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Anatomy

Expression dynamics of Crry at the implantation sites in normal pregnancy and response against miscarriage induction

Nobue KUNIYOSHI[1)], Saki HANADA[2)], Reina ANDO[2)], Lita Rakhma YUSTINASARI[3,4)], Maria KURATOMI[2)], Seizaburo KAGAWA[2)], Hiroyuki IMAI[2,3)], Ken Takeshi KUSAKABE[1–3)]*

[1)]Laboratory of Basic Veterinary Science, The United Graduate School of Veterinary Science, Yamaguchi University, Yamaguchi, Japan
[2)]Laboratory of Veterinary Anatomy, Joint Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan
[3)]Laboratory of Basic Veterinary Science, Joint Graduate School of Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan
[4)]Division of Veterinary Anatomy, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia

ABSTRACT. In mammals, immune tolerance against fetal tissue has been established for normal pregnancy progression. It is known that Crry regulates complemental activity to prevent injury of the mouse embryo and extra-embryonic tissue. This study aimed to examine the expression appearance and normal localization sites of Crry in the mouse placenta. Also, the emergency responses of Crry were verified at the time of experimental miscarriage induction. Moreover, we investigated an existing similar protein of Crry in animal placentas other than mice. *Crry* expression level showed a peak at day 8.5 of pregnancy. Trophoblast giant cells and decidual cells indicated a positive reaction to anti-Crry antibody. After treatments of interferon-γ, *Crry* expression was increased significantly in the survived implantation sites as compared with the controls. However, there was no significant difference in the miscarriage-initiated sites. It disclosed that Crry distributes from the early to middle periods of the placentas and involves complement regulation at the extraembryonic tissue and decidua basalis. Crry also showed an ability to respond to risk against external initiation for urgent miscarriage. Finally, we found anti-mouse Crry antibody-bound proteins in the placenta of many animals. It suggests a potency of Crry to make an environment of immune tolerance in many types of mammal placentas.

KEYWORDS: complement, Crry, decidual cell, trophoblast giant cell

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The complemental system belongs to natural immunity and has effects on pore formation on the target cell membrane, enhancement of vascular permeability, and initiation of opsonization [12]. Complemental factors have a risk for self-reactive injury from the complement C3 on the autologous cell membrane. The alternative pathway, one of the complemental active cascades, does not require specific recognition of the target cells likely antigen-antibody complex or lectin binding processes. Theoretically, the alternative pathway has an inherent risk for autoimmune complemental attack. Several inhibitory proteins have been found in the human complemental system, such as decay-accelerating factor (DAF or CD55), membrane cofactor protein (MCP or CD46), and an inhibitor of the membrane attack complex (CD59). These three complemental inhibitors have been found in the human placenta throughout pregnancy [7]. They seemed to engage in the specific protection of fetal tissues from maternal complemental attack, however, the fetal viability was normal even in the pregnant women who were genetically lacking DAF and CD59 genes [13]. Currently, the factors that effectively regulate complemental injury in the human pregnancy process are unknown.

In mice also, deletion of the DAF gene did not show significant phenotypes [16]. The distribution of MCP in normal mouse placentas has not been confirmed [23]. As for other candidates for complemental regulators, complement receptor 1-related gene/protein y (Crry) has been focused on. Crry can catch complement fragment of C3b and mask the complemental receptor 1 by the high affinity [22]. It was proven that Crry inhibits C3 deposition on the cell surface [11] and has a similar inhibitory mechanism to the human MCP

*Correspondence to: Kusakabe K-T: kusakabe@yamaguchi-u.ac.jp, Laboratory of Basic Veterinary Science, The United Graduate School of Veterinary Science, Yamaguchi University, 1677-1 Yoshida, Yamaguchi-shi, Yamaguchi 753-8515, Japan
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and DAF [5]. The systemic effect of Crry was confirmed to prevent deposition of C3 along with blood vessels in the lung, liver, and heart, and to maintain vascular permeability and normal blood pressure [10].

Xu *et al*. reported the indispensability of Crry in the normal progression of mouse pregnancy [23]. The Crry deficient embryos showed damage to the embryonic membrane by the maternal invasion of C3 at day 7.5 of pregnancy, and all fetuses died until day 16.5. This report showed that mid-pregnant periods are critical time points for complemental surveillance by Crry. However, the appearance dynamics of Crry have not been clarified yet throughout pregnancy.

