

Pregnancy establishment in cattle without embryonic interferon tau

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Abstract

Interferon tau (IFNT) is widely considered the indispensable embryonic signal for maternal recognition of pregnancy in ruminants^{1,2}. This paradigm, however, has never been directly tested by genetic loss-of-function. Here, we generated bovine embryos lacking all functional *IFNT* gene copies and assessed their developmental competence and ability to establish pregnancy. *IFNT*-null embryos were produced by somatic cell nuclear transfer using donor cells with complete genetic ablation of *IFNT*. The loss of IFNT had no effect on preimplantation development in vitro, with knockout embryos forming blastocysts at rates comparable to wild-type controls. As expected, IFNT protein was undetectable in conditioned media from *IFNT*-null embryos, whereas wild-type embryos secreted progressively increasing amounts of IFNT. Following transfer into synchronized recipient heifers, day-18 *IFNT*-null conceptuses were similar in size to controls but failed to induce interferon-stimulated gene expression in the maternal endometrium, confirming the absence of embryonic IFNT production. Despite this, transfer of *IFNT*-null blastocysts resulted in the establishment of pregnancies, with normal fetal and placental development observed upon recovery. All fetuses were confirmed to lack functional *IFNT* gene copies. Together, these results provide direct genetic evidence that embryonic IFNT is not required for pregnancy establishment in cattle, fundamentally revising the long-standing model of maternal recognition of pregnancy in ruminants.

Introduction

Early pregnancy loss remains a major limitation to reproductive efficiency in ruminants, with most losses occurring within the first three weeks after conception³. During this critical window, the conceptus, comprising the embryo and its extraembryonic membranes, undergoes rapid growth and elongation and initiates molecular communication with the maternal endometrium^{4,5}. This interaction leads to maternal recognition of pregnancy (MRP)^{6,7}, the critical process by which the conceptus signals its presence to ensure continued support from the maternal system. In ruminants, this signal has long been attributed to interferon tau (IFNT), a type I interferon produced exclusively by the mononuclear trophoderm of the elongating conceptus^{4,8}, and widely regarded as the central mediator of MRP^{1,2,9,10}.

IFNT was first identified in 1979 in cultured sheep conceptuses and originally termed ovine trophoblast protein-1 (oTP-1) or trophoblastin¹¹⁻¹⁵. In cattle, IFNT is secreted from approximately days 7 to 28 of gestation, with maximal production around days 18-20¹⁶. Acting through the classical type I interferon receptor complex (IFNAR1/IFNAR2), IFNT exerts several paracrine actions on the endometrium and endocrine actions on the corpus luteum (reviewed in^{1,2}). Its best-characterized role is antiluteolytic, mediated by suppression of estrogen receptor alpha (ESR1) and the oxytocin receptor (OXTR) in the endometrial luminal epithelium through interferon regulatory factor 2 (IRF2), thereby preventing oxytocin-induced prostaglandin F₂α (PGF₂α) pulses and luteolysis (reviewed in^{1,2}). IFNT may also act directly on the corpus luteum, promoting luteal cell survival, stabilizing vascular integrity, and attenuating luteolytic signaling, thereby complementing its uterine actions to extend luteal lifespan during early pregnancy¹⁷.

Beyond its antiluteolytic action, IFNT induces a broad transcriptional response in the endometrium, including robust activation of interferon-stimulated genes (ISGs) such as *ISG15*, *MX1*, *MX2*, *OAS1*, and *IRF9*, alongside cell-type-specific modulation of pathways implicated in conceptus elongation, implantation, and immune regulation^{1,2,4,7,18,19}. In cattle, endometrial ISG expression reaches peak levels around days 16-18 of gestation, corresponding to maximal IFNT signaling during the MRP window^{1,20,21}.

Cattle possess multiple copies of the *IFNT* gene, with at least three validated coding sequences on chromosome 8: *IFNT* (also known as *TP-1*, *IFNT2*, or *IFN-tau-c2*), *IFN-tau-c1* (also termed *IF1BE2*), and *IFNT3*, all encoding a 195-amino acid IFNT protein²²⁻²⁴. Despite decades of study and the near-universal acceptance of IFNT as the canonical MRP signal, definitive loss-of-function evidence demonstrating its absolute requirement for pregnancy establishment in ruminants has been lacking.

Here, we report the first complete genetic ablation of all detectable *IFNT* gene copies in cattle. Using somatic cell nuclear transfer (SCNT) from *IFNT*-mutant cells, we generated *IFNT*-knockout embryos and confirmed the complete absence of IFNT protein and antiviral activity using multiple independent assays. Unexpectedly, following embryo transfer to synchronized recipients, pregnancies were successfully established and progressed beyond the peri-implantation period in the complete absence of conceptus-derived IFNT. Two twin pregnancies were terminated at gestational days 50 and 75, respectively, and a third twin pregnancy remains ongoing, with calving expected in February 2026. Together, these findings demonstrate that conceptus-derived IFNT is not required for the establishment and maintenance of pregnancy in cattle, thereby challenging a central paradigm in ruminant reproductive biology, redefining the molecular basis of maternal recognition of pregnancy, and opening new directions for the field.

Generation of bovine embryos with mutant *IFNT* genes

To determine whether conceptus-derived IFNT is required for pregnancy establishment in cattle, we generated bovine fetal fibroblast (BFF) lines lacking all functional *IFNT* alleles, used them as nuclear donors for SCNT, verified the absence of IFNT secretion during extended in vitro culture, and transferred *IFNT*-knockout (KO) SCNT embryos or wild-type (WT) SCNT embryos to recipients to assess different stages of pregnancy (**Fig. 1a**). Candidate *IFNT*-KO single-cell clones were screened using three PCR assays targeting distinct regions of the multigene *IFNT* locus (**Fig. 1b**). PCR 1 amplified the core *IFNT* coding sequence between the two sgRNA cut sites, and loss of this amplicon indicated deletion of the shared coding sequence (CDS). PCR 2 spanned from a forward primer overlapping the sgRNA1 cut site to a reverse primer in the 3'-UTR, and the absence of this product confirmed disruption at the sgRNA1 target site. PCR 3 amplified from the 5'-UTR to a reverse primer overlapping the sgRNA2 cut site, and loss of this amplicon indicated disruption at the sgRNA2 site. *GAPDH* amplification was used as an internal control. Two independent cell clones (22 and 34) lacked all three *IFNT*-specific amplicons while retaining the *GAPDH* band, confirming complete deletion of all detectable *IFNT* copies (**Fig. 1c**). These validated *IFNT*-KO clones were used for all subsequent in vitro and in vivo experiments.

