epon solution and serially sectioned to form semi-thin sections (thickness of 1 μm). The sections were then stained with 0.3% (w/v) basic toluidine blue and examined by optical microscopy (BX60; Olympus, Tokyo, Japan).

Reverse-transcription quantitative polymerase chain reaction analysis

Each extraembryonic tissue fragment was dissected at a length of 10 mm and stored in RNA*later*™ Solution (Thermo Fisher Scientific) at −20 °C until RNA extraction. Total RNA was isolated using the ReliaPrep™ RNA Cell Miniprep System (Promega, Madison, WI, USA) and diluted to give a final concentration of 100 ng/μL after quantified using NanoDrop Lite Plus (Thermo Fisher Scientific). RNA was reverse transcribed using the ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan). Quantitative PCR (qPCR) was performed using the

THUNDERBIRD® SYBR qPCR Mix (Toyobo), LightCycler® 96 (Roche Diagnostics, Basel, Switzerland), and specific primers (Supplementary Table 1). Relative mRNA abundance was calculated using the ΔΔCq method using the arithmetic mean of *YWHAZ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta), *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase), and *ACTB* (actin beta) as the reference genes selected by geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) algorithms. All qPCR analyses were performed in triplicate.

Western blotting

Western blotting was performed following a slightly modified version of a previously described method (Murata et al., 2021). Briefly, to detect the bovine IFNT protein, lysates of extraembryonic tissue fragments from day 21 conceptuses (n = 3) and re-elongating TVs (n = 4) were prepared using 2x SDS sample buffer containing 0.125 M Tris-HCl, 4% (w/v) SDS, 10% (w/v) sucrose, 10% (v/v) 2-mercaptoethanol, and 0.01% (w/v) bromophenol blue. The lysates (50 μg/lane) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane using an iBlot™ Gel Transfer Device System (Thermo Fisher Scientific). After blocking the membranes with a PVDF blocking reagent (CanGetSignal®; Toyobo), they were treated with rabbit polyclonal anti-IFNT antibodies (1:5000, PAB862Bo01, Cloud-Clone Corp., Katy, TX, USA) or mouse monoclonal anti-beta actin antibodies (internal control, 1:5000, RRID: AB_2687938, 66009-1-Ig, Proteintech, Rosemont, IL, USA). Then, proteins were detected using secondary antibodies conjugated with horseradish peroxidase (1:25000, Amersham ECL, Cytiva, Tokyo, Japan) and EzWestLumi plus (Atto Corp., Tokyo, Japan). Densitometric quantitation was performed using the ImageJ software (version 1.53k: https://imagej.nih.gov/ij/) (Rasband, 1997).

215

216 *Statistical analysis*

217 The efficiency of TV production was evaluated using Student's *t*-test after the *F*-test to
218 confirm the equality of variance. The chi-square test was used to assess survival rate. Data from
219 RT-qPCR analysis was tested for the normality of distribution and equivariance assumptions using
220 the Shapiro-Wilk test and *F*-test, respectively. The differential expression analysis was performed
221 based on linear modeling approach and an empirical Bayes framework using the limma package
222 (Ritchie et al., 2015) of R software (http://www.r-project.org/). A moderated *t*-test was performed
223 using the ΔCq value and *P*-values were adjusted by Benjamini-Hochberg procedure. An adjusted
224 *P*-value of < 0.05 was considered statistically significant.

225

Results

Production efficiency of trophoblastic vesicles derived from the extraembryonic tissues of elongating embryos

The number of TVs produced from elongating embryos at three time points, i.e., 14, 15, and 17 days after AI, was investigated (Table 1), using two cattle animals for each time point. TV formation rate per trophoblastic fragment (92.6%) was higher in 14-day elongating embryos than in 15- and 17-day elongating embryos (54.7% and 19.2%, respectively).

Next, we compared the survival rates of frozen-thawed extraembryonic tissue fragments that cryopreserved immediately after dissection with those of frozen-thawed TVs, which were cryopreserved after forming vesicles following 24 h culture as described in a previous study (Table 2) (Isachenko et al., 1993). Approximately 60% of TVs cryopreserved after forming vesicles survived, whereas all tissue fragments cryopreserved immediately after dissection survived ($P < 0.01$).

