

Leukemia inhibitory factor stimulates primitive endoderm expansion in the bovine inner cell mass

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Abstract

Previous work determined that bovine interleukin-6 (IL6) increases inner cell mass (ICM), primitive endoderm (PE) and total cell number in *in vitro* produced (IVP) bovine blastocysts. Another IL6 family member, leukemia inhibitory factor (LIF), has the potential to produce the same effects of IL6 due to the presence of its receptor in bovine blastocysts. We compared the abilities of LIF and IL6 to increase ICM cell numbers in day 7, 8 and 9 IVP bovine blastocysts. Supplementation with 100 ng/ml LIF from day 5 onward improved blastocyst formation rates on days 7 and 8 similar to what was observed when supplementing 100 ng/ml IL6. However, LIF supplementation did not cause an increase in ICM numbers like was observed after supplementing IL6. On day 9, increases in PE cell numbers were detected after LIF supplementation, but 300 ng/ml LIF was required to achieve the same effect on PE numbers that was observed by providing 100 ng/ml IL6. Collectively, these results show that LIF can mimic at least some of the effects of IL6 in bovine blastocyst.

Introduction

Leukemia inhibitory factor (LIF) is a well-recognized pluripotency factor in mouse and human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), where it facilitates the maintenance of cells in a state reminiscent of inner cell mass (ICM) or epiblast (EPI) cells¹⁻³. In the mouse, LIF supplementation also improves embryo development⁴⁻⁶, is essential for implantation⁷, and promotes the development of primitive endoderm (PE)⁸. The roles for LIF are less well defined during early embryogenesis in cattle. Blastocyst development, hatching, post-thaw embryo recovery, and blastomere numbers were increased following LIF supplementation in some studies but not others⁹⁻¹⁴. Also, LIF supplementation did not affect ICM cell numbers in bovine blastocysts in one study and reduced ICM numbers and hypoblast development in other studies^{11,12,15}. Some of these discrepancies may be explained by the use of human and murine LIF proteins, which are only 89 and 73% similar to the bovine LIF.

Our interest in examining the actions of LIF during bovine embryogenesis was prompted by discovering that interleukin-6 (IL6), a member of the same cytokine family as LIF, contains embryotrophic activities within *in vitro*-produced (IVP) bovine embryos. IL6 did not greatly affect blastocyst development, but it altered embryo morphology by increasing ICM cell numbers and increasing PE numbers as the ICM cells differentiated into PE and EPI lineages¹⁶⁻¹⁸. These findings are noteworthy because several reports have indicated that bovine IVP blastocysts contain fewer ICM cells than *in vivo*-derived blastocysts^{19,20}, and poor post-transfer development of the embryonic disk and yolk sac, which develops from the ICM and PE, respectively, likely contributes to pregnancy failures in cattle²¹⁻²⁴.

Members of the IL6 cytokine family include IL6, LIF, other interleukins (IL11, 27, 31), cardiotrophin-1, ciliary neurotrophic factor and oncostatin M. This cytokine family utilizes a common signal-transducing receptor subunit, termed IL6ST (also known as GP130), and ligand-specific α -receptor subunits to

mediate intracellular signaling events²⁵. The bovine blastocyst contains transcripts for *IL6ST* and the α -receptor subunits recognizing IL6 (*IL6R*) and LIF (*LIFR*)¹⁸. Various downstream intracellular signaling pathways can be mediated by IL6 family cytokines, but arguably the best known signaling system for this cytokine family involves Janus kinase (JAK)-induced phospho-activation of signaling transducer and activator of transcription 3 (STAT3)²⁶. There is rapid STAT3 phosphorylation, dimerization and nuclear localization within the bovine ICM cells after exposure to IL6¹⁸. Moreover, IL6 requires JAK2 activity to influence ICM and PE development^{16,18}.

The effect of IL6 on bovine blastocysts is intriguing, and this prompted us to wonder about whether other members of the IL6 cytokine family could have a similar effect. Due to the recent availability of a bovine recombinant LIF (Kingfisher Biotech), we sought to clarify some of the previous discordance of LIF supplementation on bovine embryos, and to compare the effects of LIF with IL6. Herein we report on the ability of LIF to activate STAT3 and increase ICM and PE cell numbers in bovine preimplantation embryos.

Results

LIF Activates STAT3 in Early Morula Stage Embryos but not Blastocysts

We previously reported that IL6 activates STAT3 specifically within the ICM of day 8 blastocysts¹⁸, so a study was completed to determine if LIF also regulates STAT3 activity during bovine embryo development. To determine this, day 5 or 8 embryos were treated with 10 ng/ml LIF or IL6 for 30 minutes prior to fixation, and then were immune-stained for phospho-STAT3^{Y705} (Fig. 1). Nuclear-localized and phospho-activated STAT3 (pSTAT3^{Y705}) staining was detected in a majority of blastomeres in bovine embryos harvested at day 5 post-fertilization (early morula stage) (Fig. 1A). By contrast, no nuclear pSTAT3^{Y705} staining was observed in blastocysts following exposure to LIF (Fig. 1B).