We have found a clear elevation of adipsin, a complement activating factor, at the mouse placenta causing spontaneous abortion [8]. Along with adipsin protein, an increase of C3 protein and elevation of complement activity were confirmed in the mouse abortion placenta [17]. Treatment of recombinant adipsin protein affected elevating miscarriage occurrence rates in the early-mid mouse pregnancy period [18]. On the other hand, we simultaneously found the parallel elevation of Crry in the survived implantation sites after adipsin treatment. Similar results were obtained in the process of mouse normal pregnancy [19]. These findings made us suggest that the stable existence of Crry involves an immunotolerant environment in the placenta and can maintain a safe condition for pregnancy success. Also, flexible upregulation of Crry can improve resistant competency to abortion occurrence and exert fetal survival.

CD46 (MCP), a complemental protein firstly reported in the human placenta, has also been found in many species, including mouse, rat, guinea pig, bovine, pig, marmoset, squirrel monkey, and green monkey [20]. On the other hand, mouse Crry homologous proteins have been found only in rats. The immunity by complement factors is a phylogenetically old system that is equipped with starfishes and nematodes and is effectively used in various animals [12]. Crry may be building an underlying immune system commonly utilized in the mammal placentas. On the other hand, mammal placentas are classified into three fundamentally different structures, and their commonality is questionable.

We established here the expression pattern and localization of Crry in the mouse placenta. Our data propose here an effective period of Crry and an operational target for maternal immune tolerance. Also, we found here that experimental initiation of abortion can change the dynamics of placental Crry expression. Finally, this study verified the existence of analogous proteins with mouse Crry among several different types of placentas.

MATERIALS AND METHODS

Laboratory mice

ICR and C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan). They were bred under the controlled condition (temperature 22 ± 2°C, humidity 50 ± 10%, and 12–12 hr light-dark cycle) with water and food *ad libitum*. After 1-week acclimation, eight to ten-week-old virgin mice were paired in syngeneic in the evening. On the next morning, the mice confirmed vaginal plugs were designated as day 0.5 of pregnancy (D0.5).

ICR mice were euthanized by cervical dislocation after deep anesthesia by isoflurane. More than three pregnant mice were used at each time point and more than five pregnant implantation sites were collected from each pregnant mouse. Specimens were fixed in 10% neutral-buffered formalin for histological studies. Other samples were stored by rapid freezing for quantitative PCR and western blot.

All mice used in this study were kept under veterinary health control and received care along with the guideline for the proper implementation of animal use at the Yamaguchi University. Experimental designs were passed preliminary reviews by the Ethical Committee on Animal Experimentation at Yamaguchi University (Approval numbers: 361 and 443).

Miscarriage models

Inbred strain mice have been used for the experimental miscarriage model produced by the published method [9]. C57BL/6J strain was selected to prepare the model and to compare with our relative data [8, 17, 19]. Pregnant C57BL/6J were treated with 5,000 U recombinant murine interferon-gamma (IFN-γ, PeproTech, Rocky Hill, NL, USA) by intraperitoneal injection at one time on D6.5. Control mice were treated with the same volume of vehicle (0.2 mL of saline) at the same schedule as the experimental group. D10.5 was selected to investigate the placental appearance of Crry adjusting to the previous report [23]. Mice were administrated anesthetics by isoflurane and collected whole blood from the heart. After euthanasia, the pregnant uterus, liver, and spleen were collected. Each implantation site was divided separately, weighed, and dissected under a stereomicroscope to observe discolorization, fetal heartbeat, and absorbed conditions (Supplementary Fig. 1). Reproductive performances were scored to evaluate the effects of the treatments (Table 1).

Table 1. Influences of interferon (IFN)-γ treatment on the mouse reproduction (D10.5)

Treatment		No. of total implantation sites	Appearance rates of miscarriage sites (%)	Weight of individual implantation site (mg)	Classification in Suppl Fig. 1
Control (6)	Normal sites	4.17 ± 1.11	95.5 ± 2.83	95.1 ± 3.41	(i)
	Miscarriage sites		4.46 ± 2.83	40.0 ± 4.00	(ii)
IFN-γ (7)	Normal sites	4.57 ± 0.69	85.0 ± 4.10*	109.1 ± 5.17	(iii)
	Miscarriage sites		14.6 ± 4.10*	38.6 ± 5.10	(iv)

Parentheses show the number of examined mice. *P<0.05, vs. control.

