Increased expression of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase-3 is required for proliferation of mouse embryonic stem cells that are undergoing differentiation

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Abstract

The unlimited proliferation capacity of embryonic stem cells (ESCs) coupled with their capability to differentiate into several cell types makes them an attractive candidate for studying the molecular mechanisms regulating self renewal and transition from pluripotent state. Although the roles of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase family (PFKFB1-4) in cell survival, proliferation, and differentiation in tumor cells have been studied, their role in mESCs biology is currently unkown. In the current study, Pfkfb isozyme expressions were analyzed in undifferentiated R1 and J1 mouse embryonic stem cells (mESCs) that were cultured in the presence and absence of leukemia inhibitory factor (LIF). We report that expression of the Pfkfb3 isoenzyme was markedly increased when mESCs were promoted to differentiate with LIF removal. We then demonstrated that Pfkfb3 silencing induced the differentiation marker Brachyury suggesting that Pfkfb3 may be required for the regulation of mesodermal differentiation of mESCs. Furthermore, we show that the increase in Pfkfb3 expression is required for the proliferation of early differentiated mESCs. Although these results provide important insights into the early differentiation of mESCs with regard to Pfkfb expressions, further mechanistic studies will be needed for understanding the pathways and mechanisms involved in regulation of proliferation and early differentiation of mESCs through Pfkfb3.

Introduction

Pluripotent stem cells (PSCs) are characterized by their unlimited capacity for self-renewal and their potential to differentiate into all cell lineages of three primary germ layers. Different states of pluripotency exhibit slightly different features. The naive state represents the inner cell mass (ICM) of the early blastocyst while the primed state is representative of the early post-implantation epiblast cells. After implantation occurs, the epiblast cells progressively lose expression of pluripotency genes such as Oct4 and Nanog (Weinberger et al. 2016; Mathieu et al. 2017). Embryonic stem cells (ESCs) are derived from the ICM of pre-implantation blastocyst stage of embryos. Mouse ESCs (mESCs) resemble naive state whereas post-implantation epiblast stem cells (mEpiSCs) and human ESCs (hESCs) are considered a primed PSC. While leukemia inhibitory factor (LIF) is a crucial factor for robust self-renewal of mESCs, mEpiSCs can be grown in vitro without LIF in the presence of activin A and fibroblast growth factor (FGF) (Brons et al. 2007; Tesar et al. 2007). During culture, EpiSCs exhibit a high propensity for spontaneous differentiation (Kurek et al. 2015). The transition from the mESC to the mEpiSC state is the initial important step for ESCs to commit to differentiation. In vitro, first stages of spontaneous differentiation of mESCs can mimic this transition, but the mechanisms underlying this process are largely unknown.

PSCs exhibit a lower rate of Oxidative Phosphorylation (OXPHOS) than their differentiated counterparts; however, metabolism in naive and primed pluripotency occurs differently. Glycolysis is common to all stages of pluripotency; however, the relative contributions of glycolysis versus OXPHOS can differ in different stages of pluripotency. While mEpiSCs and hESCs are almost exclusively glycolytic, mESCs use both glycolysis and OXPHOS pathways (Lee et al. 2012; Zhou et al. 2012; Si et al. 2013; Mu et al. 2015; Cha et al. 2017). Early embryonic development occurs in a hypoxic environment which occurs as a result

of the high oxygen consumption and cellular proliferation of the rapidly growing embryo. In a hypoxic environment, the heterodimeric transcription factor hypoxia-inducible factor 1 (HIF1α), the main regulator of molecular response to hypoxia, plays a crucial role in tissue formation and stem cell homeostasis. HIF-1α upregulation stimulates the expression of glycolytic genes such as hexokinase (HK), lactate dehydrogenase (LDH), pyruvate dehydrogenase kinase (PDK1). Elucidation of the metabolic genes and pathways that are involved in pluripotency, differentiation, and proliferation of ESCs may help us better understand the molecular basis of early embryonic development as well as devise therapeutic strategies for various indications.

Glycolysis is indirectly regulated by the bifunctional 6-phosphofructo-2-kinase/ fructose-2,6-bisphosphatases isoenzymes (PFKFB1-4) which determine the intracellular concentration of fructose 2,6-bisphosphate (F2,6BP)-a shunt product of glycolysis and allosteric activator of one of the rate-limiting enzymes of glycolysis, 6-phosphofructo-1-kinase (PFK-1) (Yalcin et al. 2009). PFKFB enzymes have been shown to be involved in cell proliferation (Yalcin et al. 2014), cell survival (Domenech et al. 2015), cell differentiation (Hamanaka et al. 2017), and tumor growth (Clem et al. 2008). A recent study (Novellasdemunt et al. 2013) has revealed that the mouse Pfkfb3 mRNA becomes detectable during the blastocyst stage of embryogenesis. Chesney et al. (2005) demonstrated that the genomic deletion of Pfkfb3 results in embryonic lethality. Pegoraro et al. (2013) shows that tissue-specific, dynamic and complementary expression pattern of the PFKFB genes play an important role in the developmental stage of the Xenopus laevis (African clawed frog) embryos from blastula to tadpole stages. In another recent study by the same team (Pegoraro et al. 2015), Pfkfb4, by acting through Akt signaling pathway, has been shown to be essential in the development of frog embryos and differentiation of progenitor cells. However, studies on potential roles of Pfkfb isoenzymes in stem cell differentiation and metabolism are scarce.

In the current study, we studied the expression of Pfkfb isoenzymes in mESCs that are self-renewing (in the presence of LIF) and that are induced to spontaneously differentiate (upon LIF removal). We show, for the first time, that the differentiation of mESCs for 5 day (d) upon LIF removal resulted in an increase in Pfkfb3 expression. In addition, we demonstrate that the Pfkfb3 silencing was associated with the increased expression of the differentiation marker Brachyury. We further demonstrate that increased expression of Pfkfb3 is required for the proliferation of early differentiated mESCs in vitro.

