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Abstract

Leukemia inhibitory factor (LIF) is a cytokine which is essential for oocyte and

embryo development, embryonic stem cell, and induced pluripotent stem cell maintenance. Leukemia inhibitory factor improves the maturation of oocytes in the human and the mouse. However, feline LIF (fLIF) cloning and effects on oocytes during IVM have not been reported. Thus, we cloned complete cDNA of feline LIF and examined its biological activity and effects on oocytes during IVM in the domestic cat. The amino-acid sequence of fLIF revealed a homology of 81% or 92% with that of mouse or human. The fLIF produced by pCold® TF DNA in *Esherichia coli* was readily soluble and after purification showed bioactivity in maintaining the undifferentiated state of mouse embryonic stem cells and enhancing the proliferation of human erythrocyte leukemia cells. Furthermore, 10- and 100- ng/mL fLIF induced cumulus expansion with or without FSH and EGF ($P < 0.05$). The rate of metaphase II oocytes was also improved with 100 ng/mL fLIF ($P < 0.05$). We therefore confirmed the successful production for the first time of biologically active fLIF and revealed its effects on oocytes during IVM in the domestic cat. Feline LIF will further improve reproduction and stem cell research in the feline family.

Keywords: Recombinant protein; Feline; IVM; Leukemia inhibitory factor; Cumulus expansion; Nuclear maturation

1. Introduction

Leukemia inhibitory factor (LIF), belonging to the interleukin-6 (IL-6) family, was discovered as a cytokine that induced the differentiation of mouse myeloid leukemia cells [1]. Subsequent studies have reported various biological effects of LIF on a large range of cell types, including monocytes, megakaryocytes, neuronal cells, osteoblasts, oocytes, hepatocytes, adipocytes, embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs), which have receptors for LIF [2-7]. Most of effects exerted by LIF are similar to those of IL-6 [8].

Leukemia inhibitory factor also has further effects, not shared with IL-6. LIF supplementation during IVM of human and mouse oocytes induced cumulus expansion and in mouse enhanced cleavage rate at two-cell stage as well as birth delivery [9]. Furthermore, LIF prevented naive ESCs and iPSCs from differentiating, which have higher abilities to proliferate and differentiate [10]. These specific effects of LIF help us to improve reproductive techniques and generate naive ESCs and iPSCs, which could be used in the preservation of wild animals [11-13], regenerative medicine [14, 15] and treatment of transmissible diseases [16, 17].

Recombinant human and mouse LIF have been produced by utilizing *Esherichia coli*,

yeast and COS cells [18-20]. Protein expression in *E coli* is superior to that with yeast or COS cells in ease of manufacturing and quantity of production [21]. Some drawbacks remain, such as the dependence of the efficiency of collecting protein on its behavior and size, because of solubility and inclusion bodies, and loss of activity caused by few posttranslational modifications [22-24]. The protein expression vector pCold® TF DNA possesses a trigger factor which is known to improve the solubility of synthetic proteins [25]. In previous studies, LIF was produced by utilizing *E coli*, which showed that LIF may still be biologically active without posttranslational modifications [26].

During IVC of bovine oocytes, human LIF (hLIF) reduced the blastocyst rate, though the sequence of bovine LIF revealed a homology of 89.1% with that of hLIF [27]. Human and mouse LIF, whose aminoacid homology with that of porcine was 88% and 79%, respectively, failed to generate porcine ESCs and iPSCs, however, the cultivation with porcine LIF could establish porcine iPSCs [28, 29]. Although, ESCs of domestic cats were generated with mouse LIF (mLIF), they could not be maintained in an undifferentiated state for a prolonged period or differentiate into three germ layers [30, 31]. The iPSCs of endangered felids were also established with mLIF, whose transgene expressions were sustained [32, 33]. These studies suggest that heterospecific LIF should be carefully used instead of homospecific LIF. Biologically active LIF has been

produced only in the human [19], the mouse [1], the rat [34], the possum [35] and the chicken [36], but not in the domestic cat which is a good research model for wild felids faced with extinction. Thus, the purpose of this study was to clone the complete cDNA of feline LIF (fLIF), produce biologically active fLIF in *E coli* with effortless purification at a higher efficiency, and examine its activity in relation to mouse ESCs, human erythroleukemia cells, and domestic cat oocytes.

2. Materials and methods

2.1. Culture of feline embryonic fibroblasts

We collected domestic short-haired cat fetuses from a cat that underwent ovariohysterectomy at a local veterinary clinic at day 30 of pregnancy. The cat was privately owned, and the owner's consent was obtained before the collection of the fetuses. No cats were bred and operated upon specifically for this study. The fetuses were washed with PBS, and their heads and livers were removed. The fetuses were then cut into small pieces and cultured in Dulbecco's modified Eagle medium (Life Technologies, San Diego, CA, USA) supplemented with 10% (v:v) fetal bovine serum (FBS; PAA Laboratories, Pasching, Australia), 100-IU/mL penicillin and 100-μg/mL

1 streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 2-mM L-glutamine
2 (Sigma-Aldrich) at 37°C under 5% CO_2 in humidified air. The adherent cells were
3 passaged with 0.25% trypsin (Sigma-Aldrich). After three passages, the spindle cells
4 were used as feline embryonic fibroblasts for RNA isolation.
5 deoxyribonucleotides
6 2.2. Cloning of fLIF
7 Total RNA was extracted from cat embryonic fibroblasts with RNeasy Mini
8 (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Total RNA was
9 used in a reverse transcription (RT) reaction with a reactive solution (20 μL) containing
10 0.5-mM deoxyribonucleotides mixture (TOYOBO, Osaka, Japan), 2.5-μM random 9
11 mer (TOYOBO) and 5-U/μL ReverTra Ace (TOYOBO) in the appropriate RT buffer.
12 The RT solution was incubated at 30°C for 10 min, at 42°C for 50 min, then at 99°C for
13 5 min.
14 Following the comparison of the nucleotide sequences of LIF in human
15 (NM_002309) and rat (NM_022196), primers for cloning were designed with reference
16 to common parts between both of them. Polymerase chain reaction (PCR) was carried
17 out in 20-μL KOD buffer containing 0.5-μL RT products, 0.2-mM dNTPs, 0.02-U/μL
18 KOD plus (TOYOBO), 1- mM $MgSO_4$ (TOYOBO), and 0.5 μM of a pair of primers

