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Changes in the Gene Expression of Pyruvate Dehydrogenase Kinase Isoenzymes During Early Differentiation of Mouse Embryonic Stem Cells

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Abstract

The embryonic stem cells (ESCs) are pluripotent, self-renewing cells that are able to differentiate into any of the germ layers involved in embryogenesis. However, the molecular mechanisms that control ESC pluripotency and differentiation remain poorly understood. The family of Pyruvate dehydrogenase kinase (PDK1-4), inactivates the mitochondrial pyruvate dehydrogenase complex via phosphorylation, plays a crucial role in the control of glucose homeostasis. In the current study, gene expression levels of PDK isoenzymes were analyzed on undifferentiated mouse embryonic stem cells (mESCs) and compared to mESCs induced to differentiate by removal of leukemia inhibitory factor (LIF) for 1, 3 and 5 days. Besides, gene expression analysis of several genes related to pluripotency and differentiation were performed by real-time quantitative PCR. In addition, glucose uptake rates in early differentiated and undifferentiated mESCs were determined using a colorimetric assay kit. Differently expression level of PDK isoenzymes in pluripotent and LIF-depleted mESCs suggest that they may have roles in differentiation and pluripotency of ESCs. Furthermore, this study lays the foundation for detailed investigation of molecular mechanisms underlying the roles of PDKs in the pluripotency and transition to differentiated state of ESCs.

Keywords: Stem cell, Pyruvate Dehydrogenase Kinase, Differentiation

Introduction

Pluripotent embryonic stem cells (ESCs) are characterized by their unique ability to both unlimited self-renewal and differentiation. Pluripotency describes the capacity of a cell to differentiate into all cell lineages derived from the three germ layers of the embryo including endoderm, mesoderm, and ectoderm[1,2]. Mouse and human ESCs are isolated from the inner cell mass (ICM) of pre-implantation embryos (blastocysts stage). The early stages of embryonic development take place in a hypoxic environment[3]. Owing to the hypoxic environment in which they reside, ESCs that are highly proliferative cell types utilize glycolysis-based metabolism. Highly proliferative cells need energy, however they also need nucleotides, amino acids and lipids for biosynthesis of cellular components of daughter cell generation. Although oxidative phosphorylation (OXPHOS) is more efficient than glycolysis for generating ATP, using all available glucose solely for the purpose of generating ATP would be limiting for a proliferating cell. Instead, some glycolytic intermediates redirect into the pentose phosphate shunt. It is perhaps for this reason that ESCs, show mainly aerobic glycolytic activity, glycolysis that results into lactate generation, instead of mitochondrial oxidation of pyruvate, regardless of oxygen availability[4,5]. During the early stages of embryonic development, a metabolic shift exists from OXPHOS to glycolysis and oxidative metabolism is reinstituted at post implantation stage. Zhou et al.[6] reported that ESCs rely mostly on anaerobic glycolysis for energy supply and the mitochondria of the cells are inactive during the first stages of spontaneous differentiation. Hypoxia stimulates faster glucose consumption in an attempt to maintain ATP generation via less efficient anaerobic glycolysis.

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In a hypoxic environment, hypoxia-inducible factor 1, or HIF1α transcription factor, is the main regulatory protein, which play a crucial role in stem cell homeostasis. It was demonstrated that HIF-1α is a key factor in regulating embryonic morphogenesis and an important compensator of increased glycolytic activity in the early stages of differentiation[7,8]. HIF-1α up regulation induces the expression of glycolytic genes including pyruvate dehydrogenase kinase 1 (PDK1), lactate dehydrogenase (LDH), hexokinase (HK)[9,10]. ESCs, display Warburg effect, a shift from oxidative phosphorylation to aerobic glycolysis characteristic of cancer cells. Recent data implicate that the Warburg effect, enhanced glycolysis, is also involved in cellular immortalization and sensitizing cells to oncogenic transformation[11]. Therefore, understanding the roles of the major metabolic pathways in the maintenance and acquisition of the pluripotency and in the integration of pluripotency and glycolysis can contribute to a better understanding of molecular basis of stem cells and cancer biology.