Materials And Methods

Cell culture and mESC differentiation

Mouse embryonic stem R1 (ATCC, SCRC1036) and J1 (ATCC, SCRC 1010) cell lines were grown in tissue culture dishes (Corning, Amsterdam, The Netherlands) coated with 0.1% gelatin (Sigma, Munchen, Germany) in a Dulbecco's modified Eagle's medium (DMEM) (Sigma, Munchen, Germany) supplemented with 15% ESC-qualified fetal bovine serum (Sigma, Munchen, Germ any), 0.1mM MEM non-essential amino acids (Sigma), 0.1 mM 2-mercaptoethanol (Sigma, Munchen, Germany), L glutamin (Sigma), 100

U/ml penicillin-100 ug/ml streptomisin mix and 1000 units/ml of recombinant mouse LIF. mESCs were cultured at 37°C in a humidified atmosphere with 5% CO2. Cells were passaged every 3 or 4 d using trypsin EDTA. Media were changed every 2 d. Spontaneous differentiation was induced with the removal of LIF from media.

Real-time quantitative PCR (qPCR)

Total RNA was isolated using a commercial kit (Thermo Fisher Sci.) and reverse-transcribed using an mRNA to cDNA synthesis kit (Thermo Fisher Sci. Cat.#4387406) according to manufacturer's directions. mRNA expressions of Pfkfb1, Pfkfb2, Pfkfb3, Pfkfb4, Brachyury (T2), Nestin (Nes), Oct4, Sox2, Nanog, Klf4 mRNA expressions, were determined by qPCR using StepOnePlus (Thermo Fisher Sci., NY, USA) with TaqMan probes (Thermo Fischer Sci., Cat.#s: Pfkfb1, Mm01256237_m1; Pfkfb2, Mm00435575_m1; Pfkfb3, Mm00504650_m1; Pfkfb4, Mm00557176_m1; T2, Mm00436877_m1; NES, Mm00450205_m1; Oct4, Mm03053917_g1; Sox2, Mm03053810_s1; Nanog, Mm02019550_s1; Klf4, Mm00516104_m1; Gapdh, Mm99999915_g1). Gapdh was used as housekeeping gene control for normalization of cDNA. Cycle threshold (CT) values were taken from qPCR reactions and up/down regulation of genes of interest was determined by the ΔΔCT method using undifferentiated mESCs as a baseline (Livak et al. 2001).

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed following standard protocols. Primary antibodies specific to Pfkfb3 (Proteintech Cat.#13763-1-AP), P-Stat3 (Cell Signaling Cat.#9145), Stat3 (Cell Signaling Cat.#9139), Nanog (Cell Signaling Cat.#8822), and Gapdh (Cell Signaling Cat.#97166) proteins were used. Appropriate horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit, Cell Signaling Cat.#7074 or anti-mouse Cell Signaling Cat.#7076) were used. Signals were developed using Amersham ECL plus chemoluminescent reagent (GE Technologies). Bands on membranes were visualized with ChemiDoc MP (BioRad).

siRNA and plasmid transfection

Transfections of and Pfkfb3-specific (Thermo Fischer Sci. Cat.#100779) siRNA molecules into cells was performed in serum-free and antibiotic-free medium using Lipofectamine RNAiMAX (ThermoFisher Sci.) following the manufacturer's recommendations when cells reached approximately 50% confluency at the time of transfection. As a negative control, cells were transfected with the universal siRNA molecule, which has no homology in the human genome (Thermo Fischer Sci. Cat.# 4390846). Cells were incubated in complete medium at 37°C for 48 hours before cell harvest or further experiments. The final concentrations of siRNA molecules were 10 nM.

For ectopic expressions, pCMV6-A-BSD expression vector encoding Pfkfb3 cDNA (Origene Cat. #MG227622) was transfected into cells using Lipofectamine 3000 reagent (ThermoFisher Sci.) following the manufacturer's instructions. Cells were 70–80% confluent at the time of transfection.

Cell proliferation

Pluripotent and differentiated (for 5 d) mESCs were lifted with trypsin post-transfections and stained with trypan blue for 1 min. The numbers of viable cells were counted under an inverted microscope (Accu-Scope, China) using hemocytometer (Neubauer improved) as per standard protocol.

Fructose 2,6-bisphosphate assay

Intracellular fructose 2,6-bisphosphate (F2,6BP) levels of differentiated (for 3 and 5 d) and undifferentiated mESCs were analyzed following a Kinetic spectrophotometric coupled enzyme method described by Van Schaftingen et al. (1982). The protocol briefly as follows; mESCs were centrifuged at 270×g and resuspended in 20 volumes of 0.05 N NaOH and then 1 volume of 0.1 N NaOH vortexed for 10 s, heatted at 80°C for 5 min, and cooled in an ice bath. PH of lysates adjusted to 7.2 with ice-cold 1M acetic acid in the presence of 1M Hepes. Next, samples were incubated at 25°C for 2 min in the assay mixture. The contents of the assay mixture were as follows: 50 mM Tris, 2 mM Mg^{2}, 1 mM Fru-6-P, 10 units/liter PPi-dependent PFK1, 0.15 mM NAD, 5 kilounit/liter triose-phosphate isomerase, 0.45 kilounit/liter aldolase, and 1.7 kilounit/liter glycerol-3-phoshate dehydrogenase (Sigma). Reaction was started adding 0.5 mM Pyrophosphate and the rate of changes in absorbance (OD 339 nm) per min was analyzed in 5 min. F2,6BP levels were calculated based on a calibration curve ranging from 0.1 to 1.0 pmol standart of Fru-2,6-BP (Sigma) and normalized to total cellular protein levels.