(Table 1). The thermocycling protocol comprised preincubation at 94°C for 2 min and 35 cycles at 94°C for 15 seconds, 60°C for 30 seconds, 68°C for 1 minutes, and finally 68°C for 2 minutes. The chain reaction product was inserted into pGEM®-T Easy Vector (Promega, Madison, WI, USA) and sequenced by Sigma-Aldrich, Japan, using a BigDye® Terminator version 3.1 Cycle Sequencing Kit (Life Technologies) with a pair of primers (Table 1) and an auto sequencer (ABI 3130xl, Applied Biosystems). The nucleotide sequence of fLIF cDNA was compared with sequences of hLIF (NM_002309) and mLIF (NM_008501), and the coding region of mature protein of fLIF was predicted. This was amplified with a forward primer containing a BamHI site and a reverse primer containing HindIII site (Table 1) for subcloning and inserted into pCold® TF DNA (Takara, Shiga, Japan).

2.3. Recombinant synthetic protein expression and purification

BL21 (DE3 strain; Nippon Gene) was transformed with reconstructed pCold® TF DNA and positive clones were selected on LB agar plate with antibiotics (50-μg/mL ampicillin; TOYOBO). The selected clone was incubated in LB medium with antibiotics at 37°C until OD_{600} reached 0.5. For recombinant synthetic protein expression, isopropyl-β-D-thiogalactopuranoside (Wako, Osaka, Japan) was added to

cultures to a final concentration of 1 mM, and after stationary incubation at 15°C for 30 min, cultures were incubated at 15°C for 24 hours. Induced cells were centrifuged at 4°C for 10 min at 800 x g in an ice-cold tube, and resulting pellets were resuspended with PBS. Resuspended cells were centrifuged at 4°C for 10 min at 800 x g, and pellets were frozen at -80°C prior to protein purification.

Ice-cold PBS was added to the frozen pellets, and the mixture was sonicated on ice. This sample was centrifuged, and supernatant was harvested and used for purification. The supernatant was run through Ni-NTA Sepharose resin (Wako) which had been equilibrated with PBS. Then, to remove non-specifically bound protein, Ni-NTA Sepharose was washed with 10 column volumes of PBS. Bound proteins were harvested with 10 column volumes of PBS for which the imidazole (Nacalai Tesque, Kyoto, Japan) concentration was increasing in the series. The flow-through PBS were collected and the protein concentration was determined by the Bradford method. Thereafter, samples were digested using a Thrombin kit (Merck, Darmstadt, Germany) at 4°C for 24 h to remove His-tag and Trigger Factor and were analyzed with SDS-PAGE.

2.4. SDS-PAGE analysis

Samples of the elution fractions were vortexed with 2 μL of 5× loading buffer (Wako),

and then heated at 97°C for 3 minutes. These samples were analyzed by silver staining after SDS-PAGE with 10% or 12% separating gel.

2.5. Biological activity assay of recombinant protein

2.5.1 Ability to maintain mouse ESCs (TT2) in an undifferentiated state

Mouse ESC line, TT2 (RIKEN Bioresource Center, Japan) was cultured on gelatin-coated dishes and inactivated feeder cells in mouse ES medium comprising Dulbecco's modified Eagle medium Nutrient Mixture F-12 HAM (Sigma-Aldrich) supplemented with 20% (v:v) FBS (lot for ES culture; Life Technologies), 100-IU/mL penicillin and 100-µg/mL streptomycin, 2-mM L-glutamine, 0.1-mM MEM Non-Essential Amino Acids (Life Technologies), 0.1-mM 2-mercaptoethanol (Sigma-Aldrich), 1-mM sodium pyruvate, 0.075% (wt/vol) sodium bicarbonate (Life Technologies) and 1000-U/mL mLIF (ESGRO; Millipore, Billerrica, MA, USA) at 37°C under 5% CO_2 in humidified air. Mouse embryonic fibroblasts were collected from fetuses at day 12 of pregnancy, inactivated with mitomycin C (Kyowa Hakko Kirin, Tokyo, Japan) and used as feeder cells. The protocol was approved by the animal experiment committee of the Graduate School of Life and Environmental Sciences of Osaka Prefecture University. TT2 was

passaged with 0.25% trypsin every 2-3 days.

To assay the biological activity of fLIF produced in *E coli*, TT2 was cultured in mouse ES medium with a series of increasing concentrations of mLIF, commercial hLIF (Millipore), or fLIF (0.2 – 10 ng/ml). After five passages, the undifferentiated state of TT2 colonies was analyzed by the total number of colonies and the rate of colonies positive to alkaline phosphatase (ALP) stained with Alkaline Phosphatase Staining Kit II (Stemgent, Cambridge, MA, USA) [37]. Colonies were classified as ALP positive when the entire cells in the colony stained red. The number of colonies was counted in three high-power fields at each experiment, and the experiment was repeated three times.

2.5.2 Ability to enhance proliferation of human erythroleukemia cells (TF-1)

TF-1 cells (Health Protection Agency, London, UK) were maintained in RPMI 1640 medium (Nacalai Tesque) supplemented with 10% (v:v) FBS, 100-IU/mL penicillin and 100-μg/mL streptomycin, 2-mM L-glutamine and 1-mM sodium pyruvate (Sigma-Aldrich) at 37°C under 5% CO_2 in humidified air. Human granulocyte-macrophage colony stimulating factor (GM-CSF; Millipore) was added into the medium at a final concentration of 4 ng/mL every 48 h. TF-1 cells were

incubated without GM-CSF for 24 h before addition of a series of increasing dilution of mLIF, hLIF or fLIF (twofold serial dilution) and reseeded into a 96-well plate at a density of 2×10^4 cells/well. After 48 h incubation at 37°C under 5% CO_2 in humidified air, the proliferation rate of TF-1 cells was quantified by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium in a WST-8 colorimetric assay using a Cell Counting Kit-8 (DOJINDO) according to the manufacturer's protocol [38, 39]. In summary, WST-8 solution was supplemented with the medium at a dilution of 1:10. After 4-hour incubation at 37°C under 5% CO_2 in humidified air, the absorbance was measured at 450 nm. The proliferation rate was calculated as [(absorbance$_{450}$ sample – absorbance$_{450}$ control) / (absorbance$_{450}$ Max – absorbance$_{450}$ control)] × 100(%).