Mitochondrial multienzyme pyruvate dehydrogenase complex (PDC) catalyzes irreversible step of the oxidative decarboxylation of pyruvate to acetyl-CoA. Pyruvate dehydrogenase kinase (PDK) enzyme family inactivates the enzyme pyruvate dehydrogenase by the phosphorylation of three seryl residues in the pyruvate decarboxylase subunit, E1 and redirecting metabolism of pyruvate into lactate. PDK has four biochemically distinct isoforms (PDK1, PDK2, PDK3, and PDK4), which have diverse tissue-specific distributions and functions[9,10]. PDK isoforms are up regulated in several types of cancer including breast cancer[12], ovarian cancer[13], hepatocellular carcinoma[14], pancreatic cancer[15], leukemia[16], lymphoma[17]. Most cancer cells use glycolysis as the primary energy source and are characterized through a shift from a mitochondria-based glucose oxidation to glycolysis or lactate fermentation regardless of the presence of oxygen (Warburg effect)[18]. PDK isoforms are the key players of this metabolic shift[13,19]. Besides, stem cell metabolism which mostly relies on glycolysis, shows certain parallelism with that of cancer cells. However, due to the limited literature about the functions of PDK isoenzymes in stem cell metabolism, it is not fully known whether there are possible roles in maintaining pluripotency and there still is a lack of study how PDK family genes are expressed in pluripotent and differentiated stem cells. The current study aimed to investigate the changes of PDK isoenzymes under conditions that induce self-renewal of mESCs (in the presence of essential pluripotency mediator, Leukemia inhibitor factor-LIF), and under conditions that induce differentiation for 1, 3 and 5 days (in the absence of LIF).

Material and Method

Cell culture

Mouse embryonic stem R1 cell line (ATCC) was cultured on 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% ES cell-qualified fetal bovine serum (Sigma, Munchen, Germany), 0.1 mM 2-mercaptoethanol (Sigma, Munchen, Germany), 0.1mM MEM non-essential amino acids (Sigma), L glutamine (Sigma), 100 U/ml penicillin-100 ug/ml streptomycin mix and 1000 units/ml of recombinant mouse LIF at 37 °C in a humidified atmosphere with 5% CO2. Cells were passaged every 3 or 4 days using trypsin EDTA. Optimal cell seeding density was conserved at 40,000 cells/cm2 in mouse ESCs to maintain its pluripotent characteristics.

To induce spontaneous differentiation for five days, the ESCs were washed with 1× PBS (Sigma, Munchen, Germany) twice 12 h after the culture and then cultured in the same DMEM and supplements without LIF. Media were changed every 2 days.

Real-time quantitative PCR (RT qPCR)

Total RNA was isolated using a commercial kit (Thermo Fisher Sci.) and reverse-transcribed using cDNA synthesis kit (Thermo Fisher Sci.) according to manufacturer's directions. To analyze total PDK1, PDK2, PDK3, PDK4, SOX2, Brachyury (T2), Nestin (NES) mRNA expressions, real-time quantitative PCR was performed with StepOnePlus (Thermo Fisher Sci., NY, USA) using TaqMan probes (Thermo Fischer Sci., Cat #: PDK1, Mm00554300_m1; PDK2, Mm00446681-m1; PDK3, Mm00455220-m1; PDK4, Mm01166879-m1; SOX2, Mm03053810_s1; T2, Mm00436877_m1; NES, Mm00450205_m1; GAPDH, Mm99999915_g1). GAPDH was used as housekeeping gene control for normalization of cDNA. While Brachyury and Nestin were analyzed as differentiation markers, SOX2, was evaluated as pluripotency marker.

Glucose uptake assays

The glucose uptake colorimetric assay kit (BioVision, Milpitas, CA, USA) was used to detect relative glucose uptake of differentiated and pluripotent ESCs in accordance with the manufacturer's instructions. The principle of this assay is briefly as follows: 2-deoxyglucose (2-DG) 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.

Statistical analysis

The Statistical Package for the Social Sciences version 23.0 (SPSS, Chicago, IL, USA) was used to analyze the gene expression ratios and relative glucose uptake. Cycle threshold (CT) values were taken from qPCR reactions and regulation of genes of interest (up/down) was determined by the ΔΔCT method, using GAPDH as internal control gene, and with undifferentiated mESCs as a baseline. Data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using Student`s t-test and $p < 0.05$ was considered significant.