In utero morphological characterization of trophoblastic vesicles

Frozen-thawed extraembryonic tissue fragments were cultured for 24 h for TV formation. TVs with an average length above 0.9 ± 0.1 mm were transferred into recipient cow uteri 12 days after estrus and were allowed to recover for 7 days after the transfer (Figure 1). Recovered TVs exhibited a tubular morphology, indicating their re-elongation in cow uteri (Figure 2A). The average length of the recovered TVs was 34.4 ± 8.9 mm (n = 7), with the longest and shortest of them being 70.0 mm and 3.5 mm in length, respectively. The average length of the control conceptus on day 21 could not be measured because it could not be recovered intact; however, based on the fragment recovered in the best condition, it was estimated to be approximately 150

mm (Figure 2A). We further compared the tissue sections of the re-elongating TVs with the trophectoderms of elongated conceptuses at day 21. Both trophectoderm membranes had a bilaminar structure consisting of an outer trophoblast layer and an inner hypoblast layer (Figure 2B). Thus, these results indicated that frozen-thawed TVs could grow to a length approximately 40 times that of pre-transplant TVs, with the development of a bilaminar structure in utero. The ratios of hypoblast-to-trophoblast cell counts in the samples of re-elongating TVs and day-21 conceptuses with hypoblast set to 1 were 8.0 ± 2.5 and 4.3 ± 0.9 (mean ± SEM), respectively (Figure 3).

Expression patterns and levels of cell-specific transcription factors

We performed an immunostaining analysis for the primary differentiation-associated transcription factors, CDX2 (for trophoblasts), SOX2 (for epiblasts), and GATA6 (for hypoblasts). Each expression pattern in the re-elongated TVs was compared with that in the extraembryonic tissues of conceptuses at day 21. CDX2 and GATA6 protein distributions in the re-elongated TVs were similar to those in extraembryonic tissues derived from the control conceptus (Figure 3). As expected, no SOX2 protein signal was observed in cells in either sample. In RT-qPCR analysis, there were no significant differences in *CDX2* and *GATA6* expression levels between re-elongated TVs and the control trophectoderm, and *SOX2* mRNA expression were not detected in each sample (Figure 4A).

IFNT expression analyses

We evaluated IFNT expression levels in the re-elongated TVs using qPCR and western blot techniques. *IFNT* mRNA expression levels in the TVs were significantly lower than those in the control trophectoderm; however, protein expression levels were similar between the two groups (Figure 4).

Discussion

Bovine embryo elongation is an important process for embryo attachment to the uterine epithelium; however, the mechanisms involved remain unclear and elucidating them could help understand early embryogenesis in mammalian species. In this study, we focused on TVs derived from the trophectodermal membranes of elongating embryos and investigated whether TVs could recapitulate the elongation process with trophoblast-specific characteristics, as a means of quality assessment.

Effects of cryopreservation and thawing on trophoblastic vesicle survival

First, we examined the most efficient time for the collection of elongating embryos for TV production. Consequently, we were able to prepare most TVs using elongating embryos recovered 14 days after AI. This result was unexpected, as we anticipated that more developed, and elongated embryos with large extraembryonic tissues would be more suitable for TV production. Our results revealed that the TV formation rate after culturing was higher in day-14 embryos than in later embryos (Table 1). In general, larger embryos are more susceptible to damage during uterine washing, which may account for the relatively low availability of undamaged samples suitable for TV production. In addition, more developed and elongated embryos on day 17 may possess lower cell activity and proliferation rates compared to younger and smaller embryos. Regarding the timing of cryopreservation, extraembryonic tissues frozen immediately after dissection showed a higher survival rate than cultured TVs (Table 2). The difficulty in cryopreserving TVs after vacuole formation may be due to inadequate penetration of the cryoprotectant into the luminal region of the TV. The slow freezing method using ethylene glycol, as applied in this study, appeared to reduce freezing damage to trophoblastic tissues more effectively than the previously described fragment

296 vitrification method using glycerol and propylene glycol (Isachenko et al., 1993).

