The trophectoderm acts as a niche for the inner cell mass through C/EBPα-regulated IL-6 signaling

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SUMMARY

IL-6 has been shown to be required for somatic cell reprogramming into induced pluripotent stem cells (iPSCs). However, how Il6 expression is regulated and whether it plays a role during embryonic development remains unknown. Here, we describe that IL-6 is necessary for C/EBPα-enhanced reprogramming of B cells into iPSCs but not for B cell to macrophage transdifferentiation. C/EBPα overexpression activates both Il6 and Il6ra genes in B cells and in iPSCs. In embryo development, Cebpa is enriched in the trophectoderm of blastocysts together with Il6, while Il6ra is mostly expressed in the inner cell mass (ICM). In addition, Il6 expression in blastocysts requires Cebpa. Blastocysts secrete IL-6 and neutralization of the cytokine delays the morula to blastocyst transition. The observed requirement of C/EBPα-regulated IL-6 signaling for pluripotency during somatic cell reprogramming thus recapitulates a physiologic mechanism in which the trophectoderm acts as niche for the ICM through the secretion of IL-6.

INTRODUCTION

In the pre-implantation embryo, totipotent cells segregate into inner cell mass (ICM) cells, which are the in vivo source of pluripotent embryonic stem cells (ESCs), and an outer layer of trophectoderm (TE) cells (Zhu and Zernicka-Goetz, 2020; Rossant and Tam, 2022). Alternatively, differentiated cells can be artificially converted into induced pluripotent stem cells (iPSCs) through enforced transcription factor overexpression (Graf, 2011). Single-cell resolution of these reprogramming processes has shown that somatic cells can also diverge from the main path toward pluripotency and that cell subpopulations appear and activate alternative gene programs, such as the embryonic TE, both in vitro (Schibinger et al., 2019; Liu et al., 2020) and in vivo (Abad et al., 2013). In addition, successful iPSC reprogramming has been reported to require interleukin-6 (IL-6) in vitro (Brady et al., 2013) and in vivo (Mosteiro et al., 2016; Chiche et al., 2017), but how IL-6 and its receptor are regulated and if they play a role in early embryogenesis remains largely unknown.

The Il6 gene has been described to be activated by C/EBPβ during inflammation (Akira et al., 1990) and cell senescence (Kuilman et al., 2008), while its close relative C/EBPα has been shown to regulate its specific receptor Il6ra in newborn hepatocytes (Mackey and Darlington, 2004). In the hematopoietic system, C/EBPα is required for the formation of granulocyte-macrophage progenitors (GMPs) (Zhang et al., 1997) and its overexpression in B cells induces their transdifferentiation into macrophages (Xie et al., 2004). A pulse of C/EBPα in B cells dramatically enhances OSKM-induced reprogramming efficiency (Di Stefano et al., 2014) through the transient generation of GMP-like cells (also called Bγ cells, Di Stefano et al., 2016). GMPs in turn are the hematopoietic precursor most susceptible to OSKM-induced reprogramming (Eminli et al., 2009; Guo et al., 2014).

Here, we show that the IL-6 pathway is strictly required for the C/EBPα-enhanced reprogramming of B cells into iPSCs and that C/EBPα directly regulates the expression of both the Il6 and Il6ra genes. During early embryo development, C/EBPα is co-expressed with Il6 in the TE while the receptor is most highly expressed in the ICM. Blocking IL-6 in embryos showed that the cytokine is required for the morula to blastocyst transition, indicating that the TE acts as niche for the ICM through the secretion of IL-6.
Figure 1. Requirement of IL-6 for C/EBPα-enhanced B cell to iPSC reprogramming and C/EBPα-induced activation of Il6

(A) Experimental strategy of C/EBPα-enhanced B cell reprogramming into iPSCs. B cells derived from a reprogrammable mouse strain containing rtTA and a doxycycline (Dox)-inducible OSKM cassette (Di Stefano et al., 2014) were infected with C/EBPαER, seeded onto MEFs, and induced with β-estriadiol (β-est) for 18 h to generate Bα0 cells. After washing out the inducer, Bα0 cells were treated with Doxy for 8 days and scored for alkaline phosphatase-positive (AP+) iPSC colonies at day 12.

(B) Il6 and Il6ra RNA levels during C/EBPα-enhanced B cell reprogramming.

(C) Effect of Il6 ablation on the number of iPSC colonies, illustrated by images of representative plates and quantification (mean values ± SD, n = 5 biological replicates). Statistical significance was calculated using Student’s t test with paired values.

(D) Effect of anti-IL-6 IgG on iPSC reprogramming. Cells were treated daily with 0.1 mg/mL BE0046 antibody, during the whole process (from day 0 to 12), at an early phase (from day 0 to 6), or during later stages (from day 6 to 12). Shown are representative plates with iPSC colonies and quantification (mean ± SD, n = 3 biological replicates for the whole treatment and n = 4 for the other conditions). Statistical significance was determined using one-way ANOVA test with paired values and Dunnett’s test for multiple comparisons relative to the control condition (ns, not significant).

(E) Browser screenshots of the Il6 (left panel) and Il6ra (right panel) loci showing C/EBPα binding, chromatin accessibility, histone marks, and gene expression in B and Bα0 cells.