Animal placentas

Afterbirth placentas were used by the research corporation from Japan Racing Association (JRA, Thoroughbred horse), Tokuyama Zoo (reticulated giraffe and Japanese macaque), Nogeyama Zoological Gardens (chimpanzee), and Japan dolphin center (bottlenose dolphin). Beagle dog placenta was collected by the Caesarean operation during the preterm period. A miniature pig in the late pregnancy period was purchased from Yamaguchi Agricultural High School. Pig sedation was conducted with 0.3 mg/kg midazolam, 0.04 mg/kg medetomidine, and 0.1 mg/kg butorphanol, and euthanasia was performed using the electrical stunning equipment (ST-EC-2CT and ST-ZK, FREUND Maschinenfabrik GmbH & Co., KG, Paderborn, Germany). Experimental designs used pigs were passed preliminary reviews by the Ethical Committee on Animal Experimentation at Yamaguchi University (Approval numbers: 319). Crown-rump lengths of the pig fetuses in the pregnant uterus were 17.5 and 19.8 cm, respectively.

Quantitative PCR (qPCR)

Total RNA was purified from each implantation site with ISOGEN2 Regent for RNA Extraction kit (Nippon Gene, Tokyo, Japan). At D6.5, one implantation site was trimmed including the embryo was used. On other days of pregnancy, an embryo was removed from each implantation site after checking for miscarriages. cDNA was synthesized by reverse-transcription reaction using the ReverTra Ace qPCR kit (TOYOBO, Osaka, Japan). Real-time PCR reaction was performed using the KAPA SYBR FAST kit (Nippon Genetics, Tokyo, Japan) and CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Primer sequences were designed based on the GenBank database (National Center for Biotechnology Information, Bethesda, MD, USA) as follows. Mouse *Crry*: 5′-ACT CAA CCT GGA CGA GTG CT (forward), 5′-CTG GGG GTA TCT CAC AAG GA (reverse); mouse *C3*: 5′-ACG GCA TCT TGC CTT TGT CTT GGA (forward), 5′-GAG AAG ATC TGC TTC GGC GCA TGT (reverse); mouse *GAPDH*: 5′-CAT GGC CTT CCG TGT TCC TA (forward), 5′-GCG GCA CGT CAG ATC CA-3′ (reverse). The amplification efficiency of the primer pairs was checked by the data from amplification plots, and the specificity of the PCR products was confirmed by the data from melting curves. The Cq values from qPCR on the target genes were corrected by the corresponding Cq values of *GAPDH* qPCR. The values of expression frequencies were calculated from the corrected Cq values by the formula of minus factorial power of 2. Final data were further converted to relative values to present a mean of the control group or the reference group as 1.0.

Histological studies

Paraffin sections were hydrophilized and treated with 20 mM Tris-HCl buffer (pH 9.0) at 120°C for 10 min. Sections were then treated with 0.3% hydrogen peroxidase, 10% goat serum, and rabbit anti-mouse Crry (Santa Cruz, Dallas, TX, USA). Antibody-binding sites were detected by the Histofine Simple Stain Mouse MAX-PO (R) kit (Nichirei, Tokyo, Japan) and the Peroxidase Stain DAB Kit (Nacalai Tesque, Kyoto, Japan). Counterstaining was performed with hematoxylin.

Western blot

Frozen samples from animal placentas were trimmed in the detergent buffer (10 m M CHAPS, 0.15 M NaCl, 0.01 M sodium phosphate, 2 mM EDTA, pH 7.2) with proteinase inhibitors (10 mg/mL aprotinin, 100 mM sodium vanadate, and 10 mg/mL phenylmethylsulfonyl fluoride). These samples were homogenized with a device of a multi-beads shocker (MB1001C, Yasui Kikai, Osaka, Japan) and spin-downed to collect the supernatant. Equalized protein specimens (10 µg/lane) were loaded to the SDS polyacrylamide gel and electrophoresed. Fractionated proteins were transferred to a polyvinylidene difluoride membrane (Tefco, Tokyo, Japan). Immunoblot was carried out with rabbit anti-mouse Crry (same as above) and horseradish peroxidase-labeled anti-rabbit IgG (Promega, Madison, WI, USA). Luminescence images were captured using the Chemi-Limi One L (Nacalai Tesque) and LAS-3000 mini (Fuji Photo Film, Tokyo, Japan). Band strength was measured using Image J software (National Institutes of Health, Bethesda, MD, USA).