Normal in vitro development of *IFNT*-null embryos

To assess whether *IFNT* ablation affects preimplantation development, *IFNT*-KO and WT embryos were generated by SCNT using KO or WT donor cells, respectively. In total, 212 *IFNT*-KO and 144 WT embryos were reconstructed. No significant differences were observed between groups in fusion rate, cleavage rate, or blastocyst formation rate, indicating that *IFNT* is dispensable for in vitro blastocyst development (**Fig. 2a**). To evaluate *IFNT* bioactivity, SCNT-derived *IFNT*-KO embryos and in vitro fertilization (IVF)-derived *IFNT*-intact embryos were cultured in vitro until day 19, and embryo-conditioned media were collected at defined time points for cytopathic effect (CPE) inhibition assays. Conditioned media from *IFNT*-KO embryos showed no detectable antiviral activity at any stage, whereas media from IVF embryos exhibited robust *IFNT*-dependent antiviral activity that increased with embryo age (**Fig. 2b**). These results were independently confirmed in a long-term culture experiment in which SCNT-derived *IFNT*-KO embryos and WT embryos, generated either by SCNT from WT cells or by IVF, were maintained in vitro through day 21. Western blot analysis revealed a complete absence of *IFNT* protein in conditioned media from *IFNT*-KO embryos, while strong *IFNT* signals were detected in media from both SCNT- and IVF-derived WT embryos (**Fig. 2c**). Peptide-based quantification of *IFNT* through mass spectrometry of the same samples corroborated these findings, demonstrating a complete absence of *IFNT* peptides in *IFNT*-KO media and high *IFNT* abundance in WT media, with peak levels observed at day 19 of in vitro culture (**Fig. 2d**; **Extended Data Table 1**).

Elongation of *IFNT*-KO embryos in the absence of a uterine interferon response

Following robust in vitro validation of *IFNT* ablation, the requirement of conceptus-derived *IFNT* for early pregnancy establishment was assessed in vivo. *IFNT*-KO and WT SCNT-derived blastocysts were transferred into synchronized recipient cows (2-3 embryos per recipient). Recipients were slaughtered at gestational day 18, and conceptuses were recovered by uterine flushing, followed by collection of endometrial biopsies (**Fig. 3a**). Conceptus recovery was 100% in the *IFNT*-KO group and 50% in the WT group, yielding a total of four *IFNT*-KO and three *IFNT*-intact conceptuses. Despite the complete absence of *IFNT*, no significant differences in conceptus elongation were observed between groups, with mean conceptus lengths of 34.0 cm in the KO group and 36.6 cm in the WT group (**Fig. 3b**). PCR-based genotyping of recovered conceptuses confirmed the absence of all *IFNT* target regions in the KO group, verifying stable and complete *IFNT* deletion (**Fig. 3c**). Consistent with these findings, western blot analysis failed to detect *IFNT* protein in *IFNT*-KO conceptuses, whereas robust *IFNT* expression was observed in WT conceptuses (**Fig. 3d**). Quantitative proteomic analysis of conceptus tissues revealed no global differences in protein abundance between *IFNT*-KO and WT groups (**Fig. 3e**; **Extended Data Table 2**); notably, no *IFNT*-derived peptides were detected in any *IFNT*-KO samples (**Extended Data Table 3**). In contrast, proteomic profiling of the corresponding endometrium revealed marked differences between groups. Endometria associated with *IFNT*-KO conceptuses exhibited a pronounced reduction in

interferon-stimulated proteins compared to those associated with WT conceptuses (**Fig. 3f, Extended Data Table 4**). Pathway enrichment analysis of downregulated proteins in the *IFNT*-KO endometrium identified significant enrichment for IFNT-associated pathways, including interferon signaling, ISG15-mediated antiviral mechanisms, IFNA/B signaling, and regulation of type I interferon responses (**Fig. 3g; Extended Data Table 5**). These data demonstrate that, although *IFNT*-KO conceptuses fail to elicit the canonical uterine interferon response, they nevertheless elongate normally and establish early pregnancy.

Establishment and progression of pregnancy in the absence of embryonic IFNT

Following the demonstration that *IFNT* ablation does not impair peri-implantation development, we next assessed whether *IFNT*-KO embryos can establish and sustain pregnancy in vivo. A second round of embryo transfers was performed using SCNT-derived *IFNT*-KO blastocysts. Ten estrus-synchronized recipient heifers received either two ($n = 8$) or three ($n = 2$) *IFNT*-KO embryos per animal. Recipient activity was continuously monitored using automated movement recording systems, and time-series activity profiles were generated to assess estrus-associated behavioral patterns (**Extended Data Fig. 1**). Two recipients (67914 and 67929), which were the only animals receiving three embryos each, exhibited a normal return to estrus. In contrast, the remaining eight recipients exhibited prolonged estrus cycles, consistent with the establishment of pregnancy (**Extended Data Fig. 1**). Pregnancy diagnosis by transrectal ultrasonography performed on days 40 (**Extended Data Video 1**), 47, and 49 post-estrus (**Fig. 4a; Extended Data Fig. 2**) confirmed twin pregnancies in three recipients. One twin pregnancy was electively terminated at day 50 of gestation, yielding two fetuses, one of which appeared morphologically normal, while the second displayed features consistent with known cloning-associated abnormalities (**Fig. 4b**). A second twin pregnancy was terminated at day 75 to assess placental development and maternal-fetal attachment. Gross anatomical examination revealed a normally sized uterus and a functional corpus luteum on the left ovary (**Fig. 4c**). Post-dissection analysis showed a well-distributed pattern of maternal caruncles throughout the uterus, with approximately 60 caruncles identified (**Fig. 4d**). Placentomes appeared morphologically normal, exhibiting appropriate attachment between fetal cotyledons and maternal caruncles (**Fig. 4e**). Approximately 72 cotyledons were evenly distributed across the chorioallantoic membrane (**Fig. 4f**). One fetus recovered at day 75 exhibited normal morphology, with no overt developmental abnormalities or cloning-associated defects (**Fig. 4g**). The other fetus showed morphological alterations most likely associated with the SCNT technology (**Extended Data Fig. 3**). PCR-based genotyping confirmed that all recovered fetuses, including those collected at days 50 and 75 of gestation, harbored a complete deletion of all *IFNT* loci, with genotypes identical to their respective donor single-cell clones (**Fig. 4h**). The third twin pregnancy is ongoing at the time of writing, with parturition expected in February 2026. Together, these findings demonstrate that embryonic IFNT is dispensable for the initiation and progression of pregnancy in cattle, fundamentally revising the long-standing paradigm of maternal recognition of pregnancy in ruminants.

Discussion

Maternal recognition of pregnancy (MRP) in ruminants has long been considered one of the most clearly defined endocrine paradigms in reproductive biology, with conceptus-derived interferon tau (IFNT) viewed as the indispensable signal that prevents luteolysis and enables pregnancy establishment. This model is supported by decades of correlative, gain-of-function, and signaling studies, which demonstrate that IFNT suppresses uterine ESR1 and OXTR expression, induces interferon-stimulated genes (ISGs), and prolongs the lifespan of the corpus luteum^{1,2,4-19}. However, despite its widespread acceptance, definitive loss-of-function evidence for the essentiality of IFNT has been lacking. Here, by generating embryos completely devoid of all functional *IFNT* gene copies and demonstrating successful pregnancy initiation and progression in their absence, we provide direct experimental evidence that challenges this long-standing paradigm.