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RESULTS

Il6 ablation blocks B cell to iPSC reprogramming

To study the role of the IL-6 pathway in somatic cell reprogramming we explored our highly efficient C/EBPα-enhanced cell conversion system (Di Stefano et al., 2014). This entails exposing pre-B cells (hereafter called B cells) to C/EBPα for 18 h, generating Bα′ cells, followed by the activation of the Yamanaka factors OCT4, SOX2, KLF4, and MYC (OSKM); (Figure 1A). RNA sequencing (RNA-seq) analysis of samples obtained at various time points showed an approximately 5-fold increase in the expression of Il6 and Il6ra in Bα′ cells with a subsequent decrease of Il6 at days 2–4 and of Il6ra at days 4–6 after OSKM induction (Figure 1B). To test whether the IL-6 pathway is functionally involved in our two-step reprogramming system we tested B cells from Il6−/− mice (Kopf et al., 1994), revealing a 10-fold decrease in the number of iPSC colonies compared with Il6+/+ mice (Figure 1C). Because B cells in these experiments are grown on a feeder layer of inactivated mouse embryonic fibroblasts (MEFs), known to produce IL-6 (Mosteiro et al., 2016), we also tested whether the ablation of Il6 in the feeders inhibits the reprogramming of B cells. However, the use of Il6−/− MEFs only caused a modest reduction in iPSC colony formation (Figure S1A). The finding that Il6 and its receptor are most highly expressed between the Bα′ and day 4−6 cell stages (Figure 1B) raised the possibility that IL-6 signaling is predominantly required in the early reprogramming phase. To test this, we added IL-6 neutralizing antibodies from either day 0 to 6 or from day 6 to 12. Cultures treated during the first half of the time course showed an approximately 2.5-fold reduction in iPSC colony numbers, while no significant effect was seen when treated later (Figure 1D). IL-6 neutralization spanning from day 0 to 12 revealed an even stronger inhibitory effect, namely a 10-fold decrease of iPSC colonies, comparable with the reduced numbers observed with Il6−/− B cell cells (Figure 1C).

Our data suggest that C/EBPα-induced B cells themselves produce IL-6 required for B cell to iPSC reprogramming, acting by an autocrine/paracrine mechanism. They also show that IL-6 signaling is only required during the first half of the reprogramming process, consistent with observations made in a reprogramming model with heterokaryons (Brady et al., 2013).

C/EBPα directly regulates Il6 and Il6ra gene expression and activates IL-6 signaling

To study the regulation of the Il6 gene we analyzed in Bα′ cells binding of C/EBPα by ChIP-seq, chromatin accessibility by ATAC-seq, the enhancer and promoter marks H3K27Ac and H3K4me3 by ChIP-seq, and RNA expression by RNA-seq. C/EBPα was found to bind to −65 and −160 kb sites upstream of Il6 (Figure 1E, left), which are putative enhancer elements, as indicated by their chromatin accessibility and H3K27ac decoration. Consistent with the observed activation of Il6 transcripts, its promoter was also decorated with H3K4me3 (Figure 1E, left). In addition, the −65 kb enhancer shows increased contacts with the Il6 promoter upon C/EBPα overexpression (Figure S1B). In these cells, C/EBPα also binds to the Il6ra promoter, where it induces increased chromatin accessibility and decoration with the H3K4me3 and H3K27ac marks, consistent with the observed upregulation of the gene (Figure 1E, right). CEBPA also activates the two genes in the human B cell line BLaER (Stik et al., 2020) (Figure S1C) and the two IL6 enhancers appear to be evolutionarily conserved since the cells exhibit two CEBPA binding sites at −65 and −150 kb (Figure S1D).

IL-6 signaling is initiated by binding to its specific receptor IL-6RA, which heterodimerizes with IL-6ST (GP130), a signaling chain shared with the leukemia inhibitory factor (LIF) receptor, leading to the phosphorylation of STAT3 and transcriptional activation of downstream targets (Hunter and Jones, 2015). To determine whether C/EBPα overexpression results in activation of STAT3, we performed a western blot of B and Bα′ cell extracts immunostained with phospho-STAT3-specific antibodies. A significant increase of STAT3-P was detected in C/EBPα-induced wild-type but not in Il6−/− Bα′ cells (Figure 1E, left). Similarly, no increased STAT3-P was detected in Il6ra−/− Bα′ cells (Figure 1F, right).

Our data show that C/EBPα directly regulates the expression of the Il6 and Il6ra genes in mouse and human B cells and activates the IL-6 signaling pathway.

The IL-6 pathway is dispensable for C/EBPα-induced B cell to macrophage transdifferentiation

The observed early requirement of IL-6 for B cell reprogramming into iPSCs and high-level expression of Il6 and Il6ra in C/EBPα-induced macrophages (Figures S1E and S1F) raised the possibility that Il6 ablation likewise affects B cell transdifferentiation into macrophages. We therefore compared the cell conversion kinetics of Il6−/− and Il6−/− B cells, grown on feeders of the corresponding genotype, monitoring by FACS the expression of the B cell marker CD19 and the macrophage marker MAC-1 (CD11b). Knockout and wild-type cells showed insignificant differences in their transdifferentiation kinetics (Figure S1G).