Glucose uptake assay

To determine glucose uptake by pluripotent and differentiated (for 5 d) mESCs, a commercial glucose uptake kit (BioVision, Milpitas, CA, USA) was used in accordance with the manufacturer's instructions. The principle of this assay is briefly as follows: 2-deoxyglucose (2-DG) is metabolized to 2-DG-6-phosphate (2-DG6P) which cannot be further metabolized, and thus accumulates in the cells. The accumulated 2-DG6P is directly proportional to 2-DG (or glucose) uptake by cells. 2-DG6P is oxidized to generate NADPH, which can be determined by an enzymatic recycling amplification reaction.

ALP staining

Alkaline phosphatase (ALP) Live Stain kit (Thermo Fischer Sci. Cat.#A14353) was used to determine ALP activity in pluripotent and differentiated mESCS. Staining was performed for 20–30 min according to manufacturer's instructions. The protocol was briefly as follows; R1 and J1 mESCs were washed twice

with sterile, fresh, pre-warmed basal DMEM/F-12 media. For differentiated and undifferentiated mESCs, 1X working solution which was prepared by diluting the 500X stock solution of the LIVE AP substrate in basal DMEM/F-12 media, was found to be optimal in providing a potent signal. The ALP live stain dye was immediately applied on to the adherent mESC culture. mESCs were incubated with the substrate for 20–30 min and washed twice with the DMEM/F-12 basal media to remove excess substrate. After the final wash, fresh basal media was added and fluorescent-labeled colonies were imaged on EVOS Imaging System (ThermoFischer Sci.). The most robustly fluorescing colonies were selected for imaging within 30–40 min of staining.

Statistical analysis

The Statistical Package for the Social Sciences version 23.0 (SPSS, Chicago, IL, USA) was used for data analyses. Values were expressed as means ± standard deviation. Statistical analysis was performed using Student's t-test and results were considered to be significant when p value was < 0.05.

Results And Discussion

LIF withdrawal induces differentiation of mESCs

The LIF/STAT3 pathway has a crucial role in maintaining the self renewal and pluripotency of the mESCs (Raz et al. 1999; Hirai et al. 2011). Upon the withdrawal of LIF, mESCs rapidly exit from the pluripotent state in vitro. A recent study in mESCs has shown that LIF removal induces transition from the naive pluripotent state to the primed-like state with expression of early differentiation markers through activation of mTOR (Cherepkova et al. 2016). First, we evaluated the morphological features of R1 and J1 mESCs growing in the absence or presence of LIF. The morphology of the cells that are induced to differentiate for 5 d acquired some morphological characteristics compared to undifferentiated mESCs. While undifferentiated R1 and J1 mESCs grew as compact, dome-shaped colonies, the cells appeared more scattered, less compact and more flattened upon LIF removal (Fig. 1A).

qPCR analysis demonstrated that the LIF withdrawal from media in R1 and J1 mESCs for 5 d led to decreases in expression levels of pluripotency genes, including Oct4 (R1, p 0.05; J1, p 0.01), Klf4 (p 0.01), Sox2 (p 0.01) and Nanog (p 0.05) (Fig. 1B). A recent study showed that the induction of early differentiation in LIF-depleted mESCs proceeds by KLF4-mediated regulation of pluripotency related pathways (Cherepkova et al. 2016). Western blot analysis was used to confirm the decrease in the Nanog protein in differentiated R1 mESCs (Fig. 1D). Consistent with downregulation of pluripotency gene marker expressions, mRNA levels of brachyrury (R1, p 0.01; J1 p 0.05), a widely used early marker of mesodermal differentiation and Nestin (R1 and J1, p 0.01) (Fig. 1C), a neuroectodermal stem cell marker, were upregulated upon LIF removal. Among the intracellular signaling pathways that are regulated by LIF, Jak-Stat3 is known to be essential for the self-renewal and pluripotency of mESCs (Niwa et al. 2009). Upon LIF stimulation, Stat3 is phosphorylated and activated by Jaks, which subsequently stimulates the expressions of various pluripotency-associated genes (Hirai et al. 2011; Cherepkova et al. 2016). We

checked the phosphorylation of the Stat3 protein in the presence or absence of LIF and found that LIF removal reduced Stat3 phosphorylation (Fig. 1D).

Alkaline phosphatase activity is another valuable marker for pluripotent mESCs. High ALP activity in pluripotent mESCs is reduced or lost upon differentiation (Piquet-Pellorce et al. 1994; Hong et al. 1996). We therefore went on to determine ALP activity in undifferentiated and differentiated mESCs. As expected, while the undifferentiated R1 and J1 mESCs displayed a robust ALP activity as asssessed by staining of the cells with a fluorescent ALP subsrate, the LIF-depleted cells displayed no activity (Fig. 1E), suggesting that LIF removal induced the differentiation of mESCs.