Using precise genome editing, we achieved complete ablation of all annotated *IFNT* loci in bovine fetal fibroblasts, produced embryos by SCNT, and rigorously validated the loss of IFNT at the genomic, proteomic, and functional levels. The convergence of multiple independent validation approaches, including highly sensitive LC-MS/MS quantification using multiple IFNT-specific proteotypic peptides, provides unequivocal evidence for the complete ablation of *IFNT*, establishing a robust foundation for the conclusions of this study. *IFNT*-KO embryos developed normally to the blastocyst stage in vitro, secreted no detectable IFNT protein during extended culture, and nonetheless established pregnancies following transfer into synchronized recipients. In vitro analyses further showed that *IFNT*-intact embryos initiated IFNT secretion as early as day 11 of development, with peak abundance at day 19, consistent with previous reports¹⁶. Importantly, *IFNT*-KO conceptuses recovered at the peri-implantation stage were morphologically indistinguishable from WT controls, exhibited comparable elongation, and progressed through early gestation. Apart from the complete absence of IFNT, no other proteins were differentially expressed between *IFNT*-KO and WT conceptuses, as determined by unbiased proteomic profiling. These findings demonstrate the high on-target efficiency and specificity of the sgRNAs used for *IFNT* ablation, suggesting that there are no biologically relevant off-target effects. Moreover, they provide strong evidence that IFNT does not exert essential autocrine, paracrine, or endocrine functions within the conceptus itself and does not regulate intrinsic conceptus signaling pathways during early development.

As expected and consistent with previous reports^{25,26}, the endometrium of recipients carrying WT embryos mounted a robust IFNT-driven response, characterized by strong upregulation of canonical interferon-stimulated proteins signaling components required for type I interferon responsiveness, including ISG15, MX1, MX2, STAT1, STAT2, and IFIT family members. Among these, ISG15 is one of the earliest and most abundant IFNT-induced proteins in the ruminant endometrium and is widely used as a biomarker of pregnancy recognition²⁷. It functions as a ubiquitin-like modifier that regulates protein stability, immune signaling, and cellular homeostasis during early gestation^{1,2,28}. The dynamin-like GTPases MX1 and MX2 are classical antiviral effectors induced by IFNT in the uterine epithelium and stroma, and their sustained expression during the peri-implantation window is thought to contribute to

immune protection of the conceptus and modulation of endometrial receptivity, and are also believed to be a biomarker of pregnancy in ruminants²⁹⁻³². STAT1 and STAT2 are central mediators of IFNT signaling downstream of IFNAR activation, forming the ISGF3 complex together with IRF9 to drive the transcription of a broad ISG network in the endometrium³³⁻³⁶. STAT-dependent signaling is essential for establishing and maintaining the IFNT-responsive endometrial state during maternal recognition of pregnancy^{36,37}. Finally, the IFIT family members (IFIT1, IFIT2, IFIT3, and IFIT5), which bind viral and non-self RNA species, are also robustly induced by IFNT in pregnant endometrium and are thought to contribute not only to antiviral defense but also to immune modulation and suppression of inflammatory responses that could otherwise compromise conceptus survival^{25,38}.

In striking contrast, recipients carrying *IFNT*-KO embryos exhibited a complete absence of endometrial interferon signaling, with markedly decreased activity of all IFNT-responsive genes and pathways relative to WT controls. Despite the absence of this canonical interferon response, pregnancies were successfully initiated, maintained, and progressed beyond the implantation stage. Twin pregnancies recovered at gestational days 50 and 75 each contained one normally developed fetus and exhibited well-organized placentation, characterized by extensive and structurally intact interactions between maternal caruncles and fetal cotyledons. The second fetus in each twin pregnancy displayed developmental abnormalities consistent with previously described artifacts associated with SCNT technology³⁹. Notably, all pregnant recipients carrying *IFNT*-KO embryos, including day 18, 50, and 75, maintained a morphologically normal corpus luteum. This sustained luteal support in the complete absence of conceptus-derived IFNT indicates that luteolysis can be prevented independently of IFNT signaling, thereby challenging the long-held view that IFNT is required for luteal maintenance during early pregnancy. Together, these findings demonstrate that neither induction of endometrial interferon-stimulated genes nor IFNT itself is required for pregnancy recognition, establishment, or placental development in cattle. Nevertheless, IFNT may still play important modulatory roles during ruminant pregnancy. Because ruminant placentation does not permit transplacental transfer of maternal immune protection, IFNT may function as a conceptus-derived antiviral defense mechanism. This concept is supported by the evolution of IFNT-evasion strategies in bovine viral diarrhea virus (BVDV), a major cause of infertility, early embryonic loss, and the birth of persistently infected offspring⁴⁰. Although this potential role warrants further investigation, no evidence of viral infection was observed in any pregnancies that progressed beyond the peri-implantation stage in this study.

From a translational perspective, our findings prompt a reassessment of current approaches to improving fertility in cattle and other ruminant species, which primarily focus on modulating IFNT signaling. Although IFNT-based diagnostics and interventions may retain utility as indicators of conceptus presence or developmental status, they should no longer be considered definitive for establishing pregnancy. Instead, our results argue for a more integrative framework in which multiple embryonic and maternal signals collectively govern early pregnancy success.

In summary, this study provides the first comprehensive loss-of-function analysis of interferon tau in a ruminant species, fundamentally revising the prevailing model of maternal recognition of pregnancy. We demonstrate that embryonic IFNT is dispensable for pregnancy initiation, luteal maintenance, peri-implantation development, and early placentation in cattle. By showing that pregnancies can be established and sustained in the complete absence of IFNT-mediated endometrial reprogramming, our findings challenge the long-standing assumption that IFNT-induced ISGs are essential determinants, or even reliable proxies, of pregnancy recognition. This work necessitates a re-evaluation of the molecular mechanisms underlying embryo-maternal communication in ruminants and opens the field to the discovery of alternative, IFNT-independent pathways that support early gestation.

Declarations

Acknowledgments

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Author contributions

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Conflicts of interest

The authors declare no conflicts of interest.

Data availability

The proteomics data were submitted to MassIVE (accession number MSV000100363) and ProteomeXchange (accession number PXD072556).

Methods

Animal use and ethical approval

In vivo procedures were conducted in accordance with the German Animal Welfare Act (Tierschutzgesetz) and with permission from the responsible authority, the Government of Upper Bavaria (ROB-55.2-2532.Vet_02-24-131). Bovine ovaries were obtained from a slaughterhouse (Münchner Schlachthof Betriebs GmbH, Munich, Germany). In vitro produced embryos were obtained from an EU-approved bovine embryo collection and production center at the Chair for Molecular Animal Breeding and Biotechnology of the LMU Munich (Moorversuchsgut Badersfeld, Oberschleißheim, Germany; approval number DE ETR 006 EWG).

Generation of *IFNT*-KO cells

Primary bovine fetal fibroblasts (BFFs) were established from early-gestation fetuses and maintained under standard culture conditions at 37°C with controlled humidity and CO₂ in fibroblast growth medium supplemented with 15% fetal calf serum (FCS), non-essential amino acids, and HEPES buffer. Multiple single-guide RNAs (sgRNAs) targeting all annotated *IFNT* loci were designed using established in silico tools to ensure high on-target efficiency and minimal predicted off-target effects. Several sgRNA pairs were tested in BFFs, and the pair exhibiting the highest editing efficiency across all *IFNT* copies (sgRNA1: catcagtagagagagcacga; sgRNA2: agtgcagagctgctccagga) was selected to generate the final *IFNT*-knockout (KO) donor cell lines for somatic cell nuclear transfer (SCNT).