297

298 *Morphological and functional assessment of frozen-thawed trophoblastic vesicles*

299 Next, TVs produced from frozen-thawed extraembryonic tissues were transferred into the
300 uteri of recipient cows 12 days after estrus to assess their morphological and functional properties.
301 The re-elongated TVs were compared with extraembryonic tissues derived from developmentally
302 matched conceptuses (Figure 1, 2A). The elongation of TVs at 7 days post-transplantation was on
303 average 40-fold that of pre-transplantation TVs, and their morphological appearance was
304 reminiscent of the trophoblast membranes in day-21 conceptuses. Microscopic analysis revealed
305 that, similar to trophectoderms from in vivo day-21 conceptuses, re-elongating TVs displayed a
306 double-layered structure consisting of trophoblasts and hypoblasts, although the ratio of hypoblast-
307 to-trophoblast cell counts tended to be lower than that in the day-21 conceptuses (Figure 2B and
308 3). The hypoblast cells were expected to have derived from the residues on the initial fragments of
309 TVs, and we confirmed the expression of differentiation-related genes in the TVs. Extraembryonic
310 tissue-specific markers, such as CDX2 and GATA6 were ectopically detected in re-elongated TVs
311 and control trophectoderms derived from day-21 conceptuses (Figure 3). In a previous study, the
312 site-specific expression of these two transcription factors was qualitatively analyzed by RT-PCR
313 (Degrelle et al., 2005); however, their protein expressions were not confirmed. Our results suggest
314 that re-elongated TVs are homologous to trophectoderms of conceptuses in vivo. Overall, these
315 results clearly indicate that TVs can elongate following in utero transfer and that a full embryo is
316 not required for trophectoderm elongation. Moreover, the day-12 uterine tissue condition supports
317 trophectoderm development even in the absence of conceptus before this time. Future studies
318 should examine the timing of these events in more detail to help understand elongation. Overall,
319 TVs may be a useful tool for research into pregnancy recognition.

These morphological observations indicate that trophoblasts in TVs transferred into the uterus can proliferate in a similar manner as trophoblasts in the whole conceptus. In addition, our preliminary data showed that no re-elongated embryo was formed when TVs were transferred into the uterus 7 days after estrus (data not shown; four TVs were transferred into two recipients, and no re-elongating TV was collected), suggesting that proper synchronization between the TV and the uterus is important for TV re-elongation. A detailed comparison of the two uterine conditions (at 7 or 12 days after estrus) may provide clues for the elucidation of the mechanism underlying trophoblast elongation. A previous study has reported that extensive transcriptional changes in the endometria may occur between days 7 and 13 of the luteal phase (Forde et al., 2011).

Our qPCR analysis showed that the re-elongated TVs exhibited lower *IFNT* levels than the trophectoderms of day-21 conceptuses; however, western blotting results showed that the corresponding IFNT protein expression levels were similar between the re-elongated TVs and day-21 conceptus trophectoderms (Figure 4). The diminished expression of *IFNT* mRNA may have resulted from the amount of serum in the culture medium, as serum supplementation disturbs epigenetic processes that affect the expression of various genes (Nava-Trujillo & Rivera, 2023). Thus, any changes to the culture medium used for TV, including any reduction or complete removal of serum supplementation, may restore *IFNT* expression to the baseline level. Nevertheless, the amount of IFNT protein in the tissue samples was enough despite the mRNA level being below the expected value. Given the observed 20-day delay in estrus regression in a non-pregnant cow when two TVs were transferred into the uterus (data not shown), TVs are presumed to possess the IFNT secretory capacity. Future studies should evaluate the secretion of IFNT protein from TVs and the adequate number of vesicles to recognize pregnancy.

These results suggest that during elongation, bovine trophoblasts can grow independently of the contribution of the embryo. In mouse embryos, contact with ICM cells is essential for the

regulation of trophoblast differentiation as contact of the polar trophectoderm with the epiblast ensures the development of the extraembryonic endoderm and prospective placental cells (Gasperowicz & Natale, 2011). However, in bovine embryos, the polar trophectoderm regresses and the mural trophectoderm, which is not in contact with ICM cells, elongates to form cotyledons at the caruncles of the endometrium, thereby forming the placenta (Maddox-Hyttel et al., 2003; Mathew et al., 2022). This fundamental difference between bovine and mouse placentation is critical for a comprehensive understanding of species-specific mechanisms in mammalian development. Further studies are needed to elucidate the mechanisms underlying embryonic elongation; however, TV-based analyses will provide a foundation for understanding the molecular mechanisms of implantation in cattle.