(F) Western blot bands corresponding to STAT3-P and STAT3 obtained from wild-type, Il6−/− and Il6ra−/− B and Bα′ cells. Densitometer-based quantification of β-ACTIN or GAPDH-normalized STAT3-P relative to total STAT3 is shown on the right (mean values ±SD of n = 3 biological replicates). Statistical significance was calculated using Student’s t test with unpaired values. See also Figure S1.
and addition of an IL-6 blocking antibody only caused a minor delay in MAC-1 activation (Figure S1H). Of note, macrophages generated in the absence of Il6 displayed full phagocytic capacity (Figure S1I). These results show that Il6 ablation does not significantly impair C/EBPz-induced B cell to macrophage transdifferentiation, implying a specific role of IL-6 in the C/EBPz-enhanced reprogramming to iPSCs.

**Il6 ablation impairs upregulation of pluripotency-associated genes during reprogramming**

To study the mechanism by which IL-6 signaling is required for iPSC reprogramming, we analyzed the impact of Il6 ablation on gene expression in our C/EBPz-enhanced reprogramming by performing RNA-seq of samples collected at various time points. Principal-component analysis revealed a divergence of the Il6+/+ and Il6−/− cell trajectories at days 4–6 of reprogramming (Figure 2A). In addition, the lack of Il6 led to a significant change in the expression of ~1,000 genes at day 4 and ~4,500 genes at day 6 (Figures S2A and S2B). K-means unsupervised clustering of differentially expressed genes (p < 0.01) yielded 10 clusters that fell into 2 major groups, with clusters I to V containing mostly downregulated genes in B cells throughout reprogramming that differed from day 4 onward and clusters VI to X containing mostly upregulated genes (Figure 2B). Genes in cluster I became downregulated by day 4 and re-activated more strongly in Il6−/− cells at day 6. This cluster included the mesenchymal-to-epithelial transition factor genes Fgf2, SnaI2, Tead1/2/3, and Smad5 (Li et al., 2010). Clusters II to V contained downregulated genes and included the B cell genes Igll1, Vpreb1, Ebf1, Pax5, and Cd19. Clusters VI to IX included upregulated genes encoding the pluripotency-associated factors Oct4, Sox2, Myc, Prdm14, Dppa3, Tet1, Klf5, Zfp42, and Nобр1 as well as some TE genes (Krt8, Tead4, and Tfap2c). Finally, cluster X also contained key pluripotency factors like Nanog or Tfcp21, which are specifically downregulated in Il6−/− cells. However, this cluster mostly showed genes upregulated in Il6−/− compared with the wild-type cells, including several laminin and collagen members, such as Lamda4, Lamb1, Lamc1, Col1a1, Col3a1, and Col5a1, known to be involved in extracellular matrix formation, as well as BMP signaling genes (e.g., Bmp1, Bmp2, and Bmp6). These findings raise the possibility that some Il6−/− intermediates may acquire identities that differ from the pluripotency phenotype.

Next, we determined the reprogramming kinetics of a group of signature genes characteristic for different lineage-restricted gene expression programs. This showed that the lack of Il6 impaired the upregulation of pluripotency genes, most notably at day 6, and mildly affected TE genes (p = 0.07 at day 6, Figure 2C). In addition, cell-cycle-associated genes were significantly impaired in knockout cells at day 6, probably reflecting their reduced proliferation compared with reprogrammed Il6+/+ cells (Figure S2C). In contrast, Il6 ablation did not appreciably affect the silencing of B cell signature genes or the upregulation of myeloid genes (Figure 2C). Examples of individual genes representative for each of these categories are shown in Figure S2D.

The observation that both ESC- and TE-associated genes become upregulated during C/EBPz-enhanced reprogramming (Figure 2D) raised the question whether this reflects the formation of distinct cell subpopulations or cells with mixed phenotypes. We therefore plotted the RNA levels

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**Figure 2. Il6 deficiency impairs the upregulation of pluripotency genes during reprogramming**

(A) Principal-component analysis (PCA) of gene expression dynamics of 38,019 genes during reprogramming of Il6+/+ and Il6−/− B cells, showing biological duplicates and average trajectories.

(B) Heatmap of unsupervised K-means clustering of genes differentially expressed between Il6+/+ and Il6−/− cells (p < 0.01) at different times during reprogramming, showing biological duplicates. Representative examples of genes within each cluster are shown on the right.

(C) Gene expression changes (RNA-seq) of pluripotency, trophectoderm, B cell, and myeloid signature genes during reprogramming of Il6+/+ and Il6−/− B cells. Thin lines represent average values of biological duplicates for each gene, thick lines the mean and shades the SD. Statistically significant differences between Il6+/+ and Il6−/− cells are indicated by p values determined using two-way ANOVA and Sidák’s multiple comparison tests (ns, not significant). Signature genes are listed in Table S1.

(D) Expression kinetics (RNA-seq) of pluripotency and trophectoderm signature genes, comparing C/EBPz pulsed B cells (C/EBPz + OSKM) with non-pulsed cells (OSKM) (RNA-seq by Di Stefano et al., 2014). Lines represent average values of biological duplicates for each gene, centered around a thicker mean line for the whole signature and shades denoting SD. Statistical significance is determined using two-way ANOVA and Sidák’s multiple comparison tests. Signature genes are listed in Table S1.

(E) Heatmap of pluripotency and trophectoderm signature genes’ expression obtained from single cells at day 8 of reprogramming (Francesconi et al., 2019), with values of individual cells ordered according to their similarity to ESCs.

