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Vitamin C promotes self-renewal of rat embryonic stem cell

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MSc by Research

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2016

Postgraduate Research Thesis Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

1. Abstract

Embryonic stem cell lines (ESCs) can be used for regenerative medicine and drug discovery since they can form any type of cell in the body and self-renew indefinitely. Nowadays, rat animals appear to be more valuable in the field of biomedicine, than mouse model organisms, in terms of their body size, blood system, brain, disease symptoms and others. Thus, rat ESC (rESC) self-renewal and pluripotency mechanisms should be investigated rigorously. Although various cell signalling pathways responsible for mouse ESC (mESC) maintenance have been studied, very little is discovered about rESC. In addition, when ESCs are cultured in the 2i medium, rESCs display higher levels of differentiation genes (e.g. GATA4), compared to mESCs. Esteban et al. (2010) reported that Vitamin C improves formation of induced pluripotent stem (iPS) cell colonies. Here, we utilised Vitamin C activity to determine if this factor can reduce the spontaneous differentiation of rESCs and shift them to a more naïve pluripotent state. Our experimental works consist of three main parts, and various research techniques will be employed such as ES cell culture, siRNA transfection, morphological analysis, qRT-PCR and flow cytometry. Thus, we will identify if Vitamin C can improve rESC self-renewal and restricts differentiation, and subsequently we will determine how Vitamin C regulates self-renewal. Our project provides new insight into the mechanisms by which Vitamin C promotes rESC self-renewal.

Lay Summary

Cells derived from embryos can be used for medicine and drug discovery since they can form any type of cell in the body (called 'pluripotency') and copy themselves indefinitely (called 'self-renewal'). Nowadays, rat animals appear to be more valuable in the field of biomedical science, than mouse model organisms, in terms of their body size, blood system, brain, disease symptoms and others. Thus, the self-renewal and pluripotency mechanisms of rat embryo cells should be investigated rigorously. Although various cell signalling pathways responsible for the maintenance of mouse embryo cells have been studied, very little is known about rat embryo cells. In addition, when incubated in their culture medium, rat embryo cells tend to become different cell types, compared to mouse embryo cells. The previously published paper reported that vitamin C improves cell conversion from skin cells to embryo cells. Here, we utilised vitamin C activity to see if this factor can fully maintain rat embryo cells in a pluripotent state for a longer time. Subsequently we will determine how vitamin C regulates self-renewal. Our project provides new insight into the mechanisms by which vitamin C promotes self-renewal of rat embryo cells.

Contents

2. Literature Review

2.1. Introduction

Embryonic stem cells (ESC) represent a valuable research tool since ESCs can form any type of cell in the body and self-renew indefinitely (Wray, Kalkan et al. 2010, Kalkan and Smith 2014). Due to this unlimited developmental and proliferative potential, they have been used extensively in the production of genetic models (Buehr et al., 2008; Meek et al., 2010), and increasingly used for regenerative medicine and drug discoveries (Lindvall and Kokaia, 2006; Jensen et al., 2009; Lindvall and Kokaia, 2010). The convenient size, physiology, behaviours and genetics of rat have led this animal into the preferred laboratory model in various fields of biomedical research (Jacob and Kwitek, 2002; Aitman et al., 2008; Iannaccone and Jacob, 2009). Since rESCs were successfully derived from the blastocyst-stage embryo using MEK and GSK3 inhibitors designated as 2i (Buehr et al., 2008; Li et al., 2008), rESCs have been employed as a unique alternative to mESC to understand pluripotency and differentiation more rigorously (Meek et al., 2013; Chen et al., 2013).

Efficient transgenesis requires improved stabilisation of rESC pluripotency (Meek et al., 2013). Indeed, the long-term culture of rESC in the 2i medium is less reliable than mESC, showing higher levels of differentiation genes (e.g. GATA4) (Buehr, Meek et al. 2008, Chen, Blair et al. 2013, Meek, Wei et al. 2013). Spontaneous differentiation of rESCs in the 2i cultures compromises their utility. We propose that changing the epigenetic profile of rESC might strengthen self-renewal of undifferentiated cells. At this point, we hypothesise that rESCs exist in a pluripotent state offset from the more stable "ground state" typical of mESC (Figure 2.1). The epigenetic modulator ascorbic acid or Vitamin C improves somatic cell reprogramming to pluripotency (Esteban and Pei, 2012). Thus, we are examining whether Vitamin C can stabilise rESCs in the "ground state".
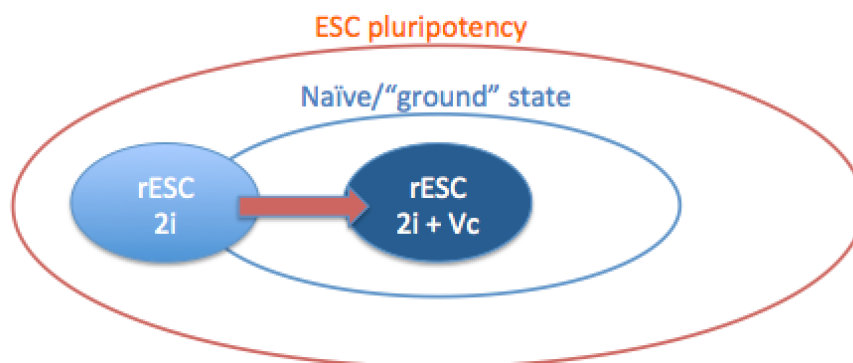
Figure 2.1. Scheme describing the hypothesis that Vitamin C shifts the state of rESCs toward a more naïve state.

2.2. Embryonic stem cells

Embryonic stem cells (ESC) are derived from inner cell mass of blastocyst-stage embryos (Figure 2.2) (Evans and Kaufman 1981). ESC displays two unique properties: self-renewal and pluripotency. Self-renewal involves generating at least one daughter cell which is identical to the mother cell after cell division, retaining the same differentiation capacity, and pluripotency describes the ability of ESC to form any type of cell in organisms (Wray, Kalkan et al. 2010, Kalkan and Smith 2014). Various intrinsic and extrinsic factors depending on the organism regulate ESC self-renewal and pluripotency (Smith, 2001; Lanza and Atala, 2014).

In 1981, mESCs were first isolated from mouse embryos and grown in culture (Evans and Kaufman, 1981; Martin, 1981). Following this discovery, mESC has been extensively investigated in biomedical research. Consequently, there are many culture techniques and applications of ESC with a remarkable potential (Solter, 2006). Theoretically, ESC research can cure most debilitating diseases, for instance, Parkinson's or Alzheimer's (Lindvall and Kokaia, 2006; Lindvall and Kokaia, 2010) and cancer (Dreesen and Brivanlou, 2007; Lanza and Atala, 2014). In addition, ESC can be employed to determine signalling pathways responsible for self-renewal and pluripotency, and to find out novel drugs (Jensen et al., 2009).
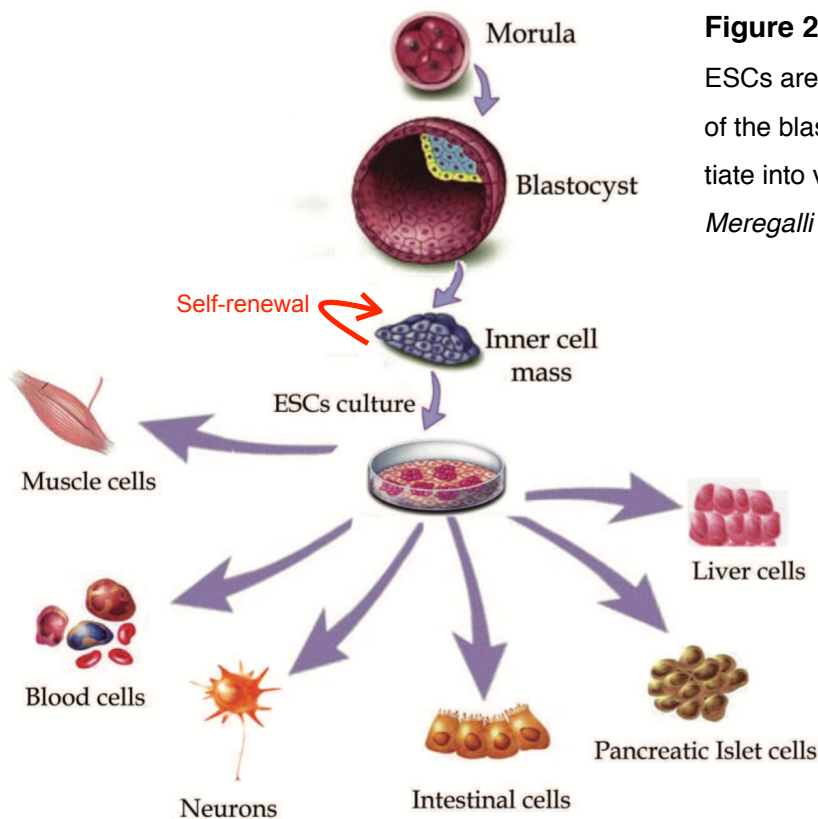
Figure 2.2. ESC derivation and potential. ESCs are established from the inner cell mass of the blastocyst. ESCs self-renew and differentiate into various cell types (*Figure taken from Meregalli et al. (2011)*).

2.2.1. Extracellular signals responsible for self-renewal of ESC

A. Leukemia inhibitory factor (LIF)

An exogenous cytokine, LIF is necessary to maintain the mESC pluripotency. LIF belongs to the interleukine-6 (IL-6) family (Gearing et al., 1987; Hirano et al., 1997; Hirai et al., 2011; Eulenfeld et al., 2012; Tang and Tian, 2013). The IL-6 family receptors make a complex with the receptor glycoprotein 130 (Gp130). The LIF receptor is composed of LIF receptor β and a Gp130 het-

erodimer (Niwa et al. 2009; Lanner and Rossant, 2010). Upon the LIF addition, three intracellular pathways are primarily activated: Grb2-mitogen-activated protein kinase (MAPK), Jak-Stat3 and PI3K (phosphatidylinositol-3-OH kinase)-Akt intracellular pathways (Figure 2.3) (Niwa et al., 2009). MAPK signalling pathway facilitates mESC differentiation, whereas Jak-Stat3 and PI3K-Akt signalling pathways promotes mESC self-renewal (Ohtsuka et al., 2015).

In terms of Jak-Stat3 pathway, artificial Stat3 activation can substitute the LIF requirement for self-renewal of mESC, highlighting that Stat3 acts as an important mediator of the LIF signal (Matsuda et al., 1999). Stat3 can maintain mESC self-renewal as demonstrated by Stat3 overexpression in serum culture conditions (Niwa et al., 1998; Matsuda et al., 1999; Raz et al., 1999). In addition, artificial Akt activation can also replace the LIF requirement. These signalling pathways deliver the LIF action into the nuclei in parallel, which upregulates the transcription factors involved in maintaining pluripotency (Niwa et al. 2009; Lanner and Rossant, 2010). However, LIF also activates the MAPK pathway, which is a negative controller of pluripotency. Some groups reported that LIF alone cannot support mESC pluripotency and initiates neural differentiation in culture conditions devoid of serum (Wilson and Edlund, 2001; Ying and Smith, 2003; Ying et al., 2003b). In the three pathways, the Grb2-MAPK and PI3K-akt pathways are controlled by not only LIF but also other extracellular factors e.g. Fibroblast growth factor (Fgf) 4 and insulin/insulin-like growth factor (Niwa et al. 2009; Lanner and Rossant, 2010).

Figure 2.3. Diagram of LIF signal.

LIF activates three intracellular pathways: Grb2-MAPK, Jak-Stat3 and PI3K-Akt intracellular pathways. Jak-Stat3 signalling upregulates Klf4, while PI3K-Akt signalling facilitates Tbx3 transcription. In addition, MAPK signalling inhibits nuclear localisation of Tbx3. The two stimulated factors, Klf4 and Tbx3 primarily upre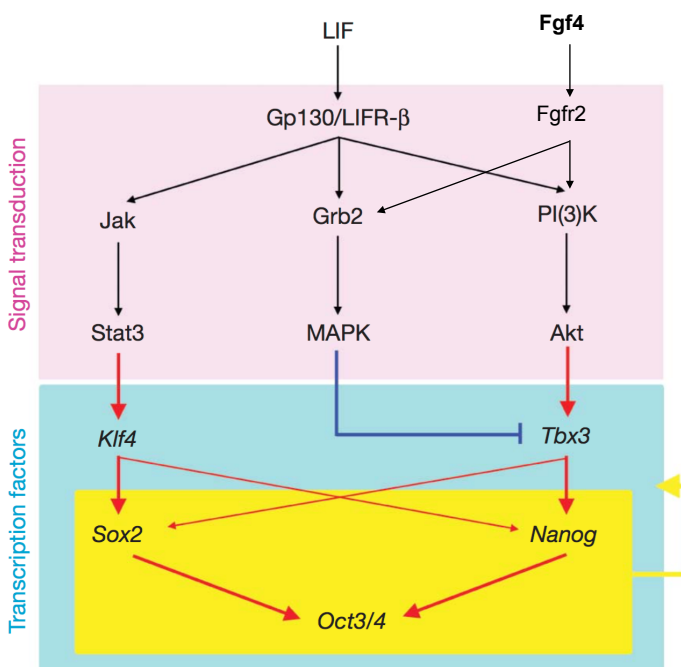gulate pluripotency factors, Sox2 and Nanog, respectively. Subsequently, this signal promotes Oct3/4 expression. Furthermore, Oct3/4, Sox2 and Nanog stimulate transcription of all these transcription factors in order to give robustness and stable activation when all signals are absent. In addition, the Fgf4 pathway is coupling to LIF signal (Niwa et al., 2009; Lanner and Rossant, 2010) (*Diagram taken from Niwa et al. (2009) with the addition of Fgf4 signalling described from Lanner and Rossant, (2010)).*

B. Bone morphogenetic protein 4 (BMP4)

A BMP4 is part of the transforming growth factor beta (TGFβ) superfamily. BMP4 is responsible for mesoderm and bone formation in mammals (Xu et al., 2002; Ying et al., 2003a). In 2002, BMP4 was initially reported to induce human ESC differentiation to trophoblast (Xu et al., 2002). However, Ying et al. (2003a) demonstrated that BMP4 supports the pluripotency of mESC by coordinating with LIF in culture conditions devoid of serum. BMPs function via hetero-dimers of type I and type II serine/threonine kinase receptors (Shi and Massague, 2003). BMP4 and BMP-relative growth and differentiation factor-6 (GDF-6) transcripts are prominent in undifferentiated ESCs. The downstream targets of BMP receptors are Smad transcription factors (von Bubnoff and Cho, 2001; Attisano and Wrana, 2002; Ying et al., 2003a). Active BMP receptor complex recruits R-Smads 1, 5, and 8 and phosphorylates them. These R-Smads subsequently combine with Smad 4 and enter the nucleus to activate inhibitors of differentiation (Id) family (Ying et al., 2003a). Id genes are known as principal targets of BMP/Smad signalling in undifferentiated ESCs. Ids are considered as negative helix-loop-helix (HLH) factors that restrict E proteins (Class I HLH proteins) for partnering with proneural bHLH transcription factors e.g. myoD, mash1, Pax and Ets, and consequently prevent ESC differentiation (Jen et al., 1992; Lyden et al., 1999; Norton, 2000, Ying et al., 2003a) (Figure 2.4).
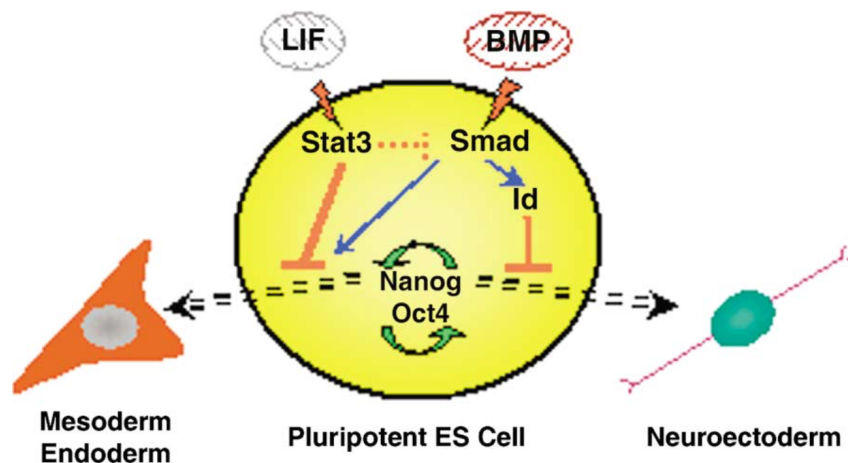
Figure 2.4. BMP/id and LIF/STAT3 signalling cooperatively restrict lineage commitment.
ESCs should suppress lineage commitment for the maintenance of self-renewal. BMP signals induce Id genes to prevent ESC from entering neural lineage differentiation, which is otherwise marginally blocked by LIF/STAT3 signalling. Concurrently, STAT3 inhibits the ability of BMP to initiate mesodermal and endodermal lineage commitment. Hence, BMP signal can be turned into the signal that induces lineage differentiation in the absence of LIF (Ying et al., 2003a) (*Image adapted from Ying et al. (2003a)*).

C. Wnt

Wnt is an autocrine stimulus for stabilising naïve pluripotency. Wnt signalling pathway is involved in maintenance of self-renewal and pluripotency acting through β catenin (Staal and Sen, 2008; Meek et al., 2013). β catenin is responsible for gene transcription as well as cell-cell adhe-

sion (Ben-Ze'ev, 1999). Huelsken et al. (2001) reported that β catenin regulates hair follicle morphogenesis and differentiation of stem cell in the skin.
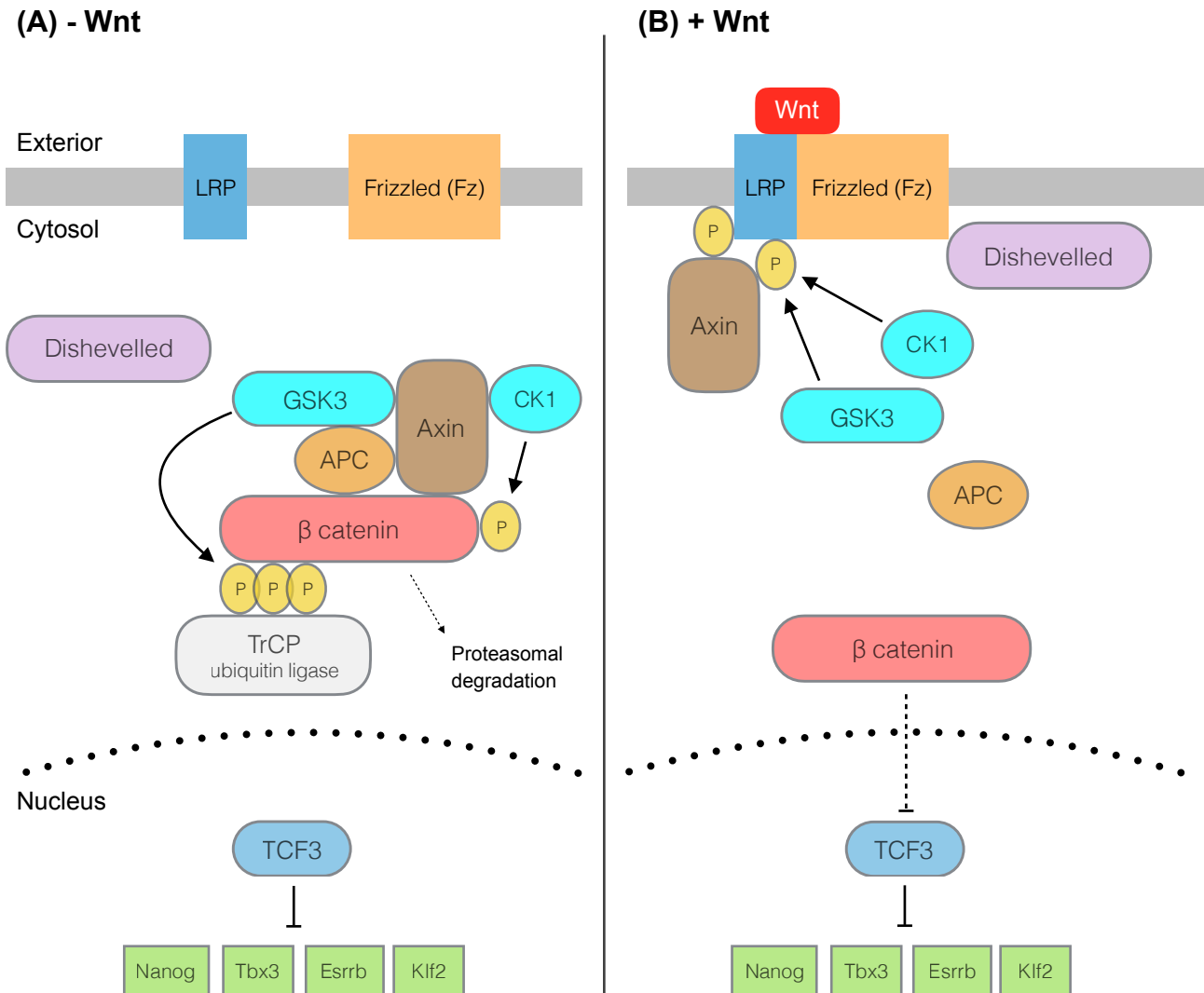
Figure 2.5. Wnt signalling pathway. The (A) diagram displays signalling pathway when Wnt is absent. β catenin is phosphorylated by GSK3 and CK1, and subsequently recognised by TrCP, which leads to proteasomal degradation. In contrast, the (B) figure exhibits signalling pathway when Wnt is present. Freed β catenin hampers the inhibitory effects of TCF3 on pluripotency markers (e.g. Nanog, Klf2, Tbx3 and Esrrb) to maintain pluripotency in ESCs (Lodish et al., 2012; Kalkan and Smith, 2014) (*Created using information from various sources such as Lodish et al., 2012; Kalkan and Smith, 2014*).

When a Wnt signal is absent (Figure 2.5), β catenin is bound to a complex of Axin, adenomatous polyposis coli (APC) protein, GSK3 and casein kinase 1 (CK1) (van Amerongen and Nusse, 2009). Sequentially, GSK3 and CK1 phosphorylate β catenin on many serine and threonine sites (Staal and Sen, 2008; Lodish et al., 2012). Now a ubiquitin-ligase protein (TrCP) can bind to these phosphorylated residues. Subsequently, β catenin is ubiquitinated and destroyed by the 26S proteasome (Lodish et al., 2012). Hence, a freed TCF3 transcriptional repressor (target of β

catenin) associates with the Nanog gene promoter, hindering its gene transcription (Pereira et al., 2006).

In the presence of the Wnt signal, Wnt binds to Frizzled and LRP co-receptor, and phosphorylates the LRP cytosolic tail with GSK3 or CK1 (van Amerongen and Nusse, 2009; Lodish et al., 2012). This tail binds to Axin, and Dishevelled disrupts the complex of Axin, APC, GSK3, CK1 and β catenin. This prevents β catenin from phosphorylation by GSK3 and CK1 and ubiquitination by TrCP ubiquitin ligase (Lodish et al., 2012). Subsequently, freed β catenin enters the nucleus and restricts the inhibitory effects of Tcf3 on pluripotency genes such as Nanog, Klf2, Tbx3 and Esrrb to stabilise naïve pluripotency (Pereira et al., 2006; Cole et al., 2008; Zhang et al., 2008; Sokol, 2011; Wray et al., 2011; Yi et al., 2011; Kalkan and Smith, 2014). Simultaneously, nuclear-localised β catenin associates with Tcf1 to upregulate its transcriptional activity (Yi, Pereira et al. 2011, Chen, Blair et al. 2013, Meek, Wei et al. 2013).

D. Fibroblast growth factor 4 (Fgf4)

A Fgf4 is an autocrine molecule for neural differentiation in ESCs (Lowell et al., 2006; Kunath et al., 2007; Revest et al., 2010). Kunath et al. (2007) demonstrated that first, Erk1/2 action in ESCs depends on FGF signal. Second, Fgf4-null and wild-type ESCs need FGFR signals for multi-lineage induction. Last, Fgf4-null and Erk2-null ESCs are deficient in neural commitment. Most of the Fgf4-null ESCs remain viable retaining Oct4 expression in the absence of LIF (Kunath et al., 2007). Fgf4 is expressed between the 8-cell and 16-cell morula in early embryos, but becomes restricted to the epiblast of blastocyst-stage embryos, and are under the regulation of Oct4 and Sox2 (Yuan et al., 1995). Fibroblast growth factor receptor 2 (Fgfr2) is predominantly expressed in the trophectoderm and primitive endoderm in the late blastocyst (Lanner and Rossant, 2010). Elimination of Fgf4 signals support self-renewal and reduces spontaneous differentiation in the serum media containing LIF (Lanner and Rossant, 2010). Fgf4-Fgfr2 acts through the Grb2-MAPK pathway to restrict pluripotency (Revest et al., 2010). MAKP inhibits nuclear localisation of Tbx3 (Niwa et al., 2009) (Figure 2.3). Conversely, the pluripotency genes (e.g. Oct3/4 and Sox2) can regulate Fgf4 expression (Lanner and Rossant, 2010).

2.2.2. Transcriptional regulation
2.2.2.1 ESC markers
A. Oct3/4

Oct4 is vital in inducing and maintaining self-renewal in embryo development (Niwa, Miyazaki et al. 2000, Niwa, Toyooka et al. 2005). For example, Oct4-null embryos cannot survive after implantation. Furthermore, since Oct4 transcriptionally represses trophectoderm-inducing genes (e.g. Cdx2), ESCs lacking Oct4 differentiate toward trophectoderm (Niwa, Toyooka et al. 2005). Interestingly, Oct4 overexpression drives ESCs into a mixture of differentiated cells, other than trophectoderm. This is because Oct4 retains its repressor function that prevents trophectoderm (Niwa et al. 2000; Niwa et al. 2005).
B. Sox2

Sox2 produces a heterodimer with Oct4, and controls various ESC genes using a positive feedback mechanism (Lanza and Atala, 2014). Sox2 regulates the expression of Oct-Sox enhancers as well as nuclear receptor family transcription factors which positively or negatively control Oct4 expression (Masui et al., 2007). In addition, Niwa et al. (2009) demonstrated that the LIF-Jak-Stat3 pathway upregulates Klf4, which subsequently activates Sox2 for Oct4 regulation (Figure 2.6).
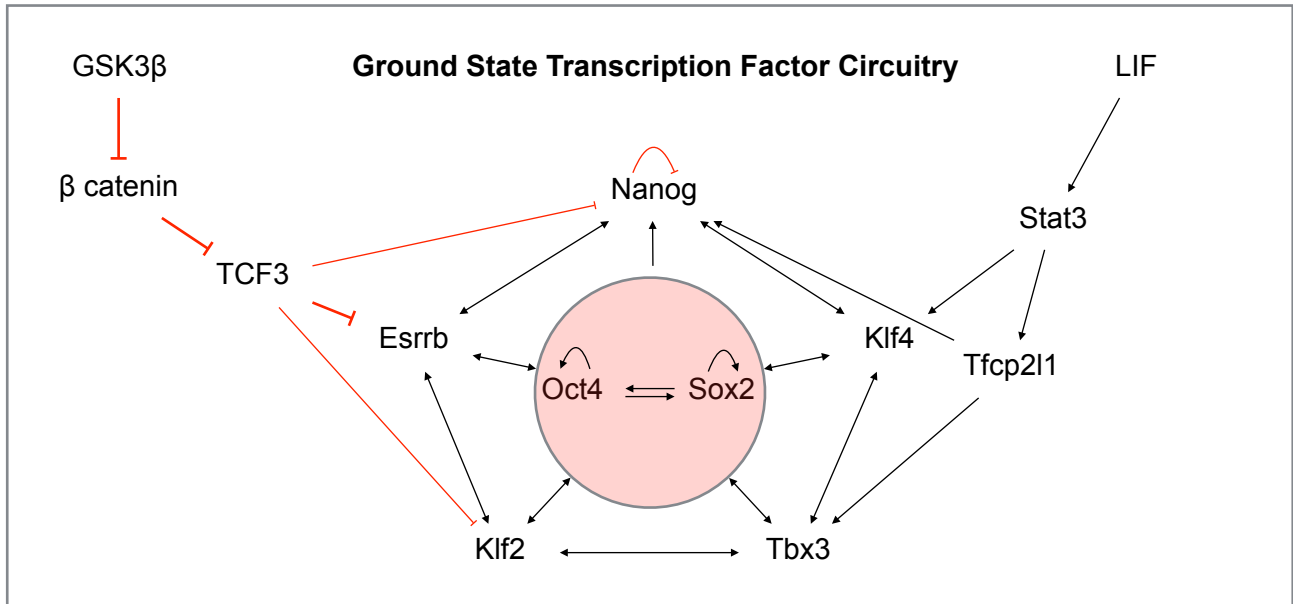
Figure 2.6. Pluripotency transcription factor network with LIF/Stat3 and GSK3/Tcf3 signalling. Pluripotency-associated transcription factors are considered to upregulate transcription each other. Arrows show how each factor controls another (Martello et al. 2012; Navarro et al. 2012; Martello et al., 2013; Takashima et al., 2014) (*Created using information form various sources such as Martello et al. 2012; Navarro et al. 2012; Martello et al., 2013; Takashima et al., 2014*).

C. Nanog

Nanog mRNA appears to be abundant in ESCs, embryonic germ cells, and embryonal carcinoma cells, but not in fibroblast, parietal endoderm and hematopoietic cells, indicating that Nanog is only expressed in pluripotent cells (Chambers et al., 2003). mESCs fluctuate between high Nanog expression state that shows high efficiency of self-renewal and low Nanog expression state that exhibits increased differentiation tendency, whereas expression of Oct4 and Sox2 appears to be uniform (Chambers et al., 2007; Navarro et al., 2012). Transient Nanog downregulation makes ESCs susceptible to differentiation but does not commit. Nanog null ESCs can self-renew indefinitely, even though these cells are prone to differentiate (Chambers et al., 2007).

In terms of Nanog regulation (Figure 2.6), Navarro et al. (2012) demonstrated that activity of Nanog in mESCs is auto-repressive and Oct4/Sox2-independent. Furthermore, Nanog effects on expression of Oct4 and Sox2 is marginal. They also argued that auto-repression would be a principal regulator of Nanog transcription switching, highlighting cellular variability in self-renewal

efficiency of the mESC pluripotency network. Nanog interacts with many transcription factors such as Oct4, Esrrb, Sall4, Nr0b1 and Tcfcp2l1 as well as Sox2-interacting factors, Chd7 and the Ncor1 complex (Gagliardi et al., 2013). Among them, Esrrb acts as a direct target of Nanog as its expression performs the function of Nanog in ESCs (Festuccia et al., 2012). In addition, elevated Nanog in mESCs makes Gp13/LIFR signalling dispensable. Nanog overexpression allows self-renewal even after LIF withdrawal, whereas forced maintenance of Oct4 or Sox2 cannot substitute for the LIF requirement in self-renewal (Chambers et al., 2003; Lanza and Atala, 2014). Moreover, Nanog overexpression with LIF signalling produces maximal self-renewal. This combination condition eliminates peripheral differentiation and renders ESC colonies highly compacted (Chambers et al., 2003).