LIF Increases Advanced Blastocyst Formation but Not ICM Cell Number

The effect of IL6 and LIF supplementation on blastocyst development and cell numbers were determined in a study where embryos were supplemented with either 100 ng/ml IL6, 100 ng/ml LIF or carrier only (1% [w/v] BSA) beginning at day 5 (Fig. 2). On day 7, neither LIF nor IL6 supplementation affected the percentage of cleaved embryos that formed blastocysts (Fig. 2A). On day 8, total blastocyst formation was not affected by LIF or IL6, but the percentage of advanced blastocysts was greater ($P < 0.05$) in both LIF and IL6-supplemented embryos when compared with controls (Fig. 2A).

A subset of representative blastocysts from each treatment were immuno-stained for CDX2 and DAPI to determine total, ICM and TE cell numbers at day 8 (Fig. 2B). Total and TE numbers were not affected by

LIF or IL6 supplementation. Number of ICM cells were not affected by LIF supplementation but were increased ($P < 0.05$) following IL6 supplementation (Fig. 2B). Also, supplementation with LIF did not affect the ICM:TE ratio whereas IL6 increased ($P < 0.05$) the ICM:TE ratio (Fig. 2C).

A follow-up study examined whether LIF or IL6 supplementation beginning on day 5 post-fertilization affected total, ICM and TE numbers in day 7 blastocysts (Fig. 3). Supplementation with LIF did not affect total, ICM or TE numbers whereas increases ($P < 0.05$) in total and ICM numbers but not TE numbers were detected in IL6-supplemented blastocysts (Fig. 3A). The ICM:TE ratio was not affected by LIF supplementation but was increased ($P < 0.05$) in IL6-supplemented blastocysts (Fig. 3B).

LIF Can Influence PE Lineage Development

Two studies were completed to examine whether LIF supplementation beginning at day 5 post-fertilization influences the number and distribution of EPI and PE cells within the ICM at day 9 (Fig. 4A&B). Representative images of immunostaining for markers of EPI (NANOG⁺), PE (GATA6⁺) and TE (CDX2⁺) are presented in Fig. 4C. Any GATA6:CDX2 dual-positive nuclei were considered TE and were not included as GATA6⁺ PE cells. In the first study (Fig. 4A), total ICM numbers (either NANOG⁺ or GATA6⁺ or dual positive) and EPI cell numbers were not different between LIF-supplemented and control blastocysts, but there was a tendency for increased ($P = 0.07$) PE cell numbers in LIF-supplemented blastocysts when compared with control blastocysts. The IL6-supplemented blastocysts exhibited greater ($P < 0.05$) ICM, PE, UN and total (206.5 ± 11.3 versus 168.2 ± 11.4 cells in the controls) cell numbers but similar EPI cell numbers when compared with control blastocysts. No differences in PE, EPI, UN or total (192.9 ± 10.2 versus 206.5 ± 11.3 cells for LIF versus IL6-treated blastocysts, respectively) cell numbers were observed between LIF- and IL6-treated blastocysts, but there was a tendency ($P = 0.09$) for IL6-supplemented blastocysts to contain more total ICM cells than LIF-supplemented blastocysts.

The second study examined whether providing a greater LIF concentration (300 ng/ml) beginning on day 5 post-fertilization could influence ICM cell numbers in day 9 blastocysts (Fig. 4B). When compared to the controls, there was a tendency ($P = 0.07$) for greater total ICM numbers in blastocysts exposed to 100 ng/ml LIF, and greater ($P < 0.05$) total ICM numbers were observed in blastocysts treated with 300 ng/ml LIF or 100 ng/ml IL6 groups. Total ICM numbers did not differ among the IL6 and two LIF treatment groups. PE cell numbers were greater ($P < 0.05$) in blastocysts supplemented with 100 ng/ml or 300 ng/ml LIF or 100 ng/ml IL6. Also, PE cell numbers were greater ($P < 0.05$) for the 300 ng/ml LIF and 100 ng/ml IL6 treatments than the 100 ng/ml LIF treatment. No changes in EPI cell numbers were observed among the treatments but increases ($P < 0.05$) in UN cell numbers were detected in blastocysts supplemented with 100 or 300 ng/ml LIF or 100 ng/ml IL6. Only treatment with IL6 increased ($P < 0.05$) total cell numbers over the controls (212.3 ± 7.0 versus 184.5 ± 8.6 cells in the controls; 100 ng/ml LIF had 200.6 ± 7.9 , and 300 ng/ml had 206.0 ± 11.7 cells).

Discussion

Published reports have failed to glean definitive actions of LIF on blastocyst formation and composition, hypoblast development, and cryoprotection in cattle⁹⁻¹⁵. Some of the problems with defining LIF actions may be attributed to differences in culture media formulations and culture conditions, the addition of undefined media components (*e.g.* serum), and the concentrations of LIF examined (range: 2 to 100 ng/ml). Also, most of the previous studies utilized recombinant human or mouse LIF preparations. Amino acid sequence identity is fairly high for LIF between these species and the bovine (89 and 73% identity of human and mouse LIF, respectively), so cross-species activities for these various LIF proteins may not be a major concern, but the use of bovine recombinant LIF in this work optimized opportunities for detecting biological effects. It also allowed us to compare LIF activity with recombinant bovine IL6. Protein concentration was used herein because the proteins have a similar mass (19.8 kDa for IL6; 20.7 kDa for LIF).