Serum specimens were measured on the protein concentration and applied to the SDS-PAGE and membrane transfer mentioned above. Anti-C3 antibody (MP Bio, Solon, OH, USA) was used for immunoblotting. C3 protein levels were evaluated by measuring the small C3b fragment (about 40 kDa) synthesized by limited degradation of the precursor protein during the activating process [12]. The values for band densities were corrected by each value of the total serum protein amount.

Statistical analysis

Each experimental group was composed of more than three mice. Statistical significance was analyzed by the two-sample *t*-test in the case of equal variances of two groups by previous analysis of the *F*-test analysis. If a difference in the variances was found, the Welch *t*-test was applied. Probability values under 0.05 were considered significant. Data were represented as mean ± standard error.

RESULTS

Crry appearances in expression and distribution

Crry mRNA was detected in mouse pregnant uteri at D6.5 (Fig. 1A). Expression intensity became in peak at D8.5, however, the variance of expression intensities was very wide among each implantation site. The *P*-value of the *F*-test was 0.0005 between D6.5 vs. D8.5. Significant differences were not detected during the time course of pregnancy (D6.5 vs. D8.5, D8.5 vs. D10.5, and D10.5 vs. D14.5). On D6.5, faint immunopositivity to anti-Crry antibodies was found at capillaries in the decidua basalis (Supplementary Fig. 2A). On D8.5, positive sites of Crry protein were found at the decidua basalis and ectoplacental cone of extraembryonic tissue (Fig. 1B). In contrast to the decidua basalis, reactivity in the decidua capsularis region was weak. At the ectoplacental cone, Crry-positive reactions were seen inside and internal spaces of trophoblast giant cell (TGC) s. Positive reactions in TGCs were also confirmed at

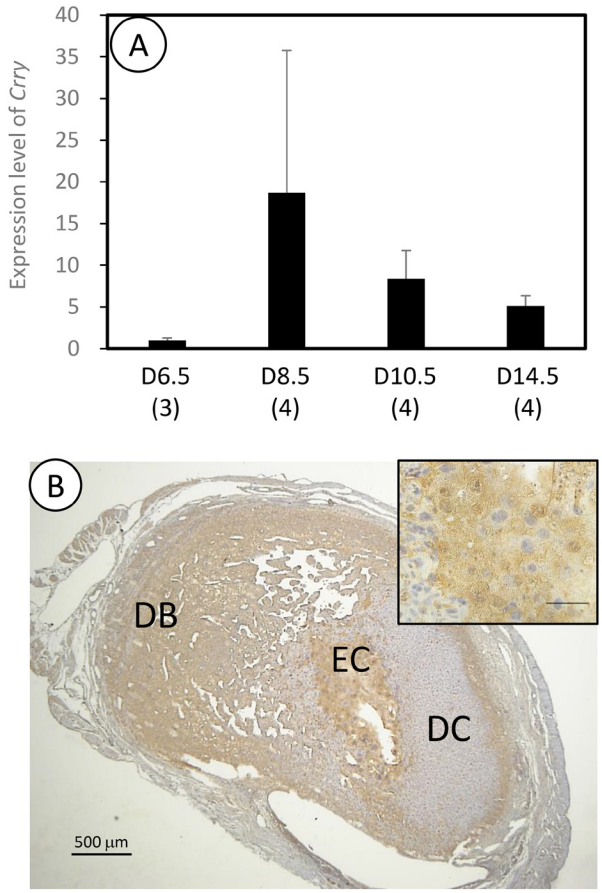

Fig. 1. (**A**) qPCR analysis for *Crry* expression at mouse implantation sites. The relative expression values were displayed based on the D6.5 expression level as 1.0. No significant differences were detected between consecutive test dates. The numbers of mice used for statistical analyses are shown in parentheses. (**B**) Immunohistochemistry for Crry at the implantation site of D8.5. DB: decidua basalis, EC: ectoplacental cone, DC: decidua capsularis. Inset: high magnification of the EC region. (Inset) Crry-positive reactions are seen around trophoblast giant cells (Bar scale=100 μm).

D10.5 but attenuated at D14.5 (Supplementary Fig. 2C and 2D). At D8.5, positive reactions were also detected in the surrounding area of blood vessels at the maternal and fetal boundary areas (Supplementary Fig. 2B).