2.2.2.2 Early differentiation markers
A. GATA4

GATA4 is a zinc-finger-possessing transcription factor which recognises the consensus sequence WGATAR (known as the GATA motif) in the promoters of many target genes (Miyamoto et al., 2008). GATA4 is a primitive endoderm marker (Morrisey, Tang et al. 1998, Fujikura, Yamato et al. 2002, Holtzinger, Rosenfeld et al. 2010). GATA4 protein regulates genes responsible for embryogenesis and myocardial differentiation (Holtzinger, Rosenfeld et al. 2010), and is essential for testicular development (NCBI, 2016). In mice, GATA4 is highly upregulated in the somatic cells of the developing gonad around the time of sex determination (Viger et al., 1998). GATA4 mutations may produce cardiac septal defects. Furthermore, aberrant gene expression may generate multiple types of tumours (NCBI, 2016).

B. Brachyury

Brachyury is a key transcription factor that develops mesoderm during gastrulation, and directly upregulated by β catenin. In naïve mESC, Brachyury expression levels are very low, but their levels are elevated in primed-type EpiSCs, representing a differentiation marker of early-stage mESC (Bernemann et al., 2011; Kelly et al., 2011).

C. Cdx2

Cdx2 was regarded as a trophoblast marker (Niwa, Toyooka et al. 2005), however Bernardo et al. (2011) cited that BMP causes pluripotent stem cells mainly to form mesoderm through Brachyury and Cdx2 transcription factors, highlighting association of Cdx2 with mesoderm formation (Bernardo et al., 2011).

2.3. Rat as a model organism

2.3.1. Rat is a better animal model than mouse?

Rats provide various advantages over mice as a human disease model. Rat ESC derivation will significantly expand their utility in medical research (Buehr et al., 2008). Rat is considered as an excellent model in various aspects for many diseases including cardiovascular disease (e.g. hypertension and stroke). Rat is physiologically and pharmacologically closer to human (Jacob and Kwitek, 2002; Aitman et al., 2008; Iannaccone and Jacob, 2009).

The animal size increases its use as a model of disease. The body size of rat is bigger, thus all the organs and cancers are bigger. This means that laboratory researchers can easily monitor their physiology for their studies including the central nervous system. In the studies of breast cancer, rat exhibits similar hormone response and premalignant stages which resemble the human disease (Iannaccone and Jacob, 2009). The vascular system and brain of rat are more relevant to those of humans than mouse. A rat heart beats approximately four times faster than a human heart, whereas a mouse heart beats almost six times faster. Rats with divergent blood pressures are used as a model organism for genetic dissection of the inherited hypertension causes (Rapp, 2000). In addition, a larger brain of rat is more complex than a mouse brain. Hence, neurological diseases such as Parkinson's and Alzheimer's can be investigated more rigorously using rat models. Furthermore, rats are superior to other animal models in cognition and memory research, since they are more intelligent, and can learn a lot of different tasks compared to mice. Also, rats are better to monitor for the research of diabetes, since they behave more similar to the human disease, including the response to environmental factors such as stress, diet and toxins. Likewise, rats appear to metabolise medicine in an analogous manner our body breaks and absorbs a substance, because of similar liver enzymes mice do not possess (Jacob and Kwitek, 2002; Li et al, 2008; Aitman et al, 2008; Iannaccone and Jacob, 2009; Phillips et al., 2013).

Although rats have various advantages over mice, they also have some disadvantages. First, rats are not easier to keep them in a laboratory and cost more than mice. The larger body size of rats requires more food and space. Second, scientists who studied a mouse model for a long time tend to keep working with the same model, since mice still offer other advantages (Li et al, 2008; Iannaccone and Jacob, 2009; Phillips et al., 2013).

2.3.2. Mouse ESC vs Rat ESC

Rats and mice look very similar, but they evolutionally separated over 12 million years ago (Gibbs et al., 2004). Thus, a rat should not be considered as just a big version of a mouse. When a certain signalling pattern is observed in mice, it does not indicates that it must be there in rats. If some patterns are observed in both rats and mice, then the chances would be much higher that it generalises to other animals, including human (Phillips et al., 2013; Till et al., 2015). Due to the large evolutionary distance, rESC maintenance is different from mESC and more challenging (Buehr et al., 2008). Since we have less information for their maintenance, we should further study rESC to extensively utilise rats as a disease model.

Although both mESC and rESC express the identical pluripotency transcription factors (e.g. Nanog, Oct4, Sox2 and Klf), there are several differences between them. First, in terms of mice,

LIF alone sustains mESC self-renewal, primarily acting through the Stat3-Tfcp2l1/Klf4 pathway (Martello et al., 2013). The addition of both LIF and BMP4 maintains mESC self-renewal in serum-free N2B27 media, although its physiological action are restricted during development (Ying et al., 2003a). Second, regarding rats, two inhibitor system, 2i (inhibitors of MEK and GSK3) or three inhibitor system, 3i (inhibitors of MEK, GSK3 and FGF receptor) can maintain rESCs in the presence of LIF. But, LIF alone does not retain rESC self-renewal (Buehr et al., 2008). Interestingly, serum induces rESC differentiation, and BMP4 has no positive influences (Li et al., 2008). Additionally, compared with the mouse cells, rat ESCs do not grow very well on feeder-free plates. One of the significant differences found between rats and mice is β catenin activity levels (Meek et al., 2013). β catenin regulates rESC self-renewal in a concentration-dependent way, which have not observed in mESC. rESC contains higher β catenin activity levels compared to mESC, since high β catenin levels caused by the high GSK3 inhibitor concentration (e.g. over 6μM CHIR) can induce differentiation in rESC but maintain self-renewal in mESC. This highlights that GSK3β inhibition is not necessarily required in rESC. Therefore, rESC self-renewal can be promoted by MEK inhibitor (1i) alone (Meek et al., 2013). Although both rat and mouse ESCs show similar signalling systems and gene expressions, they exhibit some dissimilarity regarding ESC maintenance (Figure 2.7), due to the big evolutionary distance (Gibbs et al., 2004). To efficiently derive true naïve rESCs for their broader applications, we should further investigate rESCs.

Rat ES cells	**Mouse ES cells**
• LIF + 1i, 2i or 3i systems can support self-renewal • LIF alone cannot maintain self-renewal • BMP4 has no influence • Most ESCs do not survive on feeder-free plates • Relatively high β catenin activity	• Only 2i or 3i systems can support self-renewal • LIF alone can maintain self-renewal • BMP4 with LIF promotes self-renewal in serum-free media • Most ESCs grow well on feeder-free plates • Relatively low β catenin activity

Figure 2.7. Summary of the different features of ESC maintenance between rat ESCs and mouse ESCs. Left red box exhibits the characteristics of rat ESC maintenance. Right blue box displays the characteristics of mouse ESC maintenance (Buehr et al., 2008; Li et al., 2008; Ying et al., 2003a; Martello et al., 2013; Meek et al., 2013).

2.4. Vitamin C

Vitamin C (L-ascorbic acid) is known as an essential nutrient for primates including humans. Although most mammals produce ascorbate via a metabolic pathway from glucose, some animals (e.g. primates, guinea pigs and bats) have lost the last enzyme (Vitamin C-regulated gulonolactone oxidase) in the pathway, because of mutation accumulation (Drouin et al., 2011; Monfort and Wutz, 2013). Thus, we have to take Vitamin C-containing food such as fruits and vegetables.

(A)

(B)
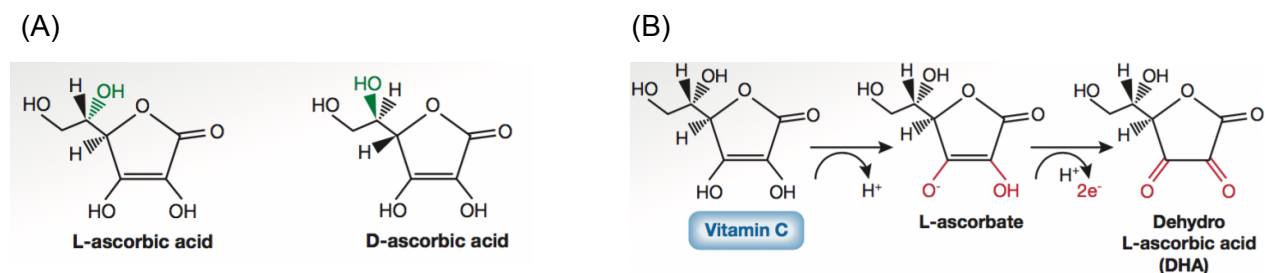
Figure 2.8. Vitamin C structure and function. (A) L-ascorbic acid indicates Vitamin C, while its optical isomer, D-ascorbic acid does not give the equivalent biological effect. But antioxidant effects are same in the L- and D-forms. (B) When Vitamin C is oxidised to DHA, it produces two electrons which act as a reducing factor in redox reactions (Monfort and Wutz, 2013) (*Image adapted from Monfort and Wutz (2013)*).

Vitamin C deficiency results in loss of collagen hydroxylase activity, which causes a structural breakdown of the epithelia and skin, called scurvy (Peterkofsky, 1991; Baron, 2009). In addition, Vitamin C controls hypoxia response by regulating HIF prolyl-hydroxylases. Vitamin C is an electron donor for changing the redox state of iron-possesing enzymes. That is, it reduces iron atom where it is oxidised to dehydro L-ascorbic acid (Figure 2.8). However, other antioxidants are also effective for this reaction, and can substitute for Vitamin C. In mice lacking Vitamin C, glutathione can replace with Vitamin C in retaining the prolyl-hydroxylase activity in the pathway of hypoxia response (Nytko et al., 2011).

In the presence of iron in the Fe(IV) state, Vitamin C produces two electrons to switch the iron to the Fe(II) state, which activates α-ketoglutarate (2OG)-dependent dioxygenases (Figure 2.9). These dioxygenases oxidatively decompose 2OG to succinate and CO_2, thereby producing an Fe(IV)-oxo intermediate. Subsequently, the activated oxygen species hydroxylate a substrate (e.g. DNA or histone), which restores the Fe(II) and removes methylation. When substrate is absent, the iron remains in a higher oxidation state that blocks further catalysis (Monfort and Wutz, 2013). Taken together, Vitamin C sustains the catalytic function of Fe(II) 2OG-dependent dioxygenases.
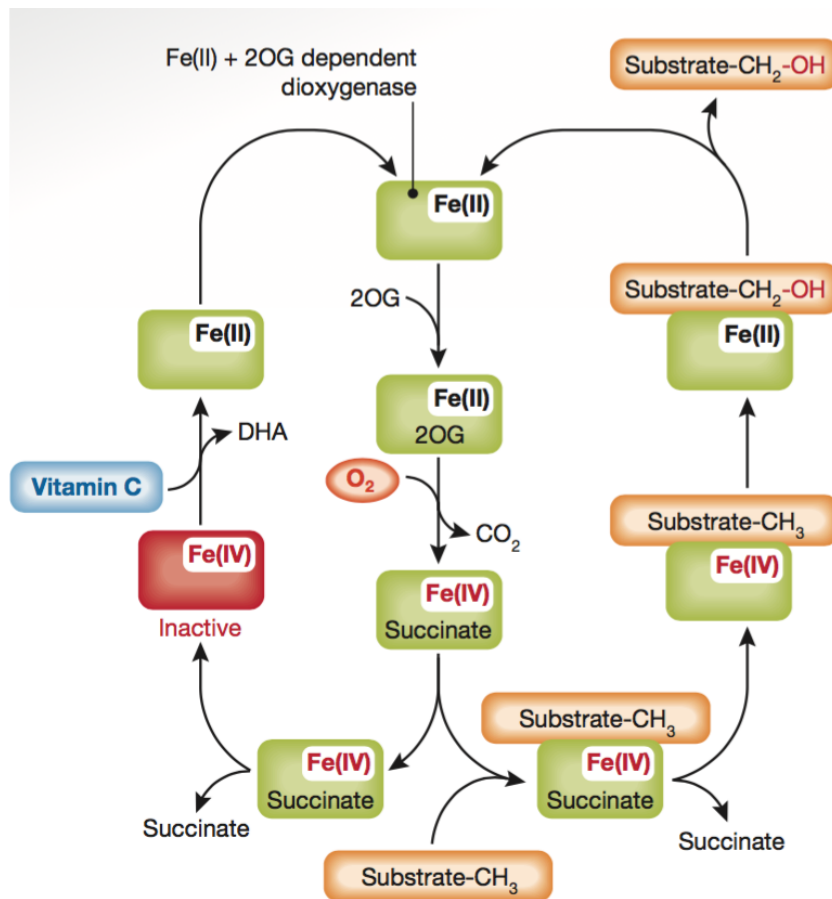
Figure 2.9. Dioxygenase catalysis. Dioxygenase catalysis acts through the substrate hydroxylation cycle or the Vitamin C-dependent regeneration cycle. Vitamin C reduces iron to Fe(II) in dioxygenases (e.g. DNA- and histone-modifying enzymes). Oxygen decarboxylates 2OG in the dioxygenases, forming an activated Fe(IV) enzyme. When substrate is present, the activated enzyme combines with substrate and releases succinate. Subsequently the dioxygenase hydroxylates the methyl part of the substrate, and Fe(IV) is concurrently reduced to Fe(II). When substrate is absent, the activated enzyme releases succinate without Fe(IV) reduction to become inactive (Monfort and Wutz, 2013) (*Image taken from Monfort and Wutz, (2013)*).

2.5. Vitamin C-mediated dioxygenases regulate epigenetics.

Fe(II) 2OG-dependent dioxygenases require Vitamin C to retain their catalytic activity, because they are very susceptible to environmental factors such as onco-metabolites (Dang et al., 2009; Ward et al., 2010; Yang et al., 2012). These dioxygenases show hydroxylase activity acting through their JmjC domain. This domain frequently associates with other domains which give selective binding, on the basis of the histone or DNA methylation status (Upadhyay et al., 2011). The catalytic JmjC domain of dioxygenases is arranged in a double-stranded β-helix fold, which is composed of eight β-strands and possesses one catalytic iron-coordinating aspartate (or glutamate) and a triad of two histidines (Clifton et al., 2006). JmjC-domain-containing histone demethylases oxidatively removes methylation of lysine residues of histones using iron Fe(II) and 2OG (Tsukada et al., 2006; Yamane et al., 2006; He et al., 2008; Horton et al., 2010; Hsia et al., 2010;

Kim et al., 2010; Qi et al., 2010; Sinha et al., 2010; Wen et al., 2010; Yu et al., 2010; Kim et al., 2012). While the flavin cofactor-dependent histone demethylases only eliminate mono- and di-methyl-lysine modifications, several JmjC-domain-containing histone demethylases can also remove methylation of tri-methylated histone lysines (Cloos et al., 2006; Fodor et al., 2006; Klose et al., 2006; Whetstine et al., 2006; Agger et al., 2007; Hong et al., 2007; Iwase et al., 2007; Klose et al., 2007; Seward et al., 2007). The cofactor-bound JmjC domain forms a very reactive oxoferryl intermediate which hydroxylates the lysine-methyl group in histone. The methyl group removal from the histone tail is completed by formaldehyde elimination (Figure 2.10). In addition to lysine methylation, there is also arginine methylation. For example, histone tails may be mono- and di-methylated on arginines. Likewise, this arginine methylation affects chromatin remodelling, thereby regulating gene expression involved in cell fate transitions (Torres-Padilla et al., 2007).
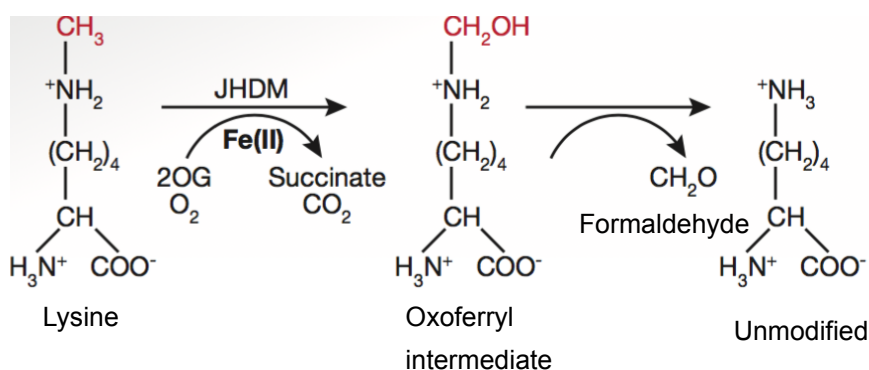
Figure 2.10. The methyl group removal from lysines of histones by histone demethylases.
JmjC-domain containing histone demethylases remove methyl parts from lysines of histones. After the formation of the unstable oxoferryl intermediate (i.e. the Fe(II) enzyme containing the substrate-CH_2-OH), this oxidised methyl parts are spontaneously released as formaldehyde (CH_2O) to regenerate unmodified bases, completing the demethylation reaction (Monfort and Wutz, 2013) (*Image taken from Monfort and Wutz, 2013*).

2.5.1. Vitamin C downstream targets

2.5.1.1. DNA hydroxylases - TET methylcytosine dioxygenases

The ten-eleven-translocation genes 1-3 (TET1-3) perform 5mC oxidation in DNA. TET enzymes oxidise 5mC (5-methyl-cytosine) to produce 5hmC (5-hydroxy-methyl-cytosine). This leads to the formation of 5fC (5-formyl-cytosine) and 5caC (5-carboxyl-cytosine). Subsequently, thymine DNA glycosylase (TDG) recognises and excises 5caC (Tahiliani et al., 2009; Ito et al., 2010; He et al., 2011; Ito et al., 2011; Wu and Zhang et al., 2011; Huang and Rao, 2014) (Figure 2.11). TET enzymes contain a DSBH fold in their catalytic domain that associates with Fe(II) and 2OG. Furthermore, intriguingly, only TET1 and TET3 possess a zinc finger domain which may stimulate interaction with DNA. In ESCs, transcription start segments of gene promoters and exons exhibit abundant 5hmC. Thus, TET proteins are considered to regulate target genes of Polycomb group pro-

teins (Pastor et al., 2011; Xu et al., 2011). In addition, mESCs show Tet1 and Tet2 expressions within which Tet3 expressions are scarcely found. In contrast, in fibroblasts, there are little expression of Tet1, intermediate expression of Tet2 and high expression of Tet3 (Monfort and Wutz, 2013). Pluripotency transcription factors regulate Tet gene expression. For example, Oct4 knockdown downregulates Tet1 and upregulates Tet3, reducing the 5hmC levels (Koh et al., 2011). This indicates that Tet1 may play an important role in mESCs.
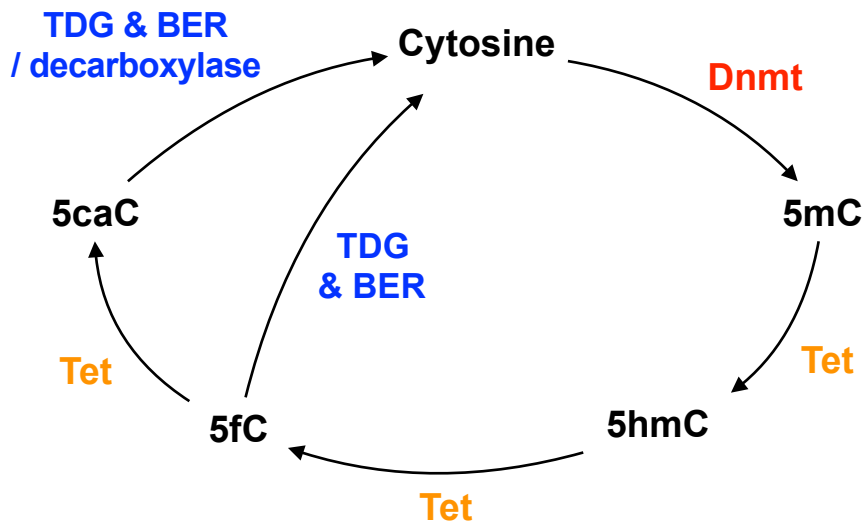
Figure 2.11. Tet-mediated DNA demethylation. DNA methyltransferases (Dnmt) establish 5mC. Tet dioxygenases hydroxylate 5mC to form sequentially 5hmC, 5fC and 5caC. DNA glycosylase (e.g. TDG)-mediated base excision repair (BER) excises 5fC and 5caC from DNA. In addition, a putative decarboxylase might directly change 5caC to cytosine (Wu and Zhang et al., 2011; Huang and Rao, 2014) *(Created using information from different sources).*

Some cancers can be developed by mutations in TET1 and TET2 with a concomitant reduction in the 5hmC levels. For instance, TET1 functions as an MLL partner in myeloid and lymphoid leukaemia. Tet2 mutations are implicated in myeloid leukaemias (Delhommeau et al., 2009). During mouse germline development, Tet1 affects DNA demethylation involved in genome-wide reprogramming (Hackett et al., 2012). Tet3 regulates 5mC hydroxylation on the sperm chromosomes in zygotes (Gu et al., 2011), while Stella binding protects the maternal chromosomes of the oocyte from Tet3 activity (Nakamura et al., 2012). This generates a differential modification of paternal chromosomes with 5hmC and maternal chromosomes with 5mC in preimplantation embryos (Monfort and Wutz, 2013).

2.5.1.2. Histone demethylases

Histone demethylase activities are frequently investigated through the iPS cell research. During reprogramming, Vitamin C reduces histone H3 Lys36 di-methylation (H3K36me2) and tri-methylation using the histone demethylases, Kdm2a and Kdm2b (or Jhdm1a and Jhdm1b) (Wang

et al., 2011), and increases H3 Lys27 tri-methylation at the INK4/ARF locus to repress this gene (He et al., 2008; Li et al., 2009; Tzatsos et al., 2009). The INK4/ARF locus (or CDKN2A gene), which is a barrier for iPSC reprogramming (Li et al., 2009), produces tumour suppressors called p16^{INK4a} and p14^{ARF} in humans (or p19^{ARF} in mice) proteins. The former INK4a protein binds to CDK4 and CDK6 proteins to block them from stimulating cell cycle progression. The latter ARF protein protects the p53 protein from being degraded in order to block tumour formation (Kim and Sharpless, 2006; Calvo et al., 2016). Normal somatic cells basally express INK4/ARF, whereas ESCs and iPSCs completely silence them (Li et al., 2009). In addition, ageing is related to increased INK4/ARF expression (Agherbi et al., 2009). Taken together, reprogramming of somatic cells is proliferation-dependent, and the expression of the INK4/ARF locus limits the reprogramming efficiency (Li et al., 2009) (Figure 2.12).

During reprogramming with Oct4 and KDM2B (JHDM1B) expression, Vitamin C upregulates the microRNA (miR or miRNA) 302/367 gene (a target of Oct4 and Sox2 in ESCs) (Wang et al., 2011; Card et al., 2008; Marson et al., 2008). The miRNAs are small non-coding RNAs that regulate gene expression post-transcriptionally. The miR-302/367 cluster consists of miR-302a, miR302b, miR-302c, miR-302d and miR-367. This intragenic cluster is located in the *LARP7* (*La-related protein 7*) gene (Gao et al., 2015). KDM2B reduces histone H3 Lys36 methylation levels around the Oct4 binding sites of the miR302/367 cluster and promotes gene expression (Wang et al., 2011). The miR302/367 gene positively controls pluripotency by inhibiting genes necessary for embryonic differentiation (Houbaviy et al., 2003). Upon differentiation, expression of miR302/367 gene quickly disappears (Suh et al., 2004). Anokye-Danso et al. (2011) reported that the miR302/367 cluster expression alone can induce reprogramming of human fibroblasts to pluripotency. This miR302/367-mediated reprogramming requires inhibition of histone deacetylase 2 and miR367 (an essential Oct4 activator). Overall, the Vitamin C potential targets, Kdm2a and Kdm2b might activate miR302/367 during iPSC generation (Esteban and Pei, 2012) (Figure 2.12).

In addition, Vitamin C can convert pre-iPSCs to fully-reprogrammed iPSCs (Esteban et al., 2010). According to the paper of Chen et al. (2012), H3K9 methylations on ESC genes repress pluripotency. Moreover, Vitamin C decreases H3K9 di-methylation, activating gene expression. In this system, Vitamin C synergises with suppression of the Lys9-targeted methyltransferases (e.g. Setdb1, G9a, Suv39h1 and Suv39h2), significantly enhancing the reprogramming efficiency. Hence, suppression of Setdb1 can also shift pre-iPSCs to the ground state of pluripotency in the absence of Vitamin C (Chen et al., 2012). In addition to the H3K36 demethylases (Kdm2a and Kdm2b), Vitamin C also upregulates the H3K9 demethylases, such as Kdm3a, Kdm3b, Kdm4b and Kdm4c in the reprogramming process; this indicates that histone H3K9 demethylation is critical for conversion of pre-iPSCs to iPSCs (Monfort and Wutz, 2013). Furthermore, Mansour et al. (2012) argued that somatic cell reprogramming is also regulated by the H3K27 demethylase, Kdm6a (Utx). In summary, Vitamin C-targeted Fe(II) 2OG-dependent histone demethylases play a significant role in inducing pluripotency.
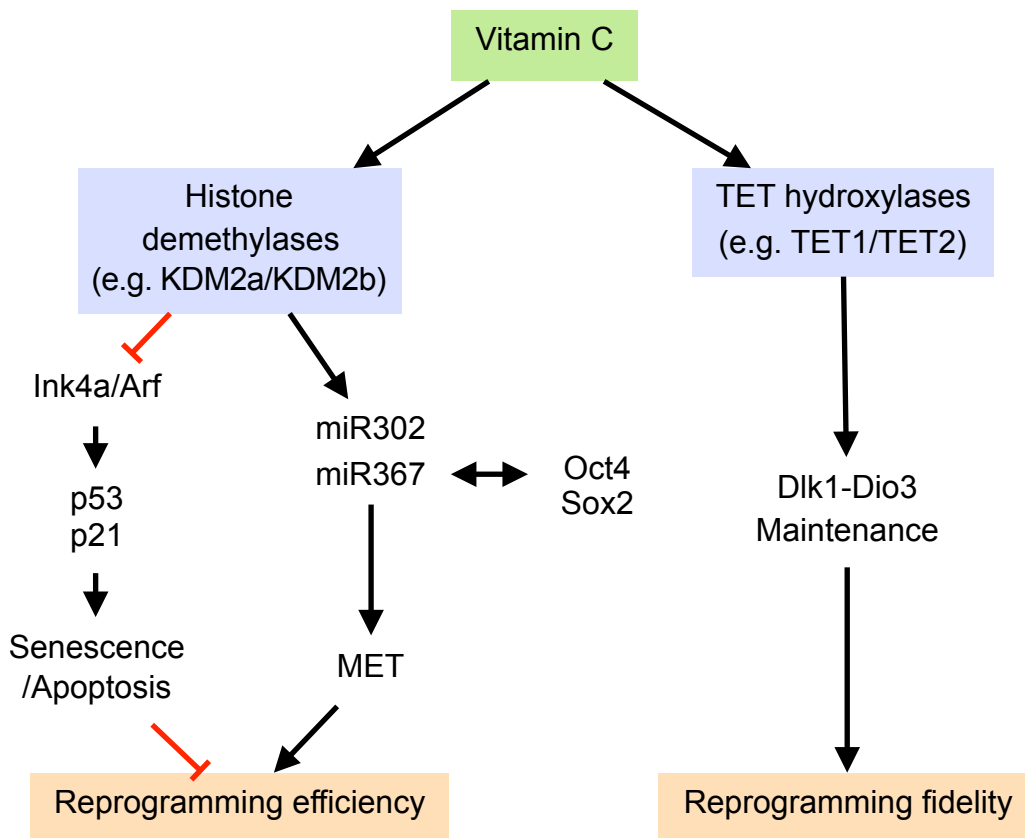
Figure 2.12. Potential Vitamin C-mediated reprogramming mechanism. Vitamin C activates histone demethylases, which increase reprogramming efficiency. Kdm2a and Kdm2b upregulate the miR302/367 cluster and inhibit the Cdkn2a locus that encodes Ink4a and Arf. The activated miR302/367 cluster induces mesenchymal-to-epithelial transition (MET). The repressed Ink4a and Arf promote p53 degradation, thereby preventing senescence and apoptosis. In addition, Vitamin C increases activity of Tet hydroxylases, which maintain the Dlk1-Dio3 locus imprinting. This activity consequently improves repro-gramming fidelity (Kawamura et al., 2009; Anokye-Danso et al., 2011; Esteban and Pei, 2012) *(Created using information form different sources such as Kawamura et al., 2009; Anokye-Danso et al., 2011; Este-ban and Pei, 2012; Monfort and Wutz, 2013).*

2.6. Vitamin C effects on inducing pluripotency.

Vitamin C-dependent enzyme activity is important during reprogramming. Somatic cells rapidly undergo senescence under culture media, partly due to accumulation of reactive oxygen species (ROS) generated by cellular metabolism (Parrinello et al., 2003). In addition to somatic cells, ROS also increases in the three factors (Sox2/Klf4/Oct4)-transduced reprogramming of mouse fibroblasts, but this is less efficient than the four factors (Sox2/Klf4/Oct4/c-Myc)-induced reprogramming, which has a lower ROS generation (Esteban et al., 2010). This suggests that antioxidant activity of Vitamin C may enhance this process. However, multiple antioxidants (e.g. Vitamin B1, Vitamin E, resveratrol, α-lipoic acid, reduced gluthatione, L-carnitine hydrochloride, amino-acetyl-cysteine and sodium selenite) do not noticeably enhance the generation of iPS cells (Esteban et al., 2010). This highlights the importance of the Vitamin C-dependent enzyme activity. Vitamin C promotes formation of iPSC colonies by decreasing p53 levels and counteracting cell senescence, a roadblock for reprogramming (Esteban et al., 2010). Generally, anti-proliferative p53 and Rb pathways are activated to induce senescence or apoptosis in aberrant cells. Deleting p53 (or its target p21 gene) enhances reprogramming of somatic cells (Kawamura et al., 2009), presumably by improving their proliferation.