2.6. Effect of fLIF on domestic cat oocytes matured *in vitro*

Ovaries from domestic short-haired cats were collected during ovariohysterectomy at a local veterinary clinic. The cats were privately owned, and owner's consent was obtained before ovaries collection. Cats were not specifically ovariohysterectomized for this study. Collected ovaries were sliced with scissors in M-199 medium (Life Technologies), and collected cumulus-oocyte complexes (COCs) were rinsed three

times in IVM medium consisting of M-199 medium supplemented with 0.4% (wt/vol) BSA (Sigma), 10-IU/mL 17β-estradiol (Sigma), 100-μg/mL gentamycin (Sigma) and 137-μg/mL sodium pyruvate. Immature oocytes with dark cytoplasm and two or more layers of cumulus cells were randomly divided into each experimental group [40]. Then, we added fLIF at each concentration (0 - 100 ng/mL) into the IVM medium to evaluate the activity of fLIF on COCs. After 28-hour incubation of COCs in IVM medium at 38.5°C under 5% CO_2 in humidified air, the cumulus layer sizes were measured with a micrometer. Furthermore, we incubated COCs in IVM medium with 0.02-IU/mL Follistim® (human recombinant FSH, MSD, Tokyo, Japan) and 25 ng/mL human recombinant EGF (Sigma) at 38.5°C under 5% CO_2 in humidified air for 28 hours to examine whether the effects of LIF on cumulus expansion are shared with those of FSH or EGF and measured the cumulus layers with a micrometer. The COCs incubated in the IVM medium with FSH and EGF were pipetted gently many times to remove the cumulus layers. Nude oocytes were collected, rinsed with PBS twice, and fixed in PBS containing 3.7% paraformaldehyde (Merck) and 1% Triton-X100 (Merck) for 15 minutes and in PBS containing 0.3% polyvinylpyrrolidone (Sigma) for 15 minutes. Fixed oocytes were placed on slides and mounted with 90% glycerol including 10-μg/mL bis-benzimide (Hoechst 33342, Sigma). The oocytes were examined with

fluorescence microscope and categorized into germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I/anaphase/telophase I (MI), metaphase II (MII) stage, parthenogenetic activation (PA), or unclassified.

2.7. Statistical Analyses

All experiments were repeated independently at least three times. The results expressed as percentages were subjected to arcsine transformation and analyzed with ANOVA, followed by Fisher's protected least significant difference post hoc analysis (Stat View; Hulinks Inc., Tokyo, Japan). A value of $P<0.05$ was taken as significant.

3. Results

3.1. Reconstruction of plasmid vectors

The cDNA of fLIF was composed of 609 base pairs (AB853322), whose nucleotide sequence revealed homology of 99% with predicted fLIF mRNA (XM_003994784) (Fig. 1). Comparison of fLIF with mLIF or hLIF revealed a homology of 81% or 92% of the aminoacid sequence of mature protein (Fig. 2). The sequence of mature protein of fLIF

was inserted into pCold® TF DNA.

3.2. Expression and purification of recombinant synthetic protein

After purification with Ni-NTA Sepharose, digestion by thrombin, and second purification with Ni-NTA Sepharose, the extracted proteins were analyzed with SDS-PAGE and silver staining (Fig. 3). The band seen around 70 kDa was divided into two bands around 50 kDa and 20 kDa. The second purification of sample digested by thrombin with Ni-NTA Sepharose led to the isolation of the band around 20 kDa. The final yield of fLIF without the tag sequence was about 1.8 mg/L of fermentation broth.

3.3. Biological activity assay of recombinant protein

3.3.1 Ability to maintain TT2 in an undifferentiated state

The total number of colonies of TT2 was less following incubation without LIF than with 0.2-, 1-, or 10-ng/mL LIF ($P < 0.01$). No differences were detected between species (Fig. 4). These were fewer ALP-positive colonies of TT2 upon incubation without LIF than with 1- or 10-ng/mL LIF ($P < 0.01$). These were fewer upon incubation with 0.2 ng/mL LIF than with 1 ng/mL or 10 ng/mL LIF ($P < 0.01$). No differences were detected

between species (Fig. 5).

3.3.2 Ability to enhance TF-1 proliferation

Proliferation of TF-1 was not seen with mLIF. However, hLIF and fLIF did enhance the proliferation of TF-1 according to the LIF concentration (Fig. 6).

3.4 Effect of fLIF on domestic cat oocytes matured in vitro

A total of 117 COCs was incubated in IVM medium with fLIF. Cumulus expansion was induced after incubation with 10- or 100-ng/mL fLIF ($P < 0.05$) (Fig. 7A). Furthermore, a total of 414 COCs was cultured with FSH, EGF and fLIF. Cumulus expansion was improved at higher concentration than 10-ng/mL fLIF ($P < 0.01$; Fig. 7B). The percentage of oocytes at MII stage were greater upon incubation with 100-ng/mL fLIF than control group ($P < 0.05$; Table 2).

4. Discussion

We cloned here for the first time fLIF cDNA. Although the cloned fLIF cDNA possesses a different aminoacid with predicted fLIF (XM_006938581), we considered the difference as individual variability. The comparison of the aminoacid sequence of

fLIF with mLIF or hLIF revealed a homology of 81% or 92%, respectively. Cross-species receptor affinity of LIF depends on aminoacids sequence and the secondary structure [41]. The most important six aminoacids of hLIF that are used in binding the hLIF receptor correspond to only five in the cat [42]. The secondary structure of the fLIF receptor is necessary to determine the hLIF affinity for fLIF receptor accurately. We predict, however, that hLIF does not bind the fLIF receptor effectively [30-33].

The 70 kDa protein, which matches the predicted size of synthetic protein, was isolated at a higher efficiency with Ni-NTA Sepharose. It was digested with thrombin into a 50- and a 20-kDa protein, which again matches the predicted size of the tag sequence and fLIF. This suggests that fLIF was indeed produced in *E coli* and that isolation was carried out accurately. Although inclusion body formation occasionally makes it difficult to isolate synthetic proteins [24], no such problems occurred with 1-mM isopropyl-β-D-thiogalactopuranoside and reconstructed pCold®-TF DNA, because of the Trigger Factor. Finally, the yield of fLIF was about 1.8 mg/L of fermentation broth, demonstrating that soluble fLIF was collected and purified at a higher efficiency [19, 24].