Early differentiation of mESCs leads to an increase in Pfkfb3 expression

Although the roles of Pfkfb isoenzymes in proliferation and survival of tumor cells are well-studied (Yalcin et al. 2014; Peng et al. 2018), potential roles of Pfkfb3 isoenzymes in proliferation and differentiation of mESCs are currently unknown. We first compared the expressions of Pfkfb isoenzymes in undifferentiated and early differentiated mESCs. J1 and R1 mESCs were cultured in the presence and absence of LIF for 5 d and mRNA levels of Pfkfb genes (Pfkfb1-4) were determined using qPCR. LIF withdrawal in both cell lines resulted in an increase in the expression of Pfkfb3 (p 0.001) and Pfkfb4 (p 0.05) isoenzymes, while no significant differences were determined in the expressions of Pfkfb1 and Pfkfb2 isoenzymes (Fig. 2A). Because the most significant increase was observed with Pfkfb3 expression, we focused our attention on this isoform. Increased Pfkfb3 mRNA expressions in differentiated R1 and J1 mESCs coincided with increases in Pfkfb3 protein levels as assessed by Western blot analyses (Fig. 2B). Consistent with the increases in mRNA and protein levels of Pfkfb3 isoenzyme, the intracellular F2,6BP level was significantly elevated in early differentiated mESCs (3 d and 5 d) relative to undifferentiated mESCs ($p < 0.001$) (Fig. 2C). LIF-depleted mouse ESCs have been shown to transit from the pluripotent state to the primed-like state, which exhibits a glycolytic phenotype (Zhou et al. 2012). Our findings show that a higher Pfkfb3 and F2,6BP levels upon LIF removal are consistent with the glycolytic phenotype reported by Zhou et al. (2012). However, we found that LIF-depleted cells exhibited a diminished glucose uptake compared with undifferentiated R1 mESCs (p 0.05) (Fig. 2D). Although it is difficult to reconcile these seemingly contradictory findings, as increased Pfkfb3 and F2,6BP levels are known to be associated with increased glucose uptake and glycolytic activity, we speculate that, given the dymanic feature of differentiation, the reliance of mESCs undergoig differentiation on glycolysis vs. OXPHOS may markedly be divergent in various stages of differentiation. For example, the highly glycoltic mESCs during the early stages of differentiation (< 5 d of differentiation) may begin to rely on OXPHOS as the cells become more differentiated (> 5 d). Lending support to this hypothesis, the study by Ando et al. (2010) that was published during the preparation of our manuscript that showed a diminished glucose uptake that coincided with a decreased Pfkfb3 level on day 6 compared with day 3 postdifferentiation in early differentiated adipocytes. However, we refrain from over-speculating regarding the data, as the setup and cell types were different in these studies. Another likely

scenario is that given that the activation of the IL-6/STAT3 pathway in LIF-containing media induces glycolysis as shown by Ando et al. (2010), and that removing LIF may have uncoupled glucose uptake and glycolysis from pluripotency signals such as STAT3. In this scenario, although glucose uptake was reduced with LIF removal, the induction of Pfkfb3 may be necessary to maintain the glucose uptake and glycolytic flux in early differentiation of mESCs. The observed upregulation of Pfkfb3 may reflect a compensatory feedback mechanism in LIF-depleted (day 5) mESCs and may diminish on further days of differentiation, which will require further investigations.

Pfkfb3 silencing induces Brachyury expression in mESCs.

The mouse Brachyury (T) is a key regulator of mesoderm formation during early embryonic development (Zhu et al. 2016) and essential for epithelial mesenchymal transition (EMT) which is important for embryonic development and EpiSCs differentiation (Kim et al. 2014; Song et al. 2016). Although there are studies suggesting the involvement of Pfkfb3 on differentiation of preadipocytes (Griesel et al. 2021) and epidermal keratinocytes (Hamanaka et al. 2017), the potential role of Pfkfb3 in early differentiation of mESCs is unknown. To investigate the requirement of Pfkfb3 for the early differentiation of mESCs, we analyzed gene expression levels of the differentiation markers brachyrury and nestin in control- or Pfkfb3-siRNA transfected cells, in the presence and absence of LIF. qPCR analyses demonstrated that the Pfkfb3 gene silencing led to an increase in the expression level of Brachyury gene in both of differentiated and undifferentiated mESC lines (p 0.05), while there were no changes in the expressions of nestin gene (Fig. 3: average mRNA fold changes of siNTCs or siPF3s; **Brachyury**: *R1 cell line* LIF+, siNTC = 1 ± 0.17 and siPF3 = 1.99 ± 0.35; LIF-, siNTC = 1,99 ± 0.55 and siPF3 = 2.55 ± 0.42; *J1 cell line* LIF+, siNTC = 1 ± 0.19 and siPF3 = 1.58 ± 0.36; LIF-, siNTC = 2,17 ± 0.19 and siPF3 = 3.05 ± 0.36; **Nestin**: *R1 cell line* LIF+, siNTC = 1 ± 0.048 and siPF3 = 0.91 ± 0.02; LIF-, siNTC = 2.52 ± 0.40 and siPF3 = 2.29 ± 0.02; J1 cell line LIF+, siNTC = 1 ± 0.03 and siPF3 = 0.98 ± 0.04; LIF-, siNTC = 5.57 ± 0.15 and siPF3 = 5.80 ± 0.18) and pluripotency gene markers, Sox2, Nanog, Oct4, Klf4 (*data not shown*). These data suggest that Pfkb3 may have a role in the regulation of barchyury-mediated mesodermal differentiation of mESCs in the early embryonic period, which is consistent with a recent study demonstrating that Pfkfb3 knockdown promoted the differentiation of epidermal keratinocytes (Hamanaka et al. 2017).

Increase in Pfkfb3 expression is required for proliferation of early differentiated mESCs

Given the known role of Pfkfb3 in cell proliferation (Yalcin et al. 2009, 2014), we then evaluated cell counts in the presence and absence of LIF after Pfkfb3 siRNA transfection and overexpression. We first confirmed the effiency of Pfkfb3 mRNA targeting by the siRNA approach using qPCR (Fig. 4A). We found that while Pfkfb3 depletion led to a decreased cell counts upon LIF withdrawal, it increased cell counts in the presence of LIF (p 0.05) (Fig. 4B). We then ectopically transfected R1 and J1 mESCs in the presence and absence of LIF with expression vector carrying Pfkfb3 (Pfkfb3-V) and empty plasmid for control.

Ectopic transfections led to marked increases in Pfkfb3 mRNA levels (Fig. 5A). In contrast to the Pfkfb3 silencing, ectopic Pfkfb3 expression reduced the proliferation of mESCs growing in the presence of LIF but stimulated the growth of mESCs that are induced to spontaneously differentiate with LIF removal (Fig. 5B). Consistent with a decrease in glucose uptake upon LIF removal, cell proliferation was diminished in LIF-depleted cells. Our observation that Pfkfb3 depletion further reduces the proliferation of early differentiated mESCs suggests that mESCs that are undergoing spontaneous differentiation may rely on increased Pfkfb3 levels to maintain glycolytic phenotype that is associated with proliferation.