Generally, when transduced with reprogramming factors, the somatic genes are efficiently repressed, whereas the pluripotency genes are induced inefficiently (Mikkelsen et al., 2008). Vitamin C de-methylates DNA on genes which are regulated by bivalent chromatin and genes which are de-methylated during reprogramming (Chung et al., 2010). Reprogramming with Vitamin C elimination decreases a iPS cell quality, causing hyper-methylation of the imprinted Dlk1-Dio3 locus (Stadtfeld et al., 2012). Vitamin C can block this aberrant methylation, although it is insufficient to remove the hyper-methylation once formed (Carey et al., 2011; Stadtfeld et al., 2012). The patterns of mRNA and miRNA expression are almost identical between ESCs and iPSCs, except transcripts of the Dlk1-Dio3 gene cluster. This gene cluster expression is abnormally reduced in iPSCs, which poorly generate chimeras and do not develop entirely iPSC-derived mice, due to the inactivity of histone H3 acetylation, H3K4me2/me3 and DNA methylation formation on the maternal allele (Stadtfeld et al., 2010). Valproic acid (a histone deacetylation inhibitor) can reactivate histone H3 acetylation and permissive H3K4 methylation on the muted Dlk1-Dio3 locus, thereby increasing the reprogramming efficiency (Anokye-Danso et al., 2011; Stadtfeld et al., 2012). In contrast, Vitamin C can block the reduction of histone H3 acetylation and H3K4 methylation, maintaining imprinted DNA methylation patterns on the Dlk1-Dio3 locus. This maintenance of correct Dlk1-Dio3 imprinting facilitates generation of iPS cells with an enhanced differentiation potential (Stadtfeld et al., 2010; Stadtfeld et al., 2012). Interestingly, the progeny of mice containing mutations in Tet1 and Tet2 carry aberrant Dlk1 imprinting in the locus. Furthermore, Tet1 and Tet2 are reported to improve reprogramming efficiency (Doege et al., 2012; Costa et al., 2013). In addition to the maintenance of Dlk1-Dio3 loci, Vitamin C can also partly overcome the epigenetic roadblocks towards ESC pluripotency by upregulating the miR302/367 and downregulating INK4/ARF (Figure 2.12). Moreover, Vitamin C extensively affects metabolic functions which might also induce indirect effects via other signalling pathways (Nelson et al., 1981; Knowles et al., 2003; Chowdhury et al., 2011; Nytko et al., 2011; Du et al., 2012).

Taken all together, Vitamin C increases enzyme activity of Fe(II) 2OG-dependent dioxyge-nases. Subsequently, these enzymes reduce histone and DNA methylation levels during somatic cell reprogramming to achieve pluripotency. Hence, it is tempting to speculate that Vitamin C can change the epigenetic profile of rat ESC and improve rat ESC pluripotency and self-renewal from the peri-naive ESC state.

2.7. Objectives of the project

Maintaining rat ESC in the "ground state" can expand their applications extensively. But, rat ESCs are sensitive to differentiation, compared with mouse ESCs. This issue should be solved to utilise rESCs for their extensive applications. Esteban et al. (2010) reported that Vitamin C boosts formation of mouse and human iPSC colonies. Our aim within this project is to establish assays of Vitamin C activity to determine whether Vitamin C improves rESC self-renewal or prevents rESCs from differentiation in the lineage-inducing conditions. Subsequently, we will identify how Vitamin C can stabilise rESC pluripotency at the molecular level. In this project, we will specifically look at the following:

- Whether Vitamin C enhances rESC proliferation.
- Whether Vitamin C prevents differentiation and supports intrinsic self-renewal.
- If it acts positively, what downstream targets are involved in the Vitamin C activity.

All of these questions would be answered following various experiments outlined below (Figure 2.13). Consequently, this project will provide insights into the process how Vitamin C promotes rat ESC pluripotency in the "ground state".

Assays of Vitamin C activity
- Improving rESC growth
- Preventing rESC differentiation

↓

Identifying Vitamin C downstream targets
- Transfection of rESCs
- siRNA validation of TET and KDM
- siRNA knockdown of TET and KDM in Vitamin C-treated rESCs

↓

Screening more targets
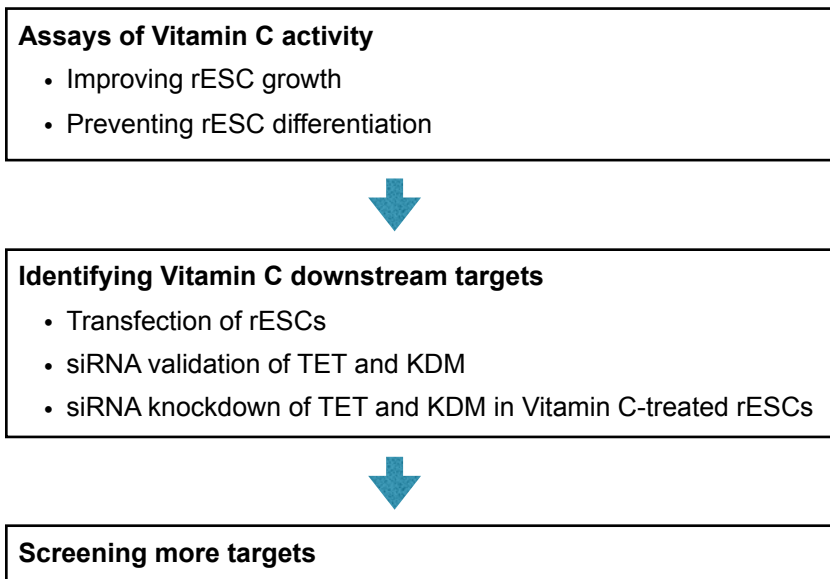
Figure 2.13. Experimental work plan. The step-by-step work in the Vitamin C project is described from top to bottom. Each box exhibits different works of the project with detailed plans.

2.8. Outline of experiments

2.8.1. Assays of Vitamin C activity

2.8.1.1. Improving rESC growth.

Rat ESCs will be cultured in 2i+LIF under the following conditions: Mock (water), Vitamin C and its isomer, D-isoascorbic acid (lacking cofactor activity). These culture conditions would allow us to determine whether Vitamin C can enhance rESC proliferation. Additionally, mouse ESC growth will be examined to compare with rat ESC proliferation.

2.8.1.2. Preventing rESC differentiation

Rat ESCs will be cultured in the differentiation-inducing condition (i.e. the 2i media containing high levels of GSK3 inhibitor) in the absence or presence of Vitamin C. This experiment will enable us to identify whether Vitamin C can rescue rESCs from differentiation. To determine their response to addition of Vitamin C, we will observe ESC morphology, the proportion of pluripotent cells and gene expression by using fluorescent microscope, flow cytometry and qRT-PCR expression profile.

2.8.2. Identifying downstream targets of Vitamin C

2.8.2.1. Transfection of rESCs

To determine how Vitamin C mediate its activity in ESCs, we will investigate potential downstream targets using siRNA knockdown. Before we conduct siRNA knockdown, transfection efficiency will be increased as near to 100% of the cells as possible to have the reliable knockdown effect of downstream targets. This efficiency will be maximised by modulating concentration of CHIR99021 (GSK3β inhibitor), Vitamin C and siRNA.

2.8.2.2. siRNA validation of TET and KDM

After transfecting siRNA into ES cells, siRNA may possibly cleave off-target mRNAs. To determine the effect of knockdown of target mRNAs, we will use qRT-PCR that measures the target mRNA levels to validate siRNA activity.

2.8.2.3. siRNA knockdown of TET and KDM in Vitamin C treated rESCs

Transfecting cells with TET & KDM siRNA and control siRNA in the presence or absence of Vitamin C will determine role of target in Vitamin C-mediated activity.

2.8.3. Screening more targets

If the siRNA knockdown of TET and KDM does not remove the the Vitamin C-mediated activity. we will further screen other potential Vitamin C-downstream targets.

2.9. Summary

Rat ESCs are extensively utilised in the field of biomedicine such as the generation of genetic models. However, this utility depends on reliable maintenance of stable ES cells in a self-renewing, pluripotent state. Although rat ESC are successfully derived from rat blastocysts using MEK and GSK3 inhibitors, spontaneous differentiation of rat ESCs in the 2i+LIF cultures limit their utility. Based on various relevant studies, Vitamin C seems to reduce this differentiation and improves self-renewal of undifferentiated ESCs. Our project will establish assays of Vitamin C activity and investigates the downstream epigenetic mechanisms regulating these changes. This will give fresh insight into the Vitamin C-mediated ES cell stabilisation.

3. Materials and Methods

3.1. Reporter of rat embryonic stem cells

In the rESC reporter, one of Rex1 alleles was replaced by GFP. Thus, this GFP is positioned under the control of the Rex1 promoter. Rex1 is found in undifferentiated ESCs, but lost in differentiated ESCs (Shi et al. 2006). This allows us to track the ESC state between differentiated and undifferentiated cells. Since the undifferentiated cells show a Rex1-GFP expression, we can observe green-positive cells under the fluorescence microscope, whereas differentiated cells are green-negative.

3.2. Culture of rat embryonic stem cells

ESCs were cultured on a monolayer of γ-irradiated (5 Gy) mouse embryonic fibroblasts (MEFs) in 2i + LIF medium in 24-well plates, and incubated at 37℃ and 5% CO_2 in humidified air. The 2i + LIF medium consists of 1µM PD0325901 (PD, acquired from Axon Medchem), 3µM CHIR99021 (CHIR, acquired from Axon Medchem), 1000U/ml mouse LIF and N2B27 in Table 1. DMSO was used to dissolve the inhibitor powder to 10mM as a stock solution and stored at -20℃. This solution was then diluted to 1mM using N2B27. Regarding feeder cells, the MEFs were treated with gamma irradiation to inactivate them.

N2B27 base medium		2i+LIF medium
DMEM/F12 medium	250 ml (50%)	N2B27 medium
Neurobasal medium	250 ml (50%)	PD0325901 (1µM)
N2 supplement	2.5 ml (0.5%)	CHIR99021 (3µM)
B27 supplement - no vitamin A	5 ml (1.0%)	Mouse LIF (1000U/ml)
L-glutamine (200mM)	5 ml (2mM)	
β-mercaptoethanol (50mM)	1 ml (0.1mM)	

Table 1. N2B27 base medium and 2i+LIF medium. Each component of N2B27 and 2i+LIF media is shown.

3.2.1 Passaging Rat Embryonic Stem Cells

ESCs were passaged every two or three days. To passage them, medium was first aspirated and 200µl TYP (0.025% trypsin, 1% chicken serum and 1 mM EDTA) was added into a 2cm² well of 24-well plates. These cells were then incubated at room temperature for 2-3 minutes, and dispersed to a single cell suspension. They were subsequently resuspended in 4ml N2B27 and pelleted by centrifugation at 1000 rpm for 3 minutes. After aspirating the supernatant, the cell pellet was resuspended in 0.5 or 1ml 2i+LIF medium. Lastly, the cells were plated at a density of 5 to 7.5 x 10⁴ / cm² onto the feeder cells, MEFs.

3.3. Cell Proliferation Assay

ESCs were plated in quadruplicate at a density of 500 per well of a 96-well plate. Cell growth was assayed utilising the CyQUANT® Direct Cell Proliferation Assay kit (supplied by Thermo Fisher Scientific) at 1, 24, 48, 72, and 96 hours, following the instructions of the manufacturer.

3.4. Inducing Differentiation of Rat Embryonic Stem Cells

In order to determine Vitamin C influence on ESC differentiation, concentrations of CHIR and PD were modulated in 2i medium to generate differentiated rESCs. As for CHIR assay, high CHIR concentration ($\geqq 4\mu M$) was applied to increase β catenin activity, which leads to upregulation of differentiation genes (e.g. LEF1). As for PD experiments, low concentration of PD ($\leqq 0.25\mu M$) was used to promote positive role of MEK on ESC differentiation.

3.5. Treatment of D- and L-ascorbic acid media

Due to the unstable nature of L-ascorbic acid in water, 2-Phospho-L-ascorbic acid trisodium salt (supplied by Sigma Aldrich) was used instead, as a stable derivative in cell culture. In addition, fresh stock was made for every experiment. For working stock, 100mg L-ascorbic acid powder was dissolved in 10ml RNase-free water. Subsequently, 32.25µl of this solution was added into the appropriate 10ml medium to produce 100µM L-ascorbic acid medium. Likewise, 100mg D-ascorbic acid (D-isoascorbic acid) powder (supplied by Sigma Aldrich) was loaded in 10ml water. Then, 17.5µl of this stock was added into 10ml medium (e.g. 2i+LIF medium) to generate 100µM D-isoascorbic acid medium.

3.6. siRNA Knockdown

In 24 well plates, ESCs were transfected with the appropriate small interfering RNA (siRNA) to knockdown the Vitamin C targets. Potentially, this target siRNA can remove the ESC phenotype rescued by Vitamin C in the pro-differentiation condition; this may identify the downstream targets of Vitamin C. Before siRNAs were employed in our experiments, we determined the optimal siRNA concentration in order to obtain the highest knockdown. Here, Lipofectamine LTX (Invitrogen) was used as a transfection reagent, following the manufacturer's instructions. Firstly, the desired number of cells were loaded in the 500ul 2i+LIF medium, and then the appropriate amount of siRNA was added in the 100ul OptiMEM medium containing 3.5ul LTX and 1ul PLUS reagent. In addition, a negative control siRNA (supplied by Ambion, 4390846) was used. After 24 hours, we replaced the liposome-transfection mix with a relevant fresh medium.

3.7. qRT-PCR

A maximum of 5×10^6 cells were harvested as a cell pellet. β-mercaptoethanol was added into cell pellet samples, since it is a reducing agent which helps to remove tannins and other polyphenols. Their RNAs were then isolated employing RNeasy Mini Kit (QIAGEN), and cDNAs were synthesised utilising AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies). The first-strand cDNA in each sample was suspended in 1ml of RNase-free water. After

this dilution,10µl of cDNA was added into each well of the 96 well-plate (qRT-PCR plate) in tripli-cate. Subsequently 15µl of master-mix (12.5µl of SYBR green mix, 1µl of 10uM forward primer, 1µl of 10µM reverse primer and 0.5µl of ROX dye) was added into each well, using SYBR Green qPCR Kit (Invitrogen) in Table S1. GAPDH was employed as a housekeeping gene, relative to which the expression level is defined. This constitutive gene is required to maintain basic cellular activity in all animal cells, since it catalyses one of the steps in glycolysis to generate energy. The qRT-PCR plate was briefly centrifuged and run on the strategene MX3000P machine following the step in Table 2.

Step	Time	Temperature	Note
First segment (1 cycle):	2 minutes	50℃	
	2 minutes	95℃	
Second segment (40 cycles):	15 seconds	95℃	
	30 seconds	60℃	Measure fluorescence
Third segment (1 cycle):	1 minute	95℃	
	30 seconds	60℃	Measure melting curve
	15 seconds	95℃	
	30 seconds	25℃	

Table 2. Thermal profile of the qRT-PCR. cDNA samples in the qRT-PCR plate were run on the strategene MX3000P machine under this following condition.

3.8. Luciferase Assay

ESCs were transfected with plasmid (360 ng) (SUPERTOP-FLASH or FOP-FLASH) and pEF1α-renilla (6.25 ng) (Hay, Sutherland et al. 2004) employing LTX, Lipofectamine and PLUS reagents (supplied by Invitrogen) at a density of 5 x 10^4 / cm^2. Cells were lysed after 2 days and analysed employing the Dual-Luciferase Reporter System (outlined in Promega, Madison, WI, http://www.promega.com). The level of luciferase was normalised to activity of renilla. Results are shown as a ratio of activity of SUPERTOP-FLASH to FOP-FLASH.

3.9. Single cell analysis (Flow cytometry)

ESCs were trypsinised using 200µl TYP and dispersed to a single cell suspension. Subse-quently, they were resuspended in 4ml N2B27 and pelleted by centrifugation at 1000 rpm for 3 minutes. The cell pellet was then resuspended in 200-500µl 2i+LIF medium and dispersed to sin-gle cells before keeping them on ice. Lastly, GFP fluorescence of single cells was measured using a BD Biosciences FacsCalibur flow cytometer and the data subsequently analysed using FlowJo software.

Chapter 4. Effects of Vitamin C on rat ESC

Rat ESCs were successfully derived from the blastocyst-stage embryo using the culture medium containing 2i plus LIF that promote self-renewal and prevent differentiation (Buehr et al., 2008; Li et al., 2008). Since this landmark discovery, rESCs have been employed as an alternative and comparative model to mESC for the study of pluripotency and differentiation (Meek et al., 2013; Chen et al., 2013). Although a range of cell signaling pathways involved in mESC maintenance have been determined, much less has been discovered about rESC. Meek et al. (2013) demonstrated that the culture of rESC in the standard 2i + LIF medium is less reliable than mESC, displaying higher expression levels of differentiation genes. Interestingly, Esteban et al. (2010) reported that Vitamin C improves the growth and formation of mouse induced pluripotent stem (iPS) cell colonies. Inspired by this study, we will document the effects of Vitamin C on rat ESCs.

To assay the pluripotent state and distinguish between differentiated and undifferentiated cells, we employed the Rex1-GFP reporter lines, in which GFP was placed under the control of the Rex1 promoter (Figure 4.1). As Rex1 is found in undifferentiated ESCs but lost in differentiated cells (Shi, Wang et al. 2006, Wang, Rao et al. 2006), their green intensity faithfully reports the expression of the pluripotency gene, Rex1, which allow us to monitor rESC self-renewal. (Meek, Wei et al. 2013).
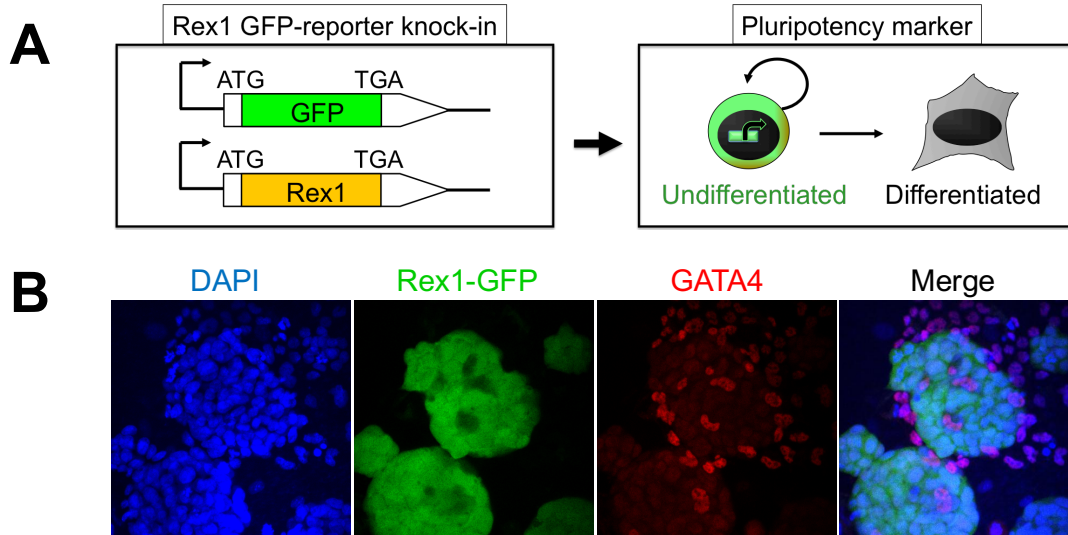
Figure 4.1. Monitoring ESC self-renewal. (A): The schematic diagram shows that one of the Rex1 alleles was replaced by the fluorescent reporter GFP. Hence, when ESCs are undifferentiated, they express the Rex1 gene as well as GFP, whereas when the cells are differentiated, the GFP is not expressed. **(B):** Observation of the Rex1-GFP expression in the 2i culture media exhibited spontaneous differentiation as shown by the early endodermal marker GATA4 expression (Meek et al., 2015) *Unpublished data from the Burdon lab.* DAPI (diamidino-2-phenylindole) indicates the DNA locations.

To investigate the effect of Vitamin C on rat ESCs in culture, we examined the effect of Vitamin C on Rex-EGFP rESCs cultured for 5 days in 2i+LIF supplemented with and without 100µM Vitamin C.
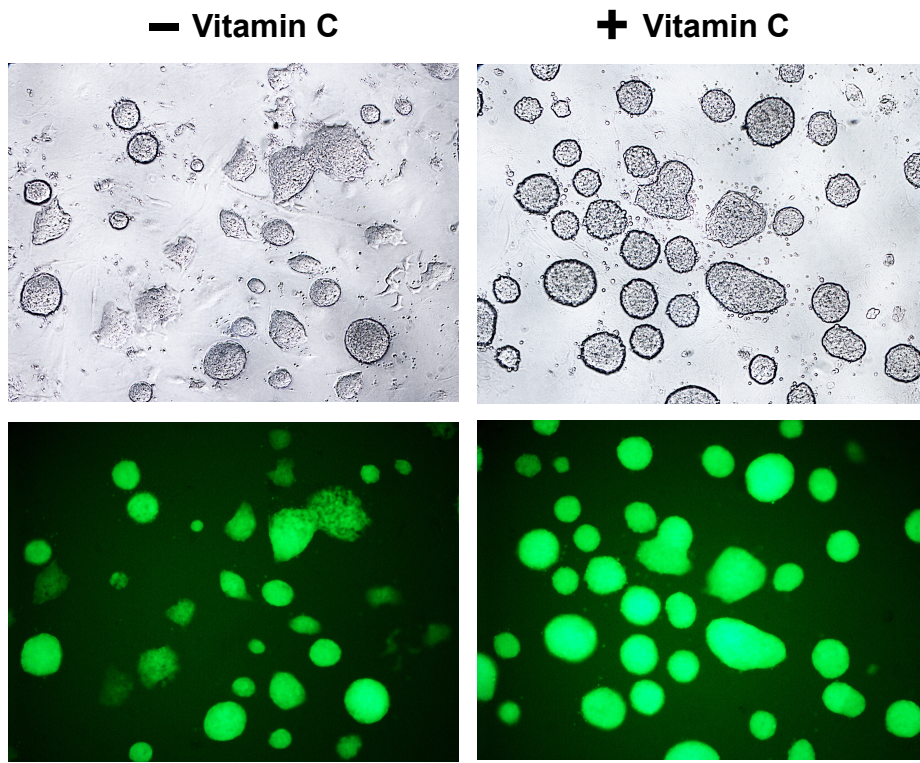
– Vitamin C **+ Vitamin C**

Figure 4.2. Vitamin C improves ESC morphology. Bright-field images (upper panels) and fluorescence images of rESCs (lower panel) cultured in 2i + LIF in the absence of Vitamin C (left panel) or presence of Vitamin C (right panel) for 5 days (magnification X10).

Interestingly, when rESCs were treated with Vitamin C (Vc), there was a noticeable change in ESC morphology and colony number. In the standard rESC culture media (2i + LIF), there were sparse ESC colonies observed, whereas more round and compact ESC morphology was found in the presence of Vitamin C. Moreover, Vitamin C increased the total number of ESC colonies as well as their colony size (Figure 4.2). In addition, the level of Rex1 expression in Vc-treated cells was higher, than those cultured in the absence of Vc (Figure 4.2). This suggests that Vitamin C reduces differentiation and improves growth of rESC.

4.1. Growth

To measure cell growth, 500 ESCs were cultured in 2i + LIF medium on the feeder cells, under three different conditions: Mock (water), D-isoascorbic acid and L-ascorbic acid. At each day, the CyQUANT® cell proliferation assay selectively detected live cells.

Figure 4.3. Vitamin C enhances growth of rat ESCs.
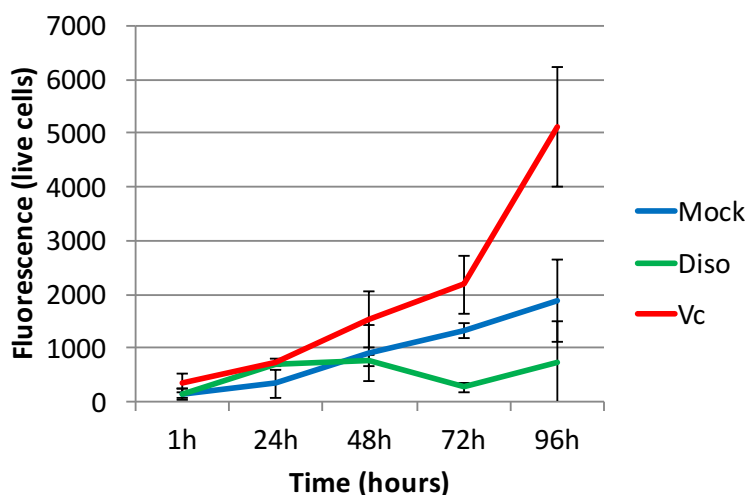
Growth of three independent rat ESC lines. These rESCs are all male lines, and 500 cells were plated in quadruplicate and cultured in 2i + LIF medium on the feeder cells in each well of 96 well plates. Mock, water; Diso, 100µM D-isoascorbic acid; Vc, 100µM Vitamin C or L-ascorbic acid. Mean and SD of three experimental replicates.

According to the measurements of live ESC numbers (fluorescence), our three independent experiments demonstrated that the growth of rESCs is improved by Vitamin C. In Figure 4.3, Vitamin C (Vc) appreciably increased the number of rat ESCs for 4 days, compared with Mock (control, water). Intriguingly, the Vc optical isomer, D-isoascorbic acid (Diso), lacking cofactor activity, did not improve rESC proliferation. Now, the intriguing question we can ask here is that 'Is this growth pattern also observed in mouse ESCs?'

Figure 4.4. Vitamin C does not improve growth of mouse ESCs.
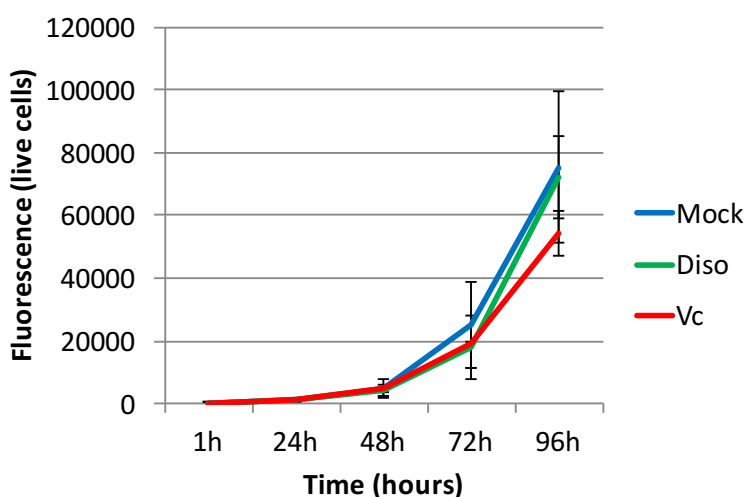
Growth of three independent mouse ESC lines. These mESCs are all male lines, and 500 cells were plated in quadruplicate and cultured in 2i + LIF medium on feeder cells in each well of 96 well plates. Mock, water; Diso, 100µM D-isoascorbic acid; Vc, 100µM Vitamin C or L-ascorbic acid. Mean and SD of three experimental replicates.

Surprisingly, rat and mouse ESCs did not respond to Vitamin C in the same way. There was no growth effects of Vitamin C in mouse ESCs (Figure 4.4). This strongly suggests that there is some unidentified mechanism in rat ESCs that we do not see in mouse ESCs. Furthermore, based on live cell fluorescence, it was observed that mouse ESCs grew much faster than rat ESCs. This rapid growth of mouse ESCs may indicate their shorter cell cycle (e.g. G1 phase), and may contribute to why they are more stable in proliferation. It could also be due to poorer survival of rESC compared to mESC at low cell density. In addition, it appears that at 48h, the mESC growth suddenly accelerates. This could be the result of reaching an optimum cell density and the resulting response to autocrine factor signaling.

4.2. Differentiation

Rat ESCs are vulnerable to sporadic differentiation (Chen, Blair et al. 2013). According to the GFP fluorescence images of Figure 4.2, the Rex1-GFP reporter was brighter in the Vc-treated cells. This suggests that Vitamin C may decrease the spontaneous differentiation of rESCs, and promote self-renewal of undifferentiated cells, thereby leading them to a more naive pluripotent state. Based on this rationale, we investigated Vitamin C influences on ESC differentiation.
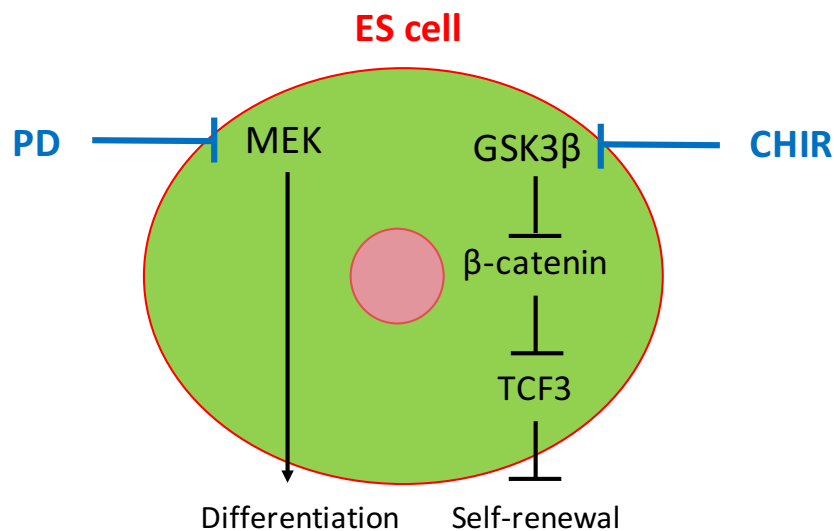
Figure 4.5. Maintenance of rat ESC self-renewal. Two inhibitor system (2i) supports self-renewal of rESCs. FGF-MEK signalling is interrupted by the presence of PD0325901 (PD), which prevents differentiation. Likewise, GSK3β is downregulated by CHIR99021 (CHIR), which retains self-renewal (Buehr et al. 2008; Lanner and Rossant, 2010; Meek et al. 2013). *Created using information from different sources.*

Rat ESCs are captured by preventing the MEK and GSK3β signalling pathways (Buehr, Meek et al. 2008, Li, Tong et al. 2008), so that they do not enter a differentiation process, but remain in a self-renewing, pluripotent state. To support this state, two inhibitors (2i), PD0325901 (PD) and CHIR99021 (CHIR or CH) are employed in the rESC culture media. Leukaemia inhibitory factor (LIF) is also incorporated in the standard rESC media (2i + LIF) to prevent differentiation and maintain Nanog, Oct4 and Klf4 (Buehr, Meek et al. 2008, Niwa, Ogawa et al. 2009). To test Vitamin C effects on differentiation, we induced differentiation by manipulating the rESC culture system (Figure 4.5).

4.2.1. Vitamin C influence on MEK-induced differentiation

First of all, the MEK inhibitor, PD was removed in the standard 2i+LIF media to allow MEK-induced differentiation. To maximise this MEK-induced differentiation, the pluripotency-facilitator LIF was also eliminated.

In Figure 4.6, when cultured in the standard ESC media (2i+LIF), there were many Rex1GFP-positive colonies observed with a round ESC morphology (2i + LIF, left panels in Figure 4.6A), whereas, in the absence of PD, Rex1GFP fluorescence was greatly reduced (-PD, middle panels). Under this same condition, Vitamin C was added in order to see if Vitamin C can rescue those cells from MEK-induced differentiation (-PD + VC, right panels in Figure 4.6A). The Vc-treated ESCs were also differentiated in the absence of PD, as demonstrated by the loss of GFP. This suggests that Vitamin C does not prevent rESCs from MEK-induced differentiation.