Results

LIF withdrawal induces the expression of differentiation genes but suppresses the expression of pluripotency gene SOX2

The cytokine LIF plays a crucial role to sustain prolonged proliferation of mESCs while maintaining their undifferentiated state (self-renewal) and pluripotency. First, the morphological characteristics of mESCs R1 cell line culturing in the absence versus presence of LIF were demonstrated. The morphology of early differentiating cells (5 days after removal of LIF) showed some morphological changes compared to undifferentiated mESCs. mESCs cultured in the absence of LIF were independently growing cells, less compact and more flattened while undifferentiated mESCs grew as compact dome-shaped colonies (Figure 1a).

qRT-PCR data showed that the removal of LIF leads to a reduction in the expression levels of pluripotency gene marker SOX2 within 3 day ($p<0.01$) (Figure 1b). Concomitantly with downregulation of SOX2 gene expression, Nestin ($p<0.01$) (Figure 1c), a neuroectodermal stem cell marker, and brachyrury ($p<0.01$) (Figure 1d), earliest marker of mesodermal and also endodermal differentiation, progressively upregulated.

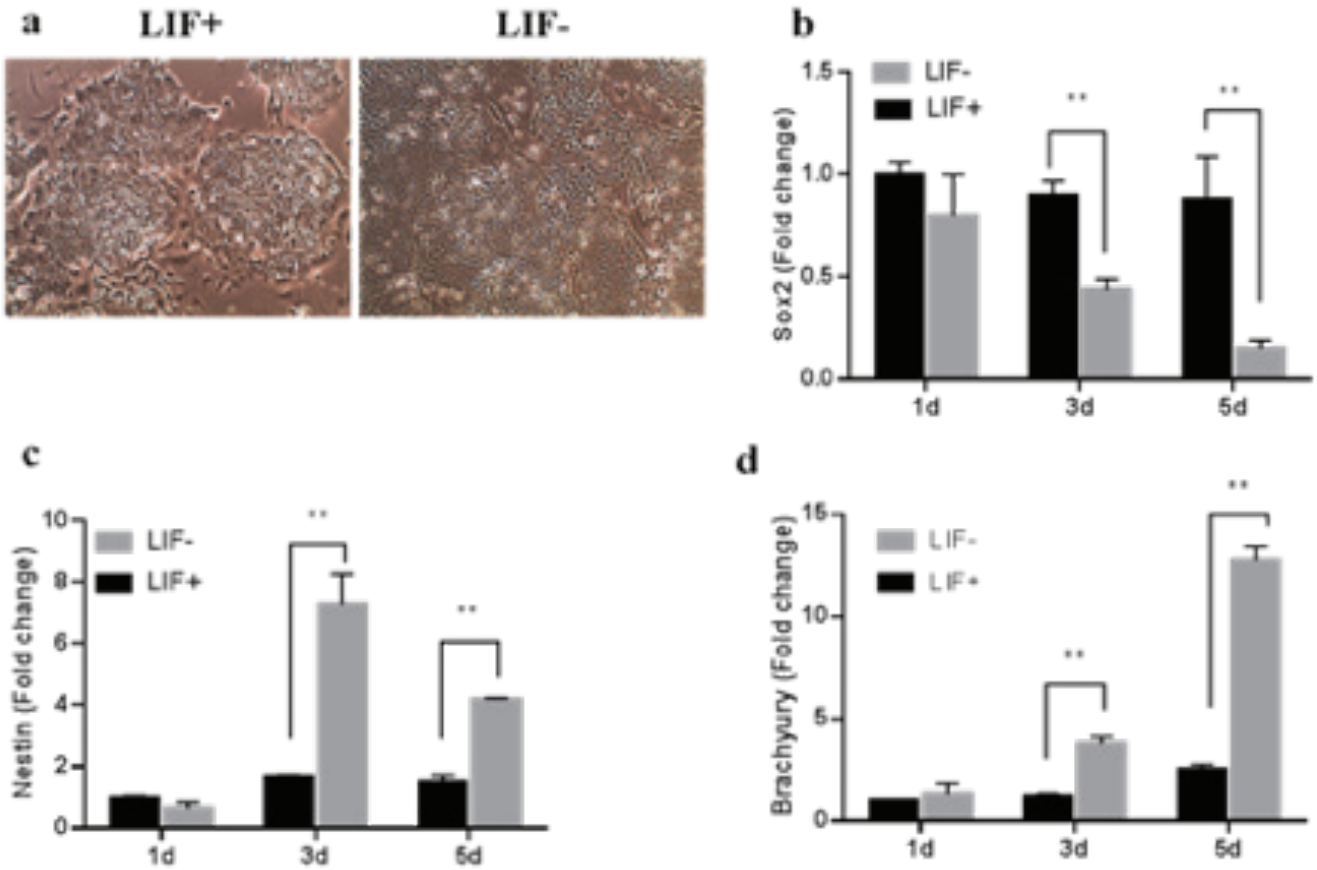

Figure 1 LIF withdrawal induces the expression of differentiation genes but suppresses the expression of pluripotency gene SOX2 **(a)**Morphology of mESCs grown in the presence (LIF+) and absence (-LIF) of LIF for 5 days. **(b)** mRNA expression