In conclusion, we described the intrauterine behavior of TVs prepared from bovine elongating embryos by investigating the morphological and functional properties of frozen-thawed TVs. Although TVs lack an embryo per se, they showed significant elongation concomitant with the expression of developmental markers, particularly that of the representative fetal–maternal recognition signal, IFNT. The establishment of trophoblast stem cells has recently been reported in cattle (Wang et al., 2023); however, trophoblast stem cell behavior in vivo remains unclear. Meanwhile, the interaction between the trophoblast and hypoblast may be necessary for elongation. This study showed that TVs with both trophoblasts and hypoblasts possess the ability to re-elongate in vivo. Thus, TVs would constitute a more appropriate material for an initial approach to investigating elongation mechanisms.

Author contributions

S.K. and M.K. designed the experiments. S.K., Y.H., and B.H. performed the experiments. S.K., B.H., and M.K. analyzed the data. B.H., M.T., and M.K. provided resources. S.K., H.B., and M.K. wrote and edited the manuscript. All authors contributed to the interpretation of the data and have read and approved the final version of the manuscript.

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Conflict of interest statement

The authors declare no conflict of interest.

Data availability statement

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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517 Table 1. Production efficiency of bovine trophoblastic vesicles at different elongated embryo
518 recovery dates

Days from insemination	Total length of conceptuses recovered from two animals† (mm)	No. of pieces of trophoblast fragments dissected	No. of TVs forming vesicles after 24-h culture	TV formation rate (%)
14 days	45.4	54	50	92.6
15 days	141.6	64	35	54.7
17 days	288.5	52	10	19.2

519 † Including damaged fragments of the extraembryonic section

520

Table 2. Effects of the timing of freezing on bovine trophoblastic vesicle viability

Timing of freezing	No. of tested fragments	No. of TVs formed	Survival rate (%)
After 24 h of incubation (conventional)	33	20	60.6 [a]
Immediately after dissection	34	34	100 [b]

Different superscript letters (a, b) within the same column indicate significantly different values ($P < 0.01$).

Figure legends

Figure 1. Schematic representation of the preparation of day 7 re-elongating bovine trophoblastic vesicles

Trophoblastic vesicles (TVs) were produced from elongating embryos collected from superovulation (SOV)-treated donor cattle on day 14 after artificial insemination (AI). After dissection, extraembryonic tissue fragments were cryopreserved until transplantation. After thawing, these fragments were cultured for 24 h. TVs grown to a diameter of approximately 1 mm were transferred into the uteri of recipient cows on day 12 after estrus. On day 7 after transfer, re-elongating TVs were retrieved by uterine flushing and used for subsequent analyses.

Figure 2. Morphological features of re-elongated bovine trophoblastic vesicles

(A) Outward appearance of a re-elongated day-7 trophoblastic vesicle (TV) (left) and a day-21 conceptus (right). The TV had originated from day-14 conceptus and was collected 7 days after transplantation into the uterus. Due to damage caused by flushing, one side of the day-21 conceptus was lacking; the total length was estimated at 150 mm. Scale bar = 1 cm. (B) Microscopic structure of a re-elongated day-7 TV. Semi-thin sections of the trophectoderm of a day-7 TV (left) and a day-21 conceptus (right). Of note, hypoblastic cells (arrowheads) are underlying the trophoblast layer (arrows) in a similar way as in the control conceptus. At least three TVs and conceptuses were used for each analysis. Scale bar = 50 μm.