Figure 4.6. Vitamin C does not rescue ESCs from MEK-induced differentiation. In A and B, ESCs (135-4 cell line) were cultured in media containing 2i + LIF, 3 µM CHIR (-PD), and 3 µM CHIR + 100 µM Vitamin C (-PD + Vc) for 4 days. Panel A shows bright-field and fluorescence images of the same cells (magnification X10). In panel B, the same rESC cultures were analysed (1×10^4 cells) by flow cytometry at day 4. Abbreviations: CHIR, CHIR99021; PD, PD0325901; LIF, leukaemia inhibitory factor; Vc, Vitamin C; SSC-H, side scatter pulse height (i.e. cell granularity or internal complexity).

To qualitatively and quantitatively assay Vitamin C effects, the same cultures were analysed by qRT-PCR and flow cytometry respectively (Figure 4.6 and 4.7). In 2i+LIF, more than 80% ESCs showed RexGFP positive (left panels in Figure 4.6B), whereas when PD was eliminated, only 10% cells remained in a pluripotent state (middle panel). Surprisingly, the addition of Vitamin C shifted almost 100% ESCs to a differentiated state, leaving only 1% cells in a pluripotent state. Moreover, the results of all three independent ESC experiments showed the same pattern as demonstrated by the supplementary data (Figure S1-2).
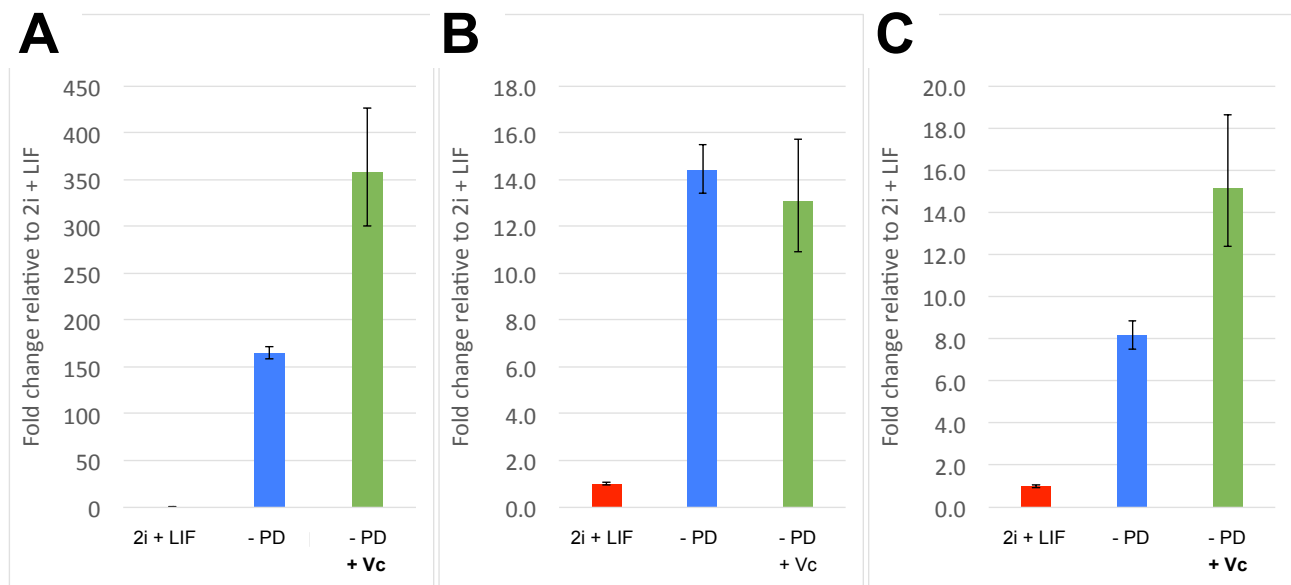

Figure 4.7. Vitamin C does not downregulate MEK-induced Gata4 expression. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis for GATA4 transcripts. The graphs indicate the level of GATA4 expressions in three independent ESC lines (135-3, A; 135-4, B; 141-5, C). These ESCs were cultured in media containing 2i + LIF (red), 3 µM CHIR (-PD, blue), and 3 µM CHIR + 100 µM Vitamin C (-PD + Vc, green) for 4 days. Abbreviations: CHIR, CHIR99021; PD, PD0325901; LIF, leukaemia inhibitory factor; Vc, Vitamin C.

We conducted qRT-PCR analysis for the differentiation marker, Gata4, on three independent rESC lines (Figure 4.7). In the absence of PD, the level of Gata4 expression in rESCs increased markedly, compared to those in the standard 2i + LIF media. Consistent with the flow cytometry data presented above, the ESC differentiation represented by Gata4 expression level was not decreased in the presence of Vitamin C. Rather, this level was increased in two of the cell lines upon Vitamin C treatment (Figure 4.7A and C).

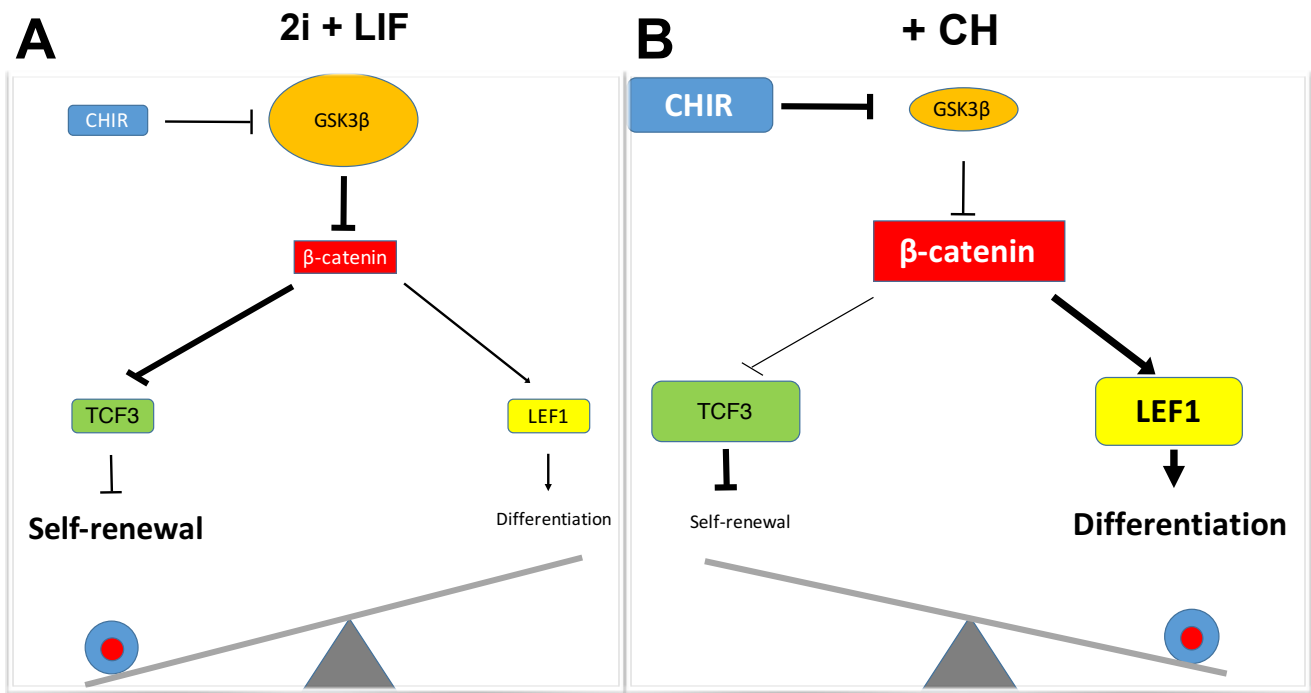## 4.2.2. Vitamin C influence on β catenin-induced differentiation

Figure 4.8. Tuning of β catenin Activity for Induction of Differentiation. GSK3β is regulated by its inhibitor, CHIR. **(A):** At lower levels of β catenin activation, the dominant β catenin-mediated activity is abrogation of TCF3 transcriptional repression and maintenance of self-renewal. **(B):** At high levels, β catenin-mediated differentiation appears to dominate via LEF1 transcriptional activation. (Chen et al. 2013; Meek et al. 2013). Abbreviations: CHIR, CHIR99021. *(Created using information from different sources).*

In addition to MEK-induced differentiation, Vitamin C effects were also examined on β catenin-induced differentiation. Canonically, rESC self-renewal is stabilised by MEK and GSK3β inhibitors (Buehr, Meek et al. 2008, Li, Tong et al. 2008). GSK3β inhibition by CHIR stabilises β catenin which antagonises the inhibitory activity of TCF3 on pluripotency genes (Chen et al. 2013). In the standard 2i+LIF medium, the level of β catenin activity is sufficient to inhibit TCF3 and prevent repression of key pluripotency genes Nanog and Esrrb (Chen et al. 2013) (Figure 4.8A). Interestingly, ESCs can also be maintained efficiently in 1i media (1µM PD) in which CHIR is absent (Meek et al. 2013).

However, when CHIR is added above the standard level (3 µM), lineage specification genes Brachyury and Cdx2 are markedly activated by high GSK3β inhibition, at the same time, the pluripotency genes Nanog, Rex1 and Esrrb are appreciably downregulated, causing ESC differentiation (Chen, Blair et al. 2013, Meek, Wei et al. 2013). Therefore, we tuned β catenin activity by adding extra CHIR, which upregulates LEF1 (Chen, Blair et al. 2013), and deliberately established rESC differentiation to interrogate Vitamin C effects on it (Figure 4.8B).
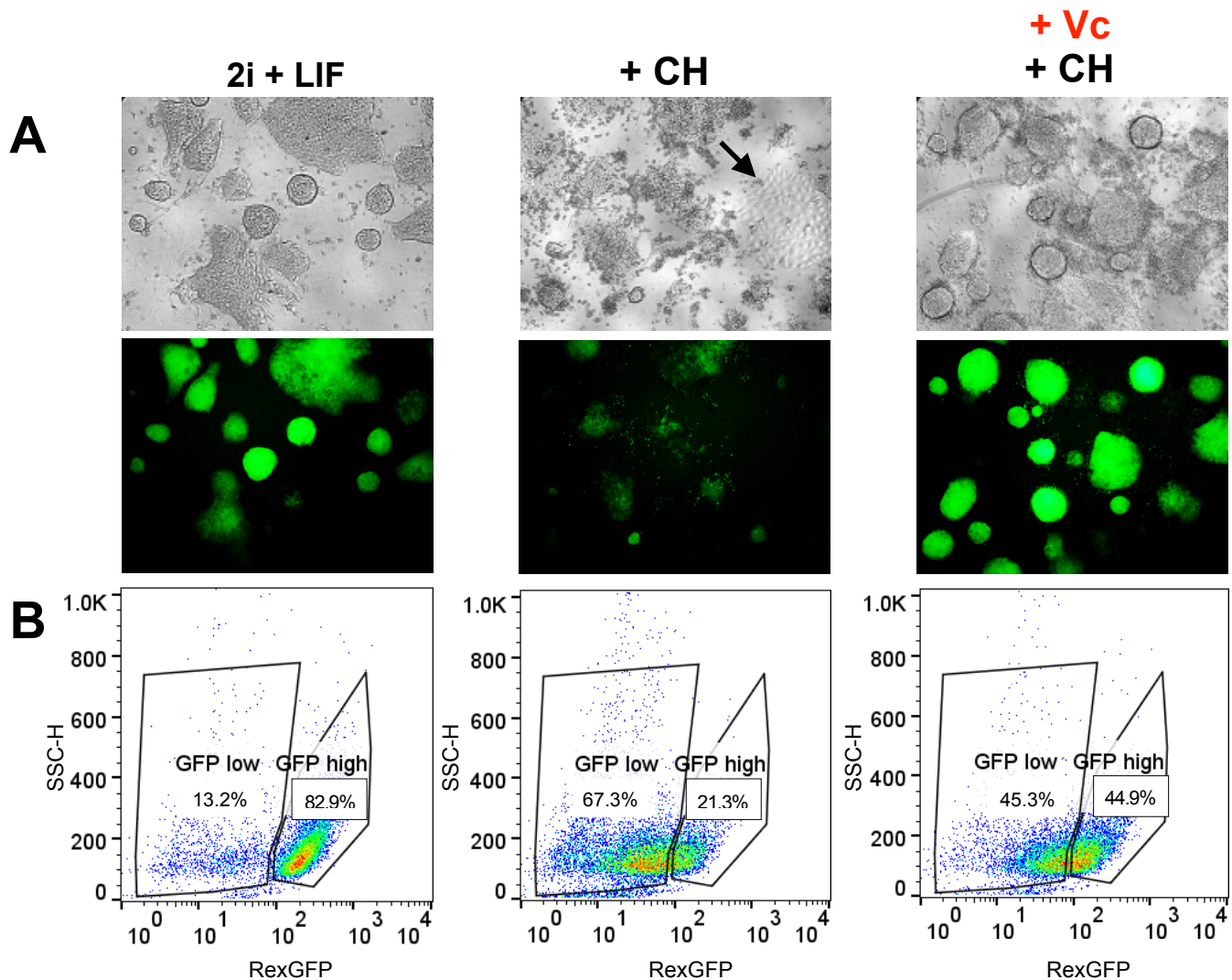
Figure 4.9. Vitamin C rescues ESCs from β catenin-induced differentiation. (A): rESCs (135-4 cell line) were cultured in media containing 2i + LIF or 2i with 5 μM CHIR (+CH), or 5 μM CHIR + 100 μM Vitamin C (+ CH + Vc) for 5 days. Panel A shows bright-field (upper) and fluorescence images (lower) of the same cells (magnification X10). **(B):** The same rESC culture conditions were used in another ESC line (135-3 line), and these cells were analysed (1x10⁴ cells) by flow cytometry at day 4. Abbreviations: CHIR/CH, CHIR99021; Vc, Vitamin C.

Under 2i+LIF conditions, rESCs exhibited tight and round morphology of Rex1GFP-positive colonies, whereas when the concentration of CHIR was increased, most of the ESCs differentiated, as demonstrated by cell morphology and loss of GFP (middle panels in Figure 4.9A). Vitamin C was administered to determine if Vitamin C can rescue those cells from CHIR-induced differentiation. Interestingly, rESCs benefited from Vitamin C treatment, showing brighter and bigger Rex-GFP-positive colonies. This suggests that Vitamin C prevents β catenin-induced ESC differentiation, promoting self-renewal.

Quantitative analysis was performed by flow cytometry. In 2i+LIF, more than 80% of the cells maintained a high level Rex1-GFP fluorescence, but when CHIR concentration increased, the majority lost fluorescence, leaving only 20% cells pluripotent (GFP high). Interestingly, this cell

population was expanded more than double by simply adding Vitamin C (Figure 4.9B). In addition, this Vitamin C plus CHIR experiment was repeated using another Rex1GFP reporter rESC line (Figure S3) as well as using two independent wild-type rESC lines (data not shown). All of which indicated the same pattern that Vitamin C prevents rESCs from CHIR-induced differentiation.
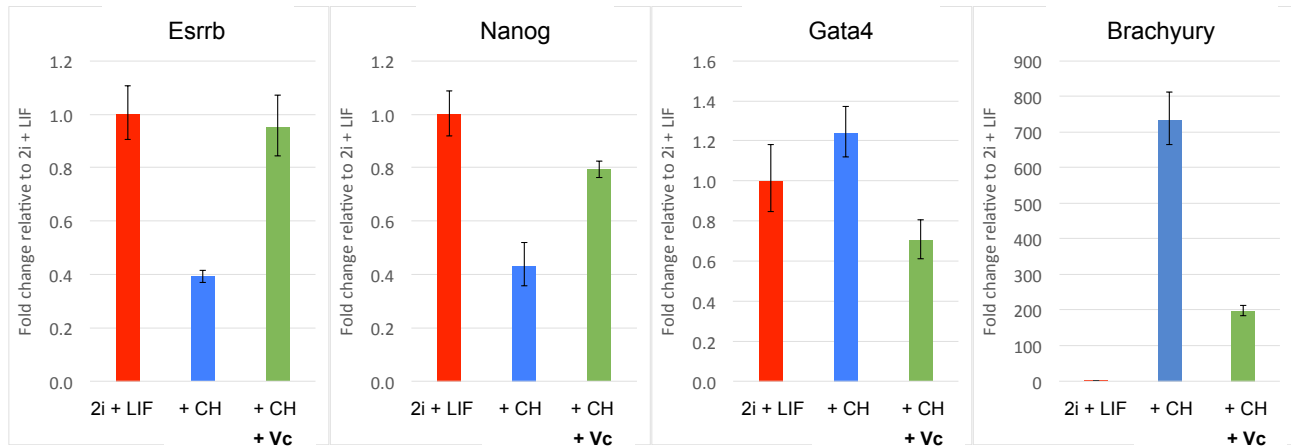
Figure 4.10. Vitamin C upregulates Esrrb and Nanog, and downregulates Gata4 and Brachyury. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis for Esrrb, Nanog, GATA4 and Brachyury genes. The graphs indicate the level of gene expressions in ESCs (135-4 line). These ESCs were cultured in media containing 2i + LIF (red) or 2i with 5 µM CHIR (+CH, blue) or 5 µM CHIR + 100 µM Vitamin C (+CH +Vc, green) for 5 days. Abbreviations: CHIR, CHIR99021; PD, PD0325901; LIF, leukaemia inhibitory factor; Vc, Vitamin C.

We performed qRT-PCR analysis to measure expression levels of ESC (Esrrb and Nanog) and differentiation markers (Gata4 and Brachyury). Under high GSK3 inhibition (+CH), the expression levels of the ESC markers, Esrrb and Nanog, were reduced by 60% and the expression of the differentiation markers, Gata4 and Brachyury increased compared to the standard 2i+LIF media. When treated with Vitamin C, rESCs upregulated Esrrb and Nanog, and downregulated Gata4 and Brachyury (Figure 4.10). This gene expression pattern was also confirmed in an independent ESC line (Figure S4).

4.2.3. D-isomer influence on CHIR-induced differentiation

As Vitamin C has both an antioxidant effect and a cofactor activity (Esteban, Wang et al. 2010, Blaschke, Ebata et al. 2013, Monfort and Wutz 2013), we examined the effect of D-isoascorbic acid (the Vitamin C isomer that lacks cofactor activity) on β catenin induced differentiation. This will allow us to determine if the Vitamin C effect results from their antioxidant effects or cofactor activities. Here, rESCs were cultured under 2i + LIF or high CHIR conditions using D-isoascorbic acid and L-ascorbic acid (Vitamin C), and analysed by flow cytometry at day 4.
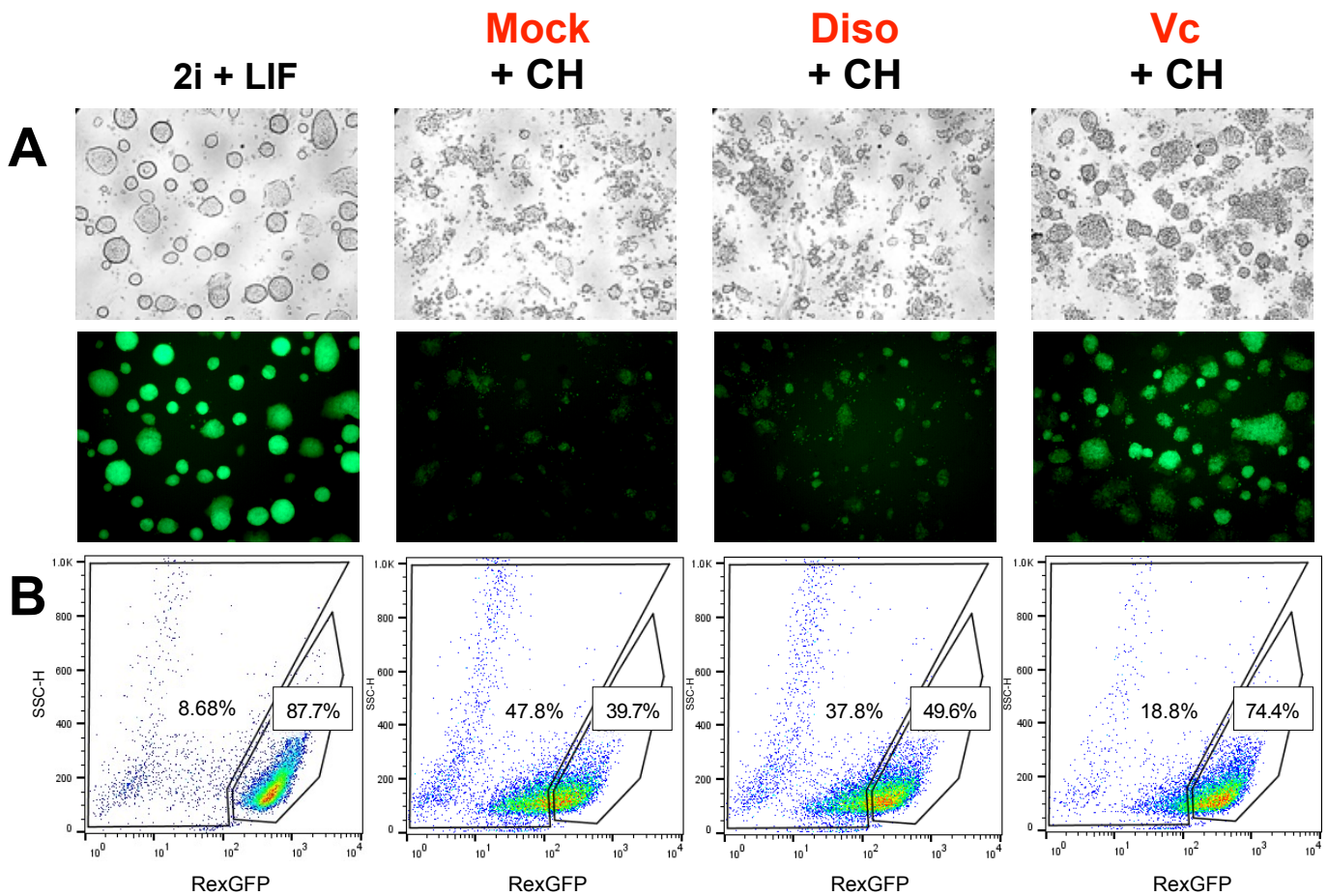
Figure 4.11. D-isoascorbic acid has little effect on β catenin-induced differentiation. (A): rESCs (123-2 RexGFP homozygous cell line) were cultured in media containing 2i + LIF, 5 µM CHIR + water (Mock + CH), 5 µM CHIR + D-isoascorbic acid (Diso + CH), or 5 µM CHIR + 100 µM Vitamin C (Vc + CH) for 4 days. The A panels shows bright-field (upper) and fluorescence images (lower) of the same cells (magnification X10). **(B):** These cells were then analysed (1×10^4 cells) by flow cytometry at day 4. Abbreviations: CH (CHIR), CHIR99021; Mock, water; Diso, D-isoascorbic acid; Vc, L-ascorbic acid (Vitamin C).

Consistent with the GSK inhibition data mentioned above (Figure 4.9), addition of extra CHIR faithfully induced ESC differentiation expanding GFP-low population by five times (yielding

nearly 50%). Likewise, Vitamin C (Vc) treatment prevented this differentiation leaving only 19% GFP-low cells. Compared with mock conditions, the addition of Vc increased the proportion of GFP-high cells by nearly 90% whereas the addition of D-isoascorbic acid (Diso, Vc isomer) only generated an increase of 25% (Figure 4.11). In short, D-ascorbic acid effects on CHIR-induced differentiation are not pronounced as much as Vitamin C effects observed on them. This suggests that the effects mediated by Vc are not exclusively due to its anti-oxidant activity.

4.2.4. Vitamin C influence on β catenin activity

It is clear that high GSK3β inhibition increases β catenin activity; β catenin binds to Lef1 and Tcf1 to promote downstream activation of their target genes which include differentiation genes (Chen, Blair et al. 2013, Meek, Wei et al. 2013). Consequently, rESCs become differentiated by these lineage-specification factors (Chen, Blair et al. 2013). However, Vitamin C prevents this progression of rESC differentiation, suggesting that the Vc downstream targets revolve around this β catenin activity in the axis of Wnt/β catenin signalling pathway. Therefore, it is very tempting to measure the β catenin activity under Vitamin C and high CHIR conditions. This will identify whether Vitamin C exerts their influence on CHIR-induced differentiation by diminishing the β catenin activity.

To measure β catenin activity, the vector DNA plasmid containing a luciferase reporter gene and a recognition binding site of TCF/LEF (β catenin direct target) was inserted into ESCs. Hence, any active endogenous TCF complexes bind to this plasmid, and drives the expression of luciferase. Hence, luciferase activity is directly correlated with the β catenin activity. Here, rESC were cultured in 2i + LIF or 2i containing 6μM CHIR in the absence or presence of vitamin C for 2 days, and quantitatively analysed using a luminometer according to light emission. In addition, the expression level of the β catenin target, Axin2 was measured using qRT-PCR to determine the Vitamin C effects on it.
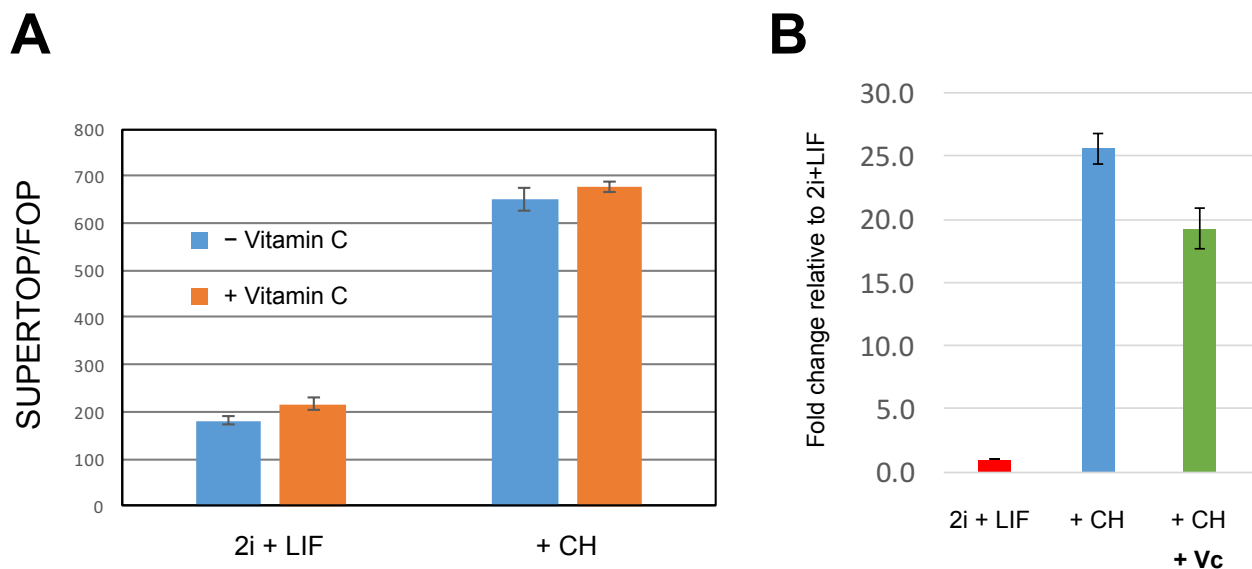
Figure 4.12 Vitamin C targets beneath β catenin/TCF. (A): No distinct change in β catenin activity. Graph indicates ratio of TOPFlash:FOPFlash activity of rESCs cultured in 2i + LIF or 2i containing 6μM CHIR (+CH) for 2 days. Abbreviations: CH, CHIR99021. **(B):** Vitamin C does not markedly affect the Axin2 expression level. qRT-PCR analysis for Axin2 gene. The graphs indicate the level of Axin2 expression in ESCs (135-3 cell line). These ESCs were cultured in media containing 2i + LIF (red) or 2i with 5 μM CHIR (+CH, blue) or 5 μM CHIR + 100 μM Vitamin C (+CH +Vc, green) for 5 days. Abbreviations: CHIR, CHIR99021; LIF, leukaemia inhibitory factor; Vc, Vitamin C.

The luciferase assay data demonstrated that, CHIR-induced β catenin activity, was not markedly reduced by Vitamin C. This indicates that Vitamin C reduces β catenin-induced differentiation by targeting other factors, not directly β catenin activity. Furthermore, our qRT-PCR analysis for Axin2 suggested that Vitamin C has no pronounced effects on the expression level of the β catenin transcriptional target Axin2 (Figure 4.12). This suggests that Vitamin C acts indirectly on targets or signalling pathways downstream of β catenin activity.

Discussion

Growth

In Figure 4.3, D-isoascorbic acid (the vitamin C optical isomer) lacking cofactor activity did not enhance proliferation of rat ESCs. This means that Vitamin C improves growth of rESCs through cofactor activities, and not exclusively via its antioxidant effects. Using the luciferase reporter assay, Meek et al. (2013) demonstrated that, in the canonical Wnt signalling pathways, its principal mediator β catenin shows a much higher activity in rat ESCs than mouse cells (Meek, Wei et al. 2013). It has been widely accepted that Wnt signalling pathways are involved in cell proliferation (Masckauchan, Shawber et al. 2005, Dejana 2010, Pei, Brun et al. 2012, Bilir, Kucuk et al. 2013, Tian, Benchabane et al. 2016, Yang, Wang et al. 2016). Thus, the different β catenin activities may address the question why rat and mouse ESCs respond to Vitamin C differently.

Naïve ESC have a very low level of DNA methylation (Leitch, McEwen et al. 2013). However, based on unpublished data from the Burdon lab in Edinburgh, rESC have higher expression levels of DNA methyltransferase-3a (Dnmt3a). Dnmt3a is known to increase DNA methylation levels (Okano, Xie et al. 1998, Okano, Bell et al. 1999, Crider, Yang et al. 2012), and this may explain spontaneous differentiation and growth differences with mESC. Indeed, most genes generally become silent as DNA methylation level increases (Bird 2002, Jones 2012, Rose and Klose 2014). We surmise that in rat ESCs, growth-facilitator genes are relatively hyper-methylated by inhibitors such as Dnmt3a, and thereby less accessible to their transcriptional enhancers. Therefore, Vitamin C may further reduce those methylation than mESCs whose methylation level is already lower.

However, this Vitamin C effect is also considered to be sex-dependent. Our unpublished data indicated that female rESC lines do not noticeably show the increased proliferation when Vitamin C is added. This highlights that other sex-dependent factors may also be involved in this Vitamin C influence. Interestingly, Ooi et al. (2010) discovered that female ESCs lose DNA methylation more rapidly, compared to male ESCs. This hypomethylation results from decreased levels of DNA methyltransferases in female ESCs, specifically DNMT3A and DNMT3B (Chen, Ueda et al. 2003, Zvetkova, Apedaile et al. 2005, Ooi, Wolf et al. 2010). Taken together, females cells are hypo-methylated relative to male cells, and thus Vitamin C may reduce methylation in male cells to a level more comparable to those in female.