levels of SOX2 gene in undifferentiated (+LIF) and LIF-depleted (LIF-) for 1, 3, 5d mESC. **(c)** mRNA expression levels of Nestin gene in undifferentiated and LIF-depleted for 1, 3, 5d mESC. **(d)** mRNA expression levels of brachyury gene of mESCs grown in the presence of LIF and without LIF for 1, 3, 5d

PDK isoenzymes are expressed differently in LIF-supplemented and LIF-depleted mESCs.

Next, the changes in the expression of the four PDK genes of mESCs during the first 5 days of differentiation were investigated. Quantitative gene expression analysis demonstrated that LIF withdrawal for 5 days, PDK1 mRNA levels decreased gradually ($p<0.05$, on day 3 and 5) (Figure 2a). Contrastly, LIF withdrawal led to an increase in the mRNA level of PDK2 gene on day 3 and 5 of differentiation ($p<0.05$) (Figure 2b). Expression of PDK3 was also increased at day 5 in the differentiated mESCs compared with undifferentiated mESCs (Figure 2c). The early differentiated mESCs showed lower PDK4 expression levels compared with undifferentiated mESCs but this difference was only statistically significant at day 3 ($p<0.01$) (Figure 2d).

LIF withdrawal decreases glucose uptake in mESCs

In addition, the effect of LIF withdrawal on glucose uptake which is the indicator of glucose metabolism was evaluated. Early differentiated ESCs (for 5 days) showed a decrease in terms of glucose uptake ($P < 0.05$ vs. their undifferentiated counterparts) (Figure 3).

