

Investigating the molecular mechanisms underlying the role of mTORC2 in human embryonic stem cells

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Thesis submitted towards the fulfilment of the degree of

Doctor of Philosophy

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December 2023

Abstract

Mechanistic target of rapamycin complex 2 (mTORC2), one of the two mTOR complexes, regulates cell proliferation, metabolism and cytoskeleton remodelling and has a crucial role in embryonic development. Loss-of-function studies show that mTORC2-deficient (*Rictor*- or *Sin1*-null) mouse embryos fail to complete their development and die around E11 due to defective vasculature. However, much less is known about the underlying mechanisms. In addition, it is unclear about the potential roles of mTORC2 in pluripotent stem cells (PSCs). To explore the role of mTORC2 in embryonic development and human embryonic stem cells (hESCs), *RICTOR*-knockout (RIC-KO) hESCs have been generated in our lab. RIC-KO hESCs retain their self-renewal, maintain expression of the core pluripotent markers, and express markers of three germ layers after embryoid body (EB) differentiation. However, their differentiation to mesoderm and endoderm lineages is reduced compared to wildtype (WT) controls.

In this study, I have further characterised RIC-KO hESCs and found that their colonies are less compact with reduced cell-cell interactions and altered cytoskeleton. RNA-seq analysis reveals that downregulated genes in RIC-KO hESCs are associated with mesoderm and endoderm differentiation. To investigate the role of mTORC2 in mesoderm and endoderm specification, I employ bone morphogenic protein-4 (BMP4)-induced differentiation and show that the mesendoderm (ME) lineage is largely impeded in RIC-KO cells compared to WT controls. Interestingly, forced activation of the WNT/ β -catenin pathway by GSK3 inhibitor completely rescues their defect in ME differentiation, indicating that RIC-KO possibly affects BMP4-induced activation of the WNT signalling. Furthermore, I demonstrate that impaired ME differentiation in RIC-KO hESCs results from inadequate upregulation of the canonical WNT genes, which is associated with reduced cell-cell interactions. These findings suggest that RICTOR/mTORC2 is crucial in ME differentiation in hESCs through regulating cellular interaction-mediated activation of the WNT genes and demonstrate the importance of cell-cell interactions in BMP4-induced activation of the WNT genes.

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Acknowledgements

First, I am immensely grateful to my supervisor, Dr Wei Cui, who has furnished me with comprehensive guidance, profound knowledge and insightful advice throughout the course of my PhD. Her ceaseless passion for science and relentless quest for perfection have motivated and encouraged me during my four-year study and will always do in my future career. Secondly, I would like to thank my former or present lab members who have helped me a lot in scientific development as well as useful suggestions in times of difficulty or trouble. Particularly, I would like to thank Dr Yueh-Ho Chiu, Dr Huihan Zhi, and Dr Manjari Trivedi for their willingness to spread their experiences and answer my questions at the beginning of my arrival in the lab, as well as Dr Faiza Batool, Dr Faiza Farhan, Xiaolun Ma, and Priscilla Wu for being the most wonderful colleagues throughout the time of the study. I would also like to extend my gratitude to Dr Nick Dibb, Dr Santosh Atanur, Dr Tristan Rodriguez, and Dr Veronique Azuara for their critical comments and valuable advice at multiple stages of the study.

Finally, I would like to thank my dearest parents, Mr. Yunhong Tong and Ms. Qearl Zhu, who have provided me with endless care and support under any circumstances. Their positive, upbeat, and optimistic attitude towards work and life have inspired me to strive for a better outcome. Most importantly, I would like to thank my wife, Ms. Xiaqing Hua, who has been with me and on my side for all these years. We have been through together for all the ups and downs. Without her boundless love and constant encouragement would I not overcome all these obstacles and challenges posed in front of me.

This thesis is dedicated to my beloved family. Words cannot express how grateful I am to them. Thank you for backing me up and making me a stronger person. I will always love you all.

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Abbreviations

2D	Two-dimensional
4EBP	eIF4E-binding protein
AA	Activin A
ACVR	Activin receptor
AD	Armadillo domain
AFP	Alpha-fetoprotein
AJ	Adherens junction
AMH	Anti-muellerian hormone
AMOT	Angiomotin
AMPK	AMP-activated protein kinase
APC	Adenomatous polyposis coli
aPKC	Atypical PKC
AXIN	Axis inhibition protein
BMP	Bone morphogenetic protein
BMPR	BMP receptor
BRA	Brachyury
BRA+	BRA-positive
BSA	Bovine Serum Albumin
C-SMAD	Common SMAD
CaMKII	Calcium/calmodulin-dependent protein kinase II
CD	C-terminal domain
CDC42	Cell division control protein 42
CDK	Cyclin-dependent kinase
CDL	Chemically Defined Lipid
CDM2	Chemically Defined Medium 2
CHIR	CHIR99021
CI	Confidence interval
CK1 α	Casein kinase 1 α
CL	Colony
cPKC	Conventional protein kinase C
CRB3	Crumbs protein homolog 3
CRIM	Conserved region in the middle
Cryo-EM	Cryo-electron microscopy
CX	Connexin

CXCR2	Chemokine receptor 2
DAG	Diacylglycerol
DE	Definitive endoderm
DEG	Differentially expressed gene
DEPTOR	DEP domain-containing mTOR-interacting protein
DMEM	Dulbecco's Modified Eagle
DMSO	Dimethyl Sulphoxide
DPBS	Dulbecco's Phosphate Buffered Saline
DVL	Dishvelled
E-CAD	E-cadherin
EB	Embryoid body
EC	Endothelial cell
ECM	Extracellular matrix
eIF4E	Eukaryotic translation initiation factor 4E
EMT	Epithelial–mesenchymal transition
EOMES	Eomesodermin
ER	Endoplasmic reticulum
ERM	Ezrin-radixin-moesin
ExE	Extra-embryonic ectoderm
F12	Ham's F-12 Nutrient Mix
FAT	FRAP/ATM/TRRAP
FAT-C	C-terminal FAT
FBS	Foetal Bovine Serum
FC	Fold change
FDR	False discovery rate
FGF	Fibroblast growth factor
FOXA2	Forkhead box protein A2
FOXH1	Forkhead box protein H1
FOXO	Forkhead box O
FRB	FKBP-rapamycin binding
FZD	Frizzled
GDF	Growth and differentiation factor
GJ	Gap junction
GO	Gene ontology
GSC	Goosecoid
GSK3	Glycogen synthase kinase 3

HD	HEAT-like domain
hESC	Human embryonic stem cell
HM	Hydrophobic motif
ICAT	Inhibitor of β -catenin and T cell factor
ICM	Inner cell mass
ILK	Integrin-linked kinase
IMDM	Iscove's Modified Dulbecco's Medium
IRS1	Insulin receptor substrate 1
JAMs	Junctional adhesion molecules
JNK	JUN N-terminal kinase
KD	Knockdown
KSR	Knockout Serum Replacement
KSR-M	Knockout Serum Replacement Medium
LEF	Lymphoid enhancer-binding factor
LIF	Leukaemia inhibitory factor
LncRNA	Long noncoding RNA
LRP	Low-density lipoprotein receptor-related protein
MAM	Mitochondria-associated ER membrane
ME	Mesendoderm
MEF	Mouse embryonic fibroblast
MEF-CM	MEF-conditioned medium
mEpiSC	Mouse epiblast stem cell
mESC	Mouse embryonic stem cell
MIXL1	Mix-like homeodomain protein 1
mLST8	Mammalian lethal with Sec13 protein 8
mSIN1	Mammalian stress-activated map kinase-interacting protein 1
mTOR	Mechanistic target of rapamycin
mTORC1	mTOR complex1
mTORC2	mTOR complex2
NEAA	Non-essential Amino Acids
NMD	Nonsense-mediated decay
NMM II	Non-muscle myosin II
nPKC	Novel protein kinase C
NTD	N-terminal domain
P/S	Penicillin-Streptomycin
PALS1	Protein associated with Lin-7 1