No efforts were taken to ensure that LIF and IL6 contained similar specific activities (*i.e.* similar ability to act on a standardized cell line). However, the recombinant bovine LIF protein used for this work was biologically active in bovine embryos. This LIF protein induced STAT3 activation in early morulae, increased the percentage of expanded and hatched blastocysts on day 8, and increased PE and UN numbers. No dose-response studies were completed for LIF. Previous dose-response studies completed with IL6 found that concentrations lower than 100 ng/ml failed to consistently affect ICM cell numbers in bovine blastocysts¹⁷. Therefore, this work focused on comparing this effective concentration for IL6 with equal or greater concentrations of LIF.

This work found that LIF cannot mimic IL6's ability to influence ICM development in blastocysts at days 7 and 8 post-fertilization. This lack of response may have been caused by LIF's inability to stimulate STAT3 at the blastocyst stage. Although early morula stage embryos responded to LIF supplementation, no STAT3 activation occurred following LIF exposure at the blastocyst stage. Previous work noted that IL6 stimulated STAT3 activation at both stages of development, and STAT3 activation occurred specifically within the ICM at the blastocyst stage¹⁸. Activation of STAT3 is a JAK2-dependent in bovine embryos, and JAK2 activation is required for normal ICM development in bovine embryos^{18,27}. Therefore, the lack of STAT3 activation by LIF at the blastocyst stage could explain its failure to influence ICM cell numbers at days 7 and 8. The scarcity of *LIFR* transcripts offers a potential reason why LIF failed to act in day 7 and 8 blastocysts. RNA-sequencing detected *LIFR* transcripts in day 8 bovine blastocysts, but its abundance was low and was approximately 60-fold less than the abundances of *IL6R* and *IL6ST*¹⁸. However, LIF promoted blastocyst expansion and hatching in day 8 bovine blastocysts, so sufficient receptor abundances must have existed. Perhaps *LIFR* expression only occurs in the TE in blastocysts.

It is noteworthy to mention that consistent responses in ICM and total cell numbers were observed after IL6 and LIF supplementation across studies examining blastocysts at days 7, 8 or 9. Variations in cell numbers were detected across these studies. Such study-to-study variations are not uncommon. However, they may also reflect a phenomenon we have observed in previous work, where a decrease in ICM numbers is observed with extended blastocyst culture in SOF-BE1 medium²⁸, the same media used in

this work. We suspect this reflects insufficiencies in the SOF-BE1 formation to support ICM development. It will be interesting to explore how profound ICM responses to IL6 treatment using media formulations reported by others that support ICM development in blastocysts^{29,30}.

A second notable outcome of this work was observing that LIF can mimic IL6's actions on PE development. Greater LIF concentrations were required to elicit an effect that was similar to IL6, but nonetheless, LIF was able to increase PE numbers in day 9 blastocysts. Further work is needed to determine if increasing IL6 concentrations would produce greater PE cell number responses, although a previous study found no added benefit to supplementing 200 ng/ml IL6 when compared with the 100 ng/ml treatment¹⁷. Determination of PE and EPI occurs randomly throughout the ICM at days 8 and 9 in bovine blastocysts based on ICM cell sensitivity to FGF2 and FGF4^{31,32}. Initially, the EPI and PE lineages produce a scattered, "salt & pepper" distribution of differentiated cells within the ICM when stained for PE and EPI markers (see Fig. 4C)^{31,33}. However, soon after their specification, PE cells migrate to the base of the ICM to form the hypoblast layer, which will then expand underneath the TE to form the yolk sac³⁴. The present work did not extend the blastocyst cultures to the point where hypoblast formation occurred, but another group failed to see a positive effect of LIF supplementation on hypoblast development in IVP bovine blastocysts cultured for extended periods³⁴, although a significantly lower LIF concentration was tested in that study (20 ng/ml). Moreover, IVP embryo degeneration occurred in these cultures, suggesting that embryo transfer may be needed to adequately test the potential benefits of IL6 and LIF on hypoblast and yolk sac development.

It is interesting to note that redundancies in IL6 and LIF action on PE has also been observed in mice. Both IL6 and LIF can control PE lineage development in PE lines derived from primed mouse ESCs⁸. However, LIF appears to be the primary player in controlling mouse PE development. Greater IL6 concentrations are needed to produce the same effects as LIF on these cells⁸. The opposite was seen in the present work, where IL6 could increase PE development at lower concentrations than LIF. Therefore, the actions of IL6 and LIF on PE development are similar in the mouse and cow, but the primary mediator of this action differs between the two species. It is not clear in the cow or mouse whether IL6 family member signaling is necessary for normal yolk sac development. Loss of *Il6st* function in the mouse causes embryonic lethality, although a link with poor yolk sac development has not been explored³⁵. Loss of *Lifr* function contains a partial embryonic lethal, with an underrepresentation of *Lifr*^{-/-} embryos in pregnancies at embryonic day 3.5 (blastocyst stage) and 9.5 (post-implantation)³⁶. No adverse pregnancy events occur in *Il6r*-deficient mice³⁷.