(F) Correlation plots between Il6ra–Il6 gene expression pairs in single cells at various time points during reprogramming. Pearson’s correlation coefficients (R), p values (P), and tendency lines are depicted in each plot. Correlation coefficients are color coded in brown and red, respectively, corresponding to zero and negative values.

(G) Model of the role of C/EBPz and the IL-6 pathway during the C/EBPz-enhanced reprogramming of B cells into pluripotent and TE-like cells. See also Figure S2.
of pluripotency and TE signature genes of day 8 induced cells in comparison with that of ESCs, using a single-cell gene expression dataset (Francescon et al., 2019). The observed inverse correlation between the expression of PSC- and TE-associated genes (Figure 2E) suggests a segregation into distinct subpopulations, despite a large degree of variability at the single-cell level. Similarly, we explored whether the ligand-receptor expression between the initially upregulated Il6 and Il6ra (Figure 1B) also corresponded to distinct populations. For that, we pairwise correlated the expression levels of Il6 and Il6ra from B cells to day 4, around which their expression drops. After the pulse of C/EBPα, Il6 and Il6ra start to negatively correlate and this is maintained until day 4, suggesting that cells predominantly express either Il6 or Il6ra already at an early time point (Figure 2F).

Our data are compatible with the notion that IL-6 is required in a paracrine fashion among B cells for the robust upregulation of pluripotency genes, while having a minor effect on the upregulation of TE genes. They also suggest that, during C/EBPα-enhanced reprogramming, pluripotent-like cells segregate from TE-like cells (Figure 2G).

**Cebpa and Il6 are co-expressed in the TE while Il6ra is predominantly expressed in the ICM**

The finding that C/EBPα-enhanced iPSC reprogramming requires IL-6 and that both Il6 and Il6ra genes are regulated by C/EBPα raised the possibility that the C/EBPα-IL-6 axis is also involved in embryo development (Figure 3A). We therefore analyzed RNA-seq datasets of mouse pre-implantation embryos when totipotent cells first differentiate into pluripotent (ICM) and TE layers (Deng et al., 2014; Guo et al., 2010). This showed that, within the mouse blastocyst, both Cebpa and Il6 are selectively expressed in the TE while Il6ra expression is enriched in the ICM (Figure 3B). Likewise, within developmentally earlier morula stage embryos, Cebpa was enriched in the outer-layer cells of morulas along with the TE markers Gata3 and Krt8, whereas inner-layer cells were enriched for Nanog and Sox2 expression (Figures 3A and 3B).

Strikingly, the observed gene expression pattern is evolutionarily conserved, as revealed by our analysis of human blastocyst single-cell data (Petropoulos et al., 2016). CEBPA and IL6 are co-expressed within the TE while IL6RA is enriched in the ICM, as validated by the expression of lineage markers (Figures 3C and S3C). Of note, none of CEBPB, CEBPD, or CEBPE showed an enrichment in the TE (Figure S3D). Resolving the ICM of E6.5 human blastocysts into epiblast and primitive endoderm cells, showed that IL6RA expression is significantly higher in both ICM layers than in TE cells (Figure S3E). A similar but slightly less pronounced selective expression pattern of these three markers was also observed in E6.5 blastocysts (Figure S3F).

Since TE cells can be subdivided into polar and mural subsets (Gardner, 2000), we also analyzed existing datasets for the distribution of our genes of interest. While coverage of Il6 expression in mouse embryos (Nakamura et al., 2015) was insufficient, human blastocysts showed a significant enrichment of IL6 and CEBPA expression in the polar TE subpopulation of E6.5 and E7.5 blastocysts, although less pronounced than the polar TE marker CCR7 (Figure 3D).

Similarly, mouse blastocysts stained with an antibody against C/EBPα revealed strong expression in the TE, both in polar and mural areas (Figure 3E).

It has been reported that human ESCs can be induced to differentiate into TE-like cells using two experimental approaches. In the first, hESCs were treated with BMP4 (Krendl et al., 2017); in the second, naïve hESCs were exposed to a combination of the MEK inhibitor PD0325901 and the TGF-β inhibitor A-83-01 (PD + A83) (Guo et al., 2021). In both TE differentiation strategies, CEBPA and IL6 become upregulated while IL6RA becomes downregulated (Figures 3F and 3G). As expected, GATA3 and KRT8 were upregulated and NANOG and SOX2 downregulated (Figures 3F and 3G), validating the described hESC to TE conversion.

These results show that CEBPA and IL6 are co-expressed in TE cells and that IL6RA is mostly expressed in the ICM in both mice and humans. The observed upregulation of CEBPA and IL6 and downregulation of IL6RA during the induced differentiation of hESCs into TE cells further supports the selective expression of the IL-6 pathway genes within the first two blastocyst lineages.