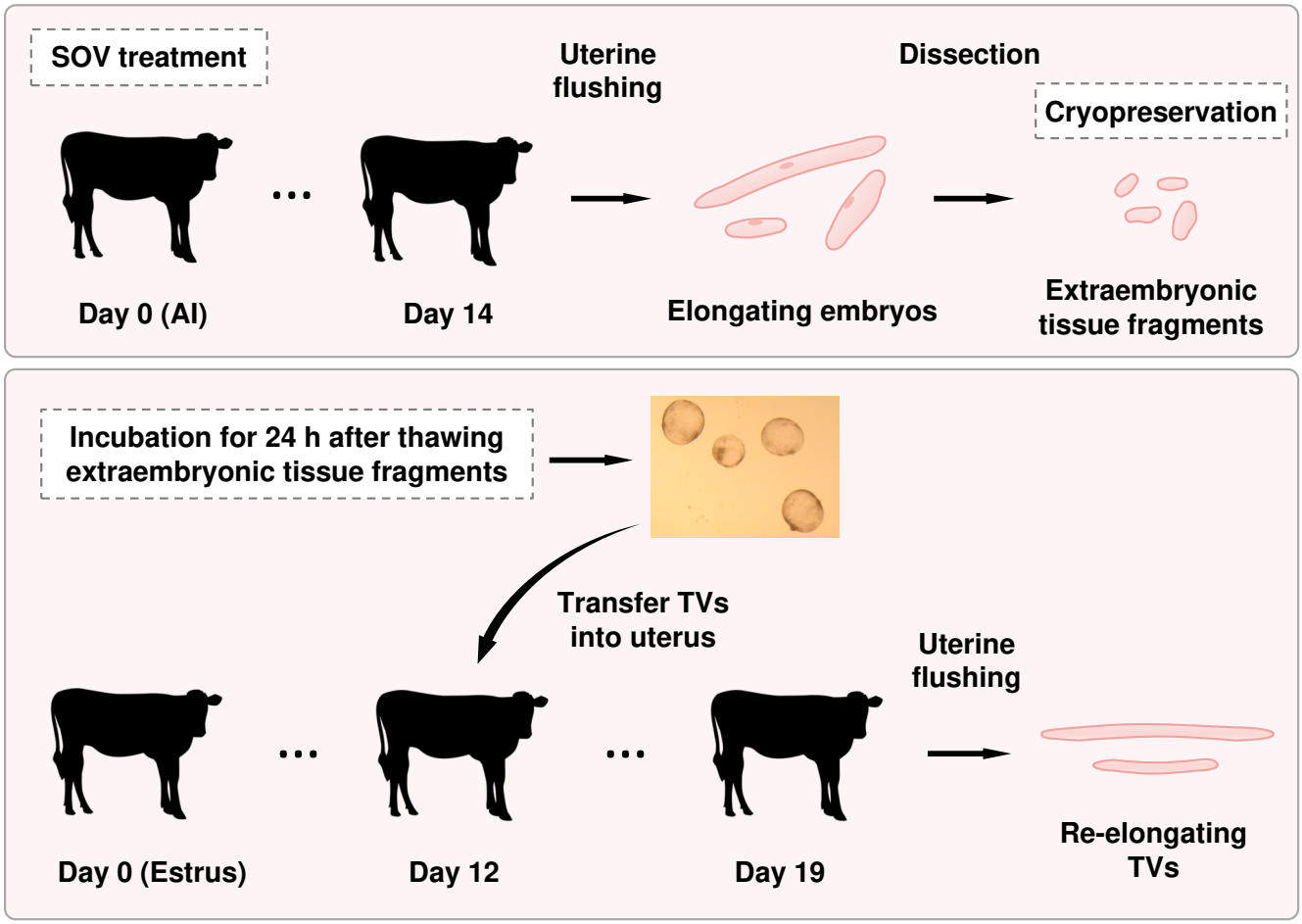
Figure 3. Immunostaining for the CDX2, SOX2, and GATA6 proteins in the trophoblast and hypoblast layers of re-elongated bovine trophoblastic vesicles (TVs)

Nuclei were counter-stained with Hoechst 33342. As in the controls, CDX2 and GATA6 were

predominant in the trophoblast and hypoblast layers of re-elongated TVs, respectively. SOX2 was absent both in TVs and the trophectoderms of control conceptuses. The hypoblast-to-trophoblast cell count ratios in the tissues of re-elongating TVs and day-21 conceptuses with hypoblast as 1 were 8.0 ± 2.5 and 4.3 ± 0.9 (mean ± SEM), respectively. At least three TVs and conceptuses were used for each analysis. Scale bar = 50 μm.