It has been reported that mouse and human LIF influenced the undifferentiated state

and proliferation of mouse ESCs by activating the signal transducer and activator of transactivation (STAT) 3 signaling pathway [3, 8, 43]. TT2 is one of major mouse ESC lines used in previous studies [44-46]; therefore, we utilized TT2 to assay the biological activity of fLIF. It has been reported that there are no differences between mLIF and hLIF affinity to mLIF receptor [41, 42]. Similarly, fLIF produced in this study influenced the undifferentiated state and the proliferation of TT2, as indicated by affinity to mLIF receptor. Although mouse ESCs are usually cultured with 10-ng/ml LIF [44-46], the total number of colonies of TT2 during incubation with 0.2-ng/mL LIF did not change. The number of ALP-positive colonies was significantly reduced; however, we assume that these results arise because of lack of the desired LIF concentration leading to loss of the undifferentiated state of TT2, which eventually caused TT2 to slow the proliferation activity.

TF-1 was established from a human erythroleukemia patient; it is well known for its characteristic of proliferation stimulated by various cytokines, such as GM-CSF or LIF, following their concentration, and has been utilized in many experiments [21, 23]. In the previous study, hybrid LIF of the human and mouse revealed that mLIF has no affinity to hLIF receptor and at least six amino-acids are important for hLIF to bind hLIF receptor [41]. Moreover, the secondary structure models of LIF and its receptor

were made to explain the binding interaction of human and mouse LIF [42]. In the present study, although hLIF and fLIF demonstrated the significant effects of enhancement of TF1 proliferation, mLIF did not possess any such activity. These results therefore indicate that fLIF possesses the similar characteristics to hLIF and mLIF and will help us to establish naive ESCs and iPSCs, which have higher abilities to proliferate and differentiate.

The addition of fLIF significantly induced oocyte cumulus expansion regardless of FSH and EGF. The effects of LIF on the cumulus expansion have been reported in porcine [47], mouse, and human [9]. Previous studies have revealed that both FSH and EGF induce cumulus expansion through mitogen-activated protein kinases (MAPK) signaling pathway in the mouse [48]. On the other hand, LIF has effects on not only MAPK but also STAT signaling pathways [8]. The activation of STAT signaling pathway also induces the cumulus expansion in the porcine [47]. These studies suggest that the supplementation of fLIF into IVM medium activates MAPK and STAT signaling pathways in cumulus cells and enhances cumulus expansion regardless of the addition of FSH and EGF.

The effects of LIF on nuclear maturation of oocytes have been reported in the mouse [49], the porcine [47], and the bovine [50]. In the present study, the addition of fLIF

significantly increased the rate of oocytes at MII stage with FSH and EGF, which agrees with previous studies. This result may come from two sources, cumulus expansion and direct effects of fLIF on oocytes. Previous studies have revealed that cumulus expansion is correlated with loss of gap junctions between cumulus cells, and leads to nuclear maturation of oocytes [51, 52]. On the other hand, the activation of STAT signaling pathways directly improves nuclear maturation in bovine oocytes [50]. These studies indicate that fLIF has direct and indirect effects on IVM of oocytes in the domestic cat. To evaluate if the beneficial effect of feline LIF on nuclear maturation and cumulus expansion is reflected on embryonic developmental competence, additional studies are required.

In the present study, we have cloned fLIF cDNA and also produced fLIF in *E coli* which is biologically active as LIF in relation to TT2, TF-1 and domestic cat oocytes. These results will improve reproduction and stem cell research in the feline family.

Acknowledgements

This study was supported in part by a Grant-in-Aid for young Scientists (B) No. 24780311 and a Grant-in-Aid for Scientific Research (B) No. 24380172 from the Japan Society for the Promotion of Science and by the Science Research Promotion Fund of

The Promotion and Mutual Aid Corporation for Private School of Japan. The authors R.K. and S.H.ccontributed equally to this work.

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1 Table 1

2 Primers for feline leukemia inhibitory factor (LIF).

Primer	Sequence (5'-3')	Appliance
Human LIF36-56	CAGCCCATAATGAAGGTCTTG	Cloning
Human LIF653-633	CTAGAAGGCCTGGGCCAACAC	
Cat-LIF-for-BamHI	AGGGATCCAGCCCCCTTCCTATCACCCC	Subcloning
Cat-LIF-rev-HindIII	ACAAGCTTGAAGGCCTGGGCCAACACAG	
Universal primer T7	TAATACGACTCACTATAGG	Sequencing pGEM®T Easy
Universal primer SP6	TATTTAGGTGACACTATAG	
pCold-TF-F1	CCACTTTCAACGAGCTGATG	Sequencing pCold® TF
pCold-TF-R	GGCAGGGATCTTAGATTCTG	

3 Primers for subcloning were added BamHI or HindIII site (underlined).

1 Table 2

2 The effect of feline leukemia inhibitory factor (fLIF) on nuclear maturation of oocytes

3 in the domestic cat.

fLIF (ng/mL)	No. of oocytes examined	Percentage of oocytes at the stage of					
		GV	GVBD	MI	MII	PA	unclassified
0	108	6.6 ± 2.4	14.1 ± 3.4	27.0 ± 6.3	48.7 ± 4.2[a]	0.7 ± 0.7	2.9 ± 2.0
1	107	3.3 ± 1.6	18.6 ± 4.0	18.8 ± 4.4	54.4 ± 5.9[ab]	0.0 ± 0.0	4.9 ± 1.9
10	95	3.1 ± 1.6	15.8 ± 5.0	16.9 ± 3.9	64.1 ± 5.9[ab]	0.0 ± 0.0	0.0 ± 0.0
100	104	2.6 ± 1.7	10.8 ± 2.4	14.4 ± 4.5	72.2 ± 4.9[b]	0.0 ± 0.0	0.0 ± 0.0

4 The oocytes were categorized into germinal vesicle (GV), germinal vesicle breakdown

5 (GVBD), metaphase I/anaphase/telophase I (MI), metaphase II (MII), parthenogenetic

6 activation (PA) or unclassified. Values present the mean ± SEM of eight independent

7 experiments and more than eight cumulus-oocyte complexes were used in each

8 experiment. Different superscripts indicate significant differences ($P < 0.05$).