PAM	Protospacer adjacent motif
PAR6	Partitioning defective 6
PATJ	PALS1-associated tight junction
PCP	Planar cell polarity
PDK1	3-phosphoinositide-dependent kinase 1
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PIKK	PI3K-related protein kinases
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PR	Phosphorylation site region
PS	Primitive streak
PSC	Pluripotent stem cell
pSMAD1	Phospho-SMAD1/5/9
qRT-PCR	Quantitative real-time Polymerase Chain Reaction
R-SMAD	Receptor-regulated SMAD
RAC1	Rac Family Small GTPase 1
RAP1	Repressor/activator protein 1
RAPTOR	Regulatory associated protein with mTOR
RBD	Ras binding domain
RHEB	Ras homolog enriched in brain
RHOA	Ras homolog family member A
RhoGDI2	Rho-GDP dissociation inhibitor 2
RIC-KO	<i>RICTOR</i> -knockout
RICTOR	Rapamycin-insensitive companion of mTOR
RIN	RNA integrity number
RLC	Regulatory light chain
RNA-seq	RNA-sequencing
RNF43	Ring finger 43
ROCK	Rho-associated kinase
ROCKi	Y27632-Rho Kinase Inhibitor
S6K	p70 S6 kinase
SC	Single cell
sFRPs	Secreted Frizzled-related proteins

SGK	Serum and glucocorticoid-induced protein kinase
sgRNA	Single-guided RNA
TAZ	Transcriptional coactivator with PDZ-binding motif
TCF	T cell factor
TE	Trophectoderm
TF	Transcription factor
TFEB	Transcription factor EB
TGF- β 1	Transforming growth factor beta 1
TIM	TOR interaction motif
TJ	Tight junction
TM	Turn motif
TSC	Tuberous sclerosis complex
ULK1	Unc-51-like autophagy-activating kinase 1
WIF1	Wnt inhibitory factor 1
WT	Wildtype
YAP	Yes-associated protein
ZNRF3	Zinc and ring finger 3
ZO-1	Zonula occludens 1
β -CAT	β -catenin

Chapter One

Introduction

1.1 mTOR signalling pathway

Mechanistic target of rapamycin (mTOR), also called mammalian target of rapamycin, is a serine/threonine kinase protein which belongs to the PI3K-related protein kinase (PIKK) family (Liu and Sabatini, 2020). mTOR is evolutionarily conserved and shares structural and functional similarities with the yeast TOR proteins (Szwed et al., 2021). This important protein was identified three decades ago in search for the target of the natural compound rapamycin, as the name of this kinase protein suggests. mTOR is targeted and inhibited by rapamycin which forms a complex with the cellular prolyl isomerase FKBP12 (Brown et al., 1994; Sabatini et al., 1994; Sabers et al., 1995). Utilising the inhibitory effect of rapamycin on mTOR, researchers have found that mTOR is a core signalling protein that coordinates the regulation of cellular metabolic processes in response to environmental cues. It senses the availability of nutrients and hormones to regulate the synthesis or degradation of cellular compartments (Liu and Sabatini, 2020). Dysregulation of the mTOR signalling disturbs cellular homeostasis, which results in adverse health conditions in humans, such as diabetes, neurodegenerative disorders, cancer, and aging (Saxton and Sabatini, 2017).

mTOR exerts its catalytic activity through two distinct protein complexes: mTOR complex1 (mTORC1) and complex 2 (mTORC2) which differ in protein composition, complex substrates, and biological functions (Liu and Sabatini, 2020). Because mTORC1 is rapamycin-sensitive and mTORC2 is insensitive, investigations on what activates mTORC1 and how it functions are much more comprehensive. However, prolonged rapamycin treatment also hinders mTORC2 activity possibly by affecting the availability of nascent mTOR protein (Sarbasov et al., 2006). A lack of mTOR complex-specific inhibitors hampers our understanding of the mTOR signalling pathway, particularly mTORC2.

1.1.1 mTORC1

1.1.1.1 Molecular structure of mTORC1

Although initial investigations considered mTORC1 to be a large multiprotein complex containing at least five protein subunits (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002), recent studies have demonstrated that mTORC1 comprises three core components: mTOR, RAPTOR (regulatory associated protein with mTOR) and mLST8 (mammalian lethal

with Sec13 protein 8) as these three core subunits are sufficient for mTORC1 to perform its activity by forming a dimer of the mTOR-mLST8-RAPTOR heterotrimer (Aylett et al., 2016; Baretić et al., 2016; Chao and Avruch, 2019; Yang et al., 2017).

The mTOR protein contains multiple domains, including N-terminal HEAT repeats followed by the FRAP/ATM/TRRAP (FAT) domain, the FKBP-rapamycin binding (FRB) domain, and the C-terminal kinase domain (Fig 1.1) (Aylett et al., 2016; Chao and Avruch, 2019; Scaiola et al., 2020; Yang et al., 2016). HEAT repeats contain Horn and Bridge domains. The Horn domain of one mTOR interacts with the Bridge domain of the other mTOR, which mediates the formation of dimeric mTORC1 (Aylett et al., 2016; Baretić et al., 2016; Yang et al., 2017; Yang et al., 2016). The FAT domain is in a large open conformation that encompasses the kinase domain (Baretić et al., 2016; Yang et al., 2017; Yang et al., 2016). The FRB domain is the site for FKBP12-rapamycin binding which acts to block access of substrates to the adjacent catalytic cleft of the kinase domain, thus inhibiting mTOR kinase activity (Fig 1.1) (Aylett et al., 2016; Yang et al., 2013). The C-terminal kinase domain has a site for mLST8 binding and a site for substrate binding and phosphorylation (Fig 1.1) (Chao and Avruch, 2019). The kinase domain also contains a C-terminal FAT (FAT-C) domain which stabilises the activation loop (Chao and Avruch, 2019). mTOR can be directly inhibited by DEPTOR (DEP domain-containing mTOR-interacting protein) which possibly prevents mTOR from adopting an active conformation (Caron et al., 2018; Heimhalt et al., 2021; Peterson et al., 2009; Wälchli et al., 2021).

RAPTOR is situated at the Horn/Bridge interface of mTOR where it stabilises mTORC1 at the surface of the lysosome (Fig 1.1) (Aylett et al., 2016; Chao and Avruch, 2019; Hara et al., 2002; Kim et al., 2002). As the defining subunit of the complex, RAPTOR contains an N-terminal caspase-like domain, followed by HEAT repeats and WD-40 repeats (Fig 1.1) (Chao and Avruch, 2019). The caspase-like domain recognises TOS motif present in certain mTORC1 substrates and mediates substrate binding (Aylett et al., 2016; Chao and Avruch, 2019; Yang et al., 2017; Yang et al., 2016). On the other hand, mLST8 is a 40-kDa protein composed mainly of WD-40 repeats (Fig 1.1) (Chao and Avruch, 2019). mLST8 binds to and may stabilise the kinase domain of mTOR (Yang et al., 2013). Interestingly, loss-of-function studies show that mLST8 deficiency does not affect mTORC1 activity in mice, indicating the possibility of an alternative mLST8 protein to be identified (Guertin et al., 2006). Regulation and function of

mTORC1 signalling have been well characterised (Battaglioni et al., 2022; Liu and Sabatini, 2020; Saxton and Sabatini, 2017; Szwed et al., 2021).