For genome editing, pre-assembled Cas9 ribonucleoprotein (RNP) complexes were prepared using TrueCut™ Cas9 Protein v2 (Thermo Fisher Scientific, Waltham, MA, USA) combined with the selected sgRNAs and delivered into BFFs via electroporation using an Amaxa Nucleofector I device. Edited populations were allowed to recover for 48-72 h before single-cell propagation. Individual clones were expanded in 96-well plates under standard culture conditions and subjected to molecular characterization. Three different primer pairs, designed to interrogate distinct regions of the multigene *IFNT* locus, were used to identify candidate knockout single-cell clones. Primer Pair 1 amplified a ~200-bp fragment within the *IFNT* coding sequence (CDS) between the two sgRNA cut sites (CD-Forward: tgaggaccacatgctaggtg; CD-Reverse: ggaagaggttgaagcactgc). Primer Pair 2 spanned from a forward primer overlapping the sgRNA1 cut site to a reverse primer in the 3'-UTR, yielding a ~774-bp product (CS1-Forward: gtgctctctctactgatggcc; 3'-Reverse: tcagggcaggcatcacttaa). Primer Pair 3 amplified a ~1,045-bp fragment from the 5'-UTR to a reverse primer overlapping the sgRNA2 cut site (5'-Forward: gtgtattccccctccctgag; CS2-Reverse: gtgcagagctgctccagga). *GAPDH* served as a housekeeping control (F: cgggtgcaattaacttgctcctt; R: cttctgggtggcagtgatgg). Clones lacking amplification across all three *IFNT*-specific primer sets, indicative of complete deletion of all *IFNT* copies, were designated KO lines. Parental, unedited, WT BFFs served as controls throughout the genotyping, SCNT, and embryo transfer procedures.

Oocyte maturation for SCNT and IVF

Unless otherwise indicated, chemicals, media, and reagents used in experiments were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ovaries were collected from Brown Swiss and Simmental cows at a slaughterhouse and transported in phosphate-buffered saline (PBS) at 25-30°C. Cumulus-oocyte complexes (COCs) were obtained by follicle aspiration, washed in TCM 199 Hepes, and transferred into four-well plates (Nunc, Life Technologies GmbH, Darmstadt, Germany) with 400 µl modified Parkers medium (MPM) containing TCM199 supplemented with L-glutamine (100 mg/L), NaHCO₃ (800 mg/L), Hepes (1400 mg/L), sodium-pyruvate (250 mg/L), L-lactic-calcium-salt (600 mg/L), gentamicin (55 mg/L), and 5% estrus cow serum (ECS) containing 0.01 U/mL b-FSH and b-LH (Sioux Biochem, Sioux Center, IA, USA). For somatic cell nuclear transfer (SCNT), oocytes were matured for 16-18 h at 39 °C in a humidified atmosphere of 5% CO₂. Following maturation, cumulus cells were removed by incubation for 5 min in modified PBS (mPBS; PBS supplemented with 4 mg mL⁻¹ bovine serum albumin) containing 3 mg mL⁻¹ hyaluronidase, followed by vortexing for 4 min and gentle pipetting. For in vitro fertilization (IVF), oocytes were matured for 20-22 h under identical temperature and atmospheric conditions.

Somatic cell nuclear transfer

Somatic cell nuclear transfer was performed at 20-25°C using Leitz micromanipulators (Leica Microsystems, Wetzlar, Germany) mounted on a Wilovert stereo microscope. Denuded oocytes with a visible polar body were incubated for 1-2 h in TCM-199 Hepes supplemented with 10% FCS (Gibco, Life Technologies GmbH, Darmstadt, Germany) and 0.6 µg/mL demecolcine prior to enucleation⁴¹. The metaphase II spindle-chromatin complex was removed in a small drop of mPBS using an enucleation pipette. Enucleated oocytes were maintained in TCM-199 Hepes with 10% FCS and used as recipient cytoplasts. A single donor cell was placed beneath the zona pellucida of each enucleated oocyte. Karyoplast-cytoplast complexes (KCCs) were aligned between two 200-µm wire electrodes in Eppendorf fusion medium and fused using an Eppendorf Multiporator (Hamburg, Germany) with two direct-current pulses of 1.95 kV/cm for 25 µs. After a 30-minute recovery in TCM-199 Hepes with 10% FCS, fused KCCs were activated by a 5-minute treatment with 7% ethanol, followed by 5 h in 10 µg/mL cycloheximide and 5 µg/mL cytochalasin B. Activated embryos were washed three times and transferred to 450-µL drops of synthetic oviduct fluid (SOF) supplemented with 2% basal medium Eagle (BME) amino acids, 1% MEM nonessential amino acids, and 5% (v/v) ECS. Culture drops were covered with paraffin oil (Merck, Darmstadt, Germany), and embryos were incubated at 39°C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂.

In vitro fertilization and culture of embryos

Matured COCs were washed three times in fertilization medium Tyrode's albumin lactate pyruvate (FERT-TALP) supplemented with sodium pyruvate (2.2 mg/mL), heparin sodium salt (2 mg/mL), and BSA (6 mg/mL) and transferred to 400 µl droplets of medium. Frozen-thawed spermatozoa that had been

subjected to the swim-up procedure for 90 min were coincubated with COCs at 2×10^6 cells/mL for 18 h in maximum humidity, 39°C, and 5% CO₂ in air. Presumptive zygotes were mechanically denuded by vortexing, washed three times in SOF culture medium with 2% BME, 1% MEM, and 5% ECS, and transferred to 400 µl droplets of medium covered with mineral oil. The culture atmosphere was 5% CO₂, 5% O₂, 90% N₂, and 39°C at maximum humidity.

Long-term culture of bovine embryos

Day 8 SCNT or IVF blastocysts were individually placed into 60 µl micro drops of Ham's F12 medium supplemented, 10^{-4} M 2-mercaptoethanol (Merck, Darmstadt, Germany) and 15% FCS. Culture was carried out at 37°C with 10% CO₂ in humidified air. On Days 11, 15, 19, and 21, 30 µl of each drop was taken for quantification of IFNT and replaced by the same amount of fresh culture medium. Samples were stored individually at -80°C until further analysis.

Western blot analysis

To confirm the *IFNT* knockout at the protein level, conditioned media from KO embryos and WT embryos (both from SCNT and IVF) were used. Western blots were also done using the protein extracts from day 18 KO and WT (SCNT) conceptuses. For IFNT protein detection, 10 µL of embryo-conditioned medium or 20 µg of protein from day 18 conceptuses was used. Samples were mixed with 2× Laemmli buffer containing 100 mM DTT and then separated on SDS-PAGE gels. Electrophoresis was performed in SDS-running buffer at 80 V until the samples entered the separating gel, and then at 100 V until complete separation was achieved. Total protein was visualized under UV using the Intas imaging system (Intas SII GmbH, Göttingen, Germany) to confirm equal loading. Proteins were transferred to nitrocellulose membranes using a wet transfer system (Bio-Rad Laboratories, Hercules, CA, USA) at 0.15 A for 75 min. Transfer efficiency was verified by UV imaging. Membranes were blocked with 5% (w/v) milk in TBS-T for 1 h at room temperature and incubated overnight at 4°C with rabbit anti-bovine IFNT polyclonal antibody (Abbexa Ltd, Cambridge, UK; cat. no. abx177051) diluted 1:100,000 for conceptus extracts or 1:50,000 for embryo-conditioned media in 2% milk/TBS-T. After two 5-minute washes with TBS-T, membranes were incubated for 1 h at room temperature with HRP-conjugated anti-rabbit secondary antibody (1:10,000; Cell Signaling Technology, Leiden, Netherlands; cat. no. 7074), followed by three washes (2 × 5 min, 1 × 10 min) with TBS-T. Proteins were detected using ECL Pico substrate (Thermo Fisher Scientific, Waltham, MA, USA) with 1 min incubation in a light-protected container, and membranes were imaged using the Intas system. For loading control, anti-actin primary antibody (1:10,000; MP Biomedicals, Irvine, CA, USA; cat. no. 691001) and HRP-conjugated anti-mouse secondary antibody (1:10,000; Cell Signaling Technology, Leiden, Netherlands; cat. no. 7076) were used.