In addition, growth benefit of Vc is more pronounced at low cell density. This suggests that other factors, such as low levels of crucial autocrine factors, may affect this cell density-dependent growth. Autocrine factors, Activin and Nodal have been reported to affect ESC proliferation (Ogawa, Saito et al. 2007, Przybyla and Voldman 2012). There is also another report that an autocrine factor, Cyclophilin A, is involved in density-dependent ESC growth (Mittal and Voldman 2011). Hence we speculate that, as mouse ESCs grow faster, mouse ESCs produces more autocrine factors, which may reduce the growth benefit of Vc, whereas less autocrine factors are generated by rat ESCs, thus Vc can largely increase the rat cell growth. If both mouse and rat cells are plated at the same density and the level of autocrine factors are similar, a different growth response could be due to a different optimum cell density, and thus the different resulting response to autocrine factor signaling. It could also be that rESC survival is poorer than mESC at low cell den-

sity. In addition, Vitamin C are generated by both mouse and rat ESCs themselves. There could be a difference in Vitamin C production, and hence they might respond to addition of Vitamin C differently. Taken all together, rat ESCs grow slower, but their growth can be improved by cofactor activities of Vitamin C.

L-ascorbic acid (Vitamin C) effects on MEK-induced differentiation

Previously there was an argument that Vitamin C does not block ERK (MEK) activation during somatic cell reprogramming to pluripotency (Shi, Zhao et al. 2010), suggesting that Vitamin C may function in an independent pathway. According to the flow cytometry data of three independent experiments (Figure 4.6 and Figure S1-2), it is apparent that Vitamin C rather accelerates MEK-induced differentiation. Numerous published papers demonstrated that Vitamin C induces de-methylation of DNA and histones with no cell-type specificity (Wang, Chen et al. 2011, Blaschke, Ebata et al. 2013, Monfort and Wutz 2013, Yin, Mao et al. 2013, Soufi 2014, Gustafson, Yang et al. 2015, Eid and Abdel-Rehim 2016). Building on this knowledge, we hypothesise that Vitamin C may open up the heterochromatin of silent genes, including differentiation genes as well as pluripotency factors (Soufi, Donahue et al. 2012, Soufi 2014, Becker, Nicetto et al. 2016). Hence, in response to Vitamin C, ESCs may likely switch any genes on randomly, but some of them may be activated according to their current cell states and their circumstances. Because our ESCs shifted to a differentiated state by MEK signals, hyper-methylated chromatin of differentiation-promoting genes (e.g. GATA4) may have become more accessible to their transcriptional activators, and Vitamin C may simply have enhanced differentiation. Therefore, we see more differentiated ESCs in the Vitamin C condition (Figure 4.6).

L-ascorbic acid (Vitamin C) effects on β catenin-induced differentiation

In Figure 4.10, our findings on up-regulation of Nanog and its direct target Esrrb by Vitamin C, support the published data that Vitamin C functionally compensates for Nanog deficiency (Schwarz, Bar-Nur et al. 2014). Furthermore, β catenin is not only known to reduce the repressive action of TCF3 on pluripotency genes (Wray, Kalkan et al. 2011), but also interacts with Lef1 to activate transcription of their target genes including early differentiation genes; CHIR stabilises β catenin and upregulates LEF1 (Chen, Blair et al. 2013, Ho, Papp et al. 2013, Meek, Wei et al. 2013). Hence it is easy to speculate that Vitamin C enables ESCs to tolerate high levels of β catenin activity, and consequently we see higher pluripotency gene expression.

In contrast to the results from the MEK-induced differentiation, Vitamin C suppresses β catenin-induced differentiation. This highlights that there are unknown interactions going on between Vitamin C and β catenin signalling. Indeed, two years ago, there was an analogous demonstration in mouse induced pluripotent stem cells (iPSCs) (Bar-Nur, Brumbaugh et al. 2014). Vitamin C and GSK3β inhibition do function synergistically during induction of pluripotency, by promoting the activation of pluripotency factors involved in a self-sustaining pluripotent state, such as, Nanog, Sox2, Zfp42, Zfp296, and miR-290-295 (Bar-Nur, Brumbaugh et al. 2014). It then raises a subse-

quent question that, 'which Vitamin C downstream effectors can support rESC self-renewal under CHIR-induced differentiation?'.

It is well-known that Vitamin C has two different functions (Monfort and Wutz 2013); one is an antioxidant effect, and the other action is a cofactor activity for DNA and histone demethylases such as TET and KDM families (Esteban, Wang et al. 2010, Blaschke, Ebata et al. 2013, Monfort and Wutz 2013). To determine by which of these mechanisms Vc mediates its effects, we also tested the influence of D-isoascorbic acid (the Vitamin C isomer that lacks cofactor activity, but retains antioxidant activity), on CHIR-induced differentiation of rESCs. This would answer the question whether the Vitamin C influence stems from their antioxidant effects or cofactor activities.

D-isoascorbic acid (Vc isomer) effects on β catenin-induced differentiation

There was some response of rESCs to D-isoascorbic acid which we expected, since this Vc isomer simply reflects a mirror structure of Vitamin C (Monfort and Wutz 2013). Therefore, this optical difference in structure may only contribute to their affinity differences with regard to associating with other target enzymes (Wilson and Dixon 1989, Monfort and Wutz 2013). That is, D-isoascorbic may still retain some cofactor activity, but reduced relative to the L-form (Vitamin C). Alternatively it suggests that the effects of Vc may be a combination of antioxidant and co-factor effects. Additionally, in accordance with other published data (Esteban, Wang et al. 2010, Blaschke, Ebata et al. 2013, Monfort and Wutz 2013), our results indicate that Vitamin C influence on β catenin-induced differentiation mostly relies on their enzymatic cofactor roles (Figure 4.11). Hence, we surmise that Vitamin C epigenetically facilitates activation of genes involved in ESC self-renewal, by acting as a cofactor (electron donor) for those Fe(II)-dependent enzymes. These enzymes are usually DNA and histone de-demethylases, such as TET and KDM families (Shi, Zhao et al. 2010, Monfort and Wutz 2013, Pera 2013).

Vitamin C influence on β catenin activity

β catenin activity is involved in both Tcf3 and Lef1 regulation (Chen, Blair et al. 2013). Optimal GSK3β inhibition mainly reduces the inhibitory effects of Tcf3 on pluripotency genes (e.g. Nanog and Esrrb) by decreasing β catenin activity; this result promotes ESC self-renewal (Chen, Blair et al. 2013, Meek, Wei et al. 2013). However, high GSK3β inhibition predominantly facilitates LEF1-mediated differentiation (Chen, Blair et al. 2013), which is correlated with up-regulation of Brachyury and Axin2, and down-regulation of Esrrb and Nanog (Figure 4.10). According to the data in Figure 4.12, Vitamin C does not affect β catenin activity directly, indicating that Vitamin C targets are present beneath β catenin. Interestingly, Brachyury was down-regulated in the presence of Vitamin C, whereas the Axin2 expression level was not markedly changed by Vitamin C. This indicates that the β catenin/TCF/LEF activity corresponds to Axin2 expression, while Brachyury may be repressed indirectly by different factors, for example, pluripotency factors such as Nanog and Esrrb.

To interrogate the role of potential downstream candidates on Vc-mediated activity, siRNA knockdown of target mRNA transcripts was employed. Previously, siRNA-mediated knockdown

was reported as an efficient approach to degrade target mRNAs (Meek et al. 2013). Thus, siRNA transfection was incorporated in our experiments to determine if knockdown of DNA and histone demethylases TET1/2 and Kdm2a/b negates the Vitamin C influence on β catenin-induced ESC differentiation.

Chapter 5. Establishing Vitamin C target assay

The results in the previous chapter show that Vitamin C suppresses CHIR-induced ESC differentiation. We therefore plan to use this GSK3β inhibition assay to interrogate potential down-stream targets of Vitamin C. In order to identify the Vitamin C targets that interfere with the effects of high β catenin activity, siRNA knockdown was performed. siRNA transfection has previously been reported as a reliable tool to knockdown mRNA targets in rESCs (Meek, Wei et al. 2013). In this chapter, we refine the siRNA transfection, differentiation and Vc conditions to increase the likelihood of identifying targets involved in Vc-mediated inhibition of CHIR induced differentiation.

5.1 Validation of siRNA activity using Lef1 knock-down

To demonstrate the efficiency of this approach, siRNA activity was first validated to target the Lef1 mRNA, because LEF1 is considered as a critical effector in driving ESC differentiation in high CHIR culture conditions (Chen, Blair et al. 2013, Ho, Papp et al. 2013). We transfected siRNA for LEF1 (50nM) into Rex-EGFP cells treated with 6µM CHIR and analyzed these cells using flow cytometry at day 5.
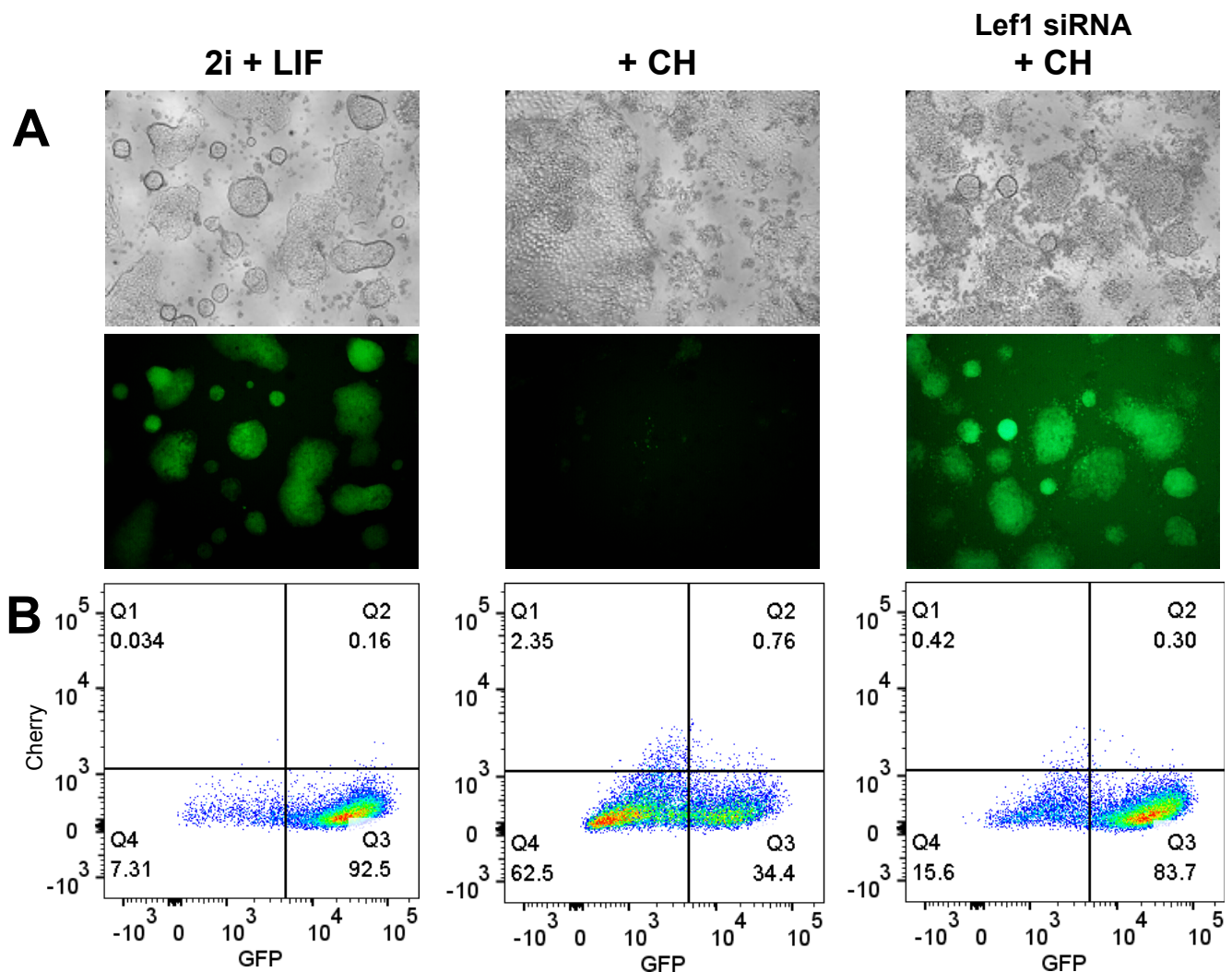
Figure 5.1 Lef1 siRNA effectively blocks ESCs from CHIR-induced differentiation. (A):
Bright-field (upper) and fluorescence (lower) images (magnification X10) of rESCs (135-4) cultured in media containing 2i + LIF or 2i with 6 μM CHIR (+CH) or 6 μM CHIR + 50 nM Lef1 siRNA (Lef1 siRNA + CH) for 5 days. **(B):** The same cells were then analysed ($1x10^4$ cells) by flow cytometry at day 5. Abbreviations: CHIR/CH, CHIR99021.

In Figure 5.1, the 2i + LIF condition generated round and tight ESC colonies with a Rex1-GFP positive population of approximately 90%. Additional CH treatment shifted the majority of Rex1-GFP positive cells to a differentiated state, exhibiting a 60%GFP-negative population. When ESCs cultured in high CHIR were transfected with Lef1 siRNA, they remained in a pluripotent state, with 85% of the cells retaining Rex-GFP expression. This experiment was repeated using a control siRNA (negative siRNA) which had no effect and additionally demonstrated that negative siRNAs have no influence on rESC differentiation, as shown by the supplementary data (Figure S6). These results clearly demonstrated siRNA knockdown and flow cytometry as a valid assay for assessment of the potential Vc targets.

5.2. Titration of CHIR concentration

To determine the optimal concentration of CHIR (GSK inhibitor), rat ESCs were cultured in media containing 2i + LIF or 2i with 5 µM CHIR, 7 µM CHIR, or 9 µM CHIR in the absence or presence of Vitamin C for 3 days.

Figure 5.2. ESCs are efficiently differentiated and rescued by Vc at 7µM CHIR. (A): Fluorescence images (magnification X10) of rESCs (123-2 RexGFP homozygous) cultured in media containing 2i + LIF or 2i with 5 µM CHIR, 7 µM CHIR, or 9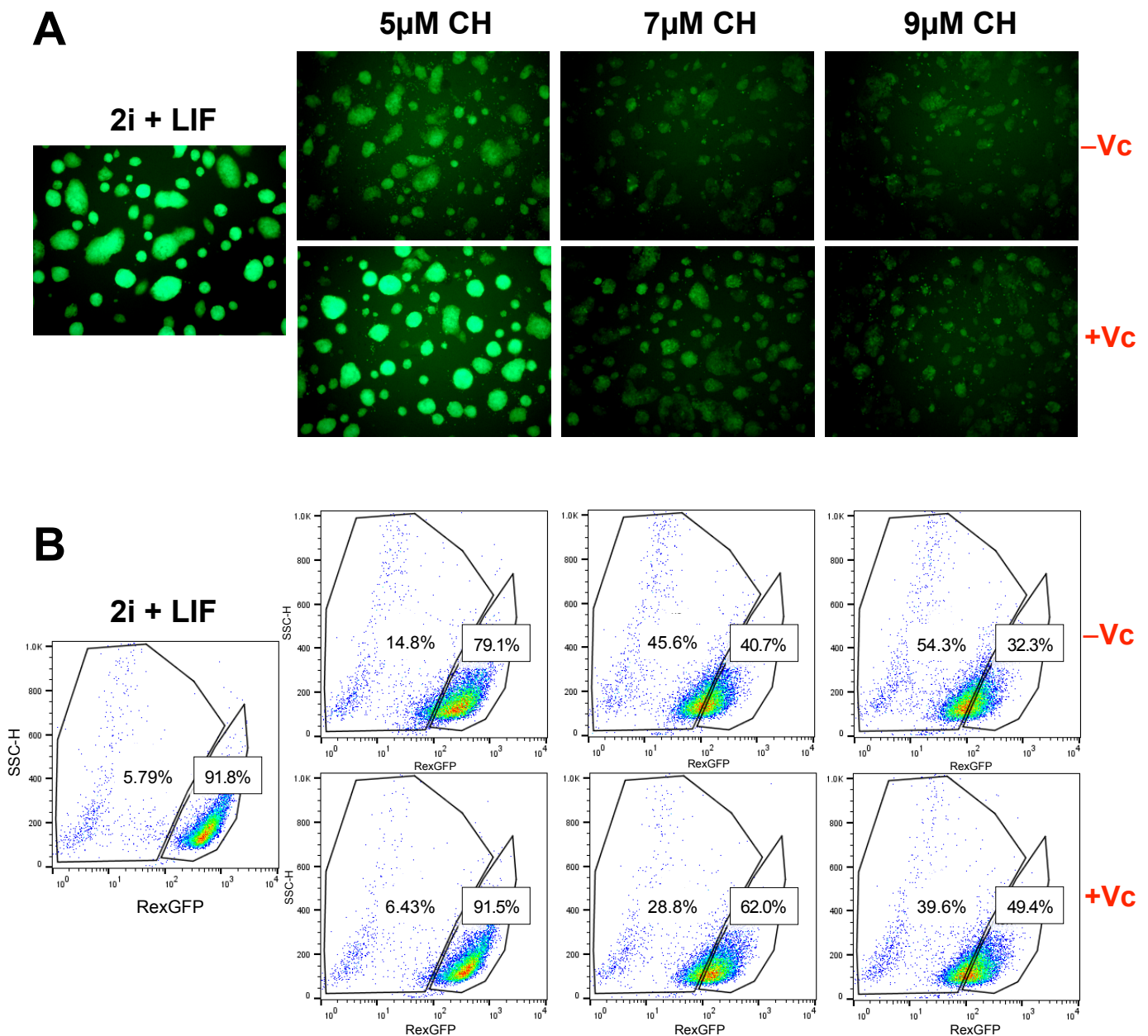 µM CHIR in the absence or presence of Vitamin C for 3 days. **(B):** These cells were then analysed (1x10^4 cells) by flow cytometry at day 3. Abbreviations: CH (CHIR), CHIR99021; Vc, L-ascorbic acid (Vitamin C).

In the 2i + LIF media, the ESC cultures contained approximately 90% RexGFP-positive and 5% negative cells (Figure 5.2). When the cultures were treated with 5µM, 7µM and 9µM CHIR each, the GFP negative populations gradually increased, indicating 15%, 46%, and 54%, respectively. When Vitamin C was administered, these Rex1 negative populations decreased showing 15% down to 6%, 46% down to 29%, and 54% down to 40%, respectively. This corresponds to a level of suppression of differentiation by Vitamin C of roughly 8%, 17%, and 15%, suggesting that Vitamin C was most effective in 7µM CHIR media. Hence, this CHIR concentration (7µM) was chosen due to pronounced differentiation and also rescued by Vitamin C.

5.3. Titration of Vitamin C concentration

To determine the optimal concentration of Vitamin C, rat ESCs were cultured in media containing 2i + LIF or 2i (7µM CHIR) along with 0µM, 50µM, 100µM, 200µM or 400µM Vc for 3 days.
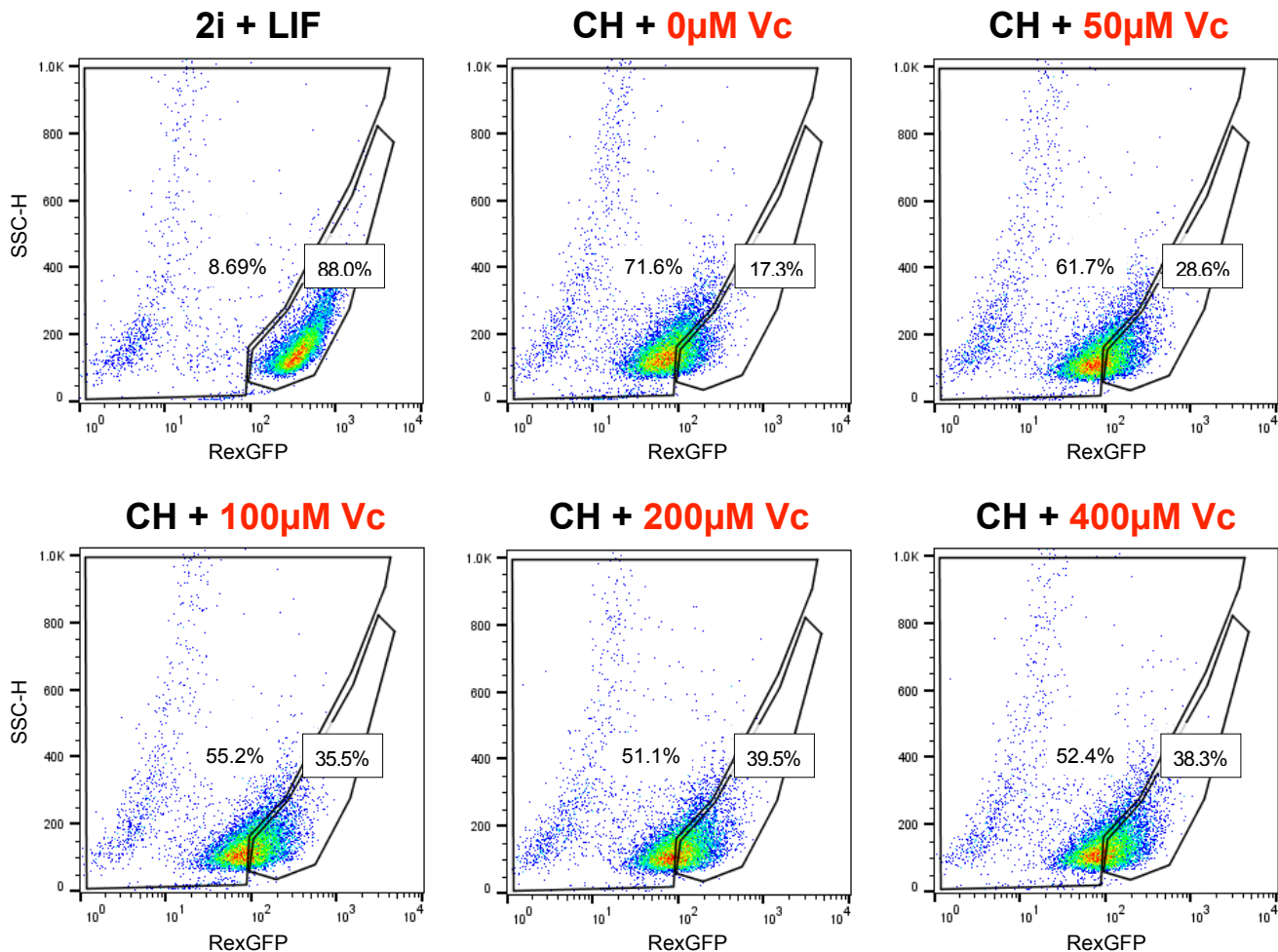
Figure 5.2. 100µM Vitamin C is effective in preventing CHIR-mediated differentiation. rESCs (123-2 RexGFP homozygous) cultured in media containing 2i + LIF or 2i (7µM CHIR) along with 0µM, 50µM, 100µM, 200µM or 400µM Vc for 3 days. These cells were analysed (1x10^4 cells) by flow cytometry at day 3. Abbreviations: CH (CHIR), CHIR99021; Vc, L-ascorbic acid (Vitamin C).

In the 2i+LIF media, 88% Rex-positive and 9% Rex-negative cells were observed, whereas, in the presence of CHIR, 17% pluripotent and 72% differentiated populations were detected (Figure 5.2). The GFP-negative cell population decreased in relation to Vc concentration up to 100µM, but with no marked benefit at concentrations greater than 100µM. Based on this experiment, 100µM Vitamin C is effective in reducing CHIR-induced differentiation. Thus, this Vc concentration was used for the Vc target assay.

In summary, we have validated siRNA activity by transfecting rESCs with Lef1 siRNA, as demonstrated by the result that most ESCs are rescued from CHIR-induced differentiation by Lef1 knockdown. In addition, CHIR concentration was titrated which demonstrated that 7µM CHIR is efficient to get ESCs differentiated, which can be delayed by Vc. Finally, titration of Vitamin C concentration was performed and revealed that 100µM Vc is the optimal concentration to prevent ESC differentiation caused by GSK3β inhibition.

Chapter 6. Roles of TET and KDM enzymes in Vitamin C effects

We discovered that Vitamin C promotes growth of rat ESCs, but not mouse ESCs. This growth effect is not observed upon treatment with D-isoascorbic acid (Vc isomer deficient in cofactor activity). In addition, Vitamin C blocks β catenin-induced differentiation, but not MEK-induced differentiation. In rESC cultures with high β catenin activity, Vitamin C upregulates pluripotency-associated genes and downregulates differentiation-related factors. Administration of D-isoascorbic acid had little effect on this β catenin-induced differentiation. This result suggests that Vitamin C influences on the ESC differentiation mostly rely on enzymatic cofactor activities (Esteban, Wang et al. 2010, Blaschke, Ebata et al. 2013, Monfort and Wutz 2013). Therefore, we speculate that Vitamin C epigenetically reconfigures chromatin structures and promotes activation of genes involved in ESC self-renewal, by acting as a cofactor (electron donor) for those iron-possessing enzymes. According to recent publications these enzymes are most likely to be DNA and histone de-demethylases (e.g. TET and KDM families) (Shi, Zhao et al. 2010, Monfort and Wutz 2013, Pera 2013).
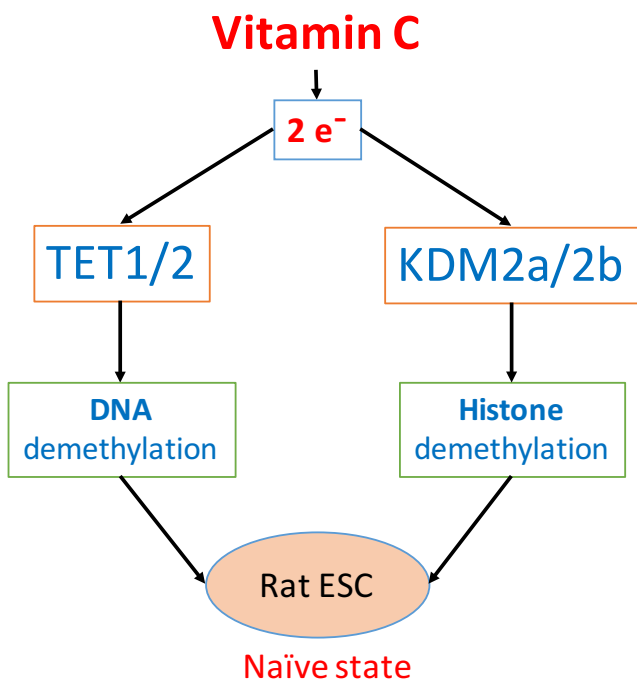
Figure 6.1. Potential downstream pathways employed by Vitamin C. Vitamin C acts as a cofactor for Fe(II)-dependent enzymes. In the presence of iron in the Fe(IV) state, Vitamin C produces two electrons to switch the iron to the Fe(II) state of iron-possessing enzymes. We hypothesise that TET1/2 activated by Vitamin C may support rESCs in a naïve state by de-methylating DNA (Blaschke et al. 2013). In addition, KDM2a/2b up-regulated by Vitamin C might facilitate rESCs in a ground state by reducing histone methylation (Wang et al. 2011). *Created using information from different sources.*

Various reports argue that Vitamin C induces pluripotency acting through TET1 and TET2 that prevent gene silencing by reconfiguring DNA structures (Blaschke, Ebata et al. 2013, Chen, Guo et al. 2013, Yin, Mao et al. 2013, Gustafson, Yang et al. 2015). Likewise, induction of pluripotency is also significantly improved by Vitamin C-dependent enzymes, such as KDM2A and KDM2B, which are involved in demethylation of H3K36me1/me2 (Tsukada, Fang et al. 2006, He, Kallin et al. 2008, Wang, Chen et al. 2011) in Figure 6.1.

To determine whether TET1/2 and KDM2A/2B are mediators of Vitamin C activity in rESCs, siRNA knockdown of their transcripts was performed. This will identify whether these enzymes are involved in preventing CHIR-induced differentiation. To illuminate this, we planned to use siRNAs as a gene-silencing tool (Figure 5.1), as previously demonstrated (Chen et al. 2013, Meek et al. 2013). Therefore, the interfering activity of commercially available siRNA sequences specific to TET1/2 and KDM2A/2B, were assessed using qRT-PCR analysis.

6.1. Validation of siRNA activity using Tet1/2 and Kdm2a/2b

siRNA activity was validated and the optimal concentration determined following transient transfection into rESC and subsequent analysis of mRNA level by qRT-PCR. As shown by the supplementary data (Figure S7), we discovered that approximately 50µM siRNA generates efficient knockdown in most of the target genes. We screened two different siRNA sequences specific for each target individually and in combination to identify the condition which produced the greatest knockdown.