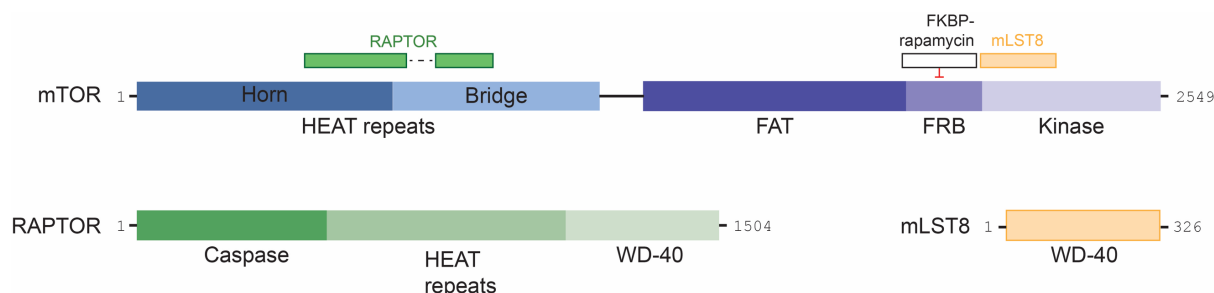


Figure 1.1 Schematic protein structure of key mTORC1 components

Linear representation of domains of the two key mTORC1 components. Interactions of mTOR with other proteins in mTORC1 are highlighted above mTOR. Horizontal bars indicate the interaction with specific proteins. FAT, FRAP ATM TRRAP; FRB, FKBP-rapamycin binding.

1.1.1.2 Upstream regulation of mTORC1

Many factors have been identified to play a role in the regulation of mTORC1 activity which can be divided into three categories, growth factors, nutrients, and energy. mTORC1 senses these factors as an input to exert its functions as summarised in Fig 1.2 (Battaglioni et al., 2022; Liu and Sabatini, 2020).

Upon stimulation of a growth factor such as insulin, phosphatidylinositol 3-kinase (PI3K) is activated which triggers the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3), leading to the binding of 3-phosphoinositide-dependent kinase 1 (PDK1) and AKT to the plasma membrane via their PH (pleckstrin homology) domain. PDK1 then activates AKT by phosphorylating its kinase domain. Activated AKT subsequently inhibits the tuberous sclerosis complex (TSC), a negative regulator of mTORC1, by specifically phosphorylating and destabilising TSC2 (Inoki et al., 2002; Manning et al., 2002; Menon et al., 2014). Inactive TSC restores activity of the small GTPase, Ras homolog enriched in brain (RHEB), and as a result, GTP-bound RHEB directly activates mTORC1 on the outer surface of the lysosome (Inoki et al., 2003; Manning et al., 2002).

The levels of nutrients such as amino acids are monitored by the small GTPase RAG-A, -B, -C, and -D which directly associate with and activate mTORC1 (Kim et al., 2008; Sancak et al., 2008). The Rags forming a set of heterodimers, A/B with C/D, are anchored to the lysosome where they bind to Ragulator (Bar-Peled et al., 2012; Condon and Sabatini, 2019; Sancak et al., 2010; Takahara et al., 2020). When the Rags are converted to their active form, that is GTP-bound A/B and GDP-bound C/D, they recruit mTORC1 to the lysosome where RHEB meets mTORC1 for its activation (Sancak et al., 2010; Takahara et al., 2020). However, in the case of low energy levels or cellular stress, AMP-activated protein kinase (AMPK) inhibits mTORC1 in two ways: either phosphorylates TSC2 at residues that activate TSC which in turn inhibits RHEB, leading to mTORC1 inactivation, or directly phosphorylates RAPTOR to inhibit mTORC1 (González et al., 2020; Gwinn et al., 2008; Van Nostrand et al., 2020).

1.1.1.3 Downstream effectors of mTORC1

Upon activation, mTORC1 phosphorylates downstream effectors to promote anabolism but limit catabolism (Fig 1.2) (Battaglionni et al., 2022; Liu and Sabatini, 2020). mTORC1 promotes mRNA translation and protein synthesis by mainly phosphorylating two effectors: p70 S6 kinase (S6K) and eukaryotic translation initiation factor 4E (eIF4E) binding protein (4E-BP). Activated S6K phosphorylates many substrates whose roles are found to be involved in cell growth, transcription and translation (Magnuson et al., 2011; Tavares et al., 2015). The other downstream effector 4E-BP, when inhibited by mTORC1, releases eIF4E and facilitates mRNA translation (Beretta et al., 1996; Brunn et al., 1997; Gingras et al., 1999; Gingras et al., 2001; Poulin et al., 1998). Moreover, mTORC1 inhibits transcription factor EB (TFEB) which regulates genes involved in lysosomal biogenesis, autophagy, and lipid production (Ramirez Reyes et al., 2021; Settembre et al., 2011). mTORC1 also inhibits unc-51-like autophagy-activating kinase 1 (ULK1) and ATG13, two key autophagy initiators which are responsible for the formation of the autophagosome (Hosokawa et al., 2009; Kim et al., 2011a; Puente et al., 2016). Therefore, mTORC1 has a critical role in the regulation of cell growth and metabolism.

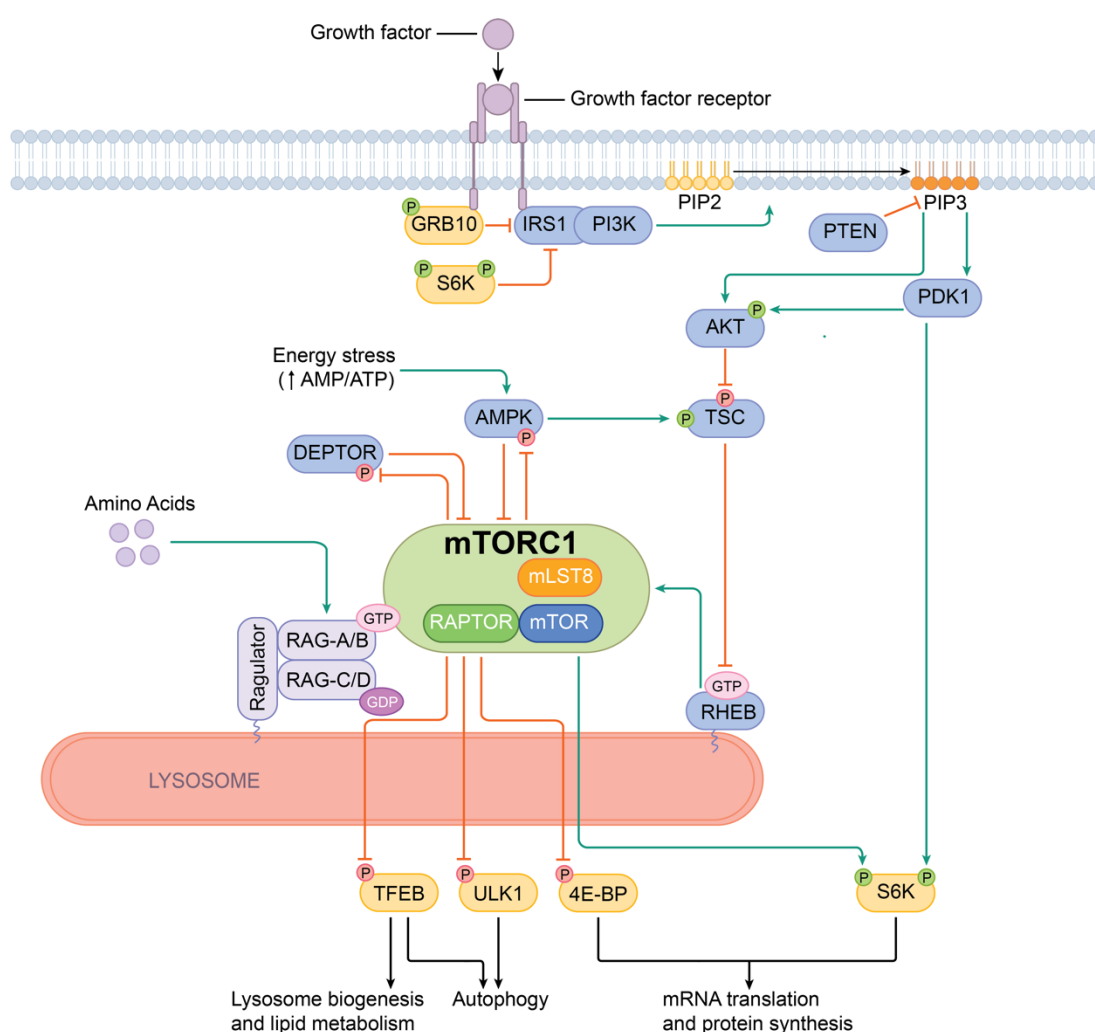


Figure 1.2 Simplified representation of mTORC1 signalling pathway

Upstream regulators and downstream effectors of mTORC1. Multiple inputs, such as amino acids, energy stress, and growth factors, regulate mTORC1 as shown. mTORC1 signalling participates in autophagy, lysosome biogenesis and lipid metabolism, and mRNA translation and protein synthesis by phosphorylating the downstream effectors shown in citrine. Green arrow ends indicate activation and red blunt ends indicate inhibition. Green Ⓟ represents activation through phosphorylation, while red Ⓟ represents inhibition through phosphorylation. Reprinted from Cell, Battagioni et al., copyright 2022, with permission from Elsevier Inc.