Conclusion

In summary, the present study demonstrates that LIF-depletion induces Pfkfb3 expression and that Pfkfb3 may be required for differentiation and proliferation of mESCs. Mechanistic studies will be needed to fully delineate the requirement of PFKFB isoenzymes for differentiation of stem cells into specialized cells. Better understanding of specific molecular mechanisms or pathways of stem cell differentiation may provide us with valuable tools that can be exploited in various disciplines of medicine.

Abbreviations

PFKFB, 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase; mESCs, mouse embryonic stem cells; LIF, leukemia inhibitory factor.

Declarations

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Ethics Declarations

Conflict of interest The authors declare that they have no conflicts of interests.

Ethics Approval Not applicable

Consent to participate Not applicable

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

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Figures

Figure 1

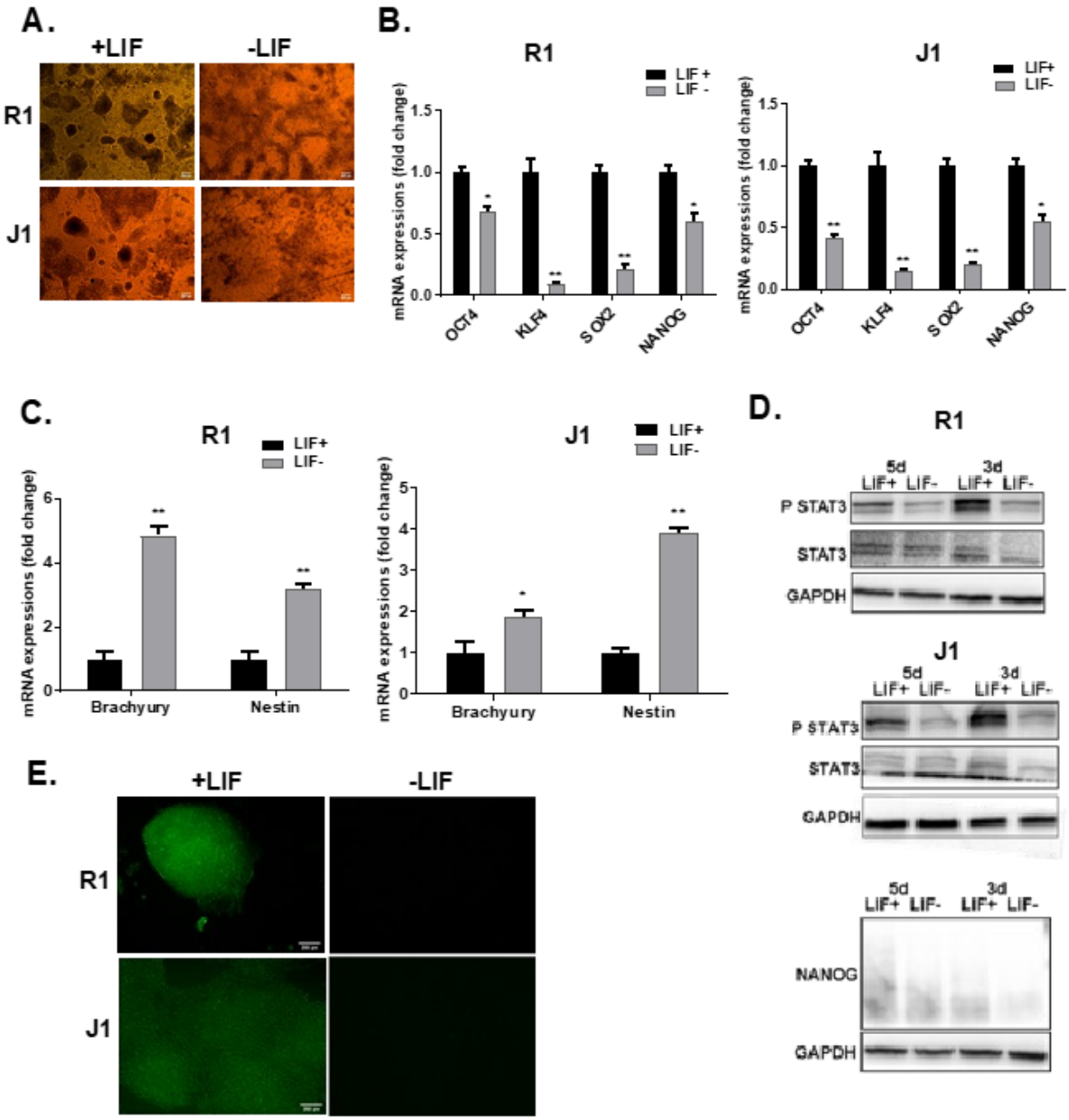

Figure 1

LIF removal induces differentiation of R1 and J1 mESCs.

(A) Morphology of R1 and J1 mESCs grown in the presence (LIF+) and absence (LIF-) of LIF for 5 d. **(B)** mRNA expression levels of pluripotency genes Oct4, Klf4, Sox2 and Nanog in undifferentiated and LIF-depleted mESCs. **(C)** mRNA expression levels of the differentiation genes Brachyury and Nestin in mESCs

grown in the presence (+) of and absence (-) of LIF for 5 d. Gapdh was used as internal control. Data are presented as mean±s.d. of an experiment that was conducted in triplicate (n=3). *p 0.05, **p 0.01 compared to LIF (+) control. **(D)** Western blot analysis of Nanog in R1 mESCs, and Stat3/p-Stat3 activity in R1 and J1 mESCs. Gapdh was used as loading control. **(E)** Alkaline phosphatase staining of R1 and J1 mESCs cultured on gelatin-coated plates with (+)/without (-) LIF.