9

Figure legends

Fig. 1. Nucleotide and aminoacid sequence of feline leukemia inhibitory factor (fLIF) cDNA. Signal sequence is underlined and the termination codon is marked with an asterisk.

Fig. 2. Aminoacid sequence of mature protein of feline leukemia inhibitory factor (fLIF). A comparison of fLIF with human LIF (hLIF) and mouse LIF (mLIF) revealed that they have a so homologous sequence of amino-acids of mature protein (81% and 92%). However, most important aminoacids of hLIF to bind the hLIF receptor (*) have difference. The predicted fLIF possesses not L but V as 176th aminoacid.

Fig. 3. SDS-PAGE analysis of proteins. The synthetic protein (about 70 kDa) was digested with thrombin and feline leukemia inhibitory factor (fLIF) was isolated by the second purification with Ni-NTA Sepharose. BT, synthetics protein before thrombin digestion; M, molecular weight marker; SP, protein after the second purification by Ni-NTA Sepharose

Fig. 4. The alkaline phosphatase-positive colonies of TT2 with leukemia inhibitory factor (LIF). (A) TT2 cultured without LIF. TT2 cultured with 10- ng/mL LIF of human (B), mouse (C) or cat (D). Mouse embryonic stem cells are recommended to culture with 10-ng/mL mouse LIF. Scale bars = 100 μm

Fig. 5. The effect of leukemia inhibitory factor (LIF) on maintenance of undifferentiated state of TT2. (A) A comparison of proliferation of mouse embryonic stem cells (ESCs) with different doses of human, mouse and feline LIF. The total colony number was counted after 5 passages. (B) A comparison of alkaline phosphatase (ALP)-positive rate of mouse ESCs with different doses of human, mouse and feline LIF. ALP-positive colonies were detected by ALP Staining Kit II. Values present the mean ± SEM of three independent experiments. Values with different letters differ by $P < 0.01$.

Fig. 6. The effect of leukemia inhibitory factor (LIF) on proliferation of TF-1. A comparison of growth rate of TF-1 cells with different doses of human, mouse and feline LIF after 48-hour incubation. Values present the mean ± SEM of three independent experiments.

1 Fig. 7. The effect of feline leukemia inhibitory factor (fLIF) on cumulus expansion in
2 the domestic cat. (A) A comparison of cumulus expansion with different doses of fLIF
3 without FSH and EGF. Four independent experiments were done and more than three
4 cumulus-oocyte complexes (COCs) were used in each experiment. (B) Cumulus
5 expansion incubated with FSH, EGF and fLIF. Eight independent experiments were
6 done and more than eight COCs were used in each experiment. Values present the mean
7 ± SEM Values with different letters differ by $P < 0.01$.

Figure

```
ATGAAGGTCTTGGCGGCAGGAGTCGTGCCCCTGCTGCTGGTTCTGCACTGGAAACATGGG
 M  K  V  L  A  A  G  V  V  P  L  L  L  V  L  H  W  K  H  G

GCGGGGAGCCCCCTTCCTATCACCCCTGTCAACGCCACCTGTGCCACACGCCACCCATGT
 A  G  S  P  L  P  I  T  P  V  N  A  T  C  A  T  R  H  P  C

CACAGCAACCTCATGAACCAGATCAGGAACCAACTGGCGCAGCTCAATGGCAGTGCCAAT
 H  S  N  L  M  N  Q  I  R  N  Q  L  A  Q  L  N  G  S  A  N

GCCCTCTTTATTCTCTATTACACGGCCCAGGGGGAGCCGTTCCCCAACAACCTGGACAAA
 A  L  F  I  L  Y  Y  T  A  Q  G  E  P  F  P  N  N  L  D  K

CTGTGCGGCCCCAACGTGACGGACTTCCCGCCATTCCATGCCAACGGCACAGAGAAGACC
 L  C  G  P  N  V  T  D  F  P  P  F  H  A  N  G  T  E  K  T

CGGTTAGTGGAGCTGTACCGCATCATCGCTTACCTTGGTGCCTCCCTGGGCAACATCACC
 R  L  V  E  L  Y  R  I  I  A  Y  L  G  A  S  L  G  N  I  T

CGGGACCAGAAGGTCCTCAATCCCAATGCCCTCAGCCTCCACAGCAAACTGAACGCCACT
 R  D  Q  K  V  L  N  P  N  A  L  S  L  H  S  K  L  N  A  T

GCAGACATCATGCGGGGCCTCCTCAGCAACGTGCTTTGCCGCCTGTGTAACAAGTATCAC
 A  D  I  M  R  G  L  L  S  N  V  L  C  R  L  C  N  K  Y  H

GTGGCCCACGTGGACGTGGCCTATGGCCCTGACACCTCAGGCAAGGACGTCTTTCAGAAG
 V  A  H  V  D  V  A  Y  G  P  D  T  S  G  K  D  V  F  Q  K

AAGAAGCTGGGCTGTCAGCTCCTGGGGAAGTATAAACAGGTCATTGCTGTGTTGGCCCAG
 K  K  L  G  C  Q  L  L  G  K  Y  K  Q  V  I  A  V  L  A  Q

GCCTTCTAG
 A  F  *
```