1.1.2 mTORC2

1.1.2.1 Molecular structure of mTORC2

Only with the recent advances on high-resolution cryo-electron microscopy (cryo-EM) and crystallographic analyses are the researchers able to decipher and visualise the structure of mTORC2. The molecular structure of mTORC2 resembles that of mTORC1, forming a homodimer of two mTORC2 complexes. However, instead of a trimeric structure in each mTORC1, mTORC2 is a tetramer in which RAPTOR is replaced by RICTOR (rapamycin-insensitive companion of mTOR) and mSIN1 (mammalian stress-activated map kinase-interacting protein 1) (Fig 1.3) (Jacinto et al., 2004; Karuppasamy et al., 2017; Sarbassov et al., 2004; Scaiola et al., 2020; Yang et al., 2006).

RICTOR, as the mTORC2-specific subunit, is a protein with a molecular weight of 190 kDa. It is composed of three interacting stacks of α -helical repeats, referred to as the Armadillo domain (AD), the HEAT-like domain (HD), and the C-terminal domain (CD) (Fig 1.3) (Scaiola et al., 2020). In the sequence space between the HD and CD lies the phosphorylation site region (PR) and phosphorylation in this region can modulate mTORC2 activity (Fig 1.3) (Scaiola et al., 2020). Through its AD and CD, RICTOR interacts and binds to mTOR at multiples sites. The AD sits between the proximal and distal mTOR subunits. The CD of RICTOR blocks the binding space of FKBP12-rapamycin in mTOR, thus explaining the rapamycin insensitivity of mTORC2 (Fig 1.3) (Chen et al., 2018; Scaiola et al., 2020). RICTOR not only physically interacts with other complex subunits to form mTORC2, but also is required for mTORC2 activity. Disruption of RICTOR by gene manipulation in various cell lines results in defective mTORC2 kinase activity (Sarbassov et al., 2005). Deletion of either the N-terminal or C-terminal domain of RICTOR leads to the failure of mTORC2 assembly and function (Aimbetov et al., 2012; Glidden et al., 2012; Ruicci et al., 2019). In addition to its function in mTORC2, RICTOR also has its own function independent of mTORC2. RICTOR forms a complex with integrin-linked kinase (ILK) which interacts with β 1-integrin and is probably crucial for transforming growth factor beta 1 (TGF- β 1)-mediated epithelial–mesenchymal transition (EMT) (McDonald et al., 2008; Serrano et al., 2013). RICTOR also interacts with MYO1C to promote cortical actin remodelling, regardless of mTORC2 activity (Hagan et al., 2008). Furthermore, RICTOR is associated with PKC ζ (protein kinase C zeta), an atypical PKC (see below), which does not require mTORC2 binding (Zhang et al., 2010). Likewise, RICTOR

suppresses Rho-GDP dissociation inhibitor 2 (RhoGDI2), a negative regulator of RHO signalling, to promote cell migration (Agarwal et al., 2013).

mSIN1 is another mTORC2-specific key subunit with a molecular weight of 59 kDa and has four domains: the N-terminal domain (NTD), the conserved region in the middle (CRIM) domain, the Ras binding domain (RBD), and the pleckstrin homology (PH) domain (Fig 1.3). mSIN1 has an important role in stabilising mTORC2 because the N terminus of mSIN1 tethers RICTOR to the mTOR-mLST8 core (Scaiola et al., 2020). The interaction between mSIN1 and mLST8 even extends from the N terminus to the CRIM domain, which strengthens mTORC2 stability; in addition, the CRIM domain is important for mTORC2 substrate recruitment (Scaiola et al., 2020; Tatebe et al., 2017). While the RBD domain binds and inhibits RAS, the PH domain is required for mTORC2 localisation to the plasma membrane (Berchtold and Walther, 2009; Liu et al., 2015; Schroder et al., 2007). Besides the longest isoform mSIN1.1, mSIN1 also has other 3 isoforms mSIN1.2, mSIN1.4, and mSIN1.5 in humans from alternative splicing (Frias et al., 2006; Schroder et al., 2004). Among the four isoforms in humans, mSIN1.4 is not able to assemble into mTORC2 and mSIN1.5 is not sensitive to insulin stimulation (Frias et al., 2006). Because of the structural difference among the isoforms, they may result in different forms of mTORC2 which may have distinct subcellular localisations and functions. Thus, the RBD and PH domains of mSIN1 still remain unresolved by cryo-EM.

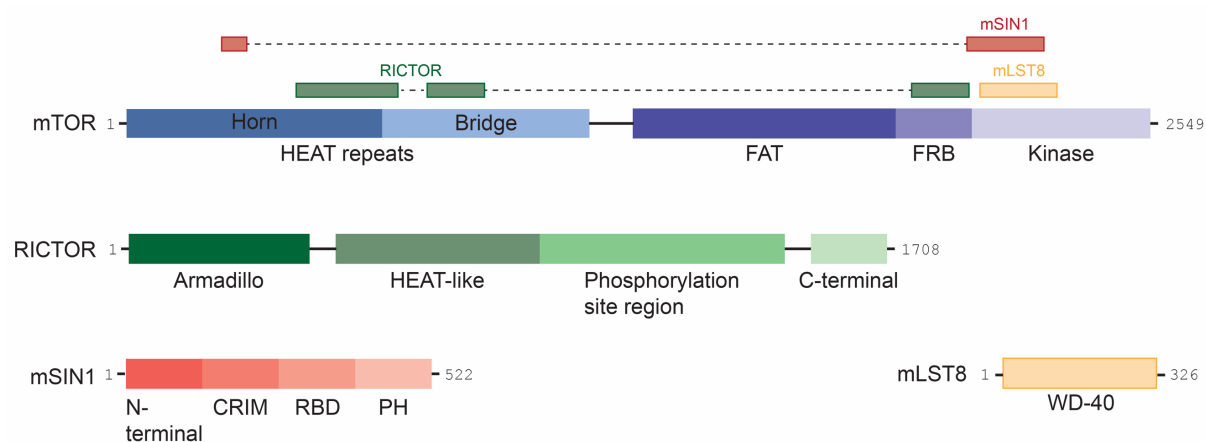


Figure 1.3 Schematic protein structure of key mTORC2 components

Linear representation of domains of the three key mTORC2 components. Interactions of mTOR with other components in mTORC2 are highlighted above mTOR. Horizontal bars indicate the interaction with specific proteins. CRIM, Conserved region in the middle; FAT, FRAP ATM TRRAP; FRB, FKBP-rapamycin binding; PH, Pleckstrin homology; RBD, Ras binding domain.