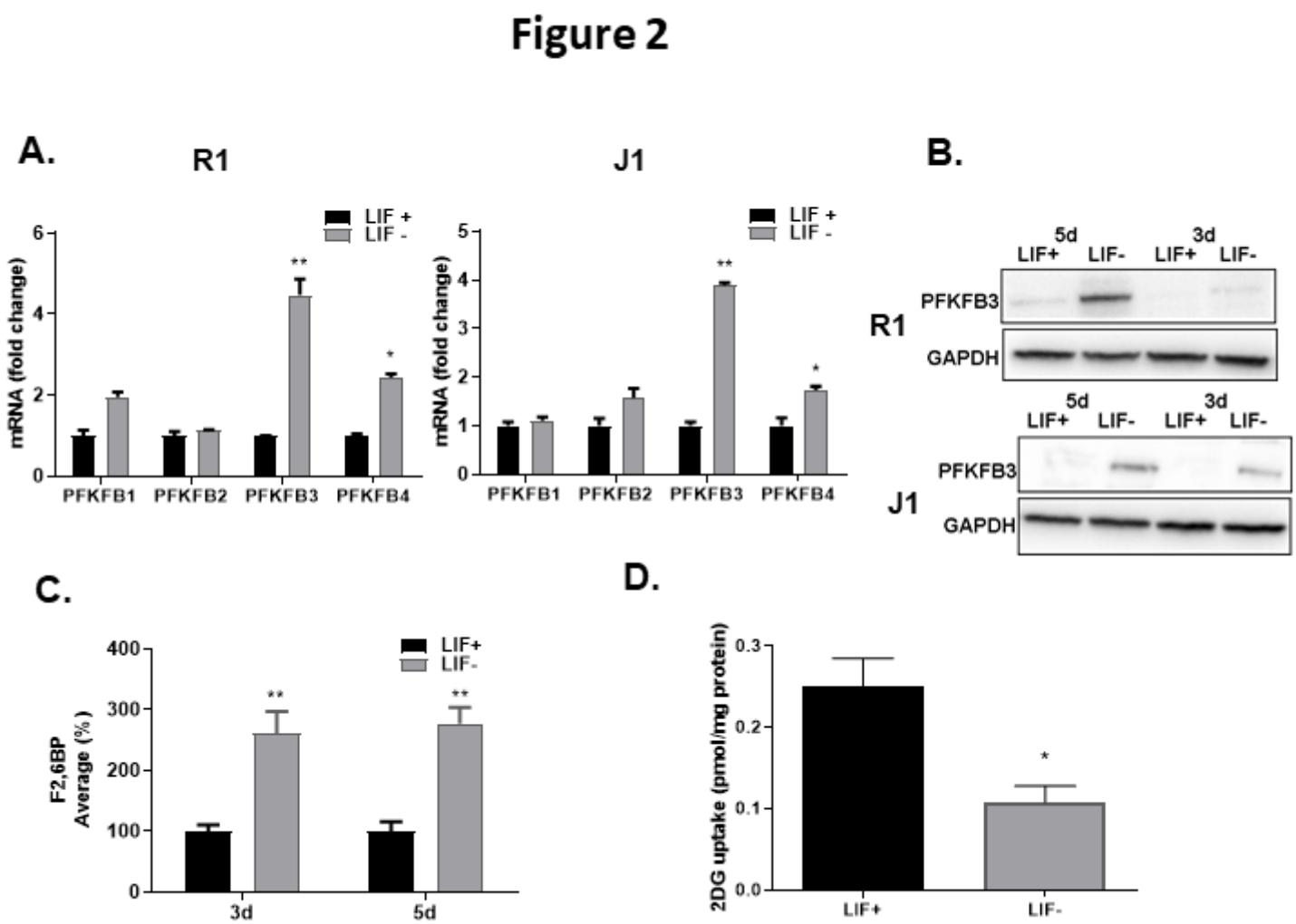

Figure 2

Differentiation of mESCs leads to an increase in Pfkfb3 expression. **(A)** mRNA expression levels of Pfkfb isoenzymes in R1 and J1 mESCs grown with (+)/without (-) LIF for 5d. **(B)** Western blot analysis of the Pfkfb3 protein in R1 and J1 mESCs grown with/without for 3 d and 5 d. **(C)** F2,6BP levels of mESCs grown grown with/without LIF for 3 d and 5 d. **(D)**2DG uptake of mESCs grown grown with/without LIF for 5 d. **p 0.05, **p 0.001* compared to LIF (+) control.