Neither LIF nor IL6 affected EPI cell numbers. The absence of IL6 effect was also observed in earlier work¹⁶. This suggests that LIF and IL6 are unable to affect EPI cell proliferation and/or survival. However, LIF and IL6 were equally effective at increasing UN cell numbers. For IL6, this could very well represent its ability to influence ICM cell numbers prior to EPI/PE specification, as observed recently in blastocysts examined at days 7 and 8 post-fertilization¹⁸. However, LIF did not have an effect on ICM numbers at day

7 or 8 in this work. Therefore, it is more likely that LIF and IL6 may be limiting or delaying ICM cell differentiation. This speculation is in-line with LIF's activity as a pluripotency factor¹⁻³.

In conclusion, LIF could not mimic IL6's ability to increase ICM cell numbers at day 8 post-fertilization but it could replicate the actions of IL6 on the PE when administered at a greater concentrations than what is required to detect an effect for IL6. Both embryokines increased PE cell numbers, although IL6 was more effective at accomplishing this than LIF. Exploring the roles for IL6, LIF and potentially other IL6 cytokine family members in yolk sac development in the cow is important because poor yolk sac development exists in a subset of transferred IVP bovine embryos^{23,24}. Also, there is a high incidence of pregnancy losses in inseminated cattle during the time when the yolk sac functions as the primary placental source of nutrients³⁸⁻⁴⁰. It remains untested whether providing IL6 or LIF to IVP before transfer will improve yolk sac development, but the notable effects of IL6 and LIF on PE suggest that this cytokine family play at least a facilitative role in mediating PE development in the bovine conceptus.

Materials And Methods

Materials

Unless specified otherwise, reagents were purchased from ThermoFisher Chemical Company (Waltham, MA).

No animals were used for this work. All studies were completed on slaughterhouse-derived materials that followed humane slaughter practices according to USDA guidelines.

Bovine embryos were produced by in vitro maturation, fertilization and culture procedures described previously^{17,41}. Cumulus-oocyte complexes (COCs) were harvested both beef and dairy ovaries purchased from Brown Packing Company (Gaffney, SC, USA) and transported to the laboratory in 0.9% [w/v] saline containing antibiotic-antimycotic mix (100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B). Upon arrival, COCs were collected by slashing small and medium-sized follicles (1–8 mm) from ovaries selected for the presence of developing follicles (*i.e.* presumably from cycling cattle) and then were placed in groups of 20–35 in 500 µl maturation media covered in light mineral oil (Cooper Surgical Inc., Trumbull, CT, USA). No effort was made to account for COC donor breed. The COCs were matured for 21 to 24 hours at 38.5°C in 5% CO₂ in humidified air. For fertilization, the COCs were washed in HEPES-SOF before being placed in groups of 150–200 in 3 ml SOF-FERT covered in light mineral oil. Sperm were isolated using a BoviPure™ density gradient (Nidacon; Spectrum Technologies Healdsburg, CA, USA), washed once in SOF-FERT, and then added to the fertilization media at a concentration of 1 million sperm/ml fertilization media. After 14–18 hours at 38.5°C in 5% CO₂ in humidified air, presumptive zygotes were denuded by gentle pipetting and before being placed in groups of approximately 25 in 50 µl SOF-BE1 covered by light mineral oil. Zygotes were incubated at 38.5°C in 5% CO₂, 5% O₂ and 90% N₂ in humidified air. Blastocyst formation was assessed on days 7 and 8 post-fertilization as the percentage of cleaved embryos that formed blastocysts. Blastocysts were then also

categorized as “regular” (blastocoel of > 50% the embryo’s mass, but no increase in embryo diameter), or “advanced” (having an obvious increase in diameter; this included both expanded and hatched blastocysts).

LIF and IL6 Stock Preparation

Concentrated stocks (10 µg/ml) of LIF (recombinant bovine; Kingfisher Biotech, St. Paul, MN) and IL6 (recombinant bovine; Kingfisher Biotech) were prepared in SOF containing 1% [w/v] bovine serum albumin (BSA). Control treatment stocks contained SOF with 1% BSA. Stocks were frozen in single-use aliquots at -80°C. Embryos were not removed from their original drops. In most studies, treatments were administered either at day 5 post-fertilization to all embryos regardless of stage of development by the addition of 2 µl of stock solution to each SOF-BEI drop containing embryos to achieve in-droplet final concentrations of 0, 100 or 300 ng/ml, depending on the study. In one study (immunofluorescence work), IL6 or LIF was administered 30 min prior to fixation and staining.

Immunofluorescence

On days 7 and 8, a representative sampling of regular and expanded blastocysts were selected (*i.e.* a similar proportion of regular or advanced blastocysts as observed for that treatment group in each replicate). On day 9, only expanded and hatched blastocysts were selected. In no studies were “early” blastocysts (having a small cavity, < 50% of the embryo’s mass) used for analysis.