1.1.2.2 Regulation of mTORC2

Activation of mTORC2 is poorly understood given its divergent cellular localisation, including the plasma membrane, ribosomes, mitochondria, Golgi, endosomes, endoplasmic reticulum (ER), and mitochondria-associated ER membrane (MAM) (Battaglioni et al., 2022). It has been considered for a long time that growth factor-activated PI3K is an upstream activator of mTORC2. As PI3K initiates generation of PIP3 which subsequently interacts with the mSIN1 PH domain, its autoinhibition on mTOR is relieved and mTORC2 is recruited to the plasma membrane, leading to its activation (Fig 1.4) (Liu et al., 2015). The notion is further supported by the findings that mTORC2 activity is repressed indirectly by mTORC1 through insulin/PI3K/AKT signalling. The mTORC1 substrate S6K promotes degradation of insulin receptor substrate 1 (IRS1), blocking insulin-mediated PI3K activation (Fig 1.4) (Harrington et al., 2004; Shah et al., 2004). mTORC1 also directly phosphorylates and activates growth factor receptor-bound protein 10 (GRB10) which inhibits insulin/PI3K signalling, leading to suppression of mTORC2 activity (Fig 1.4) (Hsu et al., 2011; Yu et al., 2011b). However, the mSIN1 isoform without the PH domain can still support mTORC2 activity, indicating that the PH domain in mSIN1 is not a necessary factor for the activation. A relatively new study using various biochemical methods demonstrates that neither growth factor depletion nor PI3K inhibition affect mTORC2 activity at the plasma membrane, suggesting that mTORC2 is constitutively active at the plasma membrane (Ebner et al., 2017). Nonetheless, growth factors may still positively regulate mTORC2 activity by promoting the interaction between membrane-bound proteins and RICTOR to increase its stability and mTORC2 activity (Xu et al., 2019). During energy stress, AMPK phosphorylates specifically mTOR and RICTOR in mTORC2 to increase its kinase activity, which serves to activate anti-apoptotic AKT signalling to promote cell survival, (Kazyken et al., 2019). This indicates that mTORC2 activation may occur on the endosomal/lysosomal membrane as well. Furthermore, it has been reported that mTORC2 not only regulates proteins through post-translational modification but also through co-translational modification at the ribosome where mTORC2 might be activated through PI3K signalling (Fig 1.4) (Oh et al., 2010; Zinzalla et al., 2011). Taken together, the regulation of mTORC2 is still unclear and its activation possibly involves multiple regulatory mechanisms.

Signalling crosstalk could also affect mTORC2 activity through regulating post-translational modifications of mTORC2 components RICTOR and mSIN1. The mTORC1 substrate S6K phosphorylates RICTOR at Thr1135 to destabilise mTORC2 (Dibble et al., 2009; Julien et al.,

2010; Treins et al., 2010). Glycogen synthase kinase 3 (GSK3) phosphorylates RICTOR at different sites: at Ser1235 to prevent the binding of AKT to mTORC2 and attenuate mTORC2 signalling in response to cellular stress and at Thr1695 to mediate the interaction between RICTOR and FBXW7 for RICTOR ubiquitylation and proteasome degradation (Chen et al., 2011; Koo et al., 2015). Acetylation of RICTOR also regulates mTORC2 activity. Several findings reveal that RICTOR acetylation promotes while its deacetylation inhibits mTORC2 activity (Glidden et al., 2012; Manning et al., 2019; Masui et al., 2015; Singh et al., 2016). Moreover, ubiquitin-specific protease 9X (USP9X), a deubiquitinase regulated by growth factors, removes ubiquitin from the AD of RICTOR to help its interaction with mTOR, promoting mTORC2 signalling (Wrobel et al., 2020). In addition, phosphorylation of mSIN1 at Thr86 and Thr398 by S6K leads to mSIN1 dissociation from mTORC2, thereby suppressing mTORC2 kinase activity (Liu et al., 2013). However, phosphorylation at Thr86 by AKT enhances mTORC2 kinase activity (Yang et al., 2015). The PH domain has a phosphoinositide-binding site that binds mTOR to autoinhibit mTORC2 as a self-regulatory mechanism. Ubiquitylation of mLST8 has been reported to prevent its interaction with mSIN1, dislodging the CRIM domain of mSIN1 required for mTORC2 substrate recruitment (Scaiola et al., 2020; Wang et al., 2017).

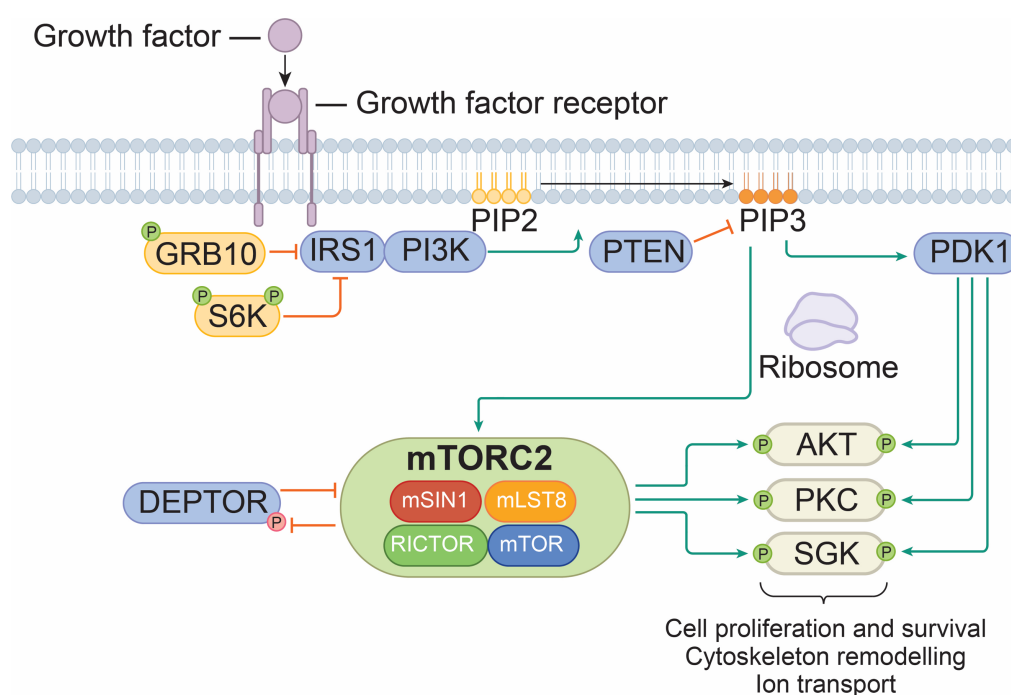


Figure 1.4 Simplified representation of mTORC2 signalling pathway

Upstream regulators and downstream effectors of mTORC2. The insulin-PI3K dependent mechanism and ribosomal activity regulate mTORC2. Only main regulators are shown and other possible regulatory mechanisms need further investigation. mTORC2 regulates cell proliferation and survival, cytoskeletal remodelling and ion transport by phosphorylating its downstream effectors shown in light green. Green arrow ends indicate activation and red blunt ends indicate inhibition. Green ③ represents activation through phosphorylation, while red ③ represents inhibition through phosphorylation. Reprinted from Cell, Battaglion et al., copyright 2022, with permission from Elsevier Inc.

1.1.2.3 Downstream targets of mTORC2

Three direct targets of mTORC2 have been identified so far, AKT (also known as protein kinase B or PKB), serum and glucocorticoid-induced protein kinase (SGK) and PKC (conventional PKCs, atypical PKCs and PKCε), all of which are members of the AGC kinase family (Fig 1.4) (Battaglion et al., 2022). The three kinases possess conserved domain structures: an N-terminal regulatory domain and a C-terminal catalytic region containing the kinase domain as well as the C-tail. In the C-terminal region, there are key phosphorylation sites, including one in the activation loop in the kinase domain and the other two in the turn motif (TM) and the hydrophobic motif (HM) at the C-tail (Fig 1.5) (Su and Jacinto, 2011). PDK1 is responsible for

phosphorylation of the activation loop while the other sites are phosphorylated mainly by mTORC2. Moreover, a recently identified motif, termed TOR interaction motif (TIM) which resides right before the TM is also phosphorylated by mTORC2 in AKT and certain PKC isoforms (Fig 1.5) (Baffi et al., 2021). TIM phosphorylation likely promotes activation loop phosphorylation by PDK1. However, these findings need further validation. Phosphorylation on these sites has an important function in the regulation of the kinase activity or stability.