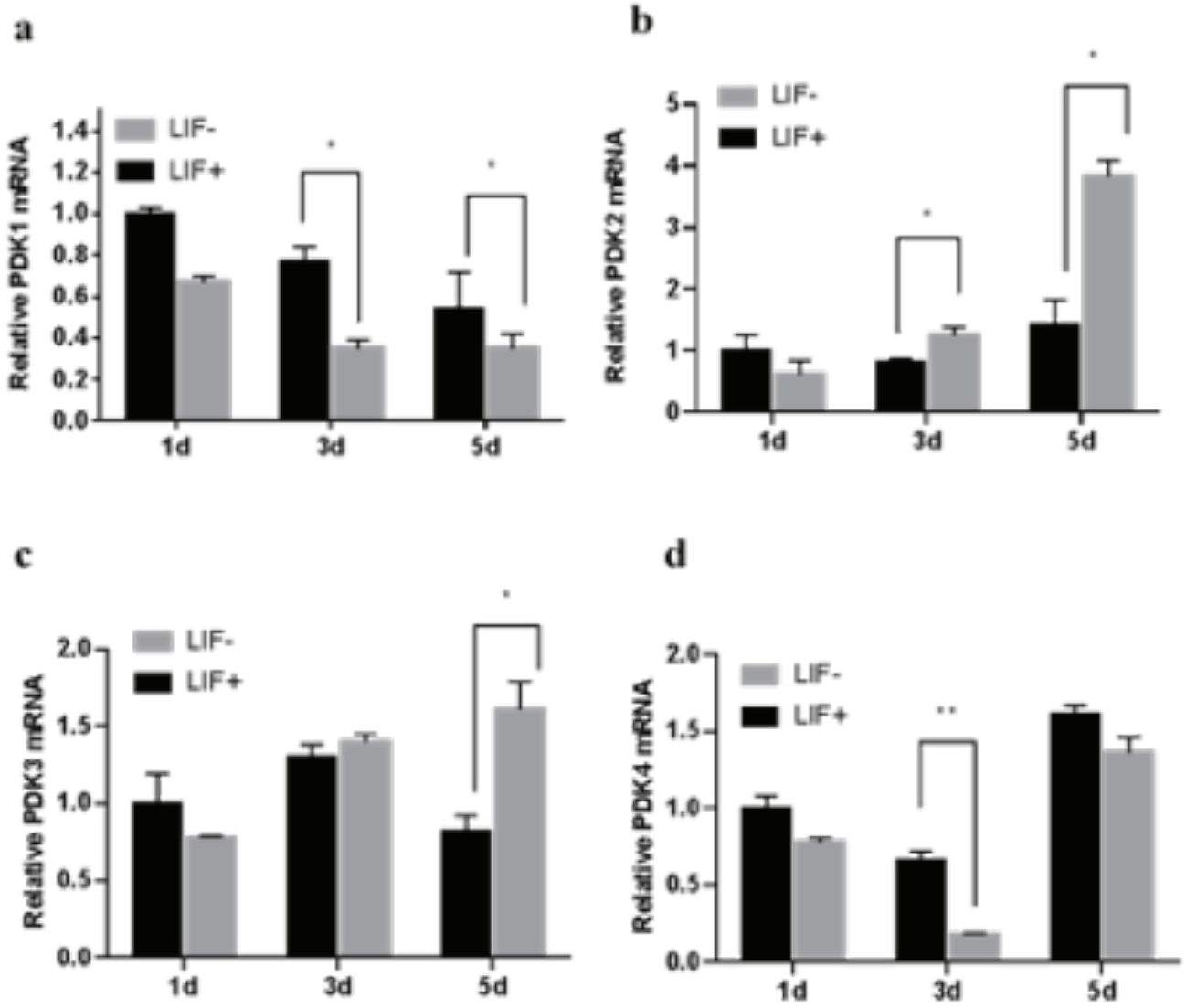

Figure 2 PDK isoenzymes are expressed differently in LIF-supplemented (LIF+) and LIF-depleted (LIF-) mESCs. **(a)** mRNA expression levels of PDK1 gene of mESCs grown in the presence of LIF and without LIF for 1, 3 and 5d. **(b)** mRNA expression levels of PDK2 gene of mESCs grown in the presence of LIF and in LIF-depleted for 1, 3 and 5d. **(c)** mRNA expression levels of PDK3 gene of mESCs grown in the presence of LIF and in LIF-depleted for 1, 3 and 5d. **(d)** mRNA expression levels of PDK4 gene of mESCs cultured in the presence of LIF and without LIF for 1, 3 and 5d.

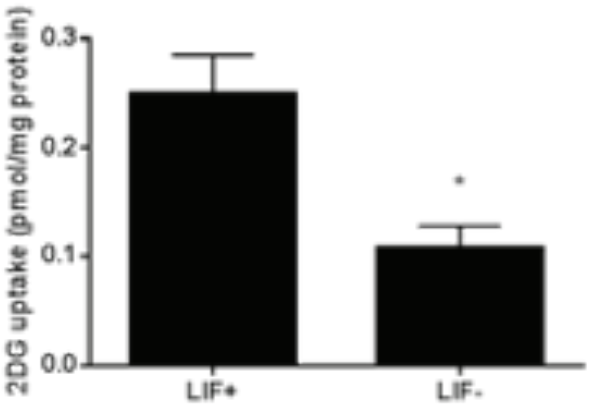

Figure 3 2-DG uptake of mESCs grown in the presence (LIF+) and absence of LIF (LIF-) for 5 days.