Figure 4. mRNA expression levels of tissue-specific markers in re-elongated bovine trophoblastic vesicles

(A) Reverse transcription quantitative polymerase chain reaction analysis of trophoblasts collected from a day-21 conceptus and a day-7 trophoblastic vesicle (TV). Relative mRNA expression level normalized with an arithmetic mean of housekeeping genes, *YWHAZ*, *ACTB*, and *GAPDH*. Data are presented as mean ± SEM (indicated by error bars). N.D. indicates not detected as under threshold cycle 40. N.S. indicates non-significant differences. Asterisks represent a significant difference (*$P < 0.05$). (B) Western blotting images for IFNT protein expression in TVs (n = 3) and control conceptuses (n = 3) (left). Actin beta (ACTB) was used as an internal control. Densitometric analysis of the bands was performed for quantitative evaluation (right). Data are presented as the mean ± SEM.



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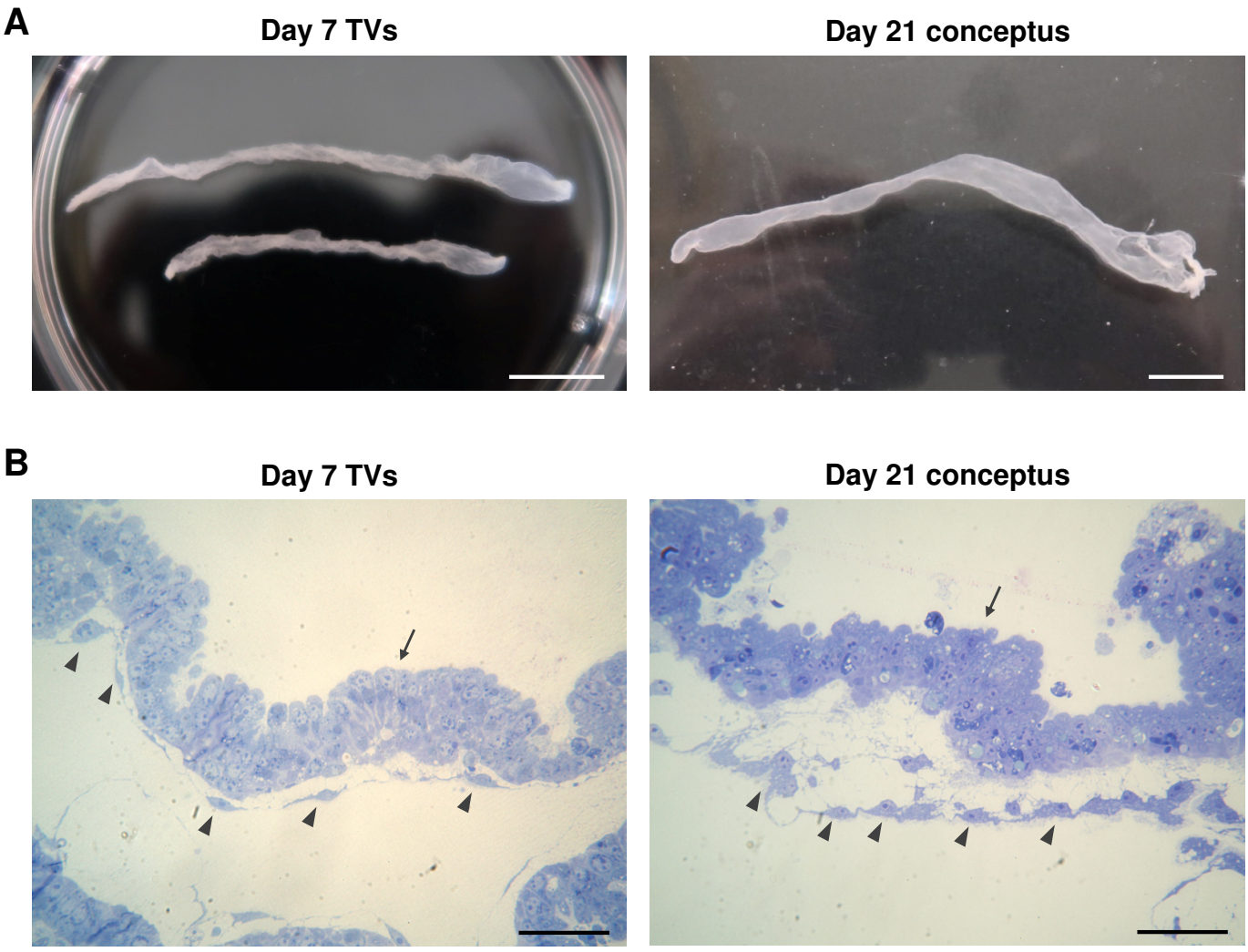