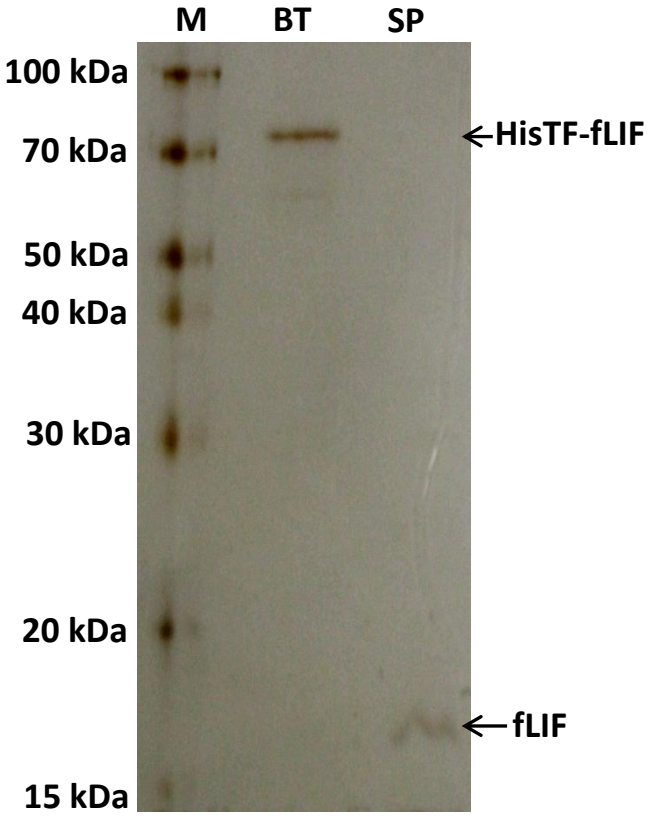
Fig. 1.

```
                                                                  *
fLIF  SPLPITPVNATCATRHPCHS NLMNQIRNQLAQLNGSANAL FILYYTAQGEPFPNNLDKLC
hLIF               I     N        S
mLIF               I     G       K                S            VE

                                                      *    **
fLIF  GPNVTDFPPFHANGTEKTRL VELYRIIAYLGASLGNITRD QKVLNPNALSLHSKLNATAD
hLIF                  AK        VV   T           I   S
mLIF  A  M    S  G      K       MV   S   T           T V  QV     I

                                          *  *
fLIF  IMRGLLSNVLCRLCNKYHVA HVDVAYGPDTSGKDVFQKKK LGCQLLGKYKQVIAVLAQAF
hLIF   L            S    G     T                         I
mLIF  V                R G     PPV  H D EA  R          T     S VV
```

Fig. 2.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SP
100 kDa
70 kDa
50 kDa
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30 kDa
20 kDa
15 kDa
HisTF-fLIF
fLIF

Fig. 3.

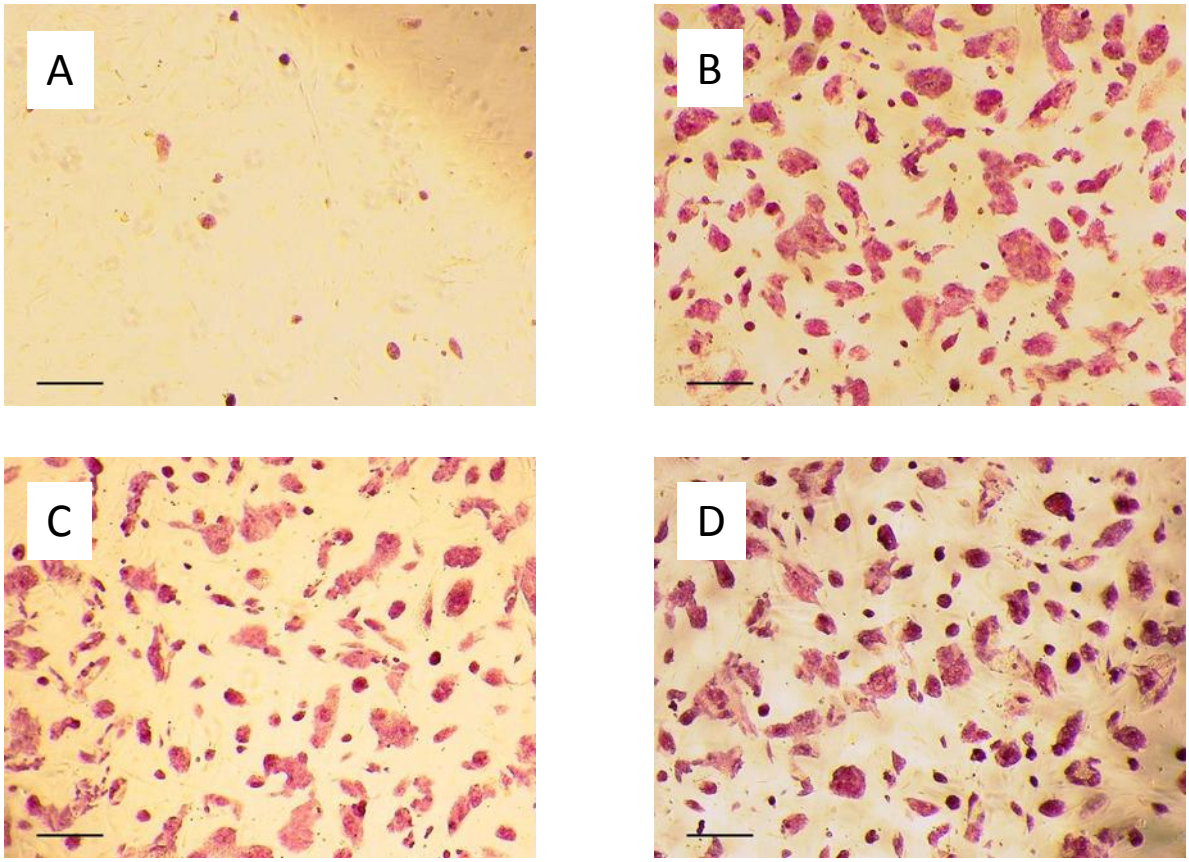


Fig. 4.