Discussion and Conclusion

PDK gene family is involved in regulation of pyruvate dehydrogenase complex (PDC). PDC catalyzes the irreversible conversion of pyruvate into acetyl-CoA, and then participates in the krebs cycle in the mitochondria[20]. Similarly to cancer cells, metabolic activities in embryonic stem cells rely mostly on glycolysis for energy supply[21] in the consistency with our study in which we demonstrated higher glucose uptake in pluripotent mESCs than in early differentiated cells. Furthermore, mitochondria in these cells are rather inactive and generation of acetyl-CoA is suppressed because PDH is phosphorylated[22,23]. The phosphorylation of PDH is catalyzed by a highly specific PDK isoenzymes which leads to a shift from OXPHOS to glycolysis[24]. Tokmakov et al.[25] demonstrated that the expression of PDK isoforms increased several-fold in the embryogenesis following mid-blastula transition in clawed frog, Xenopus laevis. Also they showed that PDK3 increased by about 3-fold during maturation while other PDK family genes are being at their lowest expression levels in the early embryos, grown-up oocytes and matured eggs. The molecular mechanisms underlying the glycolytic energy metabolism of pluripotent stem cells and pathways related to differentiation of them remain unclear. On the basis of these data, we hypothesized that PDK isoenzymes could play a possible role on pluripotency and differentiation of mESCs. Therefore we aimed to comparatively evaluate the expressions of PDK isoenzymes on pluripotent and early differentiated mESCs.

ESCs are derived from the inner cell mass (ICM) of blastocysts of embryo. Retention of pluripotency of these cells relies on expression of transcription factors including KLF4, SOX2, OCT4 and NANOG[26]. SOX2 is an essential factor in the formation of early pluripotent embryonic cells[27]. Consistent with this data, lower level of SOX2 gene expression during early differentiation was determined in this study. Furthermore, the decrease of pluripotency was accompanied with an increase of differentiation markers; Nestin and Brachyury.

In the present study, compared with undifferentiated ESCs, early differentiating ESCs displayed a lower expression of PDK1 gene. This result is in accordance with previous report that PDK1 expression is higher at the protein level in LSK cells, an early form of mouse hematopoietic stem cells (HSCs) compared with differentiated cells[28]. In addition, Halvarsson et al.[29] demonstrated that LT-HSCs (long term HSCs), ST-HSCs (Short term HSCs) and MPPs (multipotent progenitors) expressed higher levels of PDK1 compared to more differentiated cells. Takubo et al.[30] also reported that expression of murine PDK1 was higher in LT-HSCs. Interestingly, another study reported that there was no increase in PDK1 gene expression in pluripotent human embryonic stem cells vs. differentiated cells, although protein levels were higher in pluripotent lines[21].
The current study detected a certain similarity in the expressions of PDK2 and PDK3 isoenzymes; both PDKs were higher expressed in early differentiated mESCs (especially on 5th day) than pluripotent mESCs. Consistent with this result, a recent study showed that there was a positive role of PDK2 in chondrogenic differentiation of mesenchymal stem cells[31]. Contrary, Halvarsson et al.[29] showed that PDK2 and PDK3 expressions were higher in LT-HSCs than in their differentiated progeny. Klimmeck et al.[28] also showed that the protein levels of PDK3 were higher in the LSK cells compared to more differentiated cells. Takubo et al.[30] demonstrated that the expression of PDK2 is important for the quiescence and function of mouse HSCs.

It has previously been shown that the expression of PDK4 is higher in pluripotent LT-HSCs when compared to differentiated cells. Furthermore, it has also been revealed that loss of both PDK4 and PDK2 in mice suppressed glycolysis in HSCs, while overexpression of PDK in glycolysis-defective HSCs reestablished glycolysis and stem cell capacity. Thus, they revealed that PDK4 and PDK2 play a pivotal role in the transition from proliferation to differentiation of the HSCs[30]. Liu et al.[32] showed that PDK4 is the highest expressed isoform of PDKs in placental trophoblast cells and PDK4 expression is dramatically down-regulated during syncytialization. Thus, they proved that PDK4 plays a critical role during syncytiotrophoblast differentiation. Besides, it was demonstrated that PDK4 increases cell stemness properties and stem-associated genes expression in ovarian cancer cells[13]. Consistent with these studies, this

study also determined that PDK4 expression was higher (especially on 3rd day) in pluripotent mESCs than in their early differentiating counterpart. Based on obtained comparative mRNA expression profiling of PDK isoforms in mESCs and early differentiating cells, differently expression profile of both PDK2 and PDK3 from other two isoforms may be attributed to compensatory upregulation of these genes.

Current study comparatively revealed the expression levels of PDK isoforms in pluripotent mESCs (R1 cell lines) and their early differentiated progeny (for 5 day), for the first time. Given that the results indicate expression of PDK isoforms at different levels in pluripotent mESCs and LIF depleted cells, it can be envisaged that PDK isoforms may be important markers for pluripotency and early differentiation of mESCs.

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