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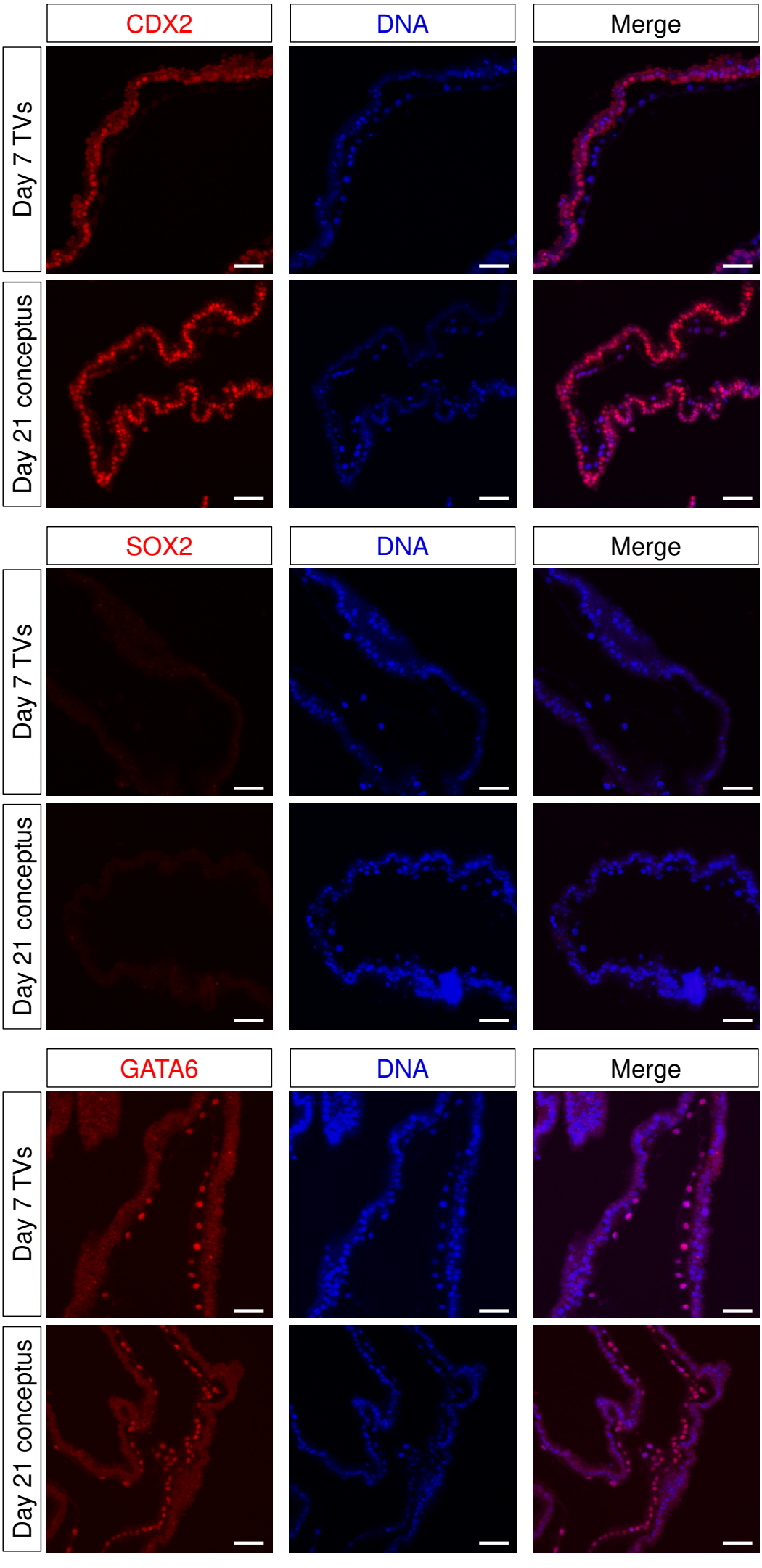
CDX2
DNA
Merge
Day 7 TVs
Day 21 conceptus
SOX2
DNA
Merge
Day 7 TVs
Day 21 conceptus
GATA6
DNA
Merge
Day 7 TVs
Day 21 conceptus