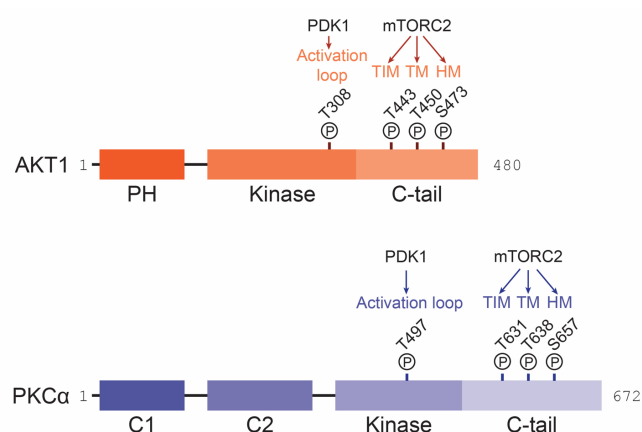


Figure 1.5 Schematic protein of key mTORC2 effectors

Linear representation of domains of the two key mTORC2 components, AKT and PKC. The isoform AKT1 and PKCα are illustrated, and their activating phosphorylation sites as well as positive regulators are indicated. HM, hydrophobic motif; PH, Pleckstrin homology; TIM, TOR interaction motif; TM, turn motif.

Through the three aforementioned direct effectors, mTORC2 mainly regulates cell proliferation and cytoskeleton remodelling (Liu and Sabatini, 2020; Saxton and Sabatini, 2017). Each effector will be discussed in more details:

i. AKT

AKT has three isoforms with AKT1 being the most characterised one. AKT1 is phosphorylated by PDK1 in the activation loop (Thr308) and by mTORC2 on three sites, which are Thr443, Thr450, and Ser473 at the TIM, TM, and HM, respectively (Fig 1.5) (Battaglioni et al., 2022). Phosphorylation of the activation loop by PDK1 is crucial for the majority of AKT activity, such as phosphorylating TSC2 and GSK3 (Guertin et al., 2006; Jacinto et al., 2006; Yang et al., 2015). TIM phosphorylation in AKT facilitates its phosphorylation in the activation loop, which is reported to be necessary and sufficient for AKT activity, by PDK1 (Baffi et al., 2021; Balasuriya et al., 2018). HM phosphorylation is required for maximal AKT activation, since defective HM phosphorylation of AKT1 on Ser473 regulates only a subset of AKT substrates,

such as forkhead box O 1/3A (FOXO1/3A) (Guertin et al., 2006; Jacinto et al., 2006; Yang et al., 2015). Ser477 and Thr479 at the C terminus of AKT1 are also phosphorylated by mTORC2 or cyclin-dependent kinase2 (CDK2) to increase AKT1 activity by facilitating Ser473 phosphorylation (Liu et al., 2014). In addition, AKT contains an N-terminal PH domain which is autoinhibited in the absence of PIP3, suggesting that correct membrane localisation is required for AKT activation (Chu et al., 2020). AKT also interacts with mSIN1 and enhances mTORC2 kinase activity by forming a positive feedback loop (Yang et al., 2015).

AKT is a central effector in PI3K signalling that promotes cell survival and proliferation through phosphorylating a number of downstream targets (Liu and Sabatini, 2020; Manning and Toker, 2017). For example, phosphorylation of FOXO1/3A results in their translocation out of the nucleus, which suppresses apoptosis, cell-cycle arrest, and growth inhibition (Webb and Brunet, 2014). AKT puts an inhibitory phosphorylation on GSK3 which prevents β -catenin from proteasome degradation, leading to nuclear localisation of β -catenin. Inhibition of GSK3 is also responsible for the activation of the prosurvival BCL-2 family member MCL1 and the transcription factor C-MYC, thereby promoting cell survival and proliferation (Maurer et al., 2006; Morel et al., 2009; Sears et al., 2000; Welcker et al., 2004). Moreover, AKT directly inhibits TSC2, thereby activating mTORC1 (Inoki et al., 2002; Manning et al., 2002), as stated above.

ii. PKC

PKC has nine isoforms which are classified into three subclasses: conventional (α , β , γ), novel (δ , ϵ , η , θ), and atypical PKCs (ζ , ι/λ) (Newton, 2018). All PKC isoforms, except three novel PKCs (δ , η , θ), are phosphorylated by mTORC2 at the TIM, TM, and HM, which is important for PKC activity (Fig 1.5) (Baffi and Newton, 2022; Battaglioni et al., 2022). At the translational stage, mTORC2 phosphorylates nascent PKC at the TIM and TM, which separates the PKC dimer and facilitates post-translational phosphorylation of the activation loop by PDK1; this process triggers HM phosphorylation by mTORC2 for full catalytic competence of PKC (Baffi and Newton, 2022). Without phosphorylation at the TIM and TM, PKC is unstable and prone to degradation.

Conventional and novel PKCs (cPKC and nPKC) have C1 and C2 at the N-terminal regulatory domain which sense diacylglycerol (DAG) and Ca^{2+} levels, respectively (Fig 1.5) (Baffi et al., 2021). Binding of membrane-bound DAG and synthesis of intracellular Ca^{2+} are required for

fully activated conventional and novel PKCs (cPKC and nPKC) (Newton, 2018). cPKCs and nPKC positively regulate cytoskeleton remodelling and cell migration by interacting with integrins to promote the cell-ECM binding in many cell types (Larsson, 2006; Sarbassov et al., 2004). PKC α and PKC ϵ interact with β 1 integrin, while PKC β associates with β 3 integrin (Ivaska et al., 2002; Ng et al., 1999; Parsons et al., 2002; Podar et al., 2002). In addition, PKC α -mediated integrin internalisation and translocation to the leading edge of the cell facilitates cell migratory effect (Parsons et al., 2002). On the other hand, PKCs regulate the structure of actin cytoskeleton through its substrates, such as fascin, ERM (ezrin-radixin-moesin) and RAC1 to promote cell migration (Anilkumar et al., 2003; Bretscher et al., 2002; Ng et al., 1999; Tu et al., 2020). Furthermore, nPKC participates in the formation of blood vessels and endothelial lumen formation (Bekhite et al., 2011; Koh et al., 2008; Monti et al., 2013). cPKC and nPKC also play an important role in the regulation of metabolic homeostasis (Kolczynska et al., 2020).

Atypical PKCs (aPKCs) plays a central role in the regulation of the apical-basal polarity complex with partitioning defective 6 (PAR6) which is vital in the maturation of epithelial junctions, asymmetric cell division, and differentiation (Chen and Zhang, 2013; Parker et al., 2021; Reina-Campos et al., 2019; Vorhagen and Niessen, 2014). Recent studies suggest that aPKCs are also important for tumorigenesis and could have both tumour-promoter and suppressive functions in context-dependent manner (Reina-Campos et al., 2019).

iii. SGK

SGK has three isoforms: SGK1, SGK2, SGK3. For its full kinase activity, SGK is phosphorylated in the activation loop by PDK1, at the TM by an unidentified kinase, and at the HM by mTORC2 (Battaglioni et al., 2022; García-Martínez and Alessi, 2008). Active SGK prevents the degradation of epithelial sodium channel which is essential in sodium homeostasis (Bhalla et al., 2005; Butterworth, 2010; Lu et al., 2010; Pearce, 2003). Moreover, SGK exerts the same inhibitory effect on FOXO1/3A as AKT to mediate the anti-apoptotic function (Jang et al., 2022; Liu et al., 2017).