**Perturbation experiments of C/EBPα in ESCs and in embryos alter the expression of IL-6 signaling genes**

The observed expression of Cebpa and Il6 in the blastocyst TE in vivo and in hESC-derived TE-like cells in vitro raised the possibility that the factor could regulate Il6 expression in ESCs and during embryo development as in B cells. To test this idea, we monitored the effect of C/EBPα overexpression in ESCs. For this purpose, we generated two stable, tamoxifen-inducible E14 ESC clonal lines expressing C/EBPαER12-dTomato. These cells were treated for 12 and 48 h with tamoxifen and subsequently analyzed by RNA-seq, revealing an approximately 4-fold upregulation of Il6 and ~30-fold of Il6ra (Figure 4A). Of note, the Il6ra levels in the induced cells were ~250 times higher than those of Il6. To determine whether C/EBPα also induces the expression of IL-6 genes in human ESCs, we analyzed an RNA-seq dataset of a human ESC line expressing a doxycycline-inducible form of CEBPA, before and after a 48 h treatment with doxycycline (Nakata et al., 2020). Here again the treated cells showed an approximately 2-fold upregulation of Il6 and approximately 4-fold upregulation of IL6RA as well as similar differences in their initial expression levels.
in hESCs (Figure 4B). To determine whether another TE-associated transcription factor is able to upregulate these genes in human ESCs, we analyzed the same dataset (Nakatake et al., 2020) for the effect of GATA3 overexpression. This revealed an approximately 3-fold upregulation of IL6, similar to that observed for CEBPA. However, GATA3 only induced a modest (∼1.5-fold) activation of the IL6RA gene (Figure S4A, left). Curiously, also CEBPB strongly upregulates IL6RA (∼9-fold) but not IL6 expression in the hESCs context (Figure S4A, right).

We next studied the impact of Cebpa ablation in cultured embryos. Since Cebpa−/− mice die perinatally (Wang et al., 1995), we crossed heterozygous animals, yielding Cebpa+/−, Cebpa−/−, and Cebpa+/+ zygotes. Isolated zygotes were cultured for 2.5 and 4.5 days, reaching morula and blastocyst stages, respectively (Figure 4C). A total of 21 morulas and 24

Figure 3. Cebpa and Il6 expression are enriched in the TE while Il6ra is enriched in the ICM
(A) Diagram depicting the segregation of the TE from the ICM layer during the morula to blastocyst transition.
(B) Cebpa, Il6, and Il6ra expression levels in the TE and ICM of mouse blastocysts by single-cell RNA-seq (Deng et al., 2014). Dots represent gene expression values of single cells in the ICM and TE; green lines represent median values, and boxplots and whiskers represent 90th and 10th percentiles, respectively. Statistical significance was determined using multiple unpaired Student’s t test applying Benjamin, Kieger, and Yekutieli’s correction methods. Ns, not significant. For the ICM group, only cells with >0 Il6ra RPKMs were selected (n = 28/35, 80%); in the TE group, only cells with >0 Il6 RPKMs were selected (n = 78/142, 54.9%).
(C) As in (B), but for human blastocysts [Petropoulos et al., 2016]). The values shown for the ICM correspond to a combination of epiblast (Epi) and primitive endoderm (PrE) cells. For the ICM group, all cells showed >0 Il6ra RPKMs; in the TE group, only cells with >0 Il6 RPKMs were selected (n = 16/57, 28%).
(D) Expression distribution of CEBPA, IL6, and CCR7 in single mural and polar TE cells from human blastocysts. Only cells with >0 Il6 RPKMs were selected (n = 185/331, 55.9% in E6.5, and n = 273/388, 70.3% in E7.5).
(E) Immunofluorescence images (3D projections and single z-planes) of C/EBPα and OCT4 in a mouse blastocyst (bright field in DIC). OCT4 stains pluripotent ICM cells. DNA was stained with DAPI.
(F) Kinetics of CEBPA, IL6, IL6RA, GATA3, KRT8, NANOG, and SOX2 gene expression during induced TE differentiation after BMP4 addition to hESCs (RNA-seq by Krendl et al., 2017).
(G) Kinetics of CEBPA, IL6, IL6RA, GATA3, KRT8, NANOG, and SOX2 gene expression during induced TE differentiation after PD + A83 addition to hESCs (RNA-seq by Guo et al., 2010). See also Figure S3.
blastocysts were subjected to RNA-seq analyses, allowing to distinguish Cebpa- and Cebpa+ embryos (Figures 4D and S4B), also detectable at the protein level (Figure 4E). At the morula stage, six embryos were found to be Cebpa-, closely matching the 5.25 embryos expected from Mendelian ratios. In contrast, at the blastocyst stage, three Cebpa- blastocysts
were detected instead of the expected six (Figure 4D). To analyze the impact of Cebpa on gene expression, we selected the top 3 Cebpa− and Cebpa+ embryos. At the morula and blastocyst stages 27 and 32 genes, respectively, showed a significantly altered expression between the Cebpa− and the Cebpa+ groups (Figures 4F and S4C). Remarkably, in blastocysts, Il6 was among the differentially expressed genes, showing a reduced expression in Cebpa+ (p = 0.048). This was accompanied by a compensatory upregulation of Cebpb (Figure 4G), while Cebpe and Cebpd remained unchanged as did Il6ra (Figure S4D). Finally, Cebpa also showed a modest positive correlation with Il6 expression and that of the TE marker Gata3 when analyzing all 24 blastocysts, while it showed a negative correlation with Il6ra expression and the ICM marker Oct4. No correlation was found at the morula stage (Figure S4E).

Together, our experiments show that overexpression of C/EBPα upregulates both Il6/IL6 and Il6ra/IL6RA genes in mouse and human PSCs. Moreover, Cebpa is required for Il6 expression in mouse blastocysts and it is non-redundant with Cebpb at this developmental stage.