Cytopathic effect inhibition assay to detect IFNT activity

To confirm the complete absence of IFNT at the functional level, embryo-conditioned media from the KO and WT (IVF) embryos were used. Interferon activity in embryo-conditioned media was quantified using a classical cytopathic-effect (CPE) inhibition assay⁴², which measures the capacity of interferons to protect susceptible cells from virus-induced lysis. The assay was performed using Madin-Darby bovine kidney (MDBK; ATCC CRL-6071) cells challenged with vesicular stomatitis virus (VSV, Indiana strain). Briefly, recently split MDBK cells were incubated for 24 h with serial dilutions (50 µL each) of embryo-conditioned media, starting at 1:10 and proceeding in duplicate through eight two-fold dilutions to a final dilution of 1:1,280. Each 96-well test plate included a recombinant bovine IFNT reference standard and virus-only cytopathic effect controls. The VSV input dose was empirically calibrated to achieve complete CPE in untreated MDBK cultures. Using this virus concentration, a checkerboard titration with recombinant IFNT (starting at 50.2 ng) was performed to establish the assay's operational dynamic range. The concentration of recombinant IFNT required to achieve 50% inhibition of VSV-induced CPE (half-maximal effective concentration; EC₅₀) was determined to be 0.26 pg/µL, and this value was used to calculate interferon activity in experimental samples. As previously reported, antiviral activity in IVF embryo supernatants reflects the secretion of IFNT⁴³. Interferon activity was expressed as the reciprocal of the highest dilution providing ≥50% protection, and samples exceeding the upper detection limit (i.e., protection maintained at the highest dilution tested) were reported as "> upper detection limit (UDL)". Data were interpreted using established analytical frameworks for antiviral interferon bioassays, allowing for a direct comparison of secreted IFNT activity between SCNT-derived *IFNT*-KO embryos and WT IVF embryos.

Estrus synchronization of recipient heifers

Cyclic heifers, mostly of the German Simmental breed and some crossbred heifers from German Simmental and Holstein-Friesian, aged between 15 and 18 months and weighing 350 to 400 kg, were housed together as a group in a straw-bedded barn. Estrus synchronization was achieved by two intramuscular administrations of prostaglandin F2α (Estrumate®; Intervet, Unterschleißheim, Germany, 2 mL) at an interval of 11 days. All animals exhibited estrus within 60 to 96 hours after both the first and the second prostaglandin treatments.

Embryo transfer and tissue collection

In the first round of embryo transfers for day-18 conceptus collections, two groups of synchronized recipients were established, KO and WT, each comprising four animals. In the WT group, recipients received either one (n = 1) or two (n = 3) WT SCNT blastocysts, whereas all recipients in the KO group received two *IFNT*-KO SCNT blastocysts each (n = 4). SOF medium containing 5% OCS with embryos was loaded into 0.25-mL straws. Before transfer, animals were restrained in a standing position and administered local epidural anesthesia using procaine hydrochloride (Procamidol®; Richter Pharma AG, Wels, Austria; 4 mL). The genital tract was cleaned under hygienic conditions, and embryos were

deposited into the uterine horn ipsilateral to the functional corpus luteum using a standard transcervical transfer catheter. At 18 days of gestational age (dGA), terminal surgeries were performed on all recipients. Conceptuses were flushed from the uterus using D-PBS supplemented with 0.4% BSA. Trophectoderm length was recorded, and tissues were snap-frozen. Uterine flushes were stored for subsequent protein extraction and proteomic analysis. Endometrial biopsies were collected from different locations in each uterine horn, washed for five seconds in a buffer (0.4 M Na_2HPO_4 , 0.1 M NaH_2PO_4 , 0.5 M NaCl) containing 10 IU/mL heparin.

To evaluate whether genetic ablation of *IFNT* affects pregnancy establishment beyond the peri-implantation period, an additional cohort of synchronized recipients received either two ($n = 8$) or three ($n = 2$) *IFNT*-KO embryos per animal. Estrus cycles and pregnancy progression were monitored longitudinally by transrectal ultrasonography (MyLab™X90VET; Esaote VET, Genoa, Italy). Two pregnant recipients of *IFNT*-KO embryos were electively euthanized at gestational days 50 and 75, respectively. Euthanasia was performed by intravenous administration of pentobarbital (Release®; WDT, Garbsen, Germany; 100 mL).

Sample Preparation for Proteomics

To remove cell debris, uterine flush solutions were centrifuged at 3,200 g for 15 min at 4°C. 0.1 M NH_4HCO_3 solution was added to the samples to a final concentration of ~50 mM NH_4HCO_3 . Endometrial tissue samples and trophectoderm samples were lysed by the addition of 8 M urea, 0.4 M NH_4HCO_3 , and ultrasonicated with 24 10-s pulses (Bandelin Sonopuls UW3200, Bandelin, Berlin, Germany). The lysates were centrifuged at 20,817 g for 15 min at 4°C, and the supernatant was recovered. Protein quantification was performed with Pierce 660 nm Protein Assay (Thermo Fisher Scientific, Rockford, IL, USA). Proteins were reduced by the addition of dithiothreitol to a final concentration of 5 mM and incubation at 37°C for 30 min. Subsequently, alkylation was performed by adding iodacetamide to a final concentration of 15 mM and incubating in the dark at room temperature for 30 min. Digestion was performed at 37°C for 4 hours using Lys-C (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany) in a protease-to-sample ratio of 1:100. For the endometrium and trophectoderm samples, the final concentration was diluted with water to 1 M urea. Digestion was completed by adding modified porcine trypsin (Promega, Madison, WI, USA) in a protease-to-sample ratio of 1:50, followed by overnight incubation at 37 °C. The digestion was stopped by adding formic acid to a final concentration of 1%.