1.1.3 mTOR signalling in mammalian embryonic development

1.1.3.1 mTORC1 and mTORC2 have distinct functions during embryonic development

Since the mTOR signalling governs the process of protein synthesis, cell growth and autophagy, it is no doubt that mTOR signalling plays an essential role in embryonic development. Previous studies have reported that germline gene deletion of key components in mTOR complexes results in embryonic lethality in mice (Gangloff et al., 2004; Guertin et al., 2006; Jacinto et al., 2004; Murakami et al., 2004; Shiota et al., 2006). However, the phenotypes are very different between the two mTOR complexes (summarised in Table 1.1).

Table 1.1 Summary of knockout mice with germline deletion of key mTOR signalling genes

Genetic manipulation	Reported phenotype	References
mTORC		
<i>Mtor</i> ^{-/-}	Embryonic lethality at E5.5-6.5; defective proliferation in the ICM and trophoblasts	(Murakami et al., 2004)
<i>Rptor</i> ^{-/-}	Embryonic lethality at E5.5-6.5; failure of expansion in implanted blastocysts	(Guertin et al., 2006)
<i>Mlst8</i> ^{-/-}	Embryonic lethality at E10.5; severe vasculature defects	(Guertin et al., 2006)
<i>Rictor</i> ^{-/-}	Embryonic lethality at E10.5; severe vasculature defects	(Guertin et al., 2006; Shiota et al., 2006)
<i>Sin1</i> ^{-/-}	Embryonic lethality at E10.5-11.5	(Jacinto et al., 2006)
4E-BP		
<i>4E-BP1</i> ^{-/-} and <i>4E-BP2</i> ^{-/-}	Born normal with no significant defects	(Le Bacquer et al., 2007)
S6K		
<i>S6k1</i> ^{-/-}	Smaller in size	(Pende et al., 2004)
<i>S6k2</i> ^{-/-}	Larger in size	(Pende et al., 2004)
<i>S6k1</i> ^{-/-} and <i>S6k2</i> ^{-/-}	Perinatal death; heart failure	(Pende et al., 2004)
ULK		
<i>Ulk1</i>	Born normal with no significant defects	(Kundu et al., 2008)

AKT

<i>Akt1</i> ^{-/-}	Mild growth retardation; increased apoptosis	(Chen et al., 2001)
<i>Akt2</i> ^{-/-}	Insulin resistance	(Cho et al., 2001)
<i>Akt3</i> ^{-/-}	Slow growth of brain	(Tschopp et al., 2005)
<i>Akt1</i> ^{-/-} and <i>Akt2</i> ^{-/-}	Postnatal death	(Peng et al., 2003)
<i>Akt1</i> ^{-/-} and <i>Akt3</i> ^{-/-}	Embryonic lethality at E10.5-11.5; possibly placental vascular defects	(Yang et al., 2005)
<i>Akt2</i> ^{-/-} and <i>Akt3</i> ^{-/-}	Combined phenotypes of the two	(Dummler et al., 2006)

PKC

<i>Prkce</i> (<i>Pkcε</i>) ^{-/-}	Born normal with no significant defects	(Carracedo et al., 2014; Lessmann et al., 2006)
<i>Prkci</i> (<i>Pkcι</i>) ^{-/-}	Embryonic lethality at E9.5-10; defects in mesodermal differentiation	(Seidl et al., 2013; Soloff et al., 2004)
<i>Prkcz</i> (<i>Pkcζ</i>) ^{-/-}	Born normal with no significant defects	(Leitges et al., 2001)

SGK

<i>Sgkl</i> ^{-/-}	Embryonic lethality at E10.5-11.5; severe vasculature defects	(Catela et al., 2010)
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Mutant embryos with a defect in mTOR or in the mTORC1-specific component Raptor die at E5.5-6.5 shortly after implantation due to defective proliferation in the inner cell mass (ICM) and trophoblasts (Guertin et al., 2006; Murakami et al., 2004). This indicates that mTORC1 activity involved in cell proliferation is required in early embryonic development.

In contrast, knockout of the mTORC2 key component, *Rictor* or *Sin1* in mice leads to embryo death around E11 mid-gestation due to defective vasculature in both the embryo proper and yolk sac/placenta (Guertin et al., 2006; Jacinto et al., 2004; Shiota et al., 2006). Surprisingly, disruption of *Mlst8* encoding mLST8, a shared subunit in both complexes, also leads to embryonic lethality at E10.5 with vascular defects, suggesting that it is only necessary for the mTORC2 activity during embryonic development (Guertin et al., 2006). Interestingly, tissue-specific deletion of *Rictor* in endothelial cells does not affect embryonic development or show any defective phenotypes unless this knockout occurs around gastrulation, which also causes embryonic lethality, similar to the phenotype of germline deletion (Aimi et al., 2015; Zhao et al., 2014). These results suggest that mTORC2 may play an essential role in early embryonic

development possibly around the gastrulation stage through regulating mesoderm lineage. However, the detailed mechanisms underlying the role of mTORC2 need to be fully elucidated.

1.1.3.2 Deficiency of different mTORC2 effectors show diverse phenotypes in mice

Loss-of-function studies of AKT in mice reveal a distinct but redundant role of AKT isoforms in early embryonic development (Table 1.1). Mice deficient in any one of the three isoforms alone survive after the birth despite the various physiological and pathological defects (Chen et al., 2001; Cho et al., 2001; Tschopp et al., 2005). Double knockouts of *Akt1/Akt2* and *Akt1/Akt3* cause embryonic lethality, with the latter being more severe which leads earlier death at E10.5-11.5, possibly due to placental vascular defects (Peng et al., 2003; Yang et al., 2005). However, deletion of *Akt2/Akt3* only exhibits mild phenotypes but not lethal ones (Dummler et al., 2006). Therefore, these findings suggest that while all three AKT isoforms are crucial for normal development and function, AKT1 plays a more important role.

The mTORC2 substrates aPKCs (ζ , ι) are essential in early embryonic development in mice (Table 1.1). Germline deletion of *Pkci* (*PKC λ* in humans) in mouse embryos results in retarded growth early in gestation due to defects in mesodermal differentiation, leading to embryonic lethality at E9.5-10.5 (Seidl et al., 2013; Soloff et al., 2004). Furthermore, tissue-specific deletion of *Pkci* in endothelial cells (ECs) leads to embryonic lethality at E11.5 despite few surviving, phenocopying germline deletion (Chen et al., 2021). Mice with double knockouts of *Pkci* and *Pkc ζ* in ECs die at E11.5 due to the defects in vascular development. The resultant phenotype of double knockouts is more severe than that of *Pkci* knockout alone, which indicates a possible compensatory role of PKC ζ . The fact that *Pkc ζ* deficient mice are born normal with no significant defects suggests a predominant role of PKC ι during embryonic development (Chen et al., 2021; Leitges et al., 2001). Likewise, disruption of *Sgkl* in mice results in embryonic lethality at E10.5-11.5 due to defective vasculature, which might be attributed to decreased Notch signalling genes (Catela et al., 2010). Collectively, these findings demonstrate that mice deficient in aPKCs or SGK1 exhibit the similar phenotypes of embryonic lethality around mid-gestation as those deficient in individual mTORC2 components (except mTOR), which is due to impaired vascular development. It suggests that the potential function of mTORC2 signalling in vascular development during early embryonic development might be mainly associated with downstream PKC signalling.

1.2 Signalling pathways that regulate mesoderm and endoderm differentiation

Mesoderm and endoderm are two germ layers that are formed in gastrulation and after the formation of the primitive streak (PS). Mesoderm gives rise to the bone, heart, vascular tissues, muscle, kidney, and limb buds, while endoderm develops to the lung, liver, pancreas, stomach, and intestines. Specification of mesoderm and endoderm in the vertebrate embryo is regulated by dynamic bone morphogenetic protein (BMP), Nodal, fibroblast growth factor (FGF), and WNT signalling gradients along the anterior-posterior axis of the PS (Arnold and Robertson, 2009; Gerri et al., 2020; Molè et al., 2020; Raffaelli and Stern, 2020; Tam and Loebel, 2007). Epiblast cells with high BMP and WNT signalling differentiate to mesoderm, and with high Nodal signalling give rise to definitive endoderm (DE).