**Blastocysts secrete IL-6 and blocking antibodies delay the morula to blastocyst transition**

Our data described so far are consistent with the notion that IL-6 secreted by TE cells in blastocysts signals to the receptor expressed in ICM cells. This notion is in line with a report describing that IL-6 blocking antibodies reduce STAT3 phosphorylation in blastocysts (Do et al., 2013). To directly demonstrate IL-6 secretion during embryo development, we cultured pools of 30 zygotes each for 1.5–4.5 days to obtain two- to four-cell embryos, morulas, and blastocysts. Using an ELISA assay, we detected ~2 pg/mL IL-6 in the two- to four-cell stage embryo and morula pools and ~10 pg/mL of IL-6 in the blastocyst pool (Figure 5A), consistent with Il6 mRNA expression in blastocysts (Figure 3B). We next determined whether the observed IL-6 secretion and the reported IL-6 signaling (Do et al., 2013) has a physiological role. For this purpose, we established embryo cultures in the presence of an IL-6 blocking IgG or an isotype-matched IgG against horseradish peroxidase used as a control. Microscopic examination of the embryos up to day 2.5 showed no differences in their development. However, at day 3.5, we observed a significantly lower proportion of embryos exhibiting a cavitation in the anti-IL-6-treated cultures, indicative of an impaired formation of early blastocysts. A slight cavitation impairment persisted at day 4.5 blastocysts (Figures 5B and 5C). Conversely, adding recombinant IL-6 in the medium of in-vitro-cultured porcine embryos has been reported to induce STAT3 activation and to enhance parthenote development (Shen et al., 2012).

In sum, our results show that cultured blastocysts secrete significant amounts of IL-6, likely produced by TE cells. The finding that neutralizing IL-6 caused a delay in the morula to blastocyst transition indicates that the IL-6 pathway is physiologically relevant for pre-implantation embryo development in mouse.

**Exogenous IL-6 binds to the ICM region in blastocysts exposed to an IL-6 reporter protein**

The experiments described so far suggest that, during the morula to blastocyst transition, IL-6 secreted by the TE signals to the ICM. However, attempts to directly visualize IL-6 binding in the embryo by IL-6 and IL-6RA immunostaining failed. As an alternative, we engineered an IL-6-Emerald fusion construct to identify binding of fluorescent IL-6 to the surface of IL-6RA-expressing cells. First we confirmed that soluble IL-6-Emerald protein binds to the surface of 293T cells (which are known to express the IL-6 receptor (Von Laue et al., 2000)), while no signal was observed in embryos treated with condition medium from control cells (Figure S5A). We then proceeded to perform the embryo experiments, by incubating blastocysts with soluble IL-6-Emerald protein for 30 min, staining the live embryos with Hoechst and analyzing them by confocal microscopy. The images obtained revealed the selective localization of the Emerald signal to the ICM area (Figure 5D) and in a similar patchy pattern as observed upon its overexpression in embryos (Figure S5B). These findings directly show that IL-6 binds selectively to the ICM region, in line with the RNA-seq experiments showing an enrichment of Il6ra expression in the ICM (Figure 3B). In addition, the ICM localization of IL-6-Emerald in mouse embryos further complements the observed increase in the number of ICM cells in bovine blastocysts cultured with recombinant IL-6 (Wooldridge and Ealy, 2019), which suggested that ICM cells might be the functional targets expressing the IL-6 receptor.

**DISCUSSION**

Here, we describe that IL-6 signaling is strictly required for C/EBPα-enhanced reprogramming of B cells into iPSCs but not for their transdifferentiation into macrophages. In this context, C/EBPα binds to both Il6 and Il6ra genes and activates their expression. In the developing embryo, we find that the TE co-expresses Cebpa and Il6, while the receptor is preferentially expressed by the ICM, in both mouse and human. On the other hand, whether the cells that initially upregulate Il6 or Il6ra turn respectively into the TE- and PSC-like cells during reprogramming (tallying the embryo situation as is speculated in Figure 2G) remains unresolved. Our finding that C/EBPα regulates the expression of Il6 in the TE layer of blastocysts indicates that the TE may act as a niche for the ICM through the secretion of IL-6.
recapitulating our observations for such regulatory axis during B cell to iPSC reprogramming. This adds to the growing list of factors that have been described to engage in a pre-implantation embryo crosstalk, including FGF4, BMP4, IL-11, Nodal, and WNT6/7B (Zhu and Zerwick-Goetz, 2020; Rivron et al., 2018). It is possible that the reason why the IL-6 crosstalk has not been reported before is that it was obscured by LIF, which is added during experimental approaches to study the requirement of soluble factors, such as during blastoid formation (Rivron et al., 2018). In addition, the fact that \( \text{Lif} \) is expressed before \( \text{Il6} \) during pre-implantation embryo development, at the two- to four-cell stage (Do et al., 2013), supports the notion that IL-6 and not LIF is the cytokine critical for blastocyst formation. Similarly, \( \text{LIF} \) is expressed at very low levels and before \( \text{IL6} \) in human embryos (Bourillot et al., 2020), raising the possibility that IL-6 signaling at this early stage of life is evolutionarily conserved.