LC-MS/MS analysis

A nanoElute 2 LC system, coupled with a timsTOF HT mass spectrometer (both from Bruker Daltonics, Bremen, Germany), was used for mass spectrometry analysis. For each sample, 400 ng of peptides were injected on a trap column (Pepmap NEO C18, 300 μm x 5 mm, ThermoScientific) and separated at a flow rate of 250 nL/min using a PepSep column (25 cm x 75 μm , IonOpticks), with the following eluents:

Eluent A was 0.1% formic acid in water and B was 0.1% formic acid in acetonitrile. The separation method consisted of an initial ramp from 2% to 25% eluent B over 25 minutes, followed by a 12-minute gradient to 37%. MS spectra were acquired in dia-PASEF mode with 21-25 m/z wide windows and an ion mobility range of 0.85-1.27 k/e. Protein identification was performed using DIA-NN 2.1.0⁴⁴ and the Bos Taurus reference proteome at UniProt.org. Data analysis and statistical evaluation were performed using Perseus and R⁴⁵. Proteins exhibiting reduced abundance in endometrial samples associated with *IFNT*-KO embryos were further analyzed for pathway enrichment using STRING (string-db.org), with Reactome serving as the reference pathway database. For IFNT quantification, multiple peptides were considered, and only peptides that were proteotypic for at least one interferon tau subtype were retained for analysis to ensure the specificity of IFNT detection and quantification.

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Figures

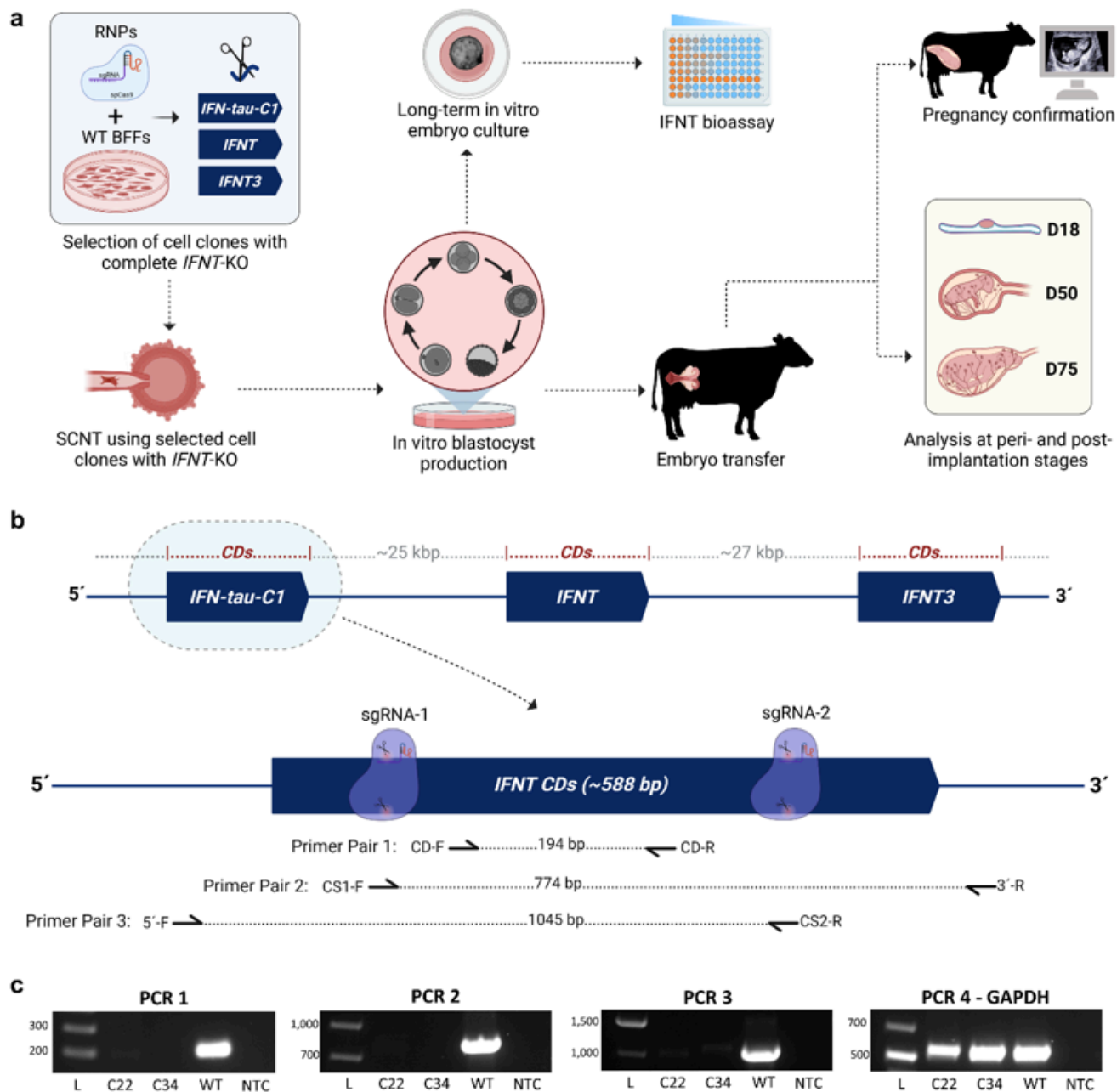


Figure 1

Study design and generation of *IFNT*-knockout donor cell lines. **a**, Schematic overview of the experimental strategy. Bovine fetal fibroblasts (BFFs) were genome-edited to delete all functional interferon-tau (*IFNT*) alleles, validated in vitro, and used as nuclear donors for somatic cell nuclear transfer (SCNT). The resulting embryos were assessed for IFNT secretion during extended in vitro culture before being transferred to synchronized recipients for analysis of pregnancy establishment at defined stages. **b**, Organization of the bovine *IFNT* multigene locus showing the locations of the two sgRNA cut sites and the three PCR assays used for genotyping. Primer Pair 1, for PCR 1, targets the *IFNT* coding sequence (CDS) between the two cut sites; Primer Pair 2, for PCR 2, spans the sgRNA1 cut site to the 3' untranslated region (UTR); Primer Pair 3, for PCR 3, spans the 5'-UTR to the sgRNA2 cut site. **c**,

PCR-based genotyping of candidate single-cell clones (C22 and C34). The absence of *IFNT*-specific amplicons across all three assays, with retention of the *GAPDH* control band, confirms the complete deletion of all detectable *IFNT* copies. L, DNA ladder; NTC, no-template control; RNP, ribonucleoprotein complex. Panels a and b created with BioRender.com.