The pSTAT3^{Y705} staining was completed as describe previously¹⁸ using rabbit anti-pSTAT3^{Y705} (Cell Signaling Technologies, Danvers, MA; 9145T; 1:100) and donkey anti-rabbit IgG (Alexafluor 555; 1:200). Differential staining for TE and ICM cells in blastocysts was completed as described previously¹⁷ using mouse anti-Caudal Type homeobox 2 (CDX2; Biogenex, San Ramon, CA, sold ready-to-use), anti-mouse IgG (Alexafluor 488; 1:200), and 4',6-diamidino-2-phenylindole (DAPI; 1 µg/ml). The CDX2, NANOG and GATA6 co-staining work was completed as described previously¹⁶ using rabbit anti-GATA6 (Cell Signaling Technology, Danvers, MA; 5851T; 1:500), mouse anti-NANOG (eBioscience; 14-5768-82; 1:200), mouse anti-CDX2 (Biogenex, San Ramon, CA), and donkey anti-rabbit and anti-mouse IgGs (Alexafluors 488, 555 and 647; 1:200).

At the end of each staining protocol, embryos were incubated with 1 µg/ml DAPI for 5 minutes at room temperature then washed in PBS-PVP and then either flattened on a glass slide lined with petroleum jelly, or not flattened (for z-series acquisition). Staining was visualized with an Eclipse Ti-E inverted microscope equipped with an X-cite 120 epifluorescence illumination system and DS-L3 digital camera. Images were captured with NIS-Elements Software (Nikon Instruments, Melville, NY), and cell counting was completed with the cell counter plugin in the program FIJI (ImageJ)¹⁷. Nuclei staining only with NANOG were considered EPI cells, while those nuclei that stained only with GATA6 were considered PE cells. Nuclei that contained staining for both NANOG and GATA6 were termed undifferentiated (UN) ICM cells. Occasionally, abnormally small, DAPI⁺ and antibody⁻ nuclei were observed. These nuclei were not counted as they usually represented sperm or cumulus cells attached to the zona pellucida. Also, many

TE cells stained weakly for GATA6. Because of this, only nuclei that were CDX2⁻ were considered as GATA6⁺ ICM cells.

Statistical Analyses

All analyses were completed by least-squares ANOVA using the general linear model of the Statistical Analysis System (Proc GLM; SAS for Windows, version 9.4; SAS Institute Inc., Cary, NC, USA). Individual comparisons were partitioned further, when necessary, by using the Probability of difference (PDIFF) test of SAS. Replicate was used as the experimental unit for blastocyst formation studies. Percentage data (e.g. blastocyst formation rates) were arcsine-transformed before analysis but are presented as non-transformed means and SEM. The Tukey honestly significant difference test was used for all blastocyst formation data. Embryo was considered the experimental unit for cell counting studies. Statistical significance was determined at $P \leq 0.05$.

Declarations

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

Alan D. Ealy conceived the studies, oversaw the completion of the studies and statistical analyses, and wrote the final version of the manuscript. Lydia K. Wooldridge assisted with conceiving the studies, performed the experiments, analyzed the data and wrote the initial draft of the manuscript.

Competing Interests

The authors declare no competing interests.