Reactivity of Crry following induction of abortion

IFN-γ treatment significantly decreased the appearance rate of normal implantation sites and elevated the rate of miscarriage (Table 1). At the miscarriage sites, no major changes were detected in the expression intensity for *Crry* expression in comparison to the normal sites from the control mice (Fig. 2A). On the other hand, *Crry* expression was elevated significantly in the normal sites after IFN-γ treatment. Crry immunostaining showed positive reaction on the membrane of TGCs at the junctional zone of the normal placenta (Fig. 2C). On the other hand, Crry localization was found mainly along vessel walls in the abortion sites (Fig. 2D). qPCR analysis for complement *C3* was performed to examine a correlation with the *Crry* expression pattern. After IFN-γ treatment, enhancement of *C3* expression was observed in the miscarriage sites (Fig. 2B). However, significant differences were not detected in comparison to the normal sites from the control mice (P=0.07) and to the normal sites from the IFN-γ-treated mice (P=0.06). C3 protein was distributed in TGCs cytoplasm (Fig. 2E) and the C3-positive TGCs were increased in the abortion sites (Fig. 2F). IFN-γ treatment also had significant effects on the maternal mice, raising *C3* gene transcription in the spleen (Fig. 3B) and the serum level of C3 protein (Fig. 3C). However, less effect was observed on the *C3* expression level in the maternal liver (Fig. 3A).

Crry analogous proteins in various animal placentas

The peptide framework of mouse Crry is 53.763 kDa in molecular weight [21]. Glycosylation of protein has been observed to increase the molecular weight to 65–70 kDa [14, 22]. Western blot analysis clarified that every protein sample purified from animal placentas showed a positive reaction to mouse Crry antibody (Fig. 4). The target sites of the first antibody were amino acids 121–300 of mouse Crry (datasheet from manufacture) and the total numbers of amino acids are 483 in the dominant isoform [21]. The existence of anti-mouse Crry antibody-bound proteins was independent of placental types (diffuse placenta 1, 2, 4; cotyledonary placenta 3; zonary placenta 5; discoid placenta 6, 7, 8). Especially, the protein band slightly above 50 kDa (a) was strongly detected in the placentas of the pig, dog, macaque, and chimpanzee. The band under 75 kDa (b) was commonly detected in many animals as well as in the mouse, except the chimpanzee. Although the under 75 kDa band was detected in horse placentas, the reactivity was too strong to separate the protein well.

DISCUSSION

We found a strong expression of *Crry* at day 8.5 of pregnancy in mouse placentas. The most prominent sites for Crry positive reactions were TGCs during days 8.5–10.5 (Fig. 1B inset, Supplementary Fig. 2C). TGCs have an invasion activity into the endometrium and contribute to the formation of the foundation of the allantochorionic placenta. Also, TGCs engage essential reproductive functions for secretion of humoral factors (placental lactogen, etc.) and iron supply to the embryo by engulfment of maternal erythrocytes. TGCs contact closely with maternal cells and blood, and in the structural and functional relevance, they are exposed to the risk of maternal immunological attack. Crry was suggested to protect TGCs from complement-derived cell injury and to bring a sustainable effect for TGC activity during placentation periods. Expression of complement suppressor in the human trophoblasts has been also reported and similar consideration has been made [9]. Also, our previous study also detected Crry not only from the cytoplasmic fraction but also from the fluid soluble fraction in the protein specimens purified from mouse placentas [19]. These data do not eliminate the possibility that Crry is secreted from TGCs and spread to the decidua basalis.

Decidua basalis also showed a positive reaction to Crry (Fig. 1B). On day 6.5, Crry positive reaction was observed around blood vessels in the decidua basalis (Supplementary Fig. 2A). The area of decidua basalis is characterized as having high vascular permeability