Figure 3

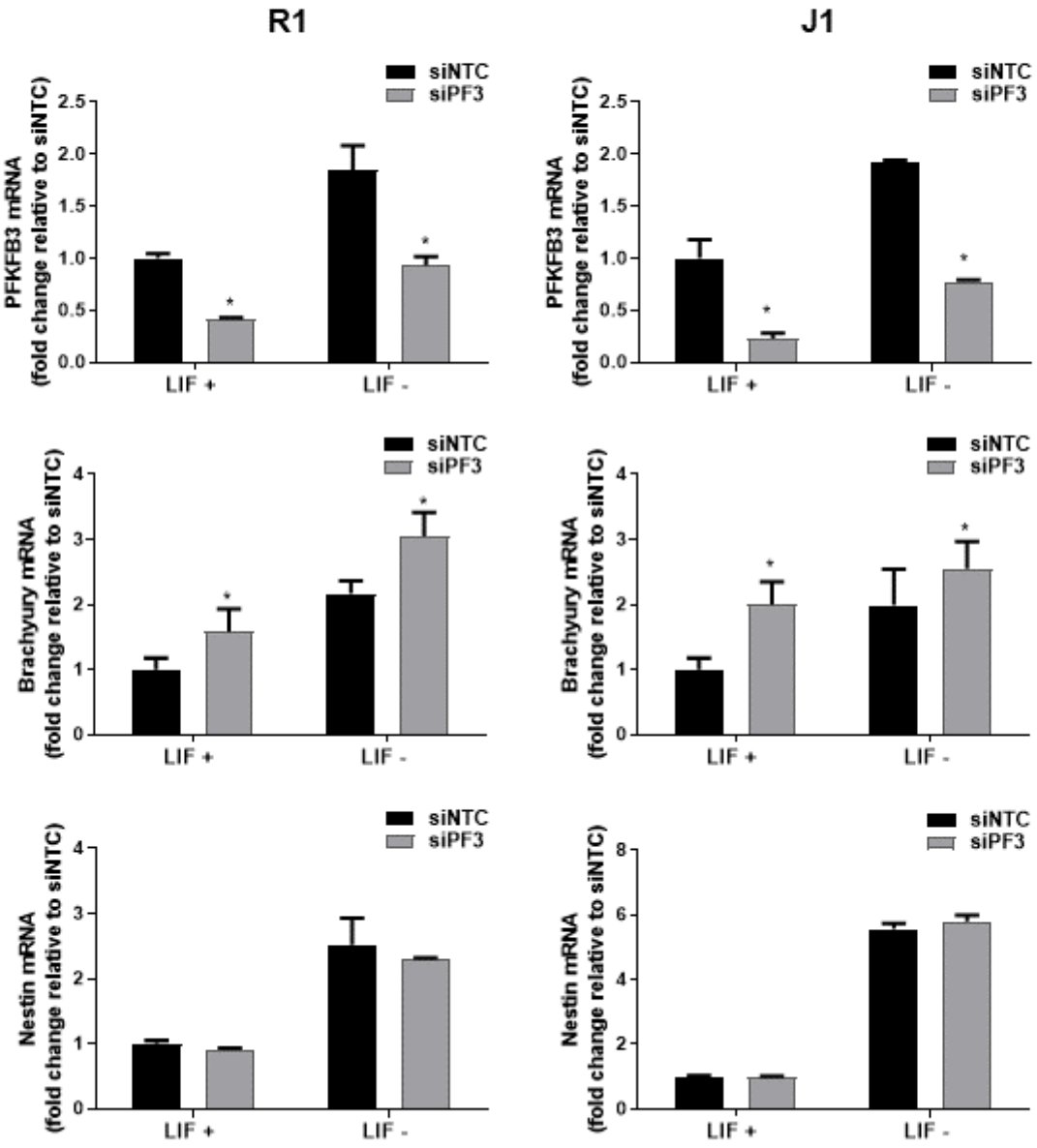

Figure 3

Pfkfb3 silencing induces differentiation marker Brachyury expression in R1and J1 mESCs.

R1 and J1 cells grown in presence of LIF (+) were transfected with either non-targeting control (siNTC) or PFKFB3-specific siRNA (siPF3) molecules. The following day, the media was replaced new media with (+)/without (-) LIF. Forthy-eight hours later, Pfkfb3, Brachyury and nestin mRNA levels were analyzed by qPCR .**p 0.05.*