Figure 3

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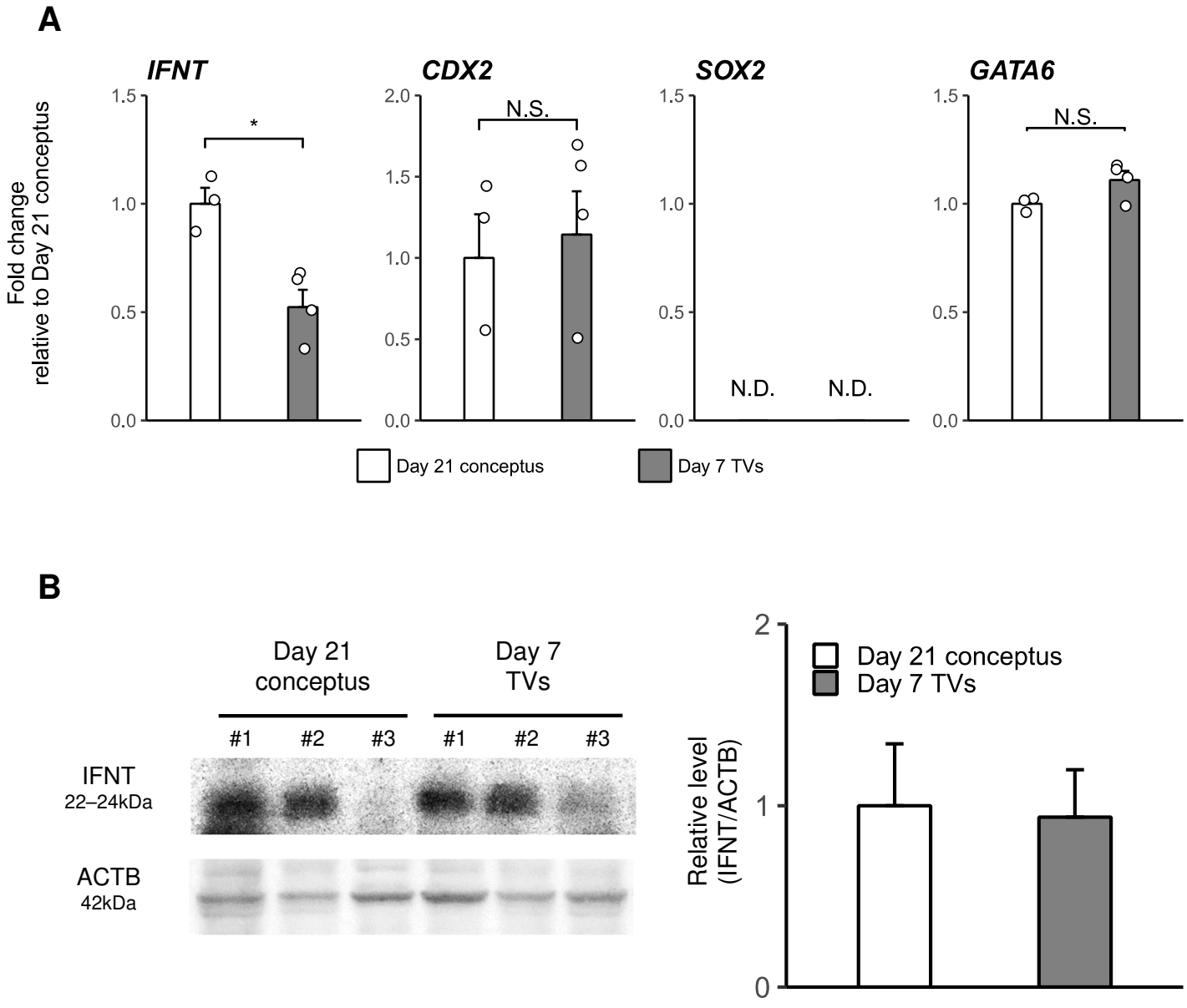

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