To validate siRNA activity, ESCs were transfected with the appropriate siRNAs and cultured in 2i + LIF medium for 3 days.
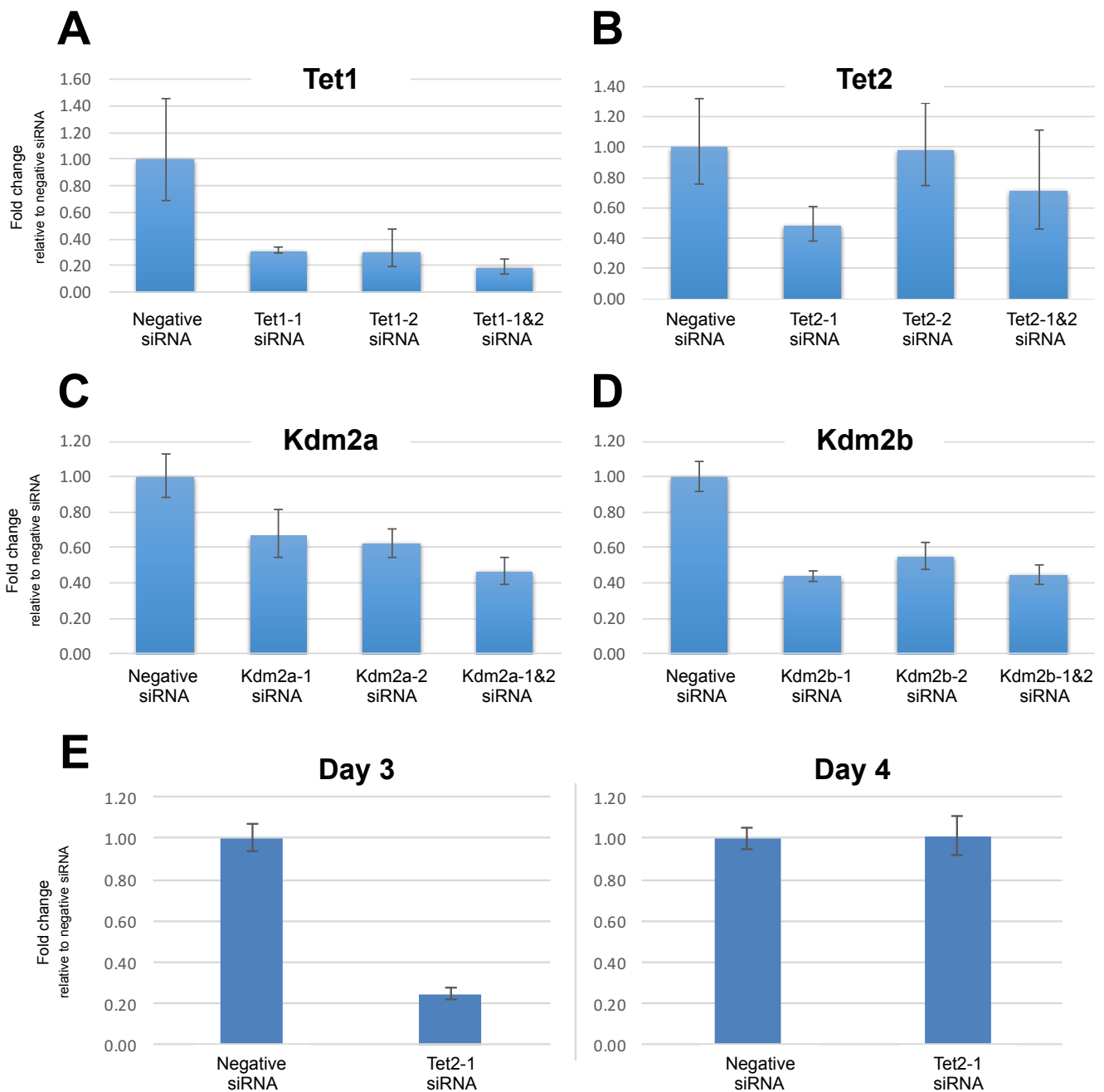
Figure 6.1 Validation of potential target siRNA activity. (A - D): Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis for Tet1, Tet2, Kdm2a and Kdm2b mRNAs. The graphs indicate the level of gene expressions in rESCs (DAK31 male) transfected with the appropriate siRNAs for 24 hours then subsequently cultured in media containing 2i + LIF for 3 days. Two different siRNAs were designed for each gene, and used individually or combined together. **(E):** Duration of siRNA activity. qRT-PCR analysis for Tet2 mRNA. The graphs indicate the level of gene expressions in rESCs (135-4 RexGFP heterozygous) transfected with the appropriate siRNAs for 24 hours then subsequently cultured in media containing 2i (6μM CHIR) and 2i (5μM CHIR) for 3 and 4 days, respectively. Abbreviation: negative siRNA, Control siRNA.

Two independent siRNAs were tested for each target. For Tet1, an equivalent knockdown of 70-80% was observed with both siRNAs whether independently or in combination (Figure 6.1A). For Tet2, optimum knockdown of 50% was achieved using siRNA 1, whereas Tet2 mRNA levels were not reduced by siRNA 2, and only by approximately 30% with both TET2 siRNAs in combination. (Figure 6.1B). For Kdm2a, a reduction of 35-40% was detected with both siRNAs independently, while both siRNAs in combination degraded 50% mRNA transcripts (Figure 6.1C). For Kdm2b, a knockdown of 45-55% was measured with both siRNAs whether independently or in combination (Figure 6.1D). Therefore, we decided to use siRNAs independently that exhibited the best knockdown level. That is, siRNAs of Tet1, Tet2, Kdm2a and Kdm2b degraded the mRNAs by 70%, 50%, 40% and 55%, respectively.

We validated siRNA activity of each gene at day 3 (Figure 6.1A - D). However, we later discovered that all siRNA activities completely perish on the following day (day 4). In Figure 6.1E, although 75% mRNA knockdown was observed under Tet2 siRNA at day 3, by day 4 no siRNA activity was observed. This suggests that siRNA knockdown starts to disappear prior to day 4. Indeed, during the validation of siRNA activity of Tet1/2 and Kdm2a/2b (Figure 6.1), one of our qRT-PCR results presented little mRNA knockdown at day 3 (data not shown). Hence, it might be difficult to see the knockdown clearly between day 3 and day 4.

6.2. siRNA Knockdown of Tet1/2 and Kdm2a/2b

If these enzymes mediate Vc effects on CHIR-induced differentiation, then we expect that Tet and Kdm siRNAs would, at least partly, hinder the Vitamin C rescue from those differentiation.

To conduct siRNA knockdown, rESCs were transfected with the appropriate siRNAs for 24 hours and subsequently cultured in media containing 2i+LIF or 2i contains 7 µM CHIR with or without 100µM Vitamin C for 3 days.

A
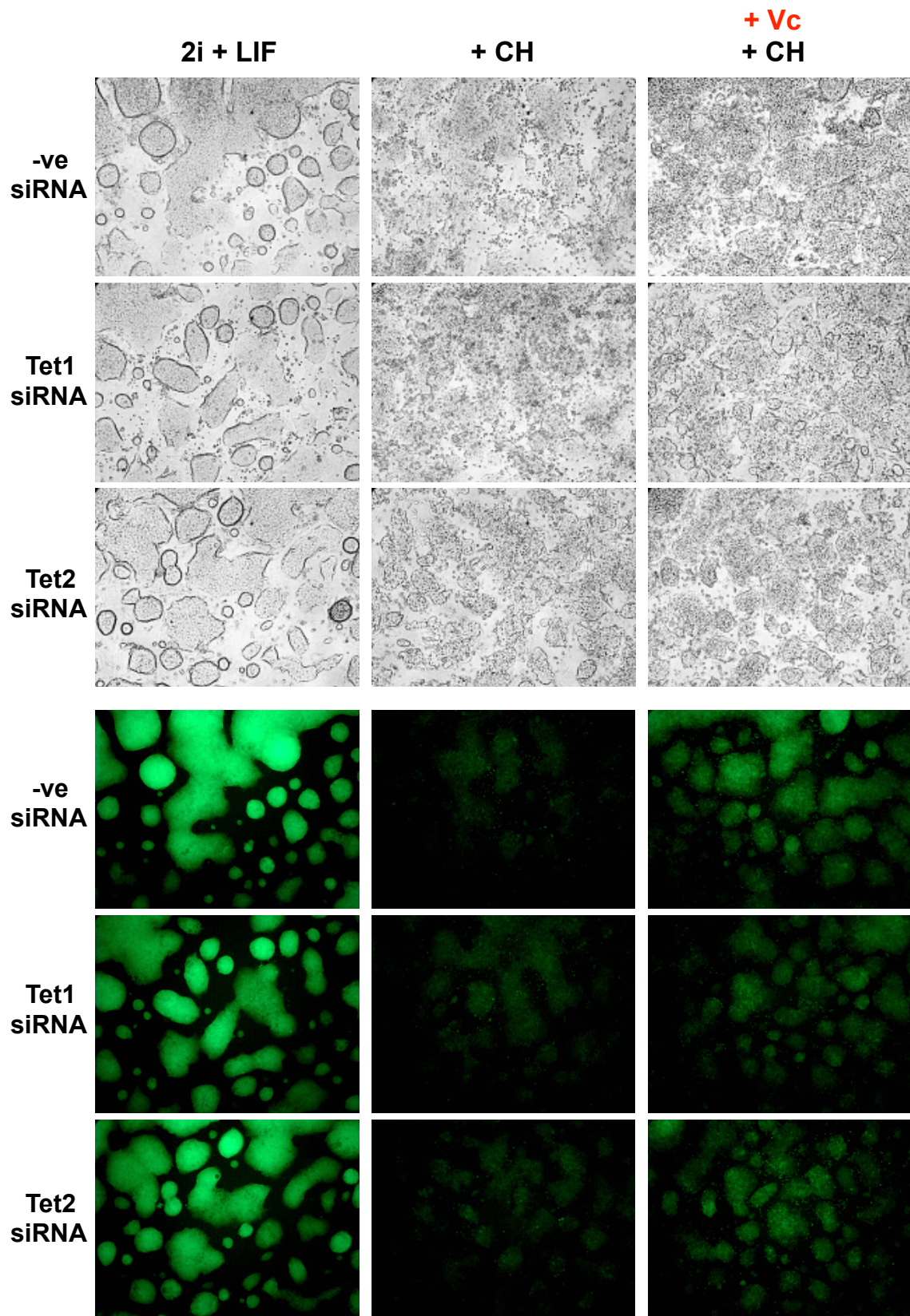
B

Figure 6.2. Effects of Tet1 and Tet2 knockdown. (A): Bright-field and fluorescence images (magnification X10) of rESCs (123-2 RexGFP homozygous) transfected with the appropriate siRNAs for 24 hours and subsequently cultured in media containing 2i + LIF, 2i (7 μM CHIR) or 2i (7 μM CHIR) + 100μM Vitamin C for 3 days. **(B):** These cells we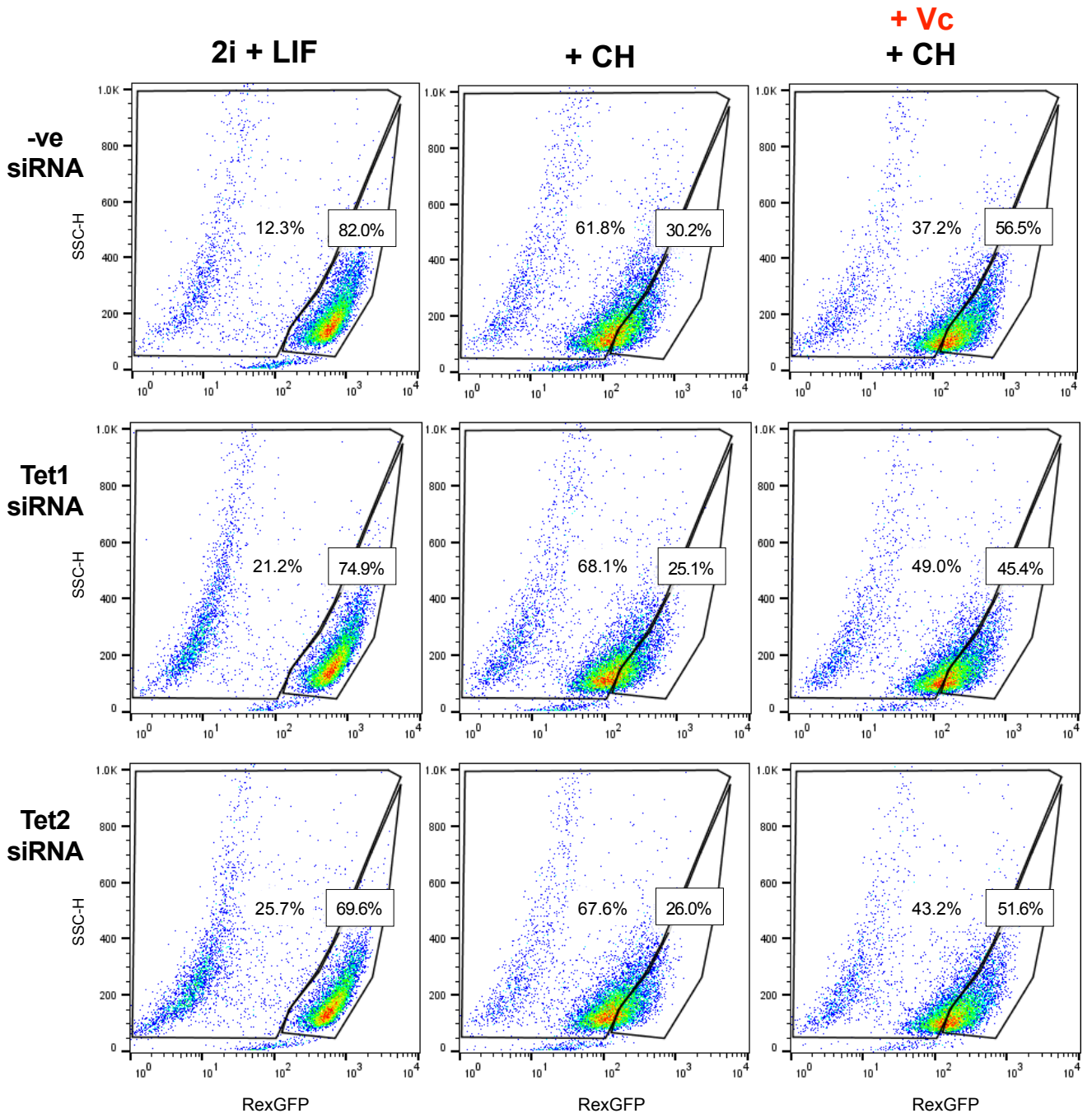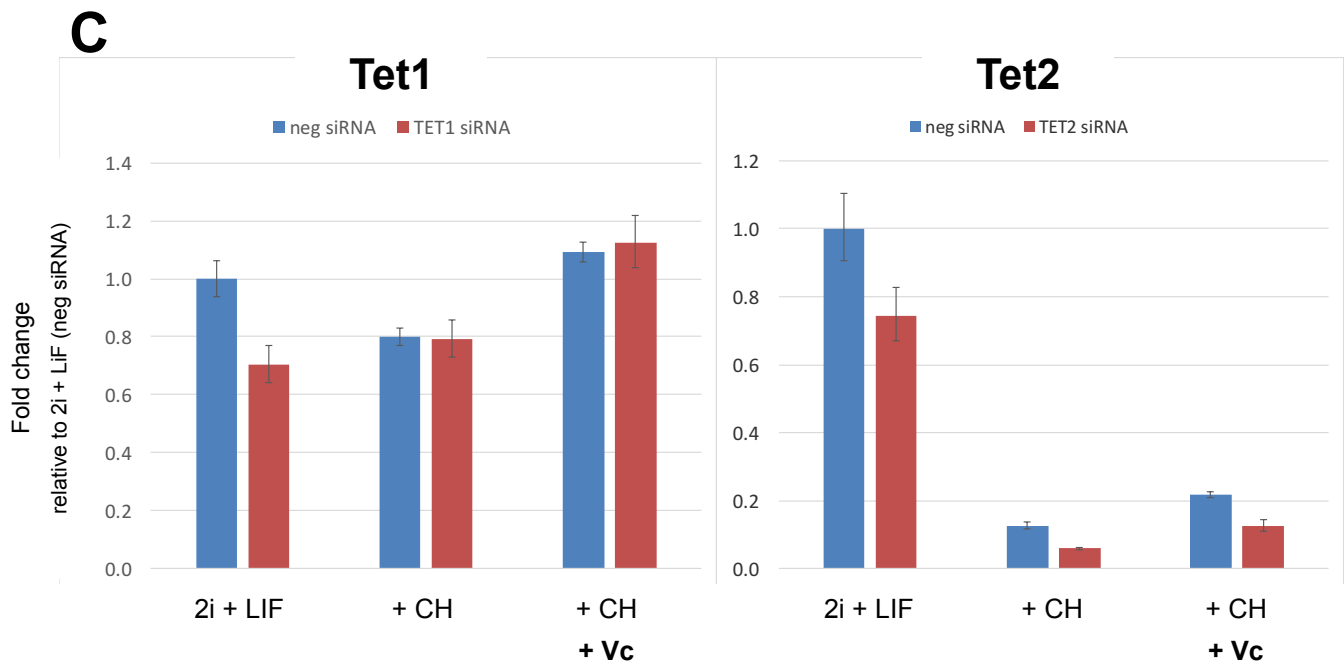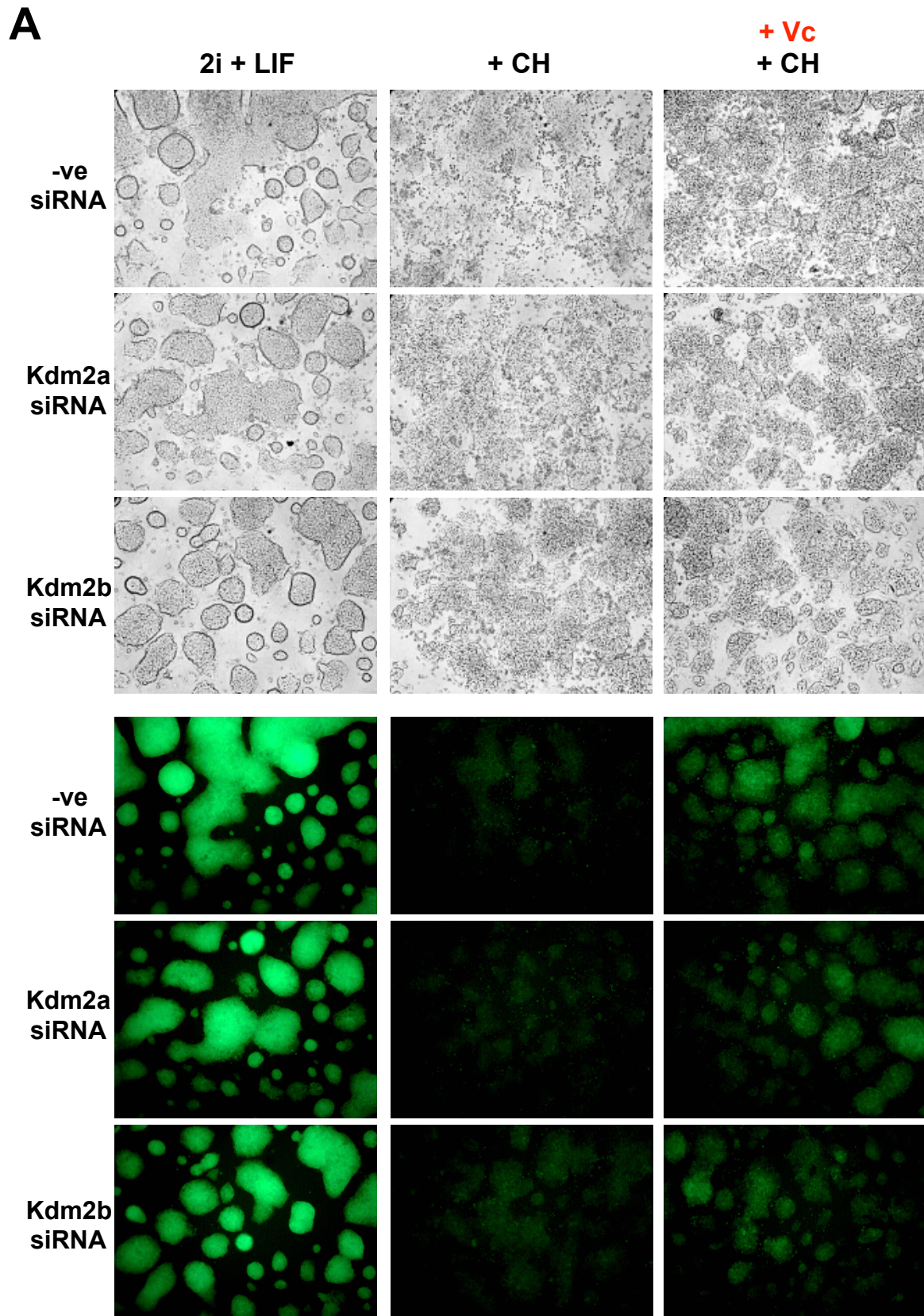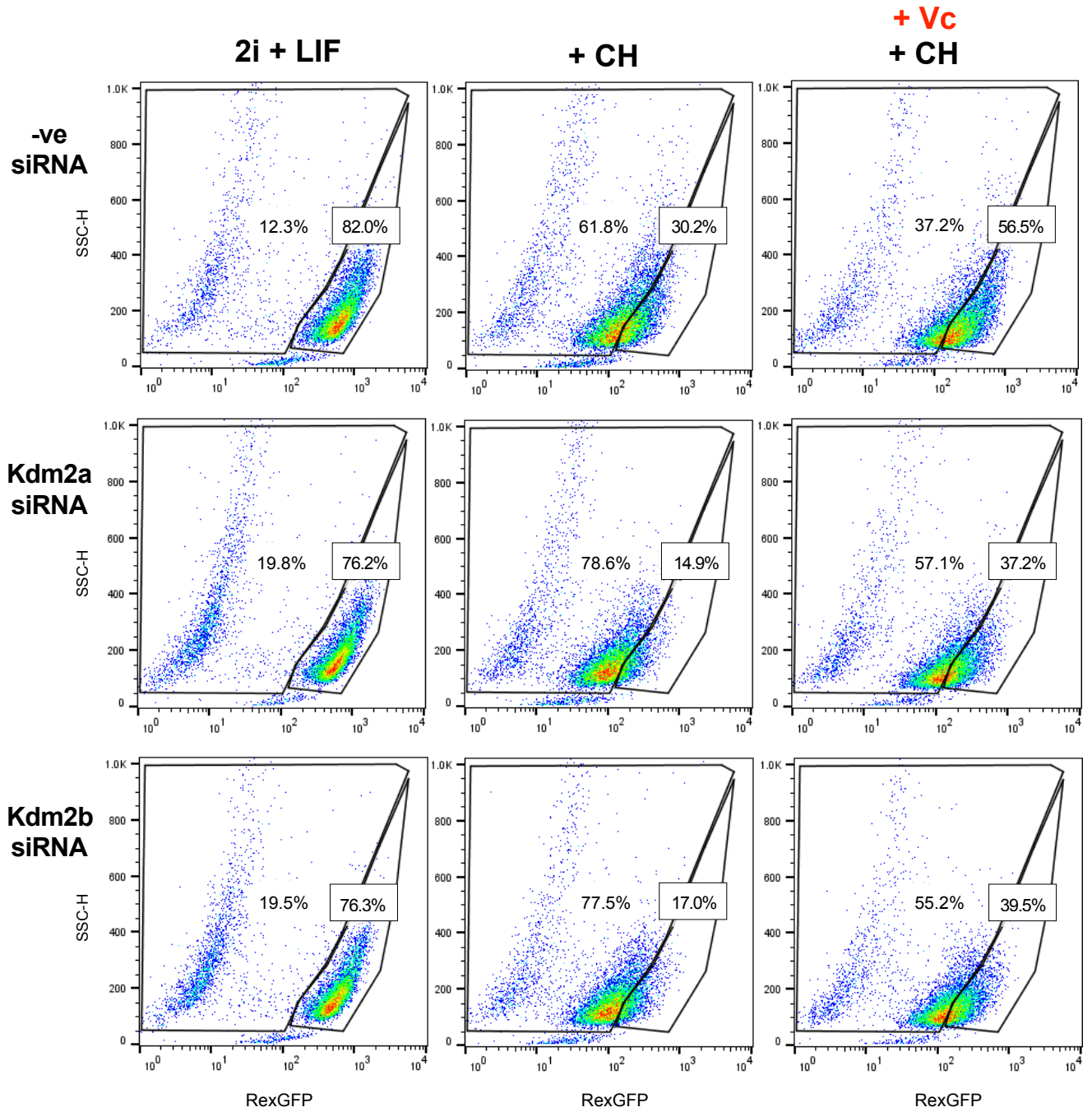re analysed (1x10^4 cells) by flow cytometry at day 3. **(C):** Validation of siRNA knockdown of Tet1 and Tet2. qRT-PCR analysis for Tet1 and Tet2 mRNAs. The graphs indicate the level of gene expressions in those transfected rESCs. Abbreviations: CH (CHIR), CHIR99021; Vc, L-ascorbic acid (Vitamin C); -ve siRNA (neg siRNA, negative siRNA), Control siRNA.

In Figure 6.2, in the presence of negative siRNA, the 2i + LIF ESC cultures contained 82% Rex1-positive cells, whereas only 30% undifferentiated population was found in the high CHIR media. When Vc was added, 57% cells remained in a pluripotent state, indicating a 1.87 fold rescue by Vc. For Tet1 and Tet2, an equivalent ESC population of 70-75% was measured in 2i+LIF with both siRNAs independently, while a Rex1-GFP positive population of 25-26% remained in high CHIR cultures with both siRNAs. This indicates loss of Rex1-GFP observed in all high CHIR conditions. Upon treatment of Vitamin C, a pluripotent cell population of 45-52% was maintained with both siRNAs indicating a 1.8-2.0 fold rescue by Vitamin C that is comparable with the –ve siRNA. In conclusion, knockdown of Tet1 and Tet2 has no significant involvement in Vc-mediated activity. This indicates Tet1 and Tet2 may not be the downstream targets of the Vitamin C effects.

In order to validate Tet knockdown, qRT-PCR analysis was performed. Upon treatment of Tet1 siRNA, there was no reduction in Tet1 mRNAs, except 30% knockdown in the 2i + LIF condition. Likewise, only 30% knockdown was observed in 2i+LIF conditions with Tet2 siRNA. Interestingly, the level of Tet2 expression was greatly reduced in all CH+ conditions, suggesting that expression of the Tet2 gene may be down-regulated under β catenin induced differentiation.

To knockdown the potential Vc targets Kdm2a & b, rESCs were transfected with the specific siRNAs for 24 hours and cultured in media containing 2i+LIF or 2i contains 7 µM CHIR with or without 100µM Vitamin C for 3 days.

B

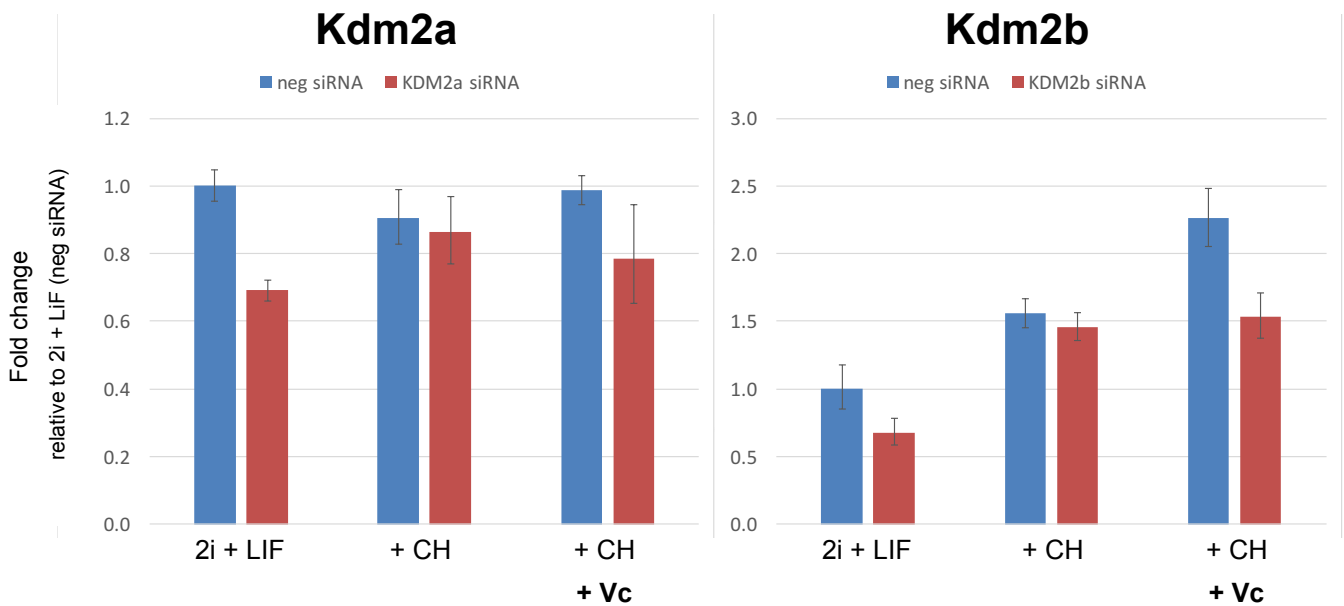|	2i + LIF	+ CH	**+ Vc** + CH
-ve siRNA	12.3% 82.0%	61.8% 30.2%	37.2% 56.5%
Kdm2a siRNA	19.8% 76.2%	78.6% 14.9%	57.1% 37.2%
Kdm2b siRNA	19.5% 76.3%	77.5% 17.0%	55.2% 39.5%

C

Figure 6.3. Effects of Kdm2a and Kdm2b knockdown. (A): Bright-field and fluorescence images (magnification X10) of rESCs (123-2 RexGFP homozygous) transfected with the appropriate siRNAs for 24 hours and subsequently cultured in media containing 2i + LIF, 2i (7 µM CHIR) or 2i (7 µM CHIR) + 100µM Vitamin C for 3 days. **(B):** These cells were analysed (1x10⁴ cells) by flow cytometry at day 3. **(C):** Validation of siRNA knockdown of Kdm2a and Kdm2b. qRT-PCR analysis for Kdm2a and Kdm2b mRNAs. The graphs indicate the level of gene expressions in those transfected rESCs. Abbreviations: CH (CHIR), CHIR99021; Vc, L-ascorbic acid (Vitamin C); -ve siRNA (neg siRNA, negative siRNA), Control siRNA.

Despite Kdm2a and Kdm2b knockdown, an equivalent Rex1-EGFP positive population of 76% was observed in 2i+LIF with both siRNAs independently (Figure 6.3). In high CHIR conditions, the pluripotent cell population reduced to only 15-17% with both siRNAs. In the presence of Vc, the Rex1-GFP positive population increased to 37-40% with both siRNAs, compared to 57% with the –ve siRNA. This indicates a 2.3-2.5 fold rescue by Vitamin C upon Kdm2a or Kdm2b knockdown, compared with only 1.87 fold with the –ve siRNA. Indeed, we expected that Vc-rescued ESC population would be diminished by a lack of expression levels of those potential Vc target genes. Interestingly, however, Kdm2 knockdown enhanced the differentiation in high CHIR conditions, suggesting that Kdm2 is a negative regulator of differentiation. Vitamin C rescued more ESC populations, as knockdown of Kdm2a and Kdm2b mRNAs decreased higher percentage of pluripotent cells in high CHIR cultures, when compared to the CH+ cultures containing negative siRNAs. The fact that the increase in differentiation is corrected by addition of Vc indicates that Kdm2 does not mediate Vc effect.

In order to validate our results above, qRT-PCR analysis for Kdm2a and Kdm2b genes was performed. In the presence of Kdm2a siRNA, there was no significant mRNA reduction in all high CHIR cultures, and only 30% knockdown in the 2i + LIF conditions. In

addition, the expression levels of Kdm2b were increased in all CH+ conditions by at least 50%, suggesting that Kdm2b may interact with β catenin activity.

Discussion

Issues of siRNA activity and duration

To knockdown target genes, siRNA was incorporated in our experiments. RNA interference (RNAi) system is found in eukaryotes to defend against RNA viruses (Hannon 2002). In the presence of a RNA viral genome, which is double-stranded RNA (dsRNA), a nuclease Dicer cleaves dsRNA into short fragments (short interfering RNA, siRNA) (Kim and Rossi 2008, Davidson and McCray 2011). Subsequently, RISC (RNA-induced silencing complex) binds this siRNA, and separates the strands (Kim and Rossi 2008, Davidson and McCray 2011). Using this siRNA strand, RISC then base-pairs with and degrades mRNA of infecting RNA virus (Hannon 2002).

The benefits of using siRNA is that it is not constitutive, and there is no need to select mutant cell lines (Wilson and Doudna 2013, Barrangou, Birmingham et al. 2015). As for siRNA transfection, negatively charged siRNAs combine with positively charged lipid vesicles (liposomes) to produce complexes of liposome-siRNAs (Shen, 2016). Since these complexes have net positive charges, they allow the cells to be transfected with the siRNAs in cell cultures (Shen, 2016). Subsequently, siRNAs induce degradation of target mRNAs during the first 24 hour-incubation (Petrocca and Lieberman 2011).