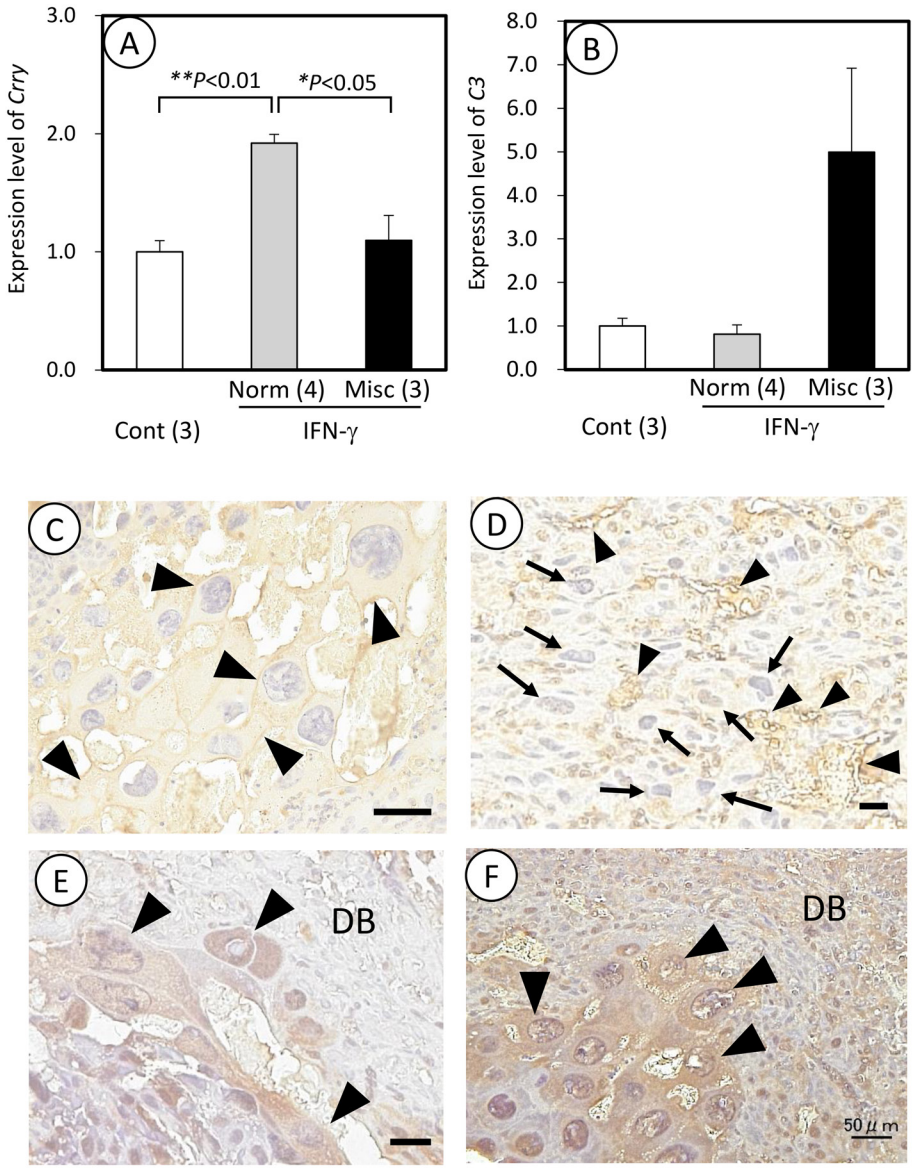

Fig. 2. qPCR analysis for *Crry* (**A**) and *C3* (**B**) expression following interferon (IFN)-γ treatment (D10.5). The relativized calculation was conducted to be 1.0 on the normal sites from the control mice (Cont). Implantation sites collected from IFN-γ treated mice were analyzed separately into normal (Norm) and miscarriage sites (Misc). The numbers of mice used for statistical analyses are shown in parentheses. In the statistical analysis in B, significant differences were not detected due to a magnitude of variant values in the group of Misc. Immunohistochemistry for Crry (C, D) and C3 (E, F) on the placentas following the IFN-γ treatment. (**C**) In the normal sites, positive reactions of Crry are shown on the membrane of trophoblast giant cells (arrowheads). (**D**) In the miscarriage sites, most of the trophoblast giant cells show negative (arrows), and positive reactions are seen on the vessel walls (arrowheads). (E, F) Positive reactions of C3 are seen in the cytoplasm of trophoblast giant cells (arrowheads). Positive ratios of the C3-positive trophoblast giant cells appeared to be higher in the miscarriage site (**F**) than in the normal sites (**E**). Positive intensity also increased at the decidua basalis (DB) in the miscarriage sites. Scale bars in C–E show 50 μm.

evidenced by blue dye incorporation [2]. C3 is generally known to be produced mainly in the liver but the gene expression has been detected in various cells and systemic organs [6]. C3 can reach implantation sites hematogenously by the route of the uterine artery and segmental arteries inflowing into the decidua basalis. Crry is also known as a name of "Complement component receptor 1-like protein" [21], which can trap C3b and C4b. In other mouse models of the experimental abortion, placental damage by C3 deposition has been commonly observed both in trophoblasts and decidual tissues [4]. On the mouse placentas that caused spontaneous abortion, the localization of complement activator adipsin was remarkable in the decidua basalis [17]. It seemed that the distribution of Crry in decidua basalis also reflects a surveillance function in the implantation sites to reduce the risk of attack from maternal complements.