Our previous work has shown that the ability of C/EBP\( \alpha \) and the closely related factor C/EBP\( \beta \) to enhance OSKM-induced reprogramming of B cells is not shared with unrelated factors capable of inducing transdifferentiation, such as with the erythroid, muscle, and neuronal fate inducing factors GATA1, MYOD, and ASCL1, respectively (Di Stefano et al., 2014). In addition, among C/EBP family members, C/EBP\( \beta \) uniquely shows a TE-specific expression in mice and is non-redundantly required for \( \text{Il6} \) expression. Moreover, the observed compensatory upregulation of \( \text{Cebp}\beta \) in \( \text{Cebp}a \) knockout embryos does not prevent a reduction in \( \text{Il6} \) expression. We therefore speculate that, in TE cells, C/EBP\( \beta \) specifically interacts with another TE-associated co-factor(s) to upregulate \( \text{Il6} \) but not \( \text{Il6}\alpha \).
It is puzzling that C/EBPα upregulates Il6ra in PSCs and in a subset of B cells, but that the receptor is not expressed in TE cells. This might be explained by the presence in the former cell types of a C/EBPα co-factor(s) required for Il6ra expression not present TE cells. It also suggests that, in the ICM, Il6ra falls under the control of alternative factors. Future experiments are needed to address the question whether IL-6 signaling is not only required for the high-level activation of pluripotency genes during reprogramming, as described here, but also for the formation of the ICM during embryo development. The IL-6-mediated crosstalk between the TE and ICM described here extends the many known functions of IL-6 in adult tissues to the earliest cell fate decision in vertebrate development.

**EXPERIMENTAL PROCEDURES**

**C/EBPα-enhanced reprogramming of B cells into iPSCs**

Reprogramming of primary B cells from wild-type or Il6−/− reprogrammable mice was performed as described previously (Di Stefano et al., 2014, 2016). In brief, freshly isolated B cells were infected with C/EBPα-Er-hCD4 retrovirus and hCD4-positive cells were plated at 500 cells/cm² in gelatinized 12-well plates onto wild-type or Il6−/− inactivated MEF feeders in 20% FBS RPMI medium with 10 ng/mL IL-7. The transcription factor was shuttled into the nucleus by the addition of 100 nM β-estradiol for 18 h. After β-estradiol washout, the cultures were switched to N2B27 medium (50% DMEM-F12 [12634010, Gibco], 50% neurobasal [21103049, Gibco], 100× N2 supplement [17502048, Gibco], and 50× B27 supplement [17504044, Gibco]) and addition of 10 ng/mL IL-4 (217-14, Preprotech, 10 ng/mL IL-7, and 2 ng/mL IL-15 [210-15, Preprotech]). The final culture medium also contained 100× MEM non-essential amino acid solution (11140068, Gibco), 1 mM sodium pyruvate (11360070, Gibco), 100 U/mL penicillin-100 ng/mL streptomycin, 2 mM L-glutamine, and 0.1 mM 2-mercaptoethanol.

The OSKM expression was activated by the addition of 2 μg/mL doxycycline (D9891, Sigma). Samples needed for western blots or RNA-seq were harvested by trypsinization followed by removal of feeder cells through differential adherence to tissue culture dishes for 40 min. iPSC colony formation was assessed at the end of the process by alkaline phosphatase staining as detailed below. Anti-IL-6 blocking treatments were performed by adding 0.1 mg/mL of antibody (BE0046, BioXCell) IgG1 or anti-horseradish peroxidase antibody (BE0088, BioXCell) IgG1 as a control in culture microdrops from the zygote stage up to late blastocysts.

**Scoring and counting of iPSC colonies**

Induced reprogrammable B cells were cultured in 12-well plates containing feeders MEFs and analyzed at day 12 after OSKM induction. Cultures were washed twice with 0.05% PBS-Tween 20 (PBSTw), fixed for 2 min in 4% PFA and washed once again in 0.05% PBSTw. Cells were then incubated in freshly prepared alkaline phosphatase staining solution at room temperature in the dark for 10–20 min and washed twice in PBS. Alkaline phosphatase expression was detected by the purple color of the iPSC colonies. Plates were scanned (Perfection V850 Pro, Epson) and colonies were counted in Fiji software. See Table S3 for iPSC staining solution composition.

**Pre-implantation mouse embryo cultures**

Zygotes were collected from the ampulla and cumulus cells removed by incubation with 300 μg/mL hyaluronidase (H4272, Sigma) in M2 medium (M7167, Sigma). After washing the embryos in a few drops of KSOM medium (MR-106-D, Millipore), they were cultured in KSOM micropipettes under mineral oil (NO-400K, Nidacon) in an incubator with 5% CO2 at 37°C. Embryos were handled with a mouth aspirator (A5177-5EA, Sigma) coupled to fire-polished glass Pasteur pipettes and collected at different stages of development from the in vitro cultures either for RNA-seq or protein immunostaining as detailed in the sections below. To track their development in control or IL-6 blocking conditions, phase contrast pictures of the developing embryos were taken with a Leica inverted microscope (DMI6000B) and embryos were counted using Fiji software.

The amounts of IL-6 produced by the various samples was assessed using an ELISA kit (M6000B, R&D Systems) according to the manufacturers’ instructions. Emissions of the samples at 450 nm wavelength were measured in a plate reader (SPECTROstar Nano, BMG Labtech). Anti-IL-6 blocking treatments were performed by adding 0.1 mg/mL of antibody (BE0046, BioXCell) IgG1 or anti-horseradish peroxidase antibody (BE0088, BioXCell) IgG1 as a control in culture microdrops from the zygote stage up to late blastocysts.