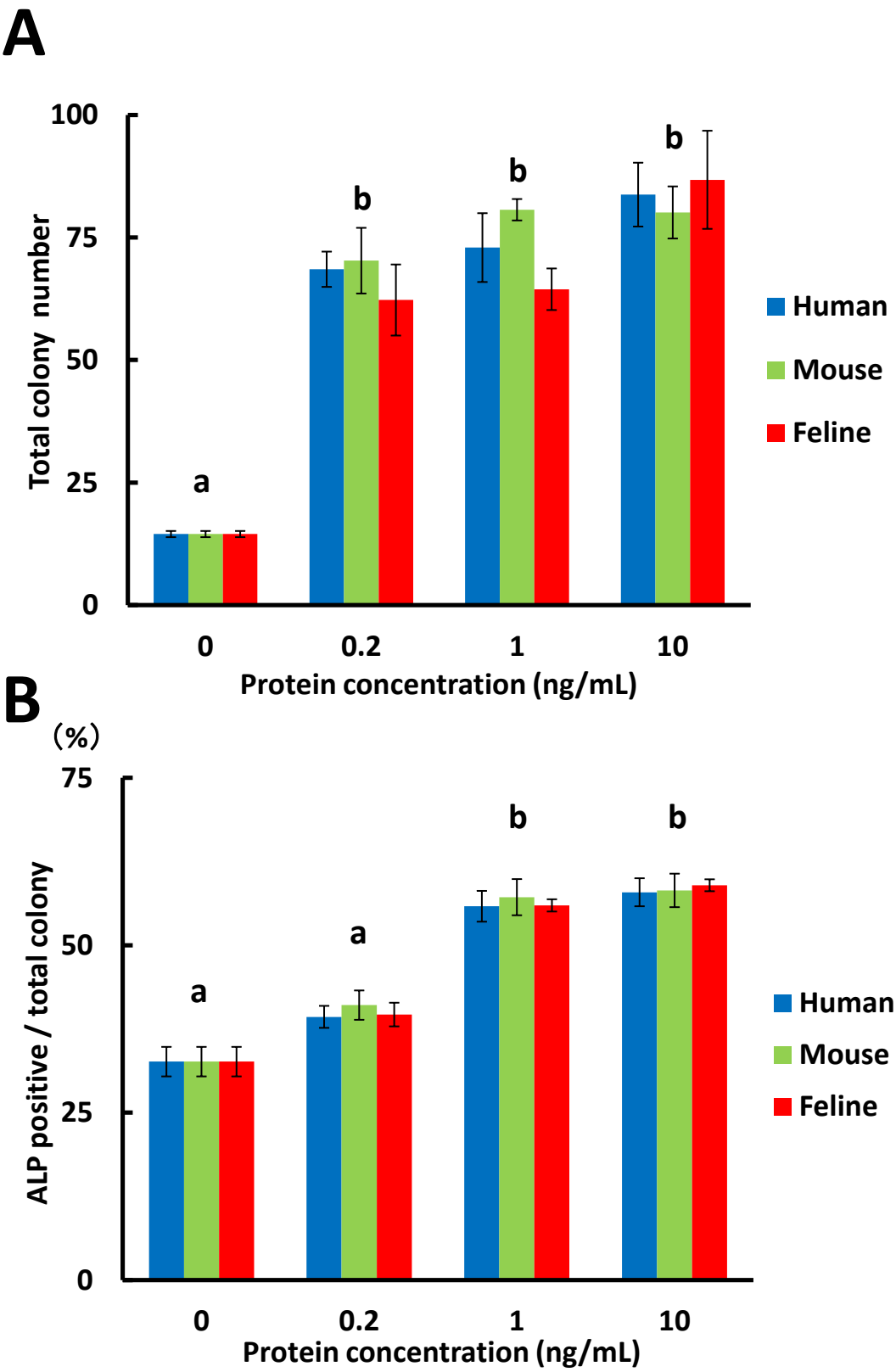

A
100
75
50
25
0
Total colony number
a
b
b
b
0
0.2
1
10
Protein concentration (ng/mL)
Human
Mouse
Feline
B
(%)
75
50
25
0
ALP positive / total colony
a
a
b
b
0
0.2
1
10
Protein concentration (ng/mL)
Human
Mouse
Feline

Fig. 5.

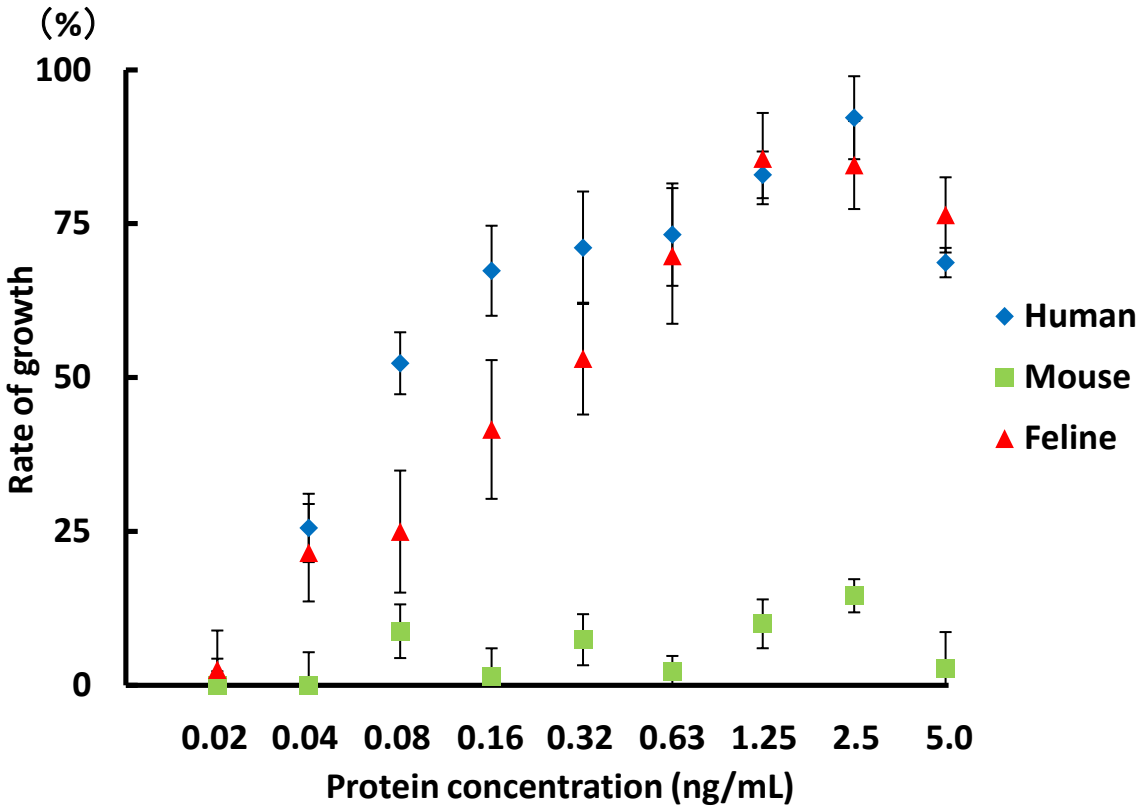
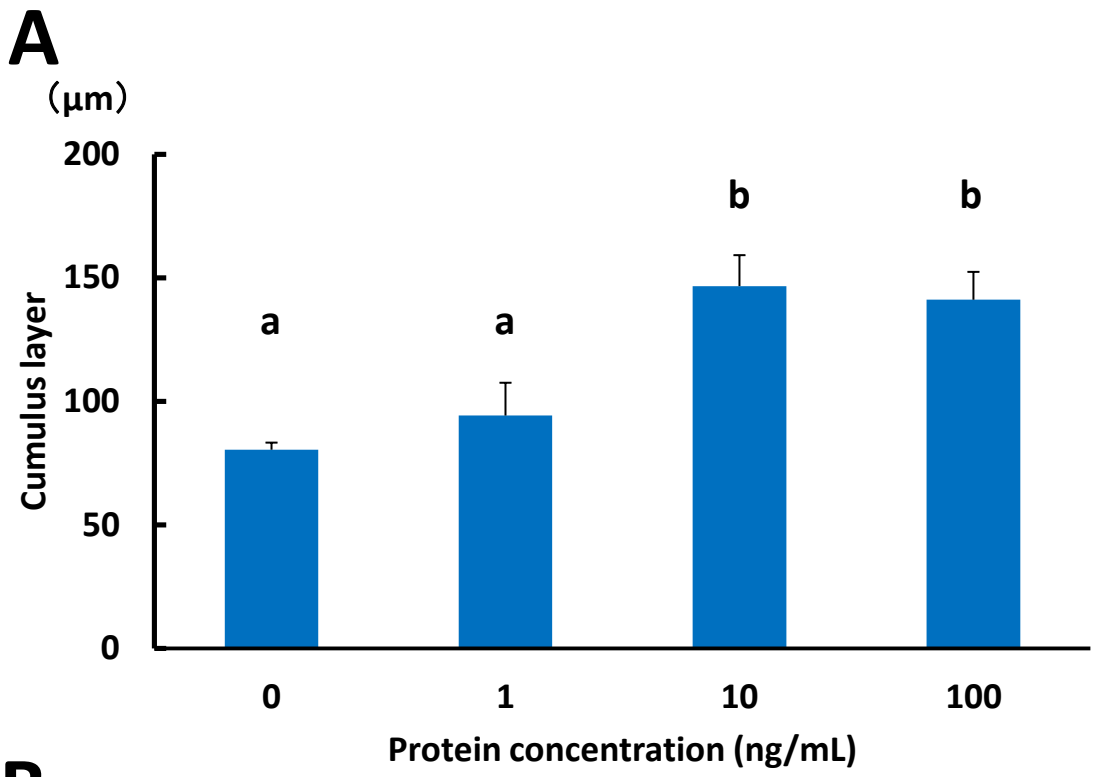