When we transfected rESCs with Lef1 siRNAs, the degree of knockdown was good enough to maintain ESC self-renewal. We also clearly validated siRNA knockdown of Tet1, Tet2, Kdm2a and Kdm2b in rESCs cultured under 2i + LIF conditions. However, we detected only limited mRNA reduction when we conducted transfection of siRNAs in the CHIR/Vc treated cells (Figure 6.2 and 6.3). Indeed, the degree of knockdown could be affected by a number of variables, for example, state of cells, cell culture media, duration of siRNA incubation, cell number, amount of transfection reagent, and amount of donor DNA (Hannon 2002, Agrawal, Dasaradhi et al. 2003, Kim and Rossi 2008, Davidson and McCray 2011, Wilson and Doudna 2013).

Practically, there are several ways we can improve efficiency of transfection and siRNA knockdown. In our experiments, we transfected ESCs with relevant siRNAs in each culture condition, in an attempt to facilitate the knock-down during the period of CHIR induced differentiation. This may have introduced different levels of knockdown in treatments despite containing the same siRNAs. Therefore, it would also be worthwhile to determine if transfecting ESCs cultures with the same siRNAs and then subdividing them into the appropriate culture conditions could reduce any variability in degree of knock-down. In this way, we can remove the different efficiency between cell culture conditions containing the identical siRNAs. However, the problem involved in this experiment is that we needed to culture the cells from day 0 in different conditions (2i+LIF culture and high CHIR culture with Vc or without Vc).

In addition, our experiment confirmed siRNA knockdown at day 3, but demonstrated no knockdown at day 4 (Figure 6.1E). This highlights that siRNA activity is transient and may last just for a couple of days, as the cells divide (Barrangou, Birmingham et al. 2015). Thus, we might not see the knockdown clearly between day 3 and day 4, although optimal knockdown may have occurred in the first 48h (Figure 6.5). Furthermore, the experimental time-window for assessing phe-

notypes is limited by the duration of the transient siRNA activity. Indeed, our experiment required at least 3 to 4 days for both Vitamin C and high CHIR effects clearly to be seen in ESCs. Although we validated siRNA activity and observed Vitamin C effects at day 3, this 72 hour-duration was too short for ESCs to be fully influenced by Vitamin C and differentiated by CHIR, because there is a delay in phenotypic expressions between DNA, mRNA and proteins. At the same time, the three/ four day culture was, on the other hand, the limit to observe sustained suppression of siRNA target activity. One of the approaches we can address this issue is to transfect cells with siRNAs every two days (Sridharan, Gonzales-Cope et al. 2013, Barrangou, Birmingham et al. 2015). In this way, we can retain siRNA knockdown throughout the experiments and can secure enough duration for Vc and CHIR treatments, although other technical variables should also be considered.
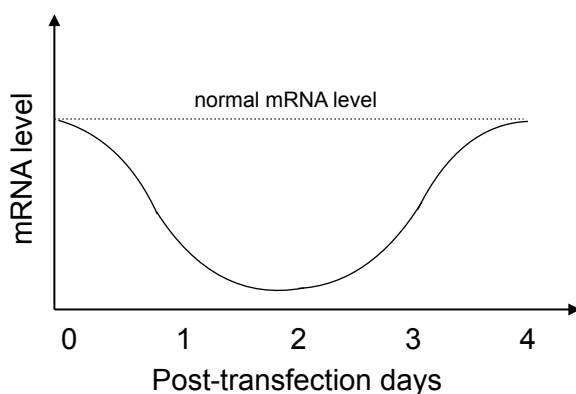

Figure 6.5 Hypothetical model of siRNA activity duration. Under the optimal ESC conditions, genes express consistently at the normal mRNA level. However, after siRNA transfection, mRNA level decreases, and its knockdown level disappears between post-transfection day 3 and 4, depending on the various factors such as duration of siRNA incubation, cell number, amount of transfection reagent and amount of donor DNA (Hannon 2002; Agrawal, et al. 2003; Kim and Rossi 2008; Davidson and McCray 2011; Wilson and Doudna 2013).

Since the discovery of siRNAs (Hamilton and Baulcombe 1999, Elbashir, Harborth et al. 2001), this approach has been reported to posses some limitations. When a high levels of of siRNAs are introduced, they can be recognised as a viral by-product and activate innate immune responses; consequently non-specific effects may occur (Whitehead, Dahlman et al. 2011). In addition, siRNA may cause off-target effects (Birmingham, Anderson et al. 2006, Shen, Sun et al. 2012). Moreover, especially as for targeting enzymes, siRNA knockdown should be carefully considered to use them. It has not been discovered how much enzyme activity is required for rat ESCs. It can be possible that just 10% activity may be enough for rESCs to respond normally (Barrangou, Birmingham et al. 2015). siRNA can also affect the viability of transfected cell cultures (Doudna and Charpentier 2014; Arunkumar, 2015).

To overcome these limitations, CRISPR-Cas9-mediated genome editing can be employed (Jinek, Chylinski et al. 2012). This CRISPR system originates from the bacterial immune system as it can defend bacteria against invading DNA and RNA viruses by destroying their viral genomes (Barrangou, Birmingham et al. 2015). In the presence of virus genome, the CRISPR domains on the bacterial chromosome are transcribed into RNA molecules, which are subsequently cleaved into segments by certain endonuclease CAS proteins (Doudna and Charpentier 2014). When this CRISPR RNA segment bound to other CAS enzymes, recognises virus RNA or DNA (target genes), their CAS proteins can cut and destroy the viral nucleic acids (Jinek, Chylinski et al. 2012,

Cong, Ran et al. 2013, Jinek, East et al. 2013, Mali, Yang et al. 2013). This bacterial immune system has been repurposed for gene editing in mammalian cells using a Cas9 nuclease that cleaves DNA flanked by a PAM (protospacer-adjacent motif) with the help of a single guide RNA (sgRNA) or a CRISPR RNA (crRNA) and a trans-activating crRNA (Brouns, Jore et al. 2008, Garneau, Dupuis et al. 2010, Deltcheva, Chylinski et al. 2011, Gasiunas, Barrangou et al. 2012, Jinek, Chylinski et al. 2012). This technology contains multiple advantages over RNA interference-based gene silencing (e.g. shRNA and siRNA). For example, the CRISPR-Cas9 based genome engineering can knockout target genes by disrupting a genes reading frame (Barrangou, Birmingham et al. 2015, Chu, Rios et al. 2015). The Cas9 nuclease generates a double-strand break (DSB) in the intended target DNA (Jinek, Chylinski et al. 2012, Cong, Ran et al. 2013, Jinek, East et al. 2013, Mali, Yang et al. 2013). The DSB can be repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Chapman et al. 2012). The NHEJ creates unpredictable small insertions or deletions, resulting in a potential inactivating mutation in the targeted DNA, whereas the HDR is utilised for precise and predictable modifications at the targeted loci (Barrangou, Birmingham et al. 2015). The Cas9 endonuclease can also act as a nickase that cleaves single-stranded DNA when one of its two nuclease domains HNH and RuvC-like is inactivated (Bolotin, Quinquis et al. 2005, Haft, Selengut et al. 2005, Makarova, Grishin et al. 2006, Barrangou, Birmingham et al. 2015). In addition, this system can be engineered to function as a repressor to suppress a target gene for a longer period of time (Doudna and Charpentier 2014, Barrangou, Birmingham et al. 2015), hence removes a requirement for the every two day delivery of siRNAs (Arunkumar, 2015).

In contrast to RNAi mechanism employed by eukaryotes, CRISPR/Cas9 systems lack in eukaryotes (Doudna and Charpentier 2014). This makes CRISPR system more important, providing different applications in that competition with endogenous eukaryotic signalling is problematic. In particular, utilising RNAi to down-regulate genes that are responsible for the RNAi signalling itself (for example, Dicer) can generate data which are complicated to analyse, because of unpredictable direct and indirect influences involved (Doudna and Charpentier 2014). Possibly, any RNA molecules utilised to reduce the expression levels of certain genes might compete with endogenous RNA-dependent gene expression. However, CRISPR-Cas9 can permanently edit the genetic code and regulate gene expression transcriptionally or post-transcriptionally; hence, this offers a great versatility in utilising alternatives, while RNAi is mainly restricted to reducing expression of genes (Doudna and Charpentier 2014). Limitation of CRISPR/Cas9 mutagenesis is that it cannot be used for essential genes, since the mutated clones cannot survive (Singh, Schimenti et al. 2015). Taken together, although RNAi system has been widely used for gene silencing over many years, several issues such as off-targeting and incomplete and transient knockdowns are the limitations of this technology (Moore, Guthrie et al. 2010, Davidson and McCray 2011), and CRISPR systems can overcome these issues as they posses multiple advantages over RNAi-based gene silencing (Harrison, Jenkins et al. 2014).

Knockdown of Tet1/2 and Kdm2a/2b

Although the degree of target gene depletion was limited in the CHIR/Vc treated cells, overall Tet and Kdm siRNAs degraded mRNAs. In the test transfections, we have demonstrated

marked reduction of target mRNA transcripts (Figure 6.1), however, in the actual experiments, when the effects of knockdown on the Vc-treated cells were measured after 3 days, we did not detect similar kind of knockdown phenotypes (Figure 6.2 and 6.3). This is because siRNA is transient, as demonstrated by data in Figure 6.1E; the level of knockdown disappears between day 3 and day 4. In addition, in the experimental knockdown, the high CHIR conditions or addition of Vitamin C may affect the level or duration of knockdown, compared with 2i+LIF conditions. In Figure 6.2C and 6.3C, while all four target siRNAs have clearly knocked down mRNAs in all 2i + LIF cultures, some of the mRNA reduction seemed to disappear in high CHIR conditions. This suggests that CHIR or Vitamin C may also affect the degree of siRNA metabolism (Abe, Goda et al. 2009, Christensen, Litherland et al. 2013, Christensen, Litherland et al. 2014); this might alter the mRNA knockdown efficiency, which varies between different experiments (Zhang, Guller et al. 2007, Davidson and McCray 2011).

It has been known that Vitamin C induces pluripotency acting through TET1 and TET2 (Blaschke, Ebata et al. 2013, Chen, Guo et al. 2013, Yin, Mao et al. 2013, Gustafson, Yang et al. 2015). However, our data in Figure 6.2 demonstrated that treatment of Vitamin C increased pluripotent cells 1.8-2.0 fold upon knockdown of Tet1 and Tet2; this ratio of Vc rescue was equivalent to that of control siRNAs (1.87 fold). However the effect of knockdown is limited by the residual activity of Tet1 and Tet2, or compensation by other TET family members (Wu and Zhang 2011). Interestingly, according to the qRT-PCR analysis for Tet genes, Tet2 expression was endogenously reduced upon all CH+ treatments. This may indicate that β catenin-induced differentiation silences Tet2 gene. Recently, it was demonstrated that Tet2 supports naïve pluripotency, and it can be repressed transcriptionally and post-transcriptionally for primed pluripotency, in contrast Tet1 facilitates primed pluripotent states (Fidalgo, Huang et al. 2016). Based on this result, Tet2 may critically function in promoting ESC self-renewal and decreasing differentiation. It would be interesting to determine if TET2 over-expression could resist CH-induced differentiation. In addition, although the TET2 transcript is reduced, we should also determine that TET2 protein is similarly reduced. The semi-quantitative technique, Western blotting could be used to test the Tet2 expression at the protein levels (Taylor and Posch 2014).

Another published paper also argued that, induction of pluripotency is markedly improved by Vitamin C-dependent enzymes, such as KDM2A and KDM2B (Wang, Chen et al. 2011). Interestingly, transfection with either Kdm2 siRNA reduced the level of Rex-EGFP in high CHIR, suggesting that Kdm2 is a negative regulator of differentiation. However, this increase in differentiation is corrected by addition of Vc, indicating that Kdm2 does not mediate Vc effect (Figure 6.3B). A recent paper by Lu et al. (2015) discovered that in the nucleus, non-phosphorylated β catenin is methylated by Kdm2, thereby leading to its ubiquitination and degradation (Fagotto 2015, Lu, Gao et al. 2015). Based on their demonstrations, it is easy to speculate that Kdm2 knockdown can stabilise and promote β catenin activity, and may upregulate differentiation markers in Figure 6.6 (Chen, Blair et al. 2013, Lu, Gao et al. 2015). This may explain why those ESCs in high CHIR treated with Kdm2a and Kdm2b siRNAs are more differentiated. In summary, the key interesting

findings here are that Kdm2 modulates β catenin signaling, such that its knockdown enhances β catenin-induced differentiation, and despite this enhanced differentiation, Vc retains the capacity to rescue self-renewal.
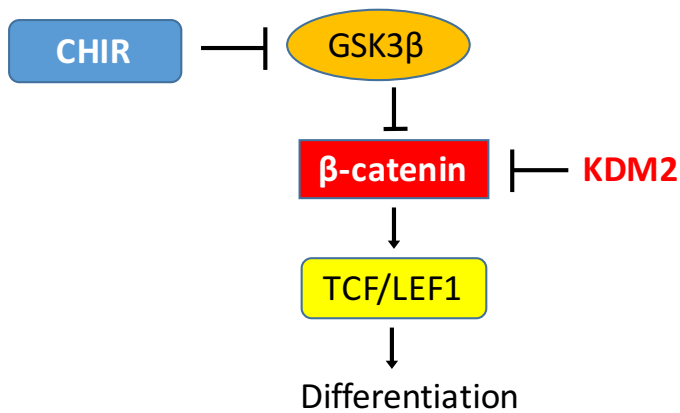

Figure 6.6. Diagram of potential β catenin control by KDM2. GSK3β activity is blocked by its inhibitor, CHIR leading to loss of phosphorylation and stabilization of β catenin. KDM2 enzymes de-methylate non-phosphorylated β catenin inducing its degradation to prevent ESC differentiation (Chen et al. 2013; Meek et al. 2013; Lu et al. 2015). Abbreviations: CHIR, CHIR99021. *(Created using information from different sources).*

In addition, using qRT-PCR analysis for Kdm2 mRNAs, we found that Kdm2b expression levels were noticeably increased in all high CHIR conditions by at least 50%. This may suggest that Kdm2b is upregulated under high β catenin activity. Recently, KDM2B enzymes were reported to suppress WNT signalling by transcriptionally regulating several ligands (Andricovich, Kai et al. 2016). In addition, downregulation of Kdm2b in Xenopus, zebrafish, and mice led to embryonic lethality (Huang, Kathrein et al. 2013, Lu, Gao et al. 2015, Andricovich, Kai et al. 2016), highlighting the significance of Kdm2b in cell development. Hence, we surmise that, in response to excessive β catenin activity caused by high CHIR treatment, KDM2B may increase to prevent the abnormal level of β catenin activity in ESCs as a negative feedback; this may help rescue developing cells from the embryonic lethality. It would be interesting to determine if over-expressing Kdm2b during high CH cultures would prevent differentiation by modulating β catenin activity.

In addition to Tet and Kdm proteins, there are many potential enzyme targets of Vitamin C. This is because Vitamin C functions as an electron donor in any iron-dependent proteins (Monfort and Wutz 2013). Therefore, it may not be a single target that drives most of the Vc-dependent activities. Rather than the single factor, a range of Vc targets may likely form a global network and associate with other factors, presenting their effects on growth (Figure 4.3) and differentiation (Figure 4.9) of rESCs.

7. Conclusions and Future Perspectives

Rat ESCs can be used to produce complex genetic models (Buehr, Meek et al. 2008, Meek, Buehr et al. 2010). They are derived from the blastocyst-stage embryo using the culture medium containing 2i plus LIF that promote self-renewal and prevent differentiation (Buehr et al., 2008; Li et al., 2008). However, the long term culture of rESC in 2i + LIF medium displays higher expression levels of differentiation genes (Meek, Wei et al. 2013). Esteban et al. (2010) demonstrated that Vitamin C improves the growth and formation of mouse induced pluripotent stem (iPS) cell colonies. The importance of Vitamin C in somatic cell reprogramming protocols suggested that this agent could also play a role in regulating the pluripotency of rESCs (Esteban, Wang et al. 2010, Shi, Zhao et al. 2010, Esteban and Pei 2012). Vitamin C is known as an antioxidant and retains the activity of iron-mediated demethylases that can change the epigenetic profile of cells (Monfort and Wutz 2013). In spite of these dioxygenases being involved in a variety of cell signalling pathways, Vitamin C is seldom used in standard ESC culture media. Here we have demonstrated the benefits of epigenetic modulator Vitamin C in rESC self-renewal and pluripotency.

Our data indicated that Vitamin C improves growth of rat ESCs. In addition, Vitamin C prevents ESCs from β catenin-induced differentiation, by upregulating pluripotency genes (e.g. Esrrb and Nanog) and downregulating differentiation genes (e.g. Gata4 and Brachyury). Although these Vitamin C effects were measured by different approaches, both two influences on growth and ESC differentiation may originate and be potentially regulated from the same Vitamin C downstream targets. It is possible that cell division cycle can affect ESC differentiation (Li, Ballabeni et al. 2012, Li and Kirschner 2014). ESCs are characterised by a rapid cell cycle structure with shortened G1 and G2 phases, and a high proportion of cells in S phase (White and Dalton 2005, Orford and Scadden 2008, Hindley and Philpott 2013, Kapinas, Grandy et al. 2013). If the G1 phase of cell cycle is truncated, and cells are driven to grow more rapidly, ESC differentiation can be suppressed (Li, Ballabeni et al. 2012, Li and Kirschner 2014, Re, Workman et al. 2014). Conversely, if G1 phase is lengthened, early multi-lineage differentiation is induced in ESCs (Calder, Roth-Albin et al. 2013). The interaction between ESC differentiation and cell cycle can be assessed by the co-regulated genes. These overlapping genes are primarily observed to be the genes responsible for cell cycle regulation, specifically, replication factors (e.g. PCNA, MCM6, CKS1B, CKS2, and CDC6) and cyclins (e.g. CCNB1, CCNA2 and CCNE2) as well as mitotic spindle assembly factors (e.g. CDC20 and TACC3) (Re, Workman et al. 2014). In addition, there are also other factors that perform dual roles in differentiation and cell proliferation, such as H1 linker histone, known to regulate mitotic chromosome and Hox gene (Maresca, Freedman et al. 2005, Zhang, Liu et al. 2012) and DNA replication inhibitor (Geminin), known to regulate transition between pluripotency and lineage commitment (Lim, Hummert et al. 2011, Re, Workman et al. 2014). More importantly, previous studies argued that the abbreviated cell cycle is functionally connected to pluripotency (Singh and Dalton 2009, Ghule, Medina et al. 2011). In particular, Nanog, Oct4 and Myc are positively correlated with cell cycle factors, such as CDKs and cohesin factors (e.g. RAD21) (Singh and Dalton 2009, Nitzsche, Paszkowski-Rogacz et al. 2011). In summary, the interaction between early lin-

eage-commitment and cell cycle regulation indicates that Vitamin C effects on growth and differen-tiation may originate from the identical targets. Therefore, in the future experiments, we should also analyse cell cycle changes in the presence of Vitamin C. This will give more specific ideas how rESCs are regulated by Vc and may highlight the potential downstream targets.

We discovered that Vitamin C does not block MEK-driven differentiation (Figure 4.6), but it does prevent β catenin-dependent differentiation (Figure 4.9). Based on the result that D-isoascor-bic acid, the Vitamin C isomer, lacking cofactor activity has little effect on β catenin-induced differ-entiation (Figure 4.11), we can conclude that the Vitamin C effect primarily results from cofactor activity. In our experiments, specific Tet and Kdm genes were knocked individually. However, knockdown of each target independently can be very weak, because there are other Tet and Kdm subtype members (Klose, Kallin et al. 2006, Tan and Shi 2012, Kohli and Zhang 2013, Dimitrova, Turberfield et al. 2015). Tet1 catalyses oxidation of the 5-position of cytosine (5-methylcytosine, 5mC) at transcription start sites and gene promoters, while Tet2 catalyses oxidation of 5mC in exon boundaries and gene bodies of actively-expressed exons and genes, respectively (Huang, Chavez et al. 2014). Despite the dissimilarity between them, it can be possible that Tet1 and Tet2 compen-sate for each other, due to their overlapping functions in catalysing oxidation of 5mC for the re-moval of DNA methylation (Tan and Shi 2012, Kohli and Zhang 2013, Hill, Amouroux et al. 2014). According to this overlap, silencing a single gene may produce no dramatic phenotype changes, unless one enzyme is vital in their cell signalling pathways. Hence it would be noteworthy to knockdown both associated members, for example, Tet1 and Tet2 together or Kdm2a and Kdm2b.

As an alternative to the use of siRNAs, there is another option of employing short hairpin RNA (shRNA) technique (Paddison, Caudy et al. 2002). shRNA is a double stranded RNA (dsRNA) containing a hairpin loop, which can silence genes through the RNAi system (Brummelkamp, Bernards et al. 2002, Paddison, Caudy et al. 2002). siRNA can occur in the cytoplasm or can be externally made and introduced into the cell, whereas shRNA is synthesised from a DNA construct delivered to the nucleus of the target cell via viral vectors (gene expression cassettes) (Rao, Vorhies et al. 2009). The DNA construct encodes a sequence of single stranded RNA (ssRNA) molecule and its complement, divided by a certain fragment that allows the ssRNA to fold back on itself, producing a dsRNA with a hairpin turn (Rao, Vorhies et al. 2009). The delivered DNA can be-come part of the target cell's own genome and make this host cell to synthesise the shRNA mol-ecule, which is subsequently processed by the endoribonuclease Dicer into siRNA and proceeds along the pathway of RNAi via RNA-induced silencing complex (RISC) to silence targets (Rao, Vorhies et al. 2009). Since shRNA assimilates into endogenous microRNA pathways, it is markedly more efficient than siRNA (Siolas, Lerner et al. 2005, McAnuff, Rettig et al. 2007, Vlassov, Korba et al. 2007) and shows low degradation of exogenously introduced shRNA. Specifically, just 1% of the introduced siRNA remains in the target cells 48 hours after transfection, whereas shRNA are con-tinuously generated by the host cell's own machinery, highlighting that its effect is more durable (Rao, Vorhies et al. 2009). In addition, siRNA concentrations required for efficient knockdown are relatively high (that is, in the nano-mole range), whereas just 5 copies of shRNA incorporated in

host DNA is sufficient to give continual knockdown effect; this low dosage indicates that shRNA is less likely to exhibit 'off-target' effects than siRNA (Rao, Vorhies et al. 2009). Also, shRNA shows lower toxicity, immune activation and inflammation (Rao, Vorhies et al. 2009). On the other hand, the limitation is the use of an expression vector that may have safety issues (Wang, Rao et al. 2011).

Since RNAi-based systems including siRNA and shRNA are comprised of RNA molecules, it is inherently fragile and naturally difficult to enter the cytoplasm; once there, it is rapidly degraded (Rao, Vorhies et al. 2009). Due to this, relatively high concentrations are usually employed for efficient gene silencing, and also the administration periodically needs to be repeated. In short, they are transient, short lived and only a small percentage of cells are transfected. Therefore, CRISPR-Cas9 gene editing is required to accurately assess prime candidates of Vitamin C for the study of their roles (Jinek et al. 2012). This CRISPR-Cas technology stems from a microbial adaptive immune system, as it can be used to protect bacteria and archaea against invading viruses by degrading their viral genomes (Ran, Hsu et al. 2013, Barrangou, Birmingham et al. 2015). With the aid of guide RNA (gRNA) containing a 20-nt targeting sequence, Cas9 nucleases can cleave the target gene flanked by the PAM sequence in Figure 6.7 (Brouns, Jore et al. 2008, Garneau, Dupuis et al. 2010, Deltcheva, Chylinski et al. 2011, Jinek, Chylinski et al. 2012, Ran, Hsu et al. 2013). This CRISPR-Cas approach is highly specific and efficient, and also relatively easy to design for genome editing for a range of cell lines (Ran, Hsu et al. 2013). CRISPR-mediated gene knockout can be conducted by designing and constructing CRISPR plasmids to chosen targets, and transfecting into ESCs. Additionally, the degree of site-specific mutation should be measured to validate and determine the efficiency of CRISPR activity. Transfecting validated CRISPR into ESC will knock-out targets only if the NHEJ generates a mutation that creates a frameshift, or the HDR is employed to make specific changes (Ran, Hsu et al. 2013, Doudna and Charpentier 2014, Barrangou, Birmingham et al. 2015). Subsequently, comparing control and knock-out cell lines in the presence or absence of Vitamin C will identify exact role of Vitamin C-mediated downstream targets.
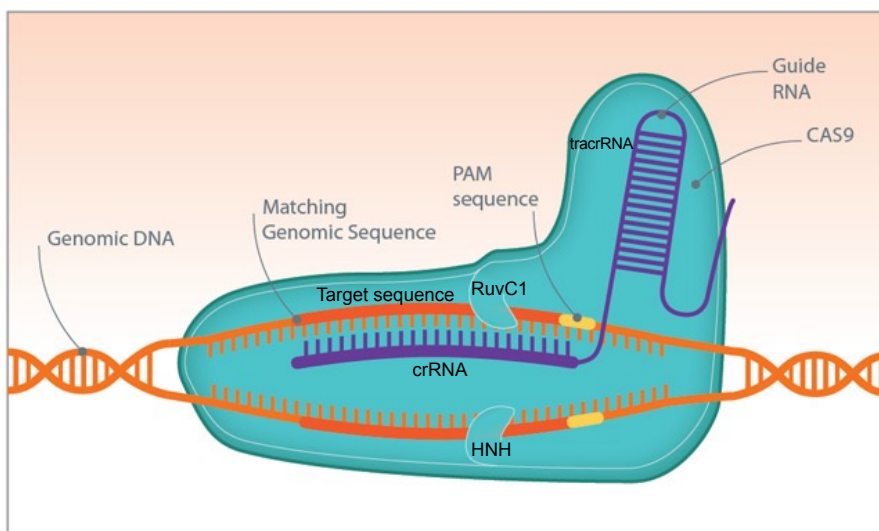

Figure 6.7. The CRISPR-Cas9 complex. Guide RNAs deliver Cas9 to the target DNA sequence followed by the PAM sequence. Cas9 then introduces a double strand break in the target DNA (Jinek, et al. 2012, Cong, et al. 2013, Jinek, et al. 2013, Mali, et al. 2013) *(Image adapted from Nash (2015) and edited).*

The CRISPR-Cas9 system can also be used to modulate expression of a gene of interest transcriptionally, not just at the level of genomic DNA (Barrangou, Birmingham et al. 2015). The CRISPR-associated protein Cas9 was identified to contain two nuclease domains HNH and RuvC-like in Figure 6.7 (Bolotin, Quinquis et al. 2005, Haft, Selengut et al. 2005, Makarova, Grishin et al. 2006). HNH domain is used to break the DNA strand which corresponds to the 20 nucleotide guide sequence of CRISPR RNA (crRNA), whereas RuvC-like domain breaks the DNA strand opposite the corresponding strand (Gasiunas, Barrangou et al. 2012, Jinek, Chylinski et al. 2012). Hence, mutating both HNH and RuvC-like domains can make a Cas9 endonuclease to act as an RNA-guided DNA binding factor (Gasiunas, Barrangou et al. 2012, Jinek, Chylinski et al. 2012). When fused to repressor or activator domains, this catalytically deactivated Cas9 (termed dCas9) can direct transcriptional suppression or activation of a target gene employing sgRNA (single guide RNA) directed against promoter sites; these techniques are called CRISPRi and CRISPRa (Cheng, Wang et al. 2013, Gilbert, Larson et al. 2013, Larson, Gilbert et al. 2013, Qi, Larson et al. 2013, Gilbert, Horlbeck et al. 2014, Konermann, Brigham et al. 2015) (advances 133-134). In terms of the CRISPRi technique, it can repress RNA polymerase binding, transcription factor binding or transcriptional elongation, according to the regions recognised by the complex of dCas9-guide RNA (Qi, Larson et al. 2013, Doudna and Charpentier 2014). Hence it contains analogous advantages to RNAi system, such as partial suppression, reversibility and transience (Guo, Barry et al. 2014, Barrangou, Birmingham et al. 2015). In addition to these similar advantages, this CRISPRi strategy was demonstrated to show no observable off-target effects (Qi, Larson et al. 2013), and to suppress multiple genes of interest simultaneously, and also its effects show reversible (Bikard, Jiang et al. 2013, Gilbert, Larson et al. 2013, Qi, Larson et al. 2013, Zhao, Dai et al. 2014).

According to our independent siRNA knockdown for each target (Figure 6.2A and 6.3A), we have not observed any significant phenotype differences with Tet1/2 or Kdm2a/2b siRNAs, compared to control siRNAs. However, using libraries of CRISPR vectors, we may identify phenotypes of cells having mutations in multiple Vitamin C-associated genes (Shalem, Sanjana et al. 2015). In previous experiments, genetic screens depended largely upon shRNA-based screens that involve extensive off-target effects and incomplete knockdown (Root, Hacohen et al. 2006, Shalem, Sanjana et al. 2015). By contrast, CRISPR technology can generate highly specific, genomic alteration in genes of interest (Barrangou, Birmingham et al. 2015). In addition, target gene recognition by a Cas9 nuclease requires only a small 20 nucleotide-homology between the target gene locus and the gRNA (Wu, Kriz et al. 2014, Tsai and Joung 2016). This aspect makes the CRISPR-Cas9 technology easy, fast and cost-effective for large scale functional genomic studies (Zhou, Zhu et al. 2014, Vidigal and Ventura 2015). CRISPR pooled libraries are composed of thousands of unique plasmid vectors, each expressing a gRNA designed to a different gene of interest (Shalem, Sanjana et al. 2014, Wang, Wei et al. 2014, Zhou, Zhu et al. 2014). In a genetic CRISPR screening experiment, the pooled library is administered to desired cells to produce a large population of mutant cells, which are then screened for a desired phenotype of interest (Shalem, Sanjana et al. 2015). Overall, CRISPR-based approach can be a powerful future approach for the identification of Vitamin C targets.

Our luciferase data demonstrated that β catenin activity is not directly regulated by Vitamin C (Figure 4.12), suggesting that it acts via an alternative pathway. In addition, our siRNA knockdown data indicated that Tet1 and Tet2 might not be actively involved in the Vitamin C-mediated activity, whereas Kdm2a and Kdm2b might be involved in regulating β catenin-induced differentiation. However the effect of knockdown is limited by the residual activity of Tet and Kdm. If those TET and KDM demethylases are accessed by the CRISPR-Cas9 afterward and it confirmed that they do not mediate Vitamin C activity, other Vitamin C-downstream candidates need to be screened.