Mouse placenta showed a Crry-enhancing response to the IFN-γ treatment. This enhancement was pronounced in the normal sites and appeared to be effective in the membrane protection of TGCs (Fig. 2C). TGC protection by membranous Crry appeared to be inadequate in the miscarriage placentas (Fig. 2D). Crry-protective abilities showed a potentiation in the mouse placenta for

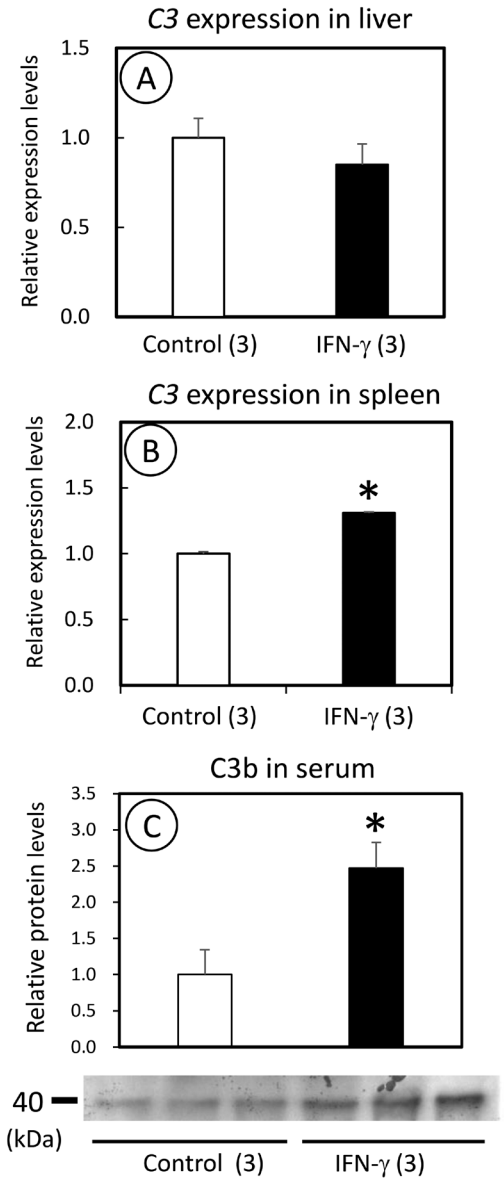

Fig. 3. Systemic changes of C3 gene expression and protein level following interferon (IFN)-γ treatment. qPCR analysis for *C3* expression in the liver (**A**) and the spleen (**B**). Western blot analysis for C3b in the serum of whole blood (**C**). The numbers of mice used for statistical analyses are shown in parentheses. All data were displayed relatively based on the control level of 1.0. **P*<0.05, vs. control.

reactivity against external stimuli that can elevate the systemic C3. "External stimuli" refers to anything involved in activating immunity, increasing complement factors, and inducing miscarriage in the pregnant mother. On the other hand, the local condition of Crry expression at 8.5 days seemed to be highly variable and unstable. The Crry elevation after IFN-γ treatment did not find in all implantation sites and could not survive all implantation sites. Perhaps since the developmental level of the implantation sites in the early-mid gestation period is not constant, the velocity of the expression of Crry was not constant, and not all implantation sites were able to maintain high expression of Crry. The regulatory mechanisms of Crry expression and localization are currently unknown, but it is possible that TGCs and decidua cells are major producers in the mouse placenta and the release may be influenced by pregnancy-related humoral factors.