(%)
100
75
50
25
0
Rate of growth
0.02
0.04
0.08
0.16
0.32
0.63
1.25
2.5
5.0
Protein concentration (ng/mL)
Human
Mouse
Feline

Fig. 6.


A
(μm)
200
150
100
50
0
Cumulus layer
a
a
b
b
0
1
10
100
Protein concentration (ng/mL)

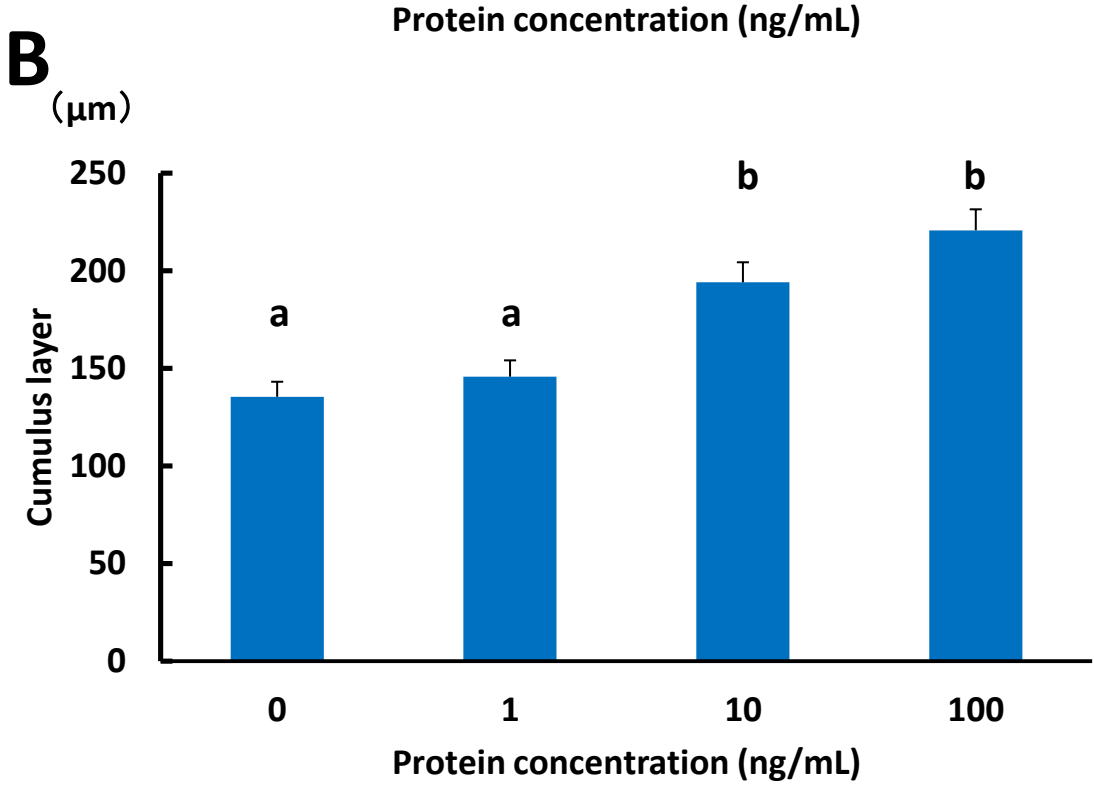

B
(μm)
250
200
150
100
50
0
Cumulus layer
a
a
b
b
0
1
10
100
Protein concentration (ng/mL)

Fig. 7.