Recently, histone 3 lysine 9 (H3K9) methylation has attracted some attention, because it acts as a potent roadblock in the process of induced pluripotency (Soufi, Donahue et al. 2012, Chen, Liu et al. 2013). Among histone demethylases that can be affected by cofactor activity of vitamin C, Kdm3a enzymes involved in spermatogenesis seem to have important roles in H3K9 methylation (Okada, Scott et al. 2007, Shi, Zhao et al. 2010). It has also been demonstrated that BIX01294, an inhibitor agent of G9a (H3K9 methyltransferase), can promote somatic cell reprogramming to induced pluripotent stem cells (iPSCs), suggesting the significance of H3K9 methylation (Feng, Ng et al. 2009, Shi, Zhao et al. 2010). Hence, it is tempting to knockdown the KDM3 families that inhibit formation of H3K9me1/me2 (Yamane, Toumazou et al. 2006, Kim, Kim et al. 2010, Kim, Kim et al. 2012) as well as KDM4 families which de-methylate H3K9me3 to see if Vitamin C effects rely on these demethylases (Cloos, Christensen et al. 2006, Fodor, Kubicek et al. 2006, Klose, Yamane et al. 2006, Whetstine, Nottke et al. 2006).

According to our results (Figure 6.3), KDM2 demethylases may be involved in maintaining ESC identity. That is, KDM2a and KDM2b may possibly retain a pluripotent state of rESCs, as their knockdown noticeably reduced the population of Rex1-GFP positive cells. It has been known that KDM2 families de-methylate the methylated lysine 36 on histone H3. According to this H3K36, other proteins such as Kdm4a, Kdm4c and Kdm8 are also involved in removing these lysine methylations on histone H3. Specifically, Kdm4a and Kdm4c target H3K36me3 (Cloos, Christensen et al. 2006, Klose, Yamane et al. 2006), whereas Kdm8 prevents formation of H3K36me2 (Hsia, Tepper et al. 2010). Therefore, it would be interesting to knockdown those demethylases to see if they are involved in promoting rESC self-renewal.

Most recently, it was demonstrated that Vitamin C enhances pluripotency of human iPSCs, acting through the histone demethylase KDM5A (JARID1A) (Eid and Abdel-Rehim 2016). This enzyme removes lysine methylation of H3K4me2/me3 (Klose, Yan et al. 2007, Eid and Abdel-Rehim 2016), which is known to be involved in haematopoietic abnormalities (Klose, Yan et al. 2007). Hence, it is also worthwhile to investigate whether this factor mediates the effects of Vitamin C in rESCs.

Inspired by the importance of Vitamin C in somatic cell reprogramming protocols, we tested whether this agent could also play a role in regulating the pluripotency of rESCs (Esteban, Wang et al. 2010, Shi, Zhao et al. 2010, Esteban and Pei 2012). Vitamin C is known to have antioxidant properties and retains the activity of iron-mediated DNA and histone demethylases (Monfort and Wutz 2013). In spite of these dioxygenases being involved in hypo-methylation that characterises

ESCs (Leitch, McEwen et al. 2013), Vitamin C is rarely administered to standard ESC culture media. This may be because the fact that rodents can generate vitamin C might have neglected it in the standard media or experiments to prove a beneficial Vitamin C influence in media have often not been convinced (Monfort and Wutz 2013). Here, we have demonstrated the benefits of epigenetic modulator Vitamin C in rESC self-renewal and pluripotency. As this enzymatic function is critical in enhancing a self-renewing, pluripotent state of ESCs, Vitamin C can be employed as a supplement to copy physiological activity levels. Our demonstrations and future experiments may suggest to overcome the limitations of the long-term culture of rESC in standard 2i + LIF media. Consequently, this will help generate better culture systems, and will then contribute to reliable maintenance of stable rat ESCs for the generation of genetic models in rats.

8. Acknowledgements

9. Supplementary data

Primer	Forward Sequence	Reverse Sequence
GAPDH	ATGACTCTACCCACGGCAAG	TGGGTTTCCCGTTGATGACC
ESRRB	CTGTCACCCGAATTTTCTTT	ATGCTTCCTCCCGTGGTCTC
NANOG	GACTAGCAACGGCCTGACTCA	CTGCAATGGATGCTGGGATA
GATA4	TGAGGGCGAGCCTGTTTGCAA	ATTCAGATTCTTGGGCTTCC
BRACHYURY	GCTCATCGGAACAACTCTCC	CTCCGAGGCTAGACCAGTTG
AXIN2	GCAGGACCCACATCCTTC	AGCCAGTCTCTTTGGCTCTT
TET1	ATAATGATCCTGCCTTTGGTGG	CGTGTGTCCCTTGTATTCTACCA
TET2	TGTTCTTCGGGACAGCAGG	GAGCTCTACTGGTTACAAAGCAA
KDM2A	GTCGATCTCCAATGTTGTGCT	TGGCATTATTTTGGGTCAGGG
KDM2A	AGCCAGAGATTGCGTGGTAC	TGACTTGGAAGAGAAGCTCCA
KDM2B	AGGCGGCCTTTAGAAGACAT	GTTTGGGACACCTTGTTCCT
KDM2B	GGAGAGTGCCACTTTTGCAA	CAGGGAAGGAGGACACAGTG

Table S1. Primer pairs of different markers for qRT-PCR. GAPDH was employed as a house-keeping gene (normaliser), relative to which the expression level is defined. Esrrb and Nanog are ESC markers. Gata4 and Brachyury are differentiation markers. Axin2 is a target of β catenin. Tet1, Tet2, Kdm2a and Kdm2b are markers of the potential Vitamin C targets.

Figure S1. Vitamin C does not rescue ESCs from MEK-induced differentiation. In the A and B, ESCs (135-3 cell line) were cultured in media containing 2i + LIF or 2i with 3 μM CHIR (-PD) or 3 μM CHIR + 100 μM Vitamin C (-PD + Vc) for 4 days. Panel A shows bright-field and fluorescence images of the same cells (magnification X10). In panel B, the same rESC cultures were analysed (1x10^4 cells) by flow cytometry at day 4. Abbreviations: CHIR, CHIR99021; PD, PD0325901; LIF, leukaemia inhibitory factor; Vc, Vitamin C; SSC-H, side scatter pulse height (i.e. cell granularity or internal complexity).
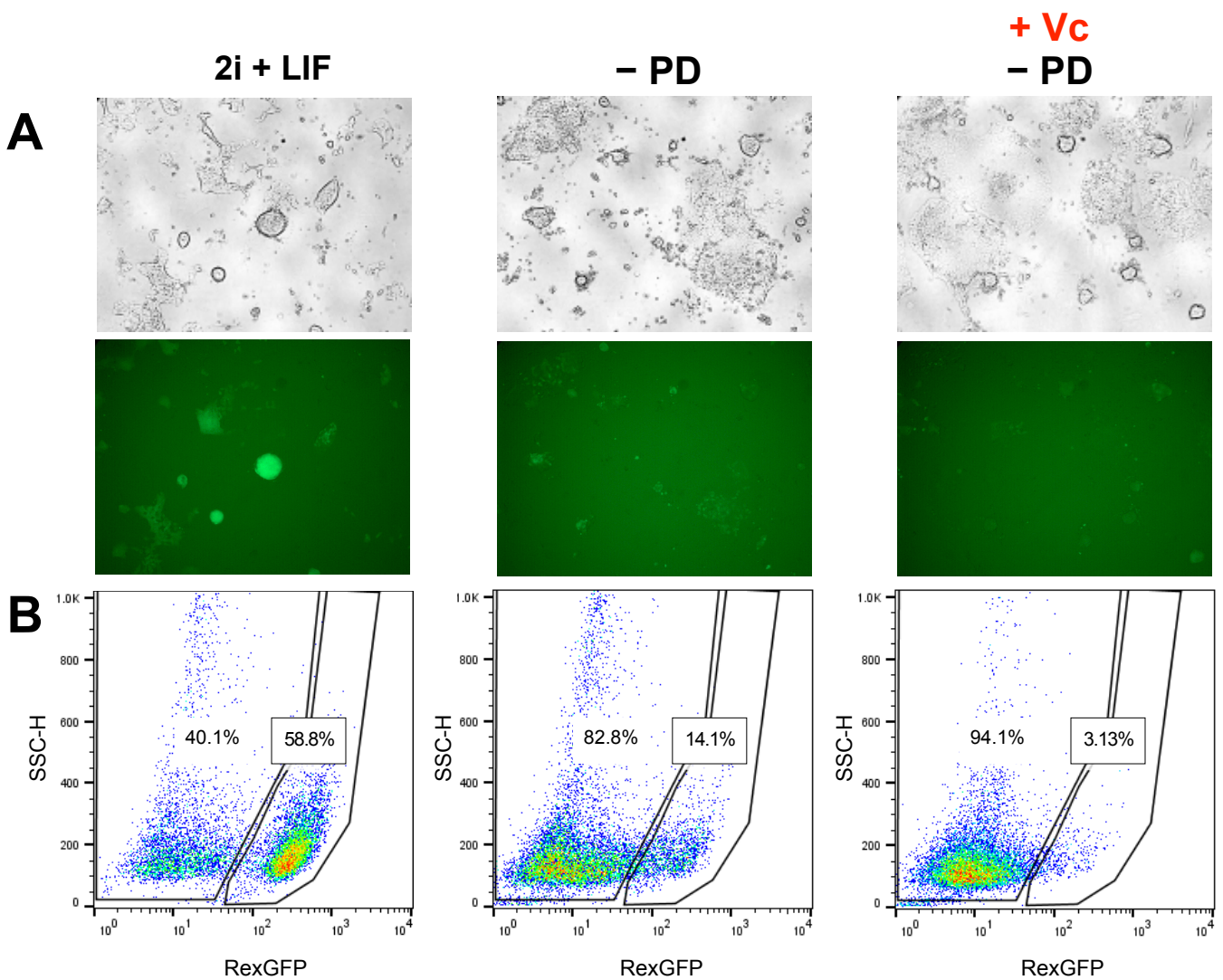
Figure S2. Vitamin C does not prevent ESCs from MEK-dependent differentiation. In the A and B, ESCs (141-5 cell line) were cultured in media containing 2i + LIF or 2i with 3 µM CHIR (-PD) or 3 µM CHIR + 100 µM Vitamin C (-PD + Vc) for 4 days. Panel A shows bright-field and fluorescence images of the same cells (magnification X10). In panel B, the same rESC cultures were analysed ($1x10^4$ cells) by flow cytometry at day 4. Abbreviations: CHIR, CHIR99021; PD, PD0325901; LIF, leukaemia inhibitory factor; Vc, Vitamin C; SSC-H, side scatter pulse height (i.e. cell granularity or internal complexity).

2i + LIF   **+ CH**   **+ Vc + CH**

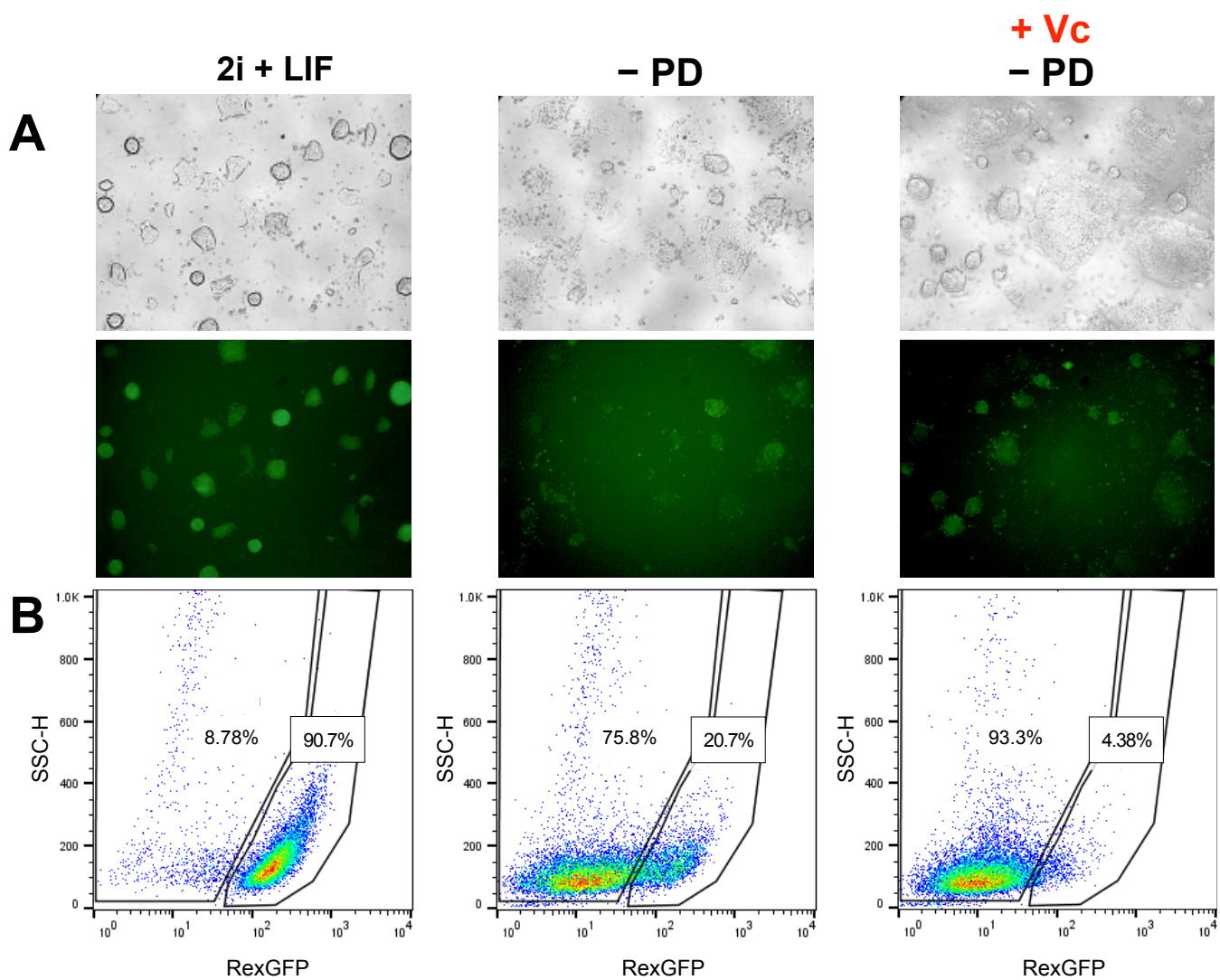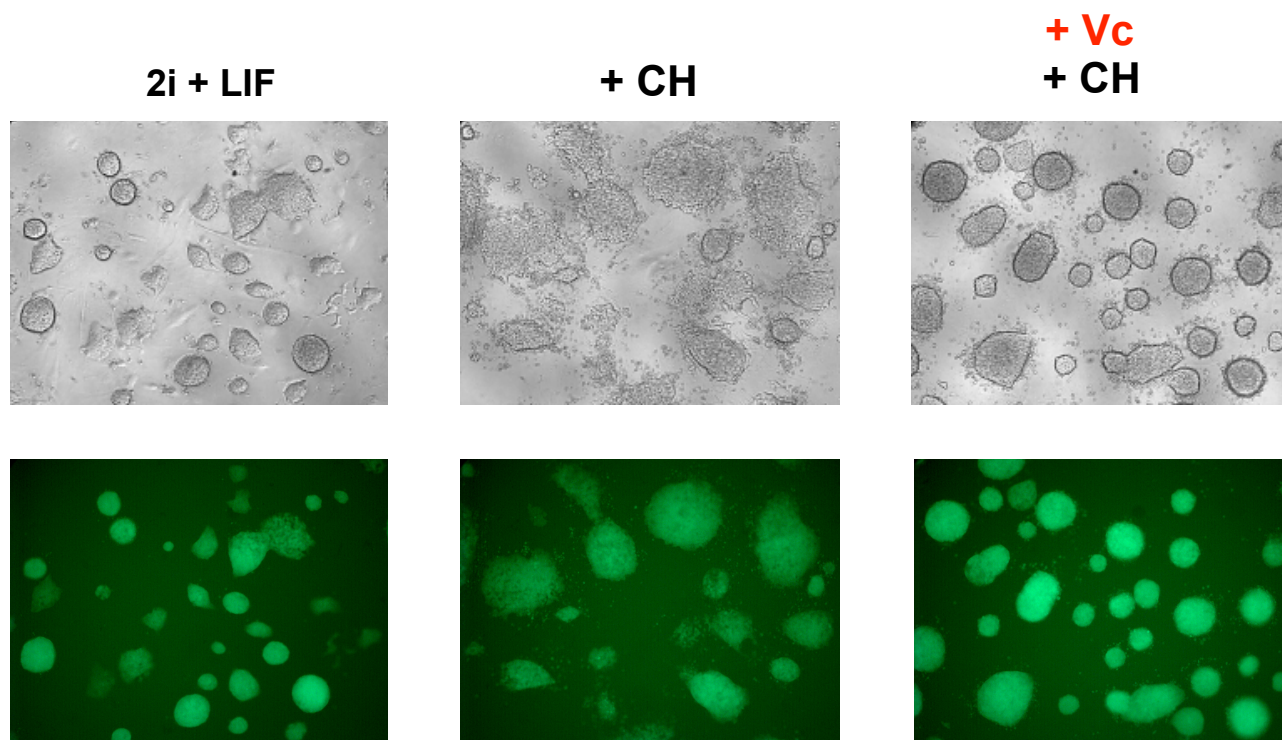**Figure S3. Vitamin C rescues ESCs from β catenin-induced differentiation. (A):** rESCs (135-3 cell line) were cultured in media containing 2i + LIF or 2i with 4 µM CHIR (+CH) or 4 µM CHIR + 100 µM Vitamin C (+ CH + Vc) for 5 days. The panels shows bright-field (upper) and fluorescence images (lower) of the same cells (magnification X10). Abbreviations: CHIR/CH, CHIR99021; Vc, Vitamin C.
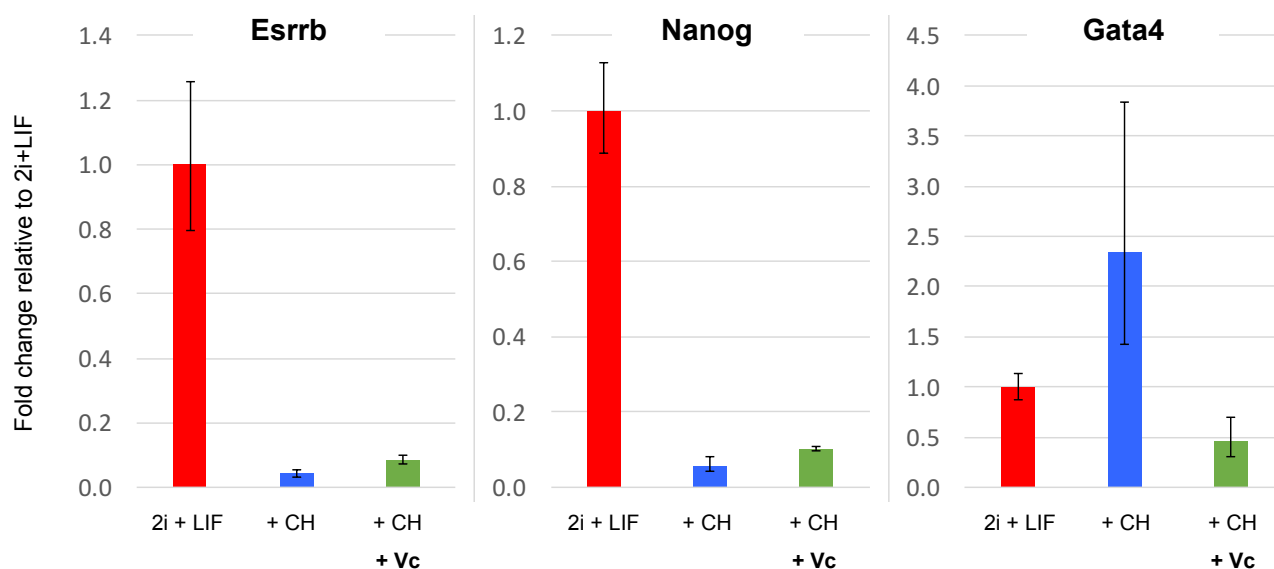
Figure S4. Vitamin C upregulates Esrrb and Nanog, and downregulates Gata4. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis for Esrrb, Nanog and GATA4 genes. The graphs indicate the level of gene expressions in ESCs (135-3 line). These ESCs were cultured in medium containing 2i + LIF (red) or 2i with 5 µM CHIR (+CH, blue) or 5 µM CHIR + 100 µM Vitamin C (+CH +Vc, green) for 5 days. Abbreviations: CHIR (CH), CHIR99021; LIF, leukaemia inhibitory factor; Vc, Vitamin C.
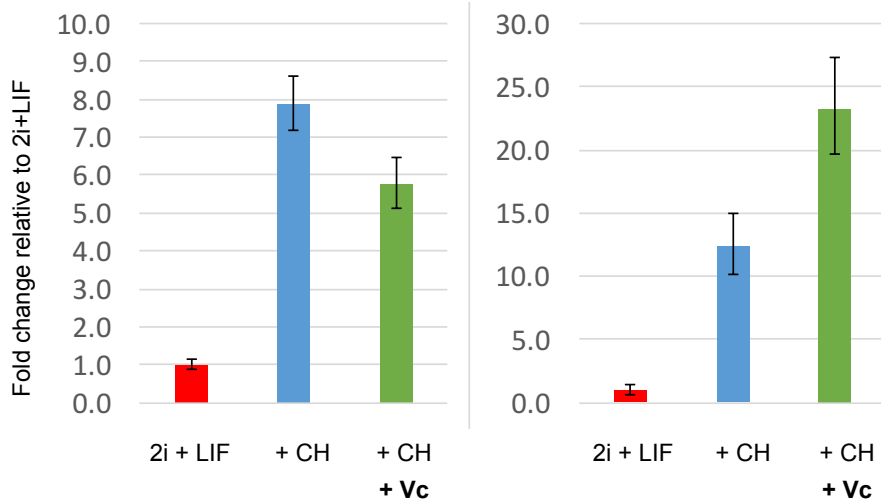
Figure S5. Vitamin C does not markedly affect the Axin2 expression level. qRT-PCR analysis for Axin2 gene. The graphs indicate the level of Axin2 expression in ESCs (135-4 and 135-6 cell lines). These ESCs were cultured in media containing 2i + LIF (red) or 2i with 5 µM CHIR (+CH, blue) or 5 µM CHIR + 100 µM Vitamin C (+CH +Vc, green) for 5 days. Abbreviations: CHIR, CHIR99021; LIF, leukaemia inhibitory factor; Vc, Vitamin C.
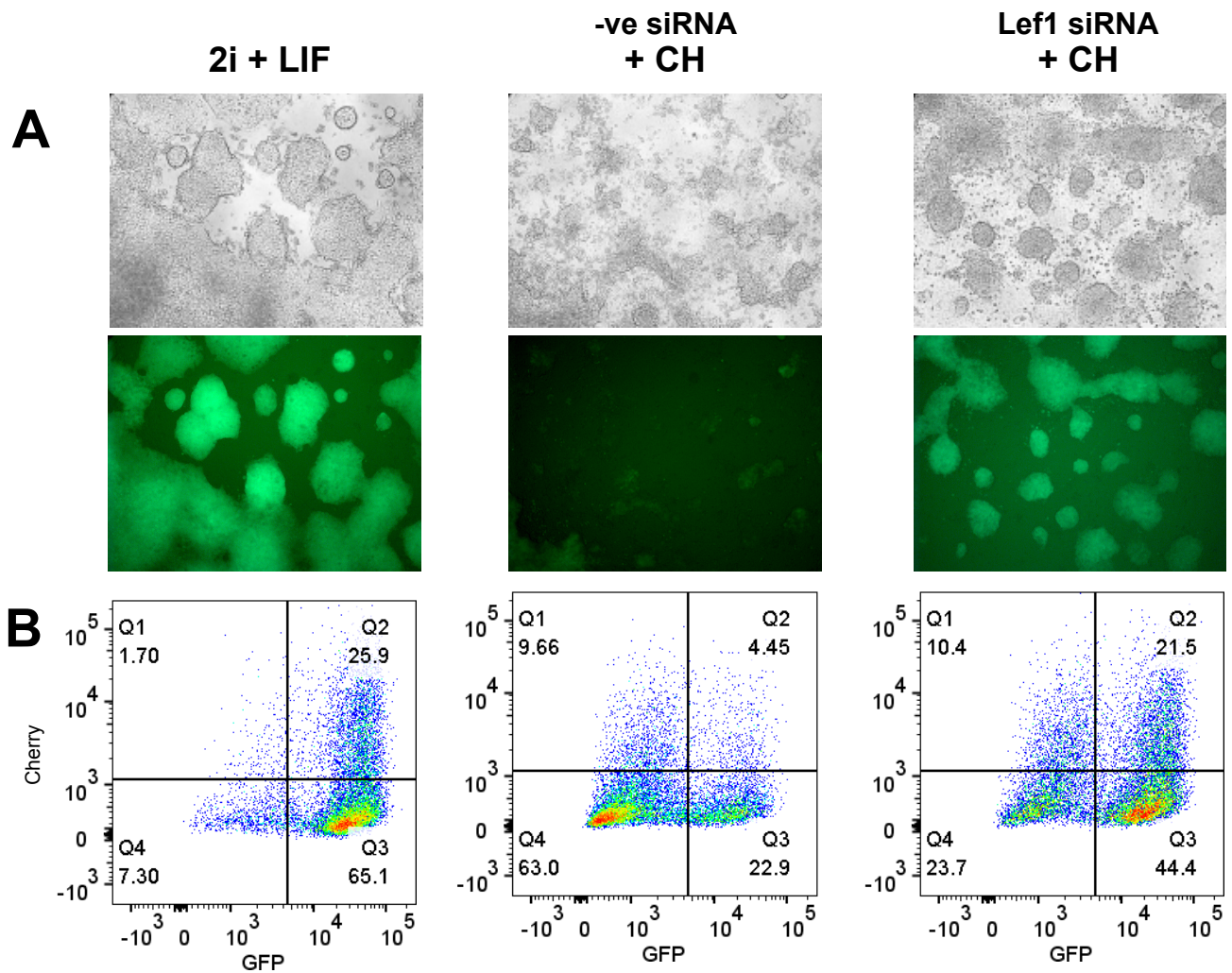
Figure S6. Lef1 siRNA can prevent ESCs from CHIR-induced differentiation. (A): Bright-field (upper) and fluorescence (lower) images (magnification X10) of rESCs (135-4) cultured in media containing 2i + LIF, 6 µM CHIR + 50 nM negative siRNA (-ve siRNA + CH) and 6 µM CHIR + 50 nM Lef1 siRNA (Lef1 siRNA + CH) for 5 days. **(B):** The same cells were then analysed (1x10^4 cells) by flow cytometry at day 5. Abbreviations: CHIR/CH, CHIR99021; Cherry, RFP; Negative siRNA (-ve siRNA), Control siRNA.
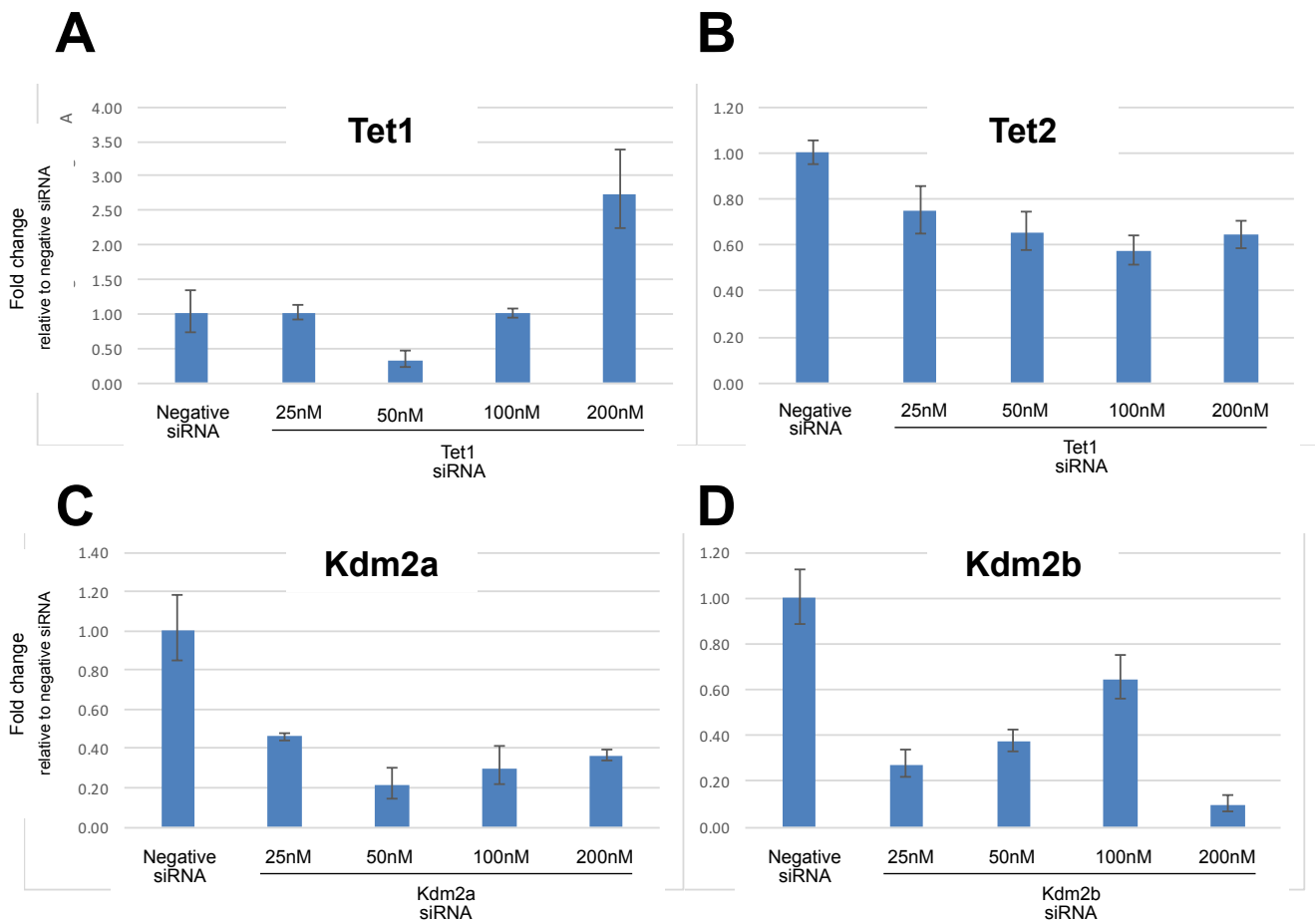
Figure S7. Titration of siRNA concentration for efficient knockdown. qRT-PCR analysis for Tet1, Tet2, Kdm2a and Kdm2b mRNAs. The graphs indicate the level of gene expressions in rESCs (135-4 cell line) transfected with the appropriate siRNAs at 25, 50, 100 and 200 nM for 24 hours and concurrently cultured in media containing 2i + LIF for 3 days. Abbreviation: negative siRNA, Control siRNA.

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