Figure 4

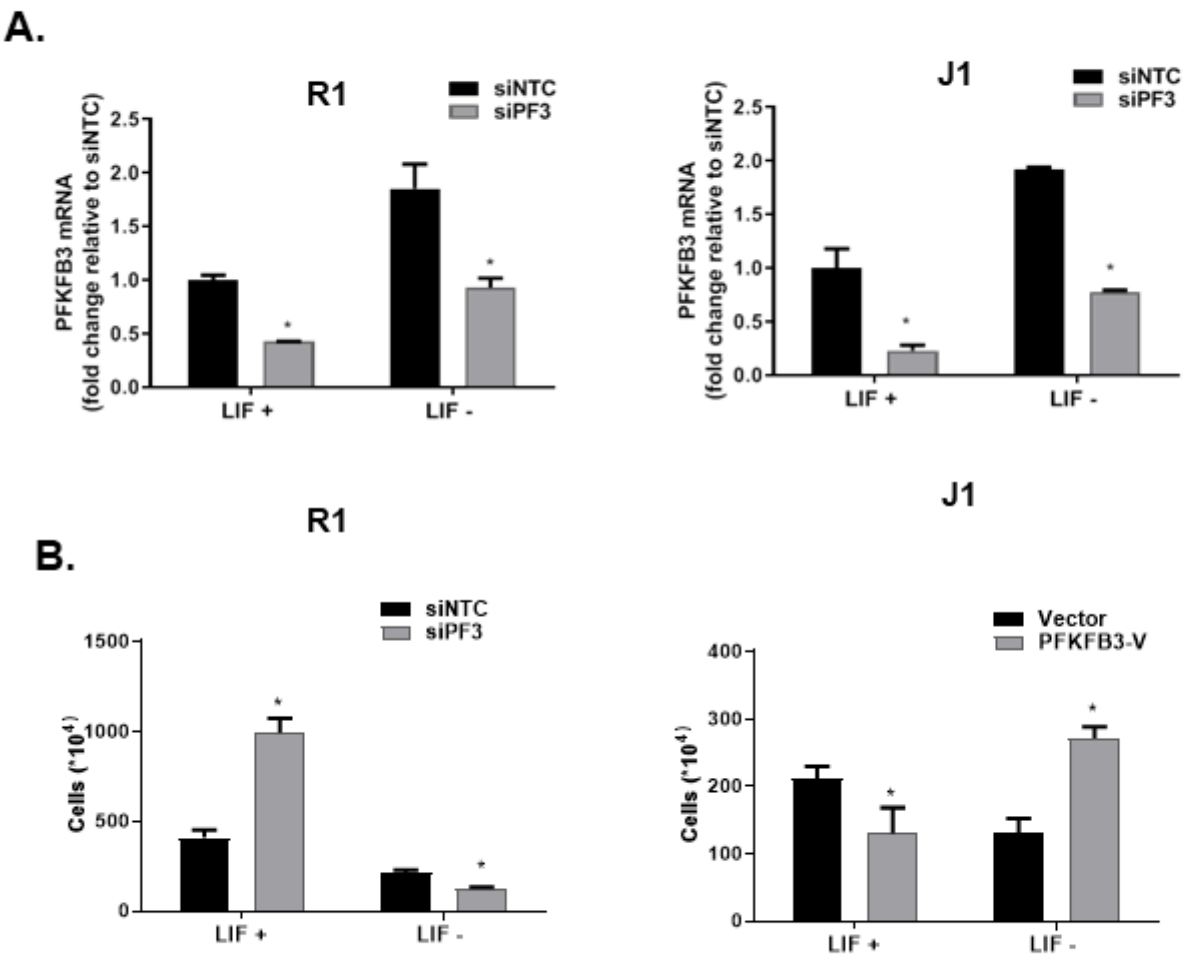

Figure 4

Increase in Pfkfb3 expression is required for proliferation of early differentiated mESCs.

(A) qPCR analyses of Pfkfb3 mRNA levels of R1 and J1 cells transfected either with non-targeting control (siNTC) or PFKFB3-specific siRNA (siPF3) molecules that were grown with (+)/without (-) LIF. **(B)** Cell counts of R1 and J1 cells transfected either with non-targeting control (siNTC) or PFKFB3-specific siRNA (siPF3) molecules that were grown with (+)/without (-) LIF. **p 0.05*.

Figure 5

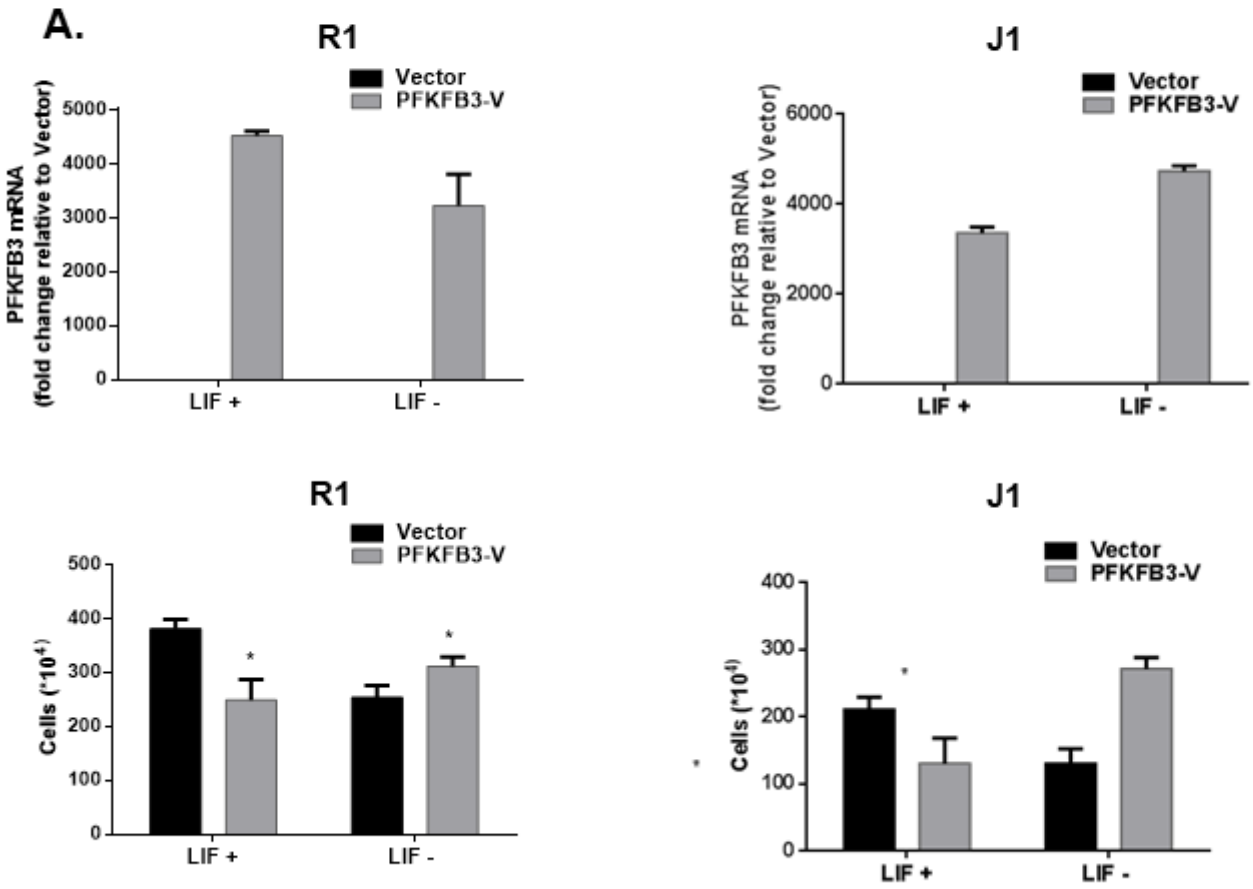

Figure 5

Forced Pfkfb3 expression stimulates the proliferation of early differentiated mESCs.

(A) qPCR analyses of Pfkfb3 mRNA levels in R1 and J1 cells that were transfected with a plasmid encoding Pfkfb3 cDNA (PFKFB3-V), or an empty vector (Vector). **(B)** Cell counts of the trasfected R1 and J1 cells that were grown with (+)/without (-) LIF. **p 0.05*