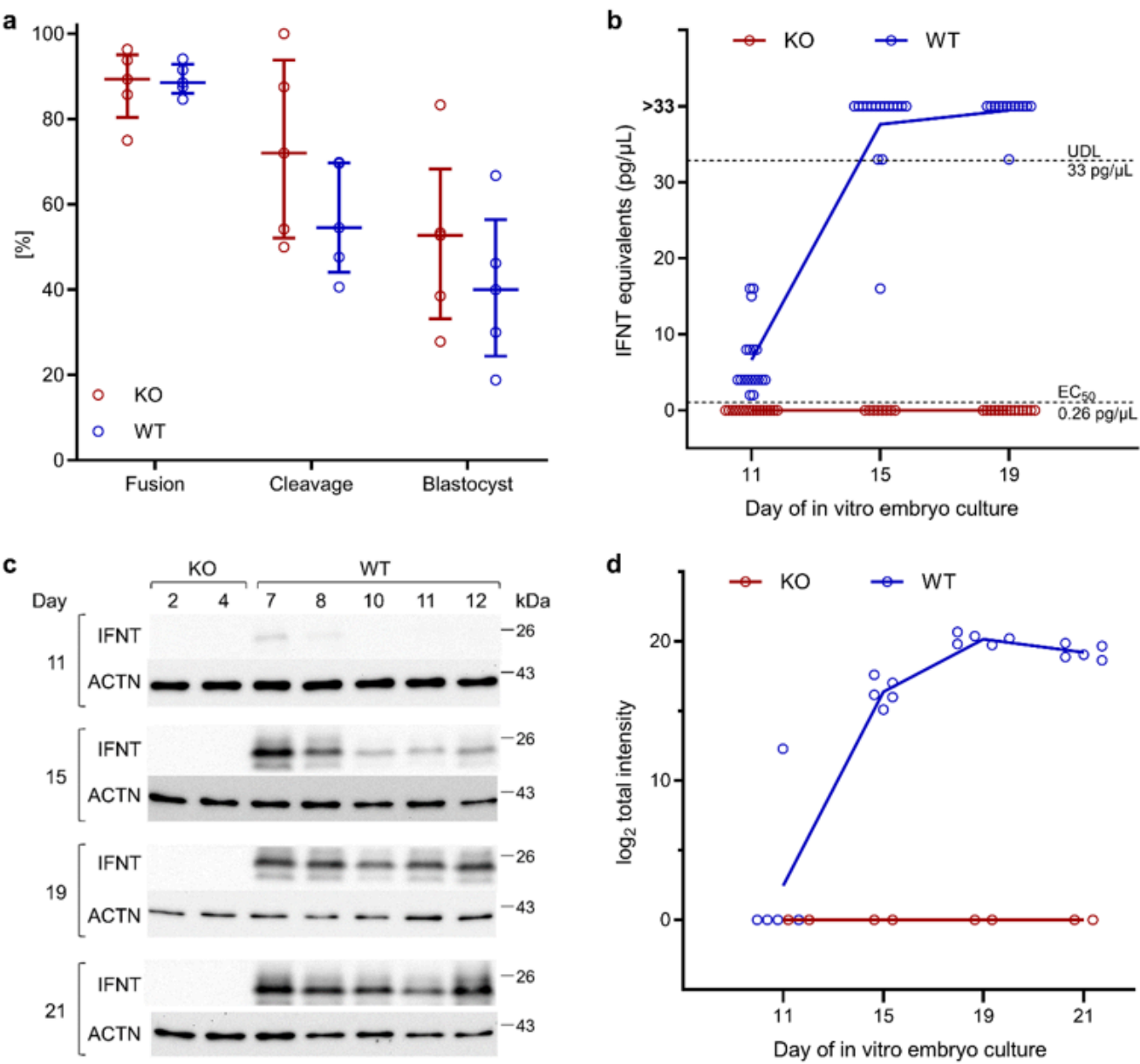


Figure 2

IFNT ablation does not impair preimplantation development. **a**, In vitro developmental competence of *IFNT*-KO and WT embryos generated by SCNT. Fusion rate, cleavage rate, and blastocyst formation rate were comparable between *IFNT*-KO and WT embryos. In addition to the results of individual experiments (circles), median and interquartile range are shown. **b**, Cytopathic-effect (CPE) inhibition assay assessing

IFNT antiviral activity in embryo-conditioned media collected at indicated time points during in vitro culture. Media from *IFNT*-KO embryos showed no detectable antiviral activity, whereas media from WT embryos exhibited robust IFNT activity that increased with embryo age. Each circle represents the IFNT activity in medium of an individually cultured embryo. **c**, Western blot analysis of IFNT protein in embryo-conditioned media from SCNT-derived *IFNT*-KO embryos (sample numbers 2 and 4) and *IFNT*-intact embryos generated by SCNT (sample number 7) or IVF (sample numbers 8, 10, 11, and 12). IFNT protein was undetectable in *IFNT*-KO samples but readily detected in WT samples. **d**, Peptide-based quantitative LC-MS/MS analysis of IFNT abundance in the same conditioned media samples. IFNT peptides were absent from *IFNT*-KO media and abundant in WT media, with peak levels observed at day 19 of in vitro culture. UDL, upper detection limit; EC₅₀, half-maximal effective concentration.

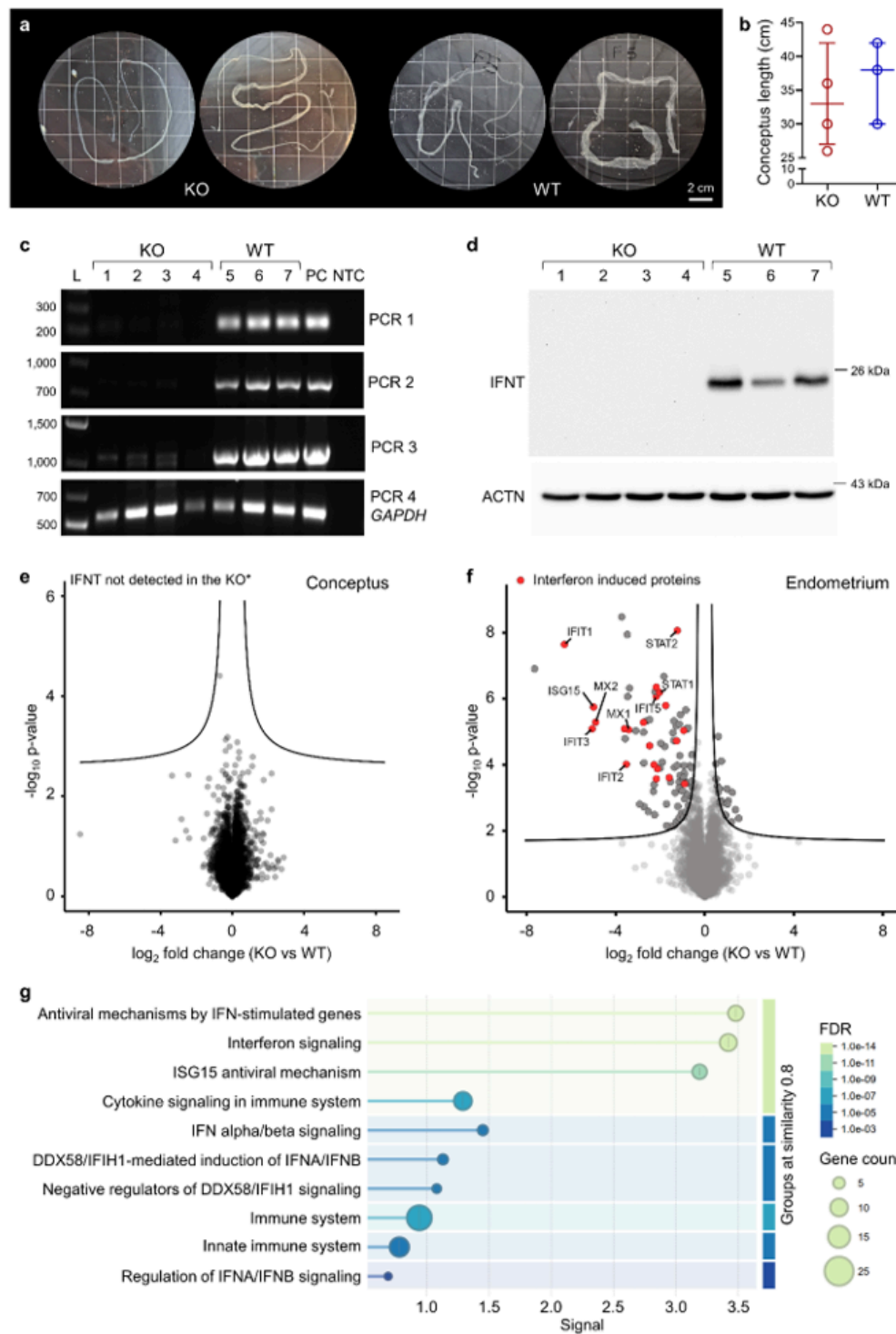


Figure 3

Early pregnancy establishment in the absence of conceptus-derived IFNT. **a**, Representative SCNT-derived *IFNT*-KO and WT day-18 conceptuses. **b**, Length of day-18 *IFNT*-KO and WT conceptuses. No significant difference was observed between the groups. **c**, PCR-based genotyping of recovered day-18 conceptuses using three primer sets targeting distinct regions of the *IFNT* locus. All *IFNT*-specific amplicons were absent in *IFNT*-KO conceptuses, while *GAPDH* amplification served as an internal control.