In the miscarriage sites, C3 localization at TGCs was dominantly found as in our previous report [8]. In the present study, expression properties of Crry and C3 after IFN-γ treatment were not correlated completely with each other at the normal and miscarriage sites. The regulatory mechanisms for both enhancements appeared to be independent. Elevation of C3 in the miscarriage placentas may be a posterior change after the miscarriage has occurred. Deposition of C3 was a prominent symptom in the placenta that caused spontaneous abortion [8, 23]. Moreover, C3 metabolites can elevate opsonization activity on macrophages and induce the elimination of dead autologous cells [14]. Concerning rapid reactivates by IFN-γ treatment, Strunk *et al.* reported C3 upregulation and secretion from the cultured fibroblasts in the combination with lipopolysaccharide treatment [15]. This paper also supports our results of the systemic C3 enhancement in the whole blood by IFN-γ. Additionally, human proximal tubular epithelial cells can produce C3 by IL-2 treatment and which means elevation of the nephritis risk [3]. In similar, a high amount of C3 influx into implantation sites was predicted to elevate the risk of injuriousness, but pre-deployment of Crry may avoid fetal tissue damage and contribute to the formation of the immunotolerance condition.

Despite its assumed importance involved in normal pregnancy maintenance, only mouse and rat have been found to possess the Crry gene. We found homologous amino acid sequences to mouse Crry protein by the BLAST analysis in the UniProtKB database for mammal proteins (as of August 9, 2022). The complement receptor isoforms in *Delphinapterus leucas* show 48.5–51.6% similarities to mouse Crry (Accession No. A0A2Y9PQW5, A0A2Y9Q1G1, A0A7F8KBU8, A0A2Y9PVK7, A0A2Y9PUN0, A0A2Y9PVK3, and A0A2Y9QV4). In *Canis familiaris*, uncharacterized 7 proteins have high homologous sores of 46.0–51.6% (Accession No. AOA8I3MIN5, AOA8I3N180, AOA8I3MKC2, AOA8I3MKY0, AOA8I3MKM4, AOA8I3N1T1, and AOA8I3MLF7). The results from our western blot and *in silico* homology analysis indicate a possibility for Crry analogous proteins distributing among many species in mammals. They also showed a potency that multiple variants of Crry analogous proteins coexist in the placenta of horses, dogs, and primates. As noted earlier in the result section, molecular sizes of each analogous protein showed no correlation with the classification of the placental barrier in each placenta. It seemed that Crry is a common protein produced in mammal placentas and has a wide range of applications for complemental regulation in the control of animal reproduction.

Recently, a fusion protein between Crry and natural antibody was developed, and its availability was proven to inhibit autoreactivity of complements in the joint cavity and relieve arthritis symptoms [1]. It is expected that Crry can be applied for a local equilibrium of complemental activity, contributing to a therapeutic agent for breeding disorders and controlling immune privilege conditions dependent on complemental activity.

This study indicated that Crry expressed strongly and transiently at day 8.5 of pregnancy, and localization sites in the mouse placenta are trophoblast giant cells (junctional zone in the matured placenta) and decidua basalis. Crry can react to the abortion initiation by IFN-γ and enhance the expression degree in the placenta. Crry may protect trophoblast giant cells against membrane injury activity from complement C3 and prevent the onset of miscarriage partially. Analogous proteins to mouse Crry seemed to exist in the placenta of other mammals. These results will contribute to a clarification of reproductive physiology and pathogenesis of miscarriage widely applicable in reproductive controls in domestic animals and exhibit animals in zoos.

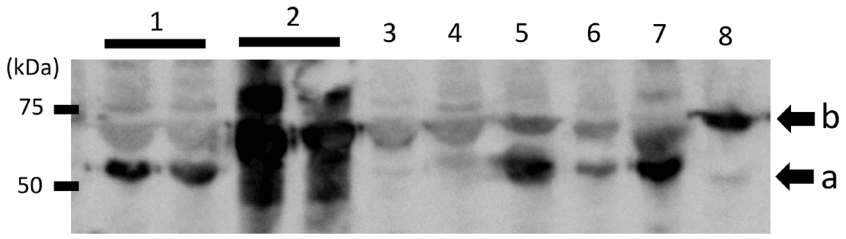

Fig. 4. Western blot analysis for the analogous proteins to mouse Crry. Protein specimens were purified from the placentas of pig (1), horse (2), giraffe (3), dolphin (4), dog (5), Japanese macaque (6), chimpanzee (7), and mouse (8). Pig placentas were collected from two implantation sites of the same dam. Horse placentas were collected from two different pregnant females. The mouse placenta was used as a positive control (D10.5). Arrows indicate reference sizes of mouse Crry protein in the forms of peptide framework (**a**) and glycosylation (**b**).

CONFLICT OF INTEREST. The authors have declared that no conflict of interest exists.

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