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Figures

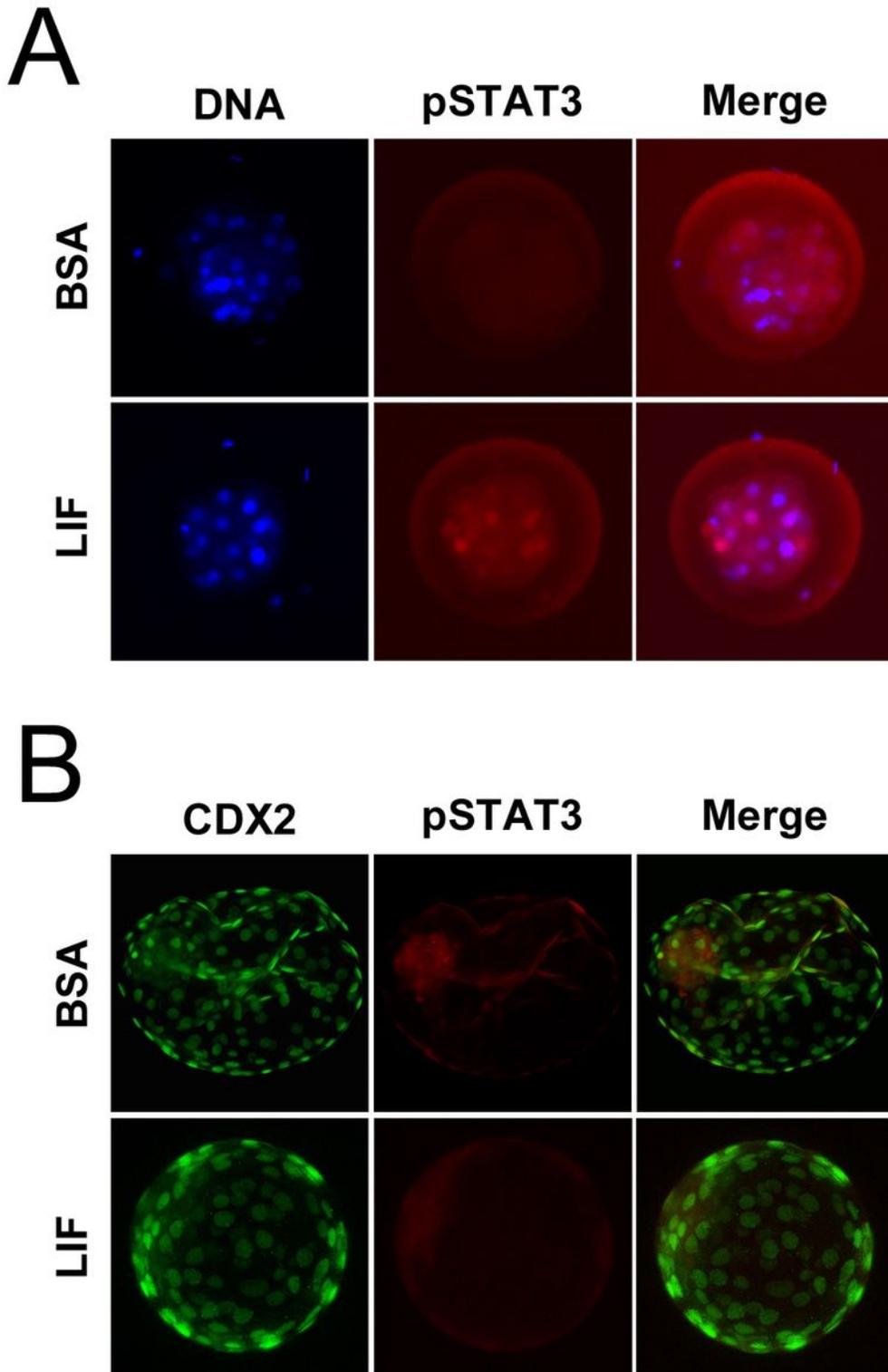


Figure 1

STAT3 activation profiles for LIF-treated bovine embryos. Panels A-B: Representative images of embryos from day 5 (>8-cell; n= 3-5/treatment) and day 8 (blastocysts; n= 3-5/treatment) receiving either 0 (BSA only) or 100 ng/ml LIF for 30 minutes before immunostaining to detect pSTAT3+ (red), total nuclei (DAPI, blue), and, for day 8 blastocysts, CDX2 (green). Panel A: Representative day 5 embryos. Panel B: Representative day 8 embryos.

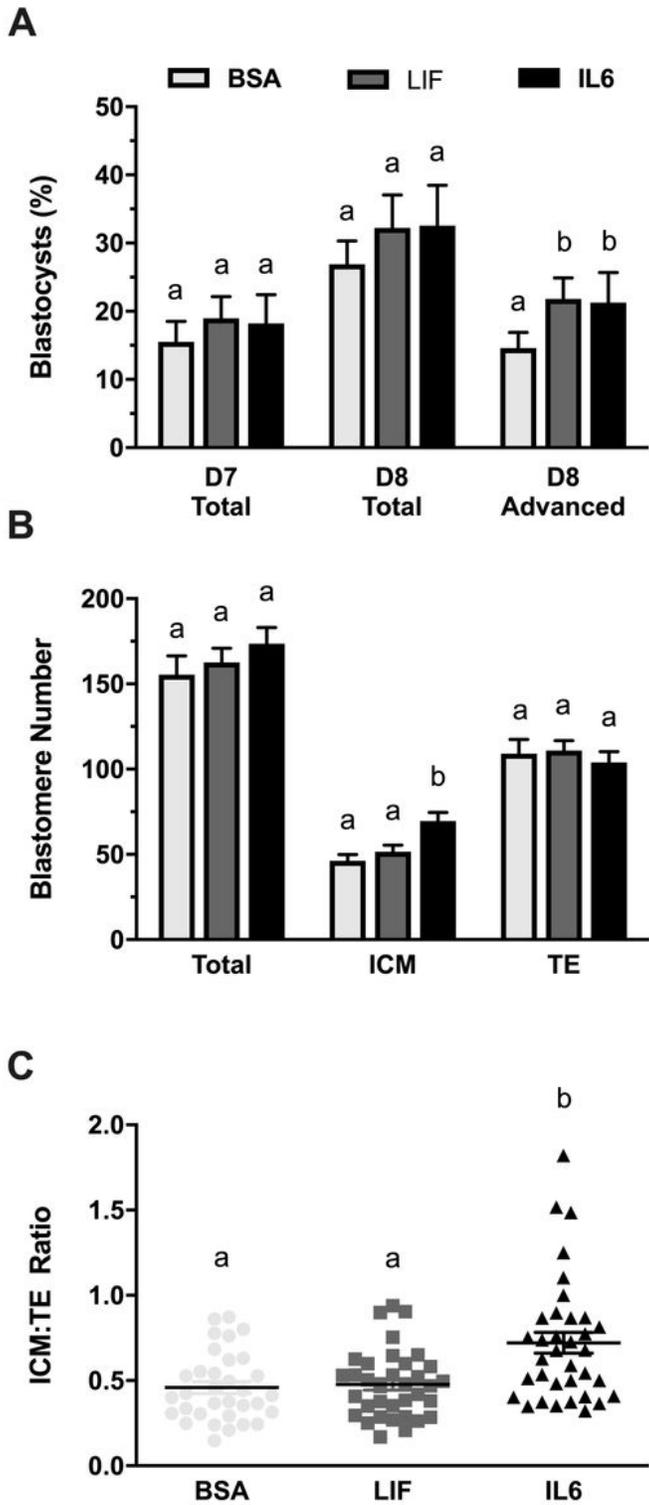
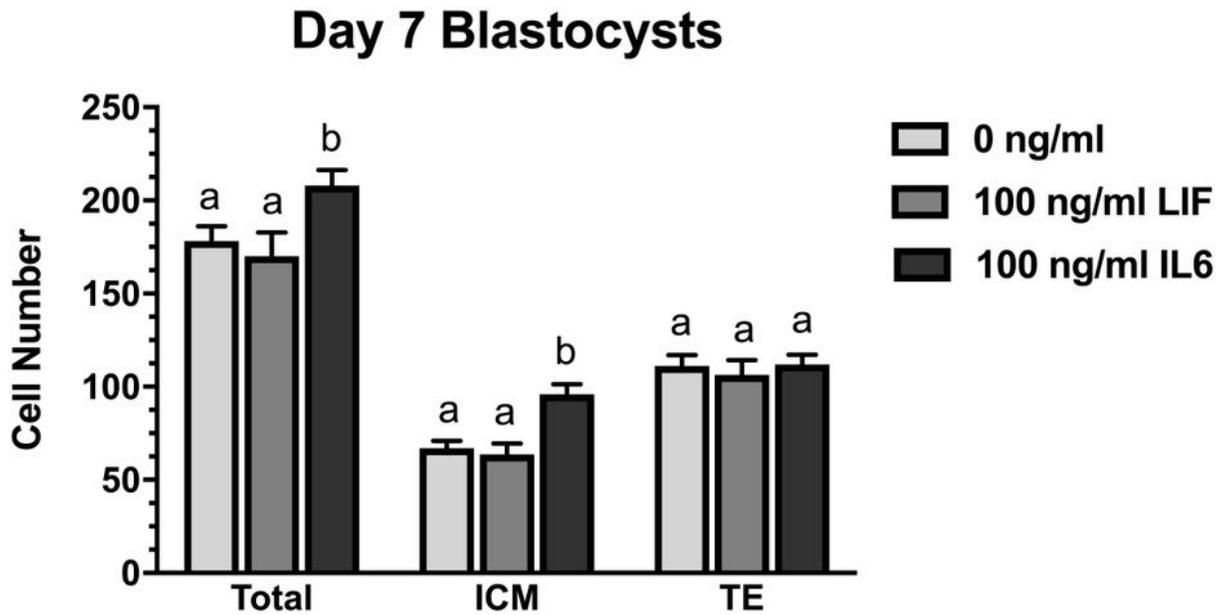


Figure 2

Comparison of LIF versus IL6 supplementation on blastocyst formation (n=18-32 zygotes/drop; 2-4 drops/treatment/replicate; 8 replicates) and day 8 cell numbers (n=33-35 blastocysts/treatment over 7 replicates). Blastocyst formation was defined as the percentage of cleaved embryos that formed into blastocysts. Panel A: Effects of IL6 and LIF supplementation total blastocyst formation (as a percentage of cleaved embryos) at day 7 and total and advanced blastocysts (includes expanded and hatched

blastocysts) formation at day 8. Panel B: Effects of IL6 and LIF supplementation on total, ICM and TE cell numbers in day 8 blastocysts. Panel C: ICM:TE ratios of day 8 blastocysts. Different superscripts within each panel indicates differences ($P < 0.05$).

A



B

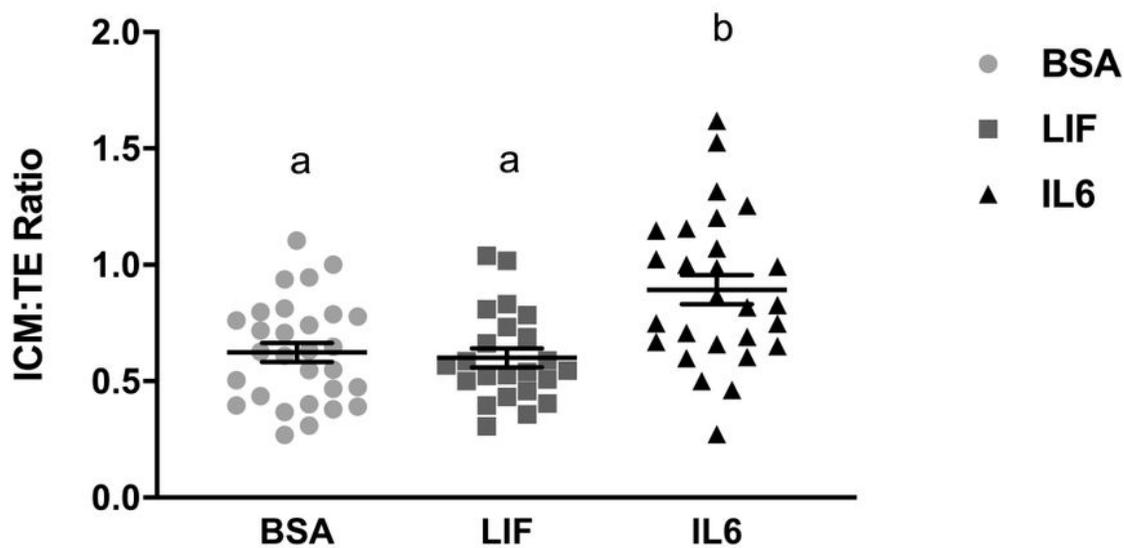


Figure 3

Comparison of LIF versus IL6 supplementation on day 7 blastocyst cell numbers (n=23-29 blastocyst/treatment/timepoint over 3 replicates). Panel A: Effects of IL6 and 100 ng/ml LIF on total, ICM

and TE cell numbers in day 7 blastocysts. Panel B: ICM:TE ratios of day 7 blastocysts. Different superscripts within each panel indicates differences ($P < 0.05$).

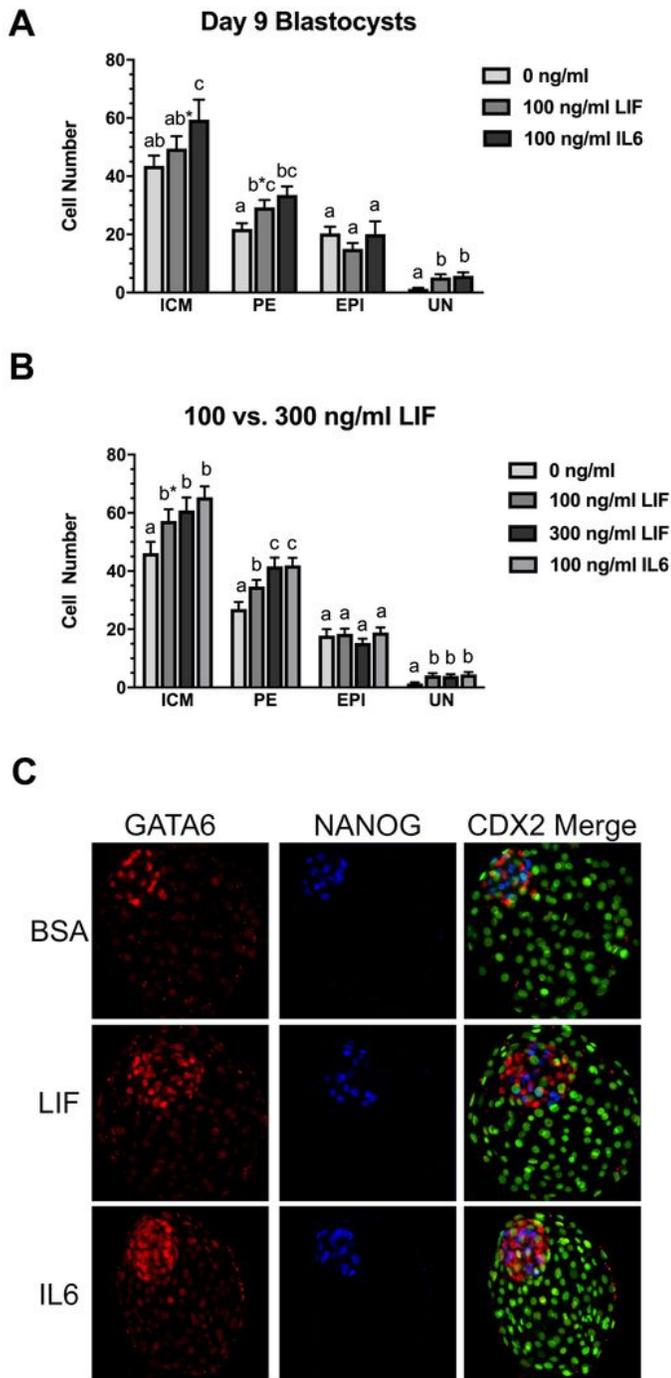


Figure 4

Comparison of LIF versus IL6 supplementation on day 9 blastocyst cell numbers. Panel A: Effects of IL6 and 100 ng/ml LIF on ICM, PE, EPI and UN cell numbers in day 9 blastocysts (n=27-29 blastocyst/treatment/timepoint over 3 replicates). Panel B: Effects of IL6 and 100 or 300 ng/ml LIF on

ICM, PE, EPI and UN cell numbers in day 9 blastocysts (n=36-45 blastocysts/treatment over 3 replicates). Different superscripts within each panel indicates differences (P<0.05). Panel C: Examples of day 9 blastocysts treated with BSA (control, no treatment), 300 ng/ml LIF (LIF) or 100 ng/ml IL6 (IL6). Green signals represent CDX2+ cells in the far-right column.