1.2.1 Gastrulation, primitive streak and mesendoderm

Gastrulation is an important process that occurs soon after implantation. During gastrulation, the pluripotent epiblast cells differentiate into three germ layers, ectoderm, mesoderm, and endoderm. This milestone event requires a complex interplay of signalling pathways involved in cell migration and cell fate determination (Steventon et al., 2021). The molecular mechanisms of gastrulation in mouse and chick embryos have been extensively studied (Arnold and Robertson, 2009; Pera, 2017; Raffaelli and Stern, 2020; Tam and Loebel, 2007), while the mechanistic studies of human gastrulation only start to emerge recently and are very limited due to the ethical restrictions of obtaining early post-implantation materials (Blakeley et al., 2015; Tyser et al., 2021). In mouse embryos, gastrulation starts at the posterior end of the epiblast epithelium with the formation of the primitive streak (PS) which is initiated by BMP4 expression secreted by the extra-embryonic ectoderm (ExE). However, due to a lack of the ExE, the amnion in human and other primates might be analogous to the ExE in the mouse, acting as a signalling hub of BMP4 activity to induce gastrulation (Shao et al., 2017; Yang et al., 2021; Zheng et al., 2019). Epiblast cells then undergo the epithelial-mesenchymal transition (EMT) and ingress through the PS to form both the definitive endoderm (DE) and mesoderm, while the remaining epiblast cells that do not ingress through the PS become the ectoderm.

During the ingress through the PS and prior to the formation of the mesoderm and DE, epiblast cells will go through EMT and acquire a transient bipotent state of mesendoderm (ME) which is marked by expression of several transcription factors (TFs), such as Brachyury (BRA) and Eomesodermin (EOMES) (Wang and Chen, 2016). Formation of the PS and ME is not a random event but rather a well-orchestrated and spatiotemporally regulated process through the synergy of key signalling pathways.

1.2.2 Key signalling pathways in the regulation of gastrulation

Increasing evidence demonstrates that four signalling pathways are crucial for the formation of the PS and gastrulation – BMP4, Nodal, WNT, and FGF. In mouse embryos, uncleaved Nodal precursors secreted from the epiblast stimulate Bmp4 expression in the ExE, which activates Wnt3 in the posterior epiblast (Ben-Haim et al., 2006). Activated Wnt signalling maintains levels of Nodal expression, thereby resulting in the BMP/WNT/Nodal signalling cascade. FGF signalling promotes EMT, cell proliferation and migration (Ciruna and Rossant, 2001; Yang et al., 2008). Although the four signalling pathways act in a cooperative manner to drive epiblast cells into ME cell fate and therefore are equally important (Arnold and Robertson, 2009; Tam and Loebel, 2007), the BMP/WNT/Nodal signalling cascade will be the main focus here.

i. BMP signalling

Bone morphogenetic proteins (BMPs) belong to the BMP subfamily of the TGF β superfamily of ligands. Several growth and differentiation factors (GDFs) and the anti-muellerian hormone (AMH) also belong to the BMP subfamily. Like all the other TGF β superfamily ligands, BMP protein precursors are synthesised as dimeric complexes which are cleaved by furin protease and other pro-protein convertases to generate the mature proteins. TGF β superfamily signalling is a typical membrane-to-nucleus process through receptor-mediated activation of TFs (David and Massagué, 2018) and their receptors are the only known serine/threonine receptors. In the canonical signalling pathway, dimeric TGF β superfamily ligands initiate the signalling transduction cascade by binding to constitutively active type II receptors to transphosphorylate and activate the type I receptors which, in turn, phosphorylate the direct downstream substrates known as receptor-regulated SMADs (R-SMADs) to induce their conformational changes. These conformational changes enable R-SMADs to interact with common SMAD (C-SMAD, SMAD4) to form the R-SMADs-C-SMAD heterotrimeric complexes. The R-SMADs-C-

SMAD complexes are able to translocate to and accumulate in the nucleus where they function as a TF and regulate gene expression. In BMP2/4 signalling, the type I receptors primarily phosphorylate SMAD1, SMAD5, and SMAD9 (SMAD1/5/9).

BMP2/4 signalling is regulated at multiples stages, from ligands to SMADs (David and Massagué, 2018). Ligand traps such as noggin, cerberus, chordin, follistatin, and gremlin bind bioactive BMPs, thereby preventing them from activating the signalling cascade. In addition, small molecule inhibitors like LDN-193189, LDN-212854, and Dorsomorphin target BMP type I receptors (Mohedas et al., 2013; Yu et al., 2008a; Yu et al., 2008b). Ligands of the BMP subfamily bind to specific type I and type II receptors. Among the type I receptors, BMP9 and BMP10 bind to ALK1, while the other members share ACVR1A (activin receptor type 1A), BMPRI1A (BMP receptor type 1A) and BMPRI1B; among the type II receptors, BMP ligands share ACVR2, ACVR2B and BMPRI2 (Fig 1.6) (David and Massagué, 2018). In certain BMPs, the binding of ligands to receptors is facilitated by membrane co-receptors, such as endoglin for BMP9 and BMP10 and repulsion guidance molecules (RGMs) for BMP2 and BMP6 (Massagué, 2012). Likewise, an antagonist ligand BMP3 inhibits BMP signalling by binding the type II receptors with higher affinity.

In addition to R-SMADs and C-SMAD, there is another group of SMADs called inhibitory SMADs, SMAD6 and SMAD7, which are localised in the cytoplasm and prevent activated BMP receptors from phosphorylating SMAD1/5/9 (Miyazawa and Miyazono, 2017). On the other hand, SMAD7 recruits the E3 ubiquitin ligase SMURF2 for TGF β receptor proteasome degradation. In the nucleus, binding of SMAD1/5/9 to DNA is controlled by specific DNA binding TFs and by chromatin reader TRIM33 which is recruited to histone H3 (Fig 1.6) (Xi et al., 2011). SMAD1/5/9 can be further phosphorylated by cyclin-dependent kinase 8 (CDK8) and CDK9 in the linker region (Fig 1.6) (Alarcon et al., 2009). This phosphorylation not only facilitates binding of transcription co-activators but also promotes GSK3 β -mediated phosphorylation. Phosphorylation by GSK3 β in the linker region triggers R-SMAD ubiquitylation and degradation (Fuentealba et al., 2007).

BMP signalling has been shown to regulate cell fates during embryonic development (David and Massagué, 2018; Gaarenstroom and Hill, 2014; Wang et al., 2014). BMP4 expression initially appears low in the mouse embryo prior to gastrulation and then reaches to the peak in the posterior PS (Lawson et al., 1999; Winnier et al., 1995; Zhao, 2003). Mice with *Bmp4* or *Bmpr* deletion result in reduced cell proliferation in the epiblast and gastrulation defects

(Lawson et al., 1999; Mishina et al., 1995; Winnier et al., 1995), and with *Smad1* and *Smad5* deletion display visceral endoderm and mesoderm defects, respectively (Chang et al., 1999; Tremblay et al., 2001; Zhao, 2003), suggesting the critical role of BMP signalling in germ layer formation. *In vitro* studies have demonstrated that BMP signalling drives both mouse and human pluripotent stem cell (PSC) differentiation into mainly trophoblast and ME lineages (Bernardo et al., 2011; Xu et al., 2002; Yu et al., 2011a). BMP-activated SMAD1/5/9 first stimulates expression of *BRA*, two of which then cooperate together to promote mesoderm differentiation but repress the endodermal fate (Bernardo et al., 2011; Faial et al., 2015; Yu et al., 2011a). Moreover, SMAD1/5/9 interacts with GATA binding proteins to induce differentiation of extra-embryonic lineages, such as haematopoietic cells (Genander et al., 2014; Trompouki et al., 2011). Therefore, given the interaction of SMAD1/5/9 with various DNA binding TFs, BMP signalling acts as an instigator of gastrulation.