**Protein immunostaining**

Embryos were fixed in 4% PFA for 10 min at room temperature. They were then washed twice in PBS for 5 min before permeabilization with 0.5% Triton X-100 PBS (0.5% PBST). Zygotes to morulas were permeabilized for 10 min while blastocysts were permeabilized for 15 min. Embryos were washed twice in 0.1% PBST for 5 min before incubation in 0.1% PBST containing 3% BSA (9048-46-8, Sigma) for 45 min at room temperature to block unspecific immunostaining. Embryos were then treated with primary antibodies diluted in 0.1% PBST containing 1% BSA overnight at 4°C inside a moistened chamber. Next morning, embryos were sequentially washed in 0.1% PBST for 5, 15, 20, and 30 min at room temperature. A second blocking was performed in 0.1% PBST containing 3% BSA for 45 min at room temperature. For the secondary staining, embryos were placed in 0.1% PBST containing 1% BSA with the corresponding antibodies and 5 μg/mL of DAPI (D1306, Invitrogen). Embryos were left in secondary staining solution for 90 min at room temperature inside a moistened chamber in the dark. Three washes in 0.1% PBST were performed before mounting the embryos in 10-μL drops of PBS on 35-mm coverglass plates (P35G-1.0-14-C, MatTek) covered in light oil (M5310, Sigma). Embryos were imaged in a Zeiss LSM 980 with Airyscan 2 inverted confocal microscopes and further processed in Fiji and Imaris software for intensity quantifications and distance measurements. Of note, all the incubation steps were performed on shaking platforms.
Mouse ESC culture and infection with C/EBPα
ESCs (E14TG2) were cultured on gelatinized plates in 15% ES-FBS (16141079, Life Technologies) Knockout medium (10829018, Gibco) containing 1,000 U/mL LIF (ESG1106, Merck Millipore). For C/EBPα expression, cells were infected with inducible C/EBPα-ERT2-dTomato lentiviral vector and single-cell clones were expanded after FACS sorting. For C/EBPα induction, cells were treated with 1 μM 4-hydroxytamoxifen (H7904, Sigma), which shuttles the factor into the cell nucleus. The final culture medium also contained 100 U/mL penicillin-100 ng/mL streptomycin, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 100× MEM non-essential amino acid solution, and 1 mM sodium pyruvate.

RNA isolation and RNA-seq
RNA was extracted from B cells and mESCs with a miRNExasy mini kit (217,004, QIAGEN), quantified with a NanoDrop spectrophotometer, and its quality examined in a fragment Bioanalyzer (Agilent 2100 Bioanalyzer DNA 7500 assay). For RNA-seq, libraries were prepared with a TrueSeq Stranded mRNA Library Preparation Kit (Illumina) followed by single-end sequencing (50 bp) on an HiSeq 2500 instrument (Illumina), obtaining at least 40 million reads per sample.

For eight-cell embryos and late blastocysts, RNA was extracted and retro-transcribed into cDNA using an SMART-Seq v4 Ultra Low Input RNA kit (634894, Takara). RNA concentration and quality was determined as above. Libraries for RNA-seq were prepared as described previously (Picelli et al., 2014) and sequenced as above.

Computational analyses of RNA-seq data
Reads were mapped using STAR (Dobin et al., 2013) (standard options) and the Ensembl mouse genome annotation version mm10vM21. Gene expression was quantified using STAR (quant-Mode GeneCounts). Sample scaling and statistical analysis were performed using the R package DESeq2 (Love et al., 2014) (R 3.3.2 and Bioconductor 3.0). Statistical power of gene expression variation at any given time point was identified using the nbinomLRT test. Log2-vsd (variance stabilized DESeq2) counts were used for further analysis unless stated otherwise. Clustering was performed using the K-means method.

Statistics
Statistical analyses were performed using Prism 9 software. To calculate significance, samples from at least three biologically independent experiments were analyzed. Two biological replicates were used for RNA-seq experiments and statistics applied to the expression of a collection of genes (see Table S1). For samples with $n \geq 3$, values shown in the figures represent median ± SD with 10–90 percentile boxplots and whiskers. One- and two-way ANOVA (with the corresponding multiple comparison analyses) and Student’s t tests were applied accordingly. p values appear indicated in each figure.

Data and code availability
The RNA-seq datasets generated and analyzed for the current study are available in the Gene Expression Omnibus (GEO) database under accession numbers GSE202611 for Il6−/− and wild-type B cell reprogramming and F1 embryos from Cebpat−/− crosses and GSE202613 for the C/EBPα overexpression in mouse ESCs.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2022.07.009.

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AUTHOR CONTRIBUTIONS
M.P.-C. and T.G. conceived the study and wrote the manuscript. M.P.-C. performed reprogramming, transdifferentiation, pre-implantation embryo manipulation, molecular biology RNA-seq experiments, and analyzed the data. G.S. and M.V.-C. processed ChIP-seq, RNA-seq, and ATAC-seq data. R.B., C.D.R.W., A.K., and M.F. analyzed single-cell expression data. C.S.-M. performed western blot and immunostaining experiments. N.A. provided bones from Il6−/− mice. L.d.A.-A. produced recombinant proteins in conditioned medium. M.G. and N.P. injected mRNA constructs and imaged mouse embryos. T.V.T. and G.T.G. performed RNA-seq experiments. M.S. provided invaluable advice and T.G. supervised the research.

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CONFLICTS OF INTEREST
The authors declare no competing interests.

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