d, Western blot analysis of IFNT protein in day 18 conceptus tissues. IFNT protein was detected in WT conceptuses but was absent in *IFNT*-KO conceptuses. **e**, Quantitative proteomic analysis of day 18 conceptus tissues. Global protein abundance profiles were comparable between *IFNT*-KO and WT conceptuses; IFNT-derived peptides were detected exclusively in WT samples. **f**, Proteomic profiling of endometrial tissue collected from recipients carrying *IFNT*-KO or WT conceptuses. Interferon-stimulated proteins were markedly reduced in endometria associated with *IFNT*-KO conceptuses. **g**, Functional enrichment analysis of proteins downregulated in *IFNT*-KO endometrium, highlighting significant enrichment of interferon-related pathways, including type I interferon signaling and ISG15-mediated antiviral responses. FDR: false discovery rate; Signal: weighted harmonic mean between the observed/expected ratio and $-\log(\text{FDR})$; gene count refers to the number of proteins included in the enriched terms. *Details of all IFNT-specific peptides used and their results are provided in **Extended Data Table 3**.

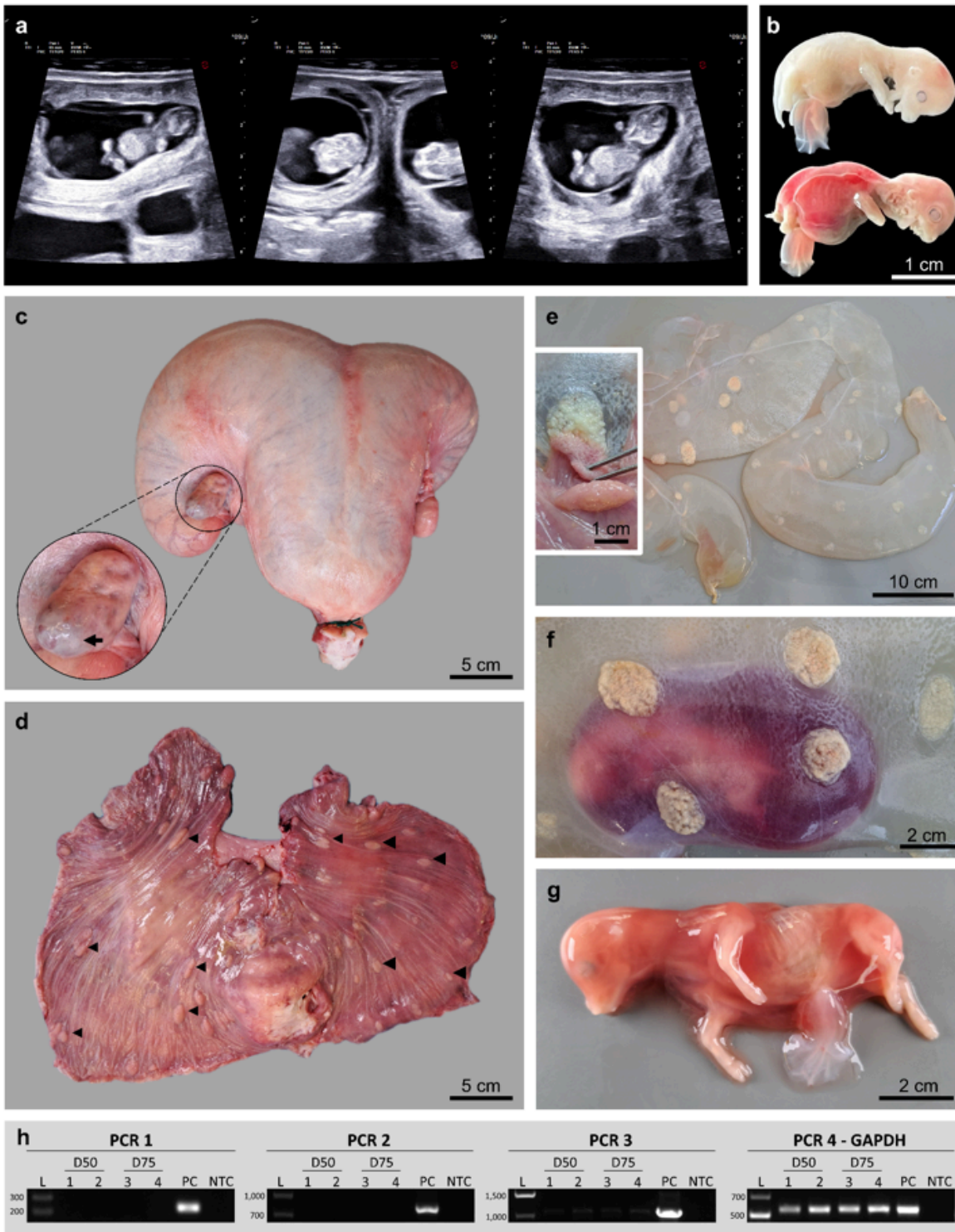


Figure 4

Establishment and progression of pregnancy in the absence of embryonic IFNT. **a**, Transrectal ultrasonography (day 49) confirming a twin pregnancy in a recipient (67927) receiving 2 *IFNT*-KO SCNT embryos. **b**, Fetuses recovered at day 50 of gestation following elective termination of another twin pregnancy (recipient 67942). One fetus displays normal morphology, while the second exhibits features consistent with known cloning-associated abnormalities. **c**, Gross anatomy of the uterus and ovary

recovered from recipient 67941 at day 75 of gestation, showing a normally developed uterus and a functional corpus luteum. **d**, Distribution of maternal caruncles throughout the uterus following dissection. **e**, Representative placentomes demonstrating normal attachment between maternal caruncles and fetal cotyledons. **f**, Chorioallantoic membrane showing evenly distributed fetal cotyledons. **g**, Fetus recovered at day 75 of gestation, exhibiting normal morphology without overt developmental abnormalities. A second fetus exhibiting cloning abnormalities is shown in **Extended Data Fig. 3**. **h**, PCR-based genotyping of fetuses recovered at days 50 and 75, confirming complete deletion of all *IFNT* loci. *GAPDH* served as an internal control.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [ExtendedDataTable1.xlsx](#)
- [ExtendedDataTable2.xlsx](#)
- [ExtendedDataTable3.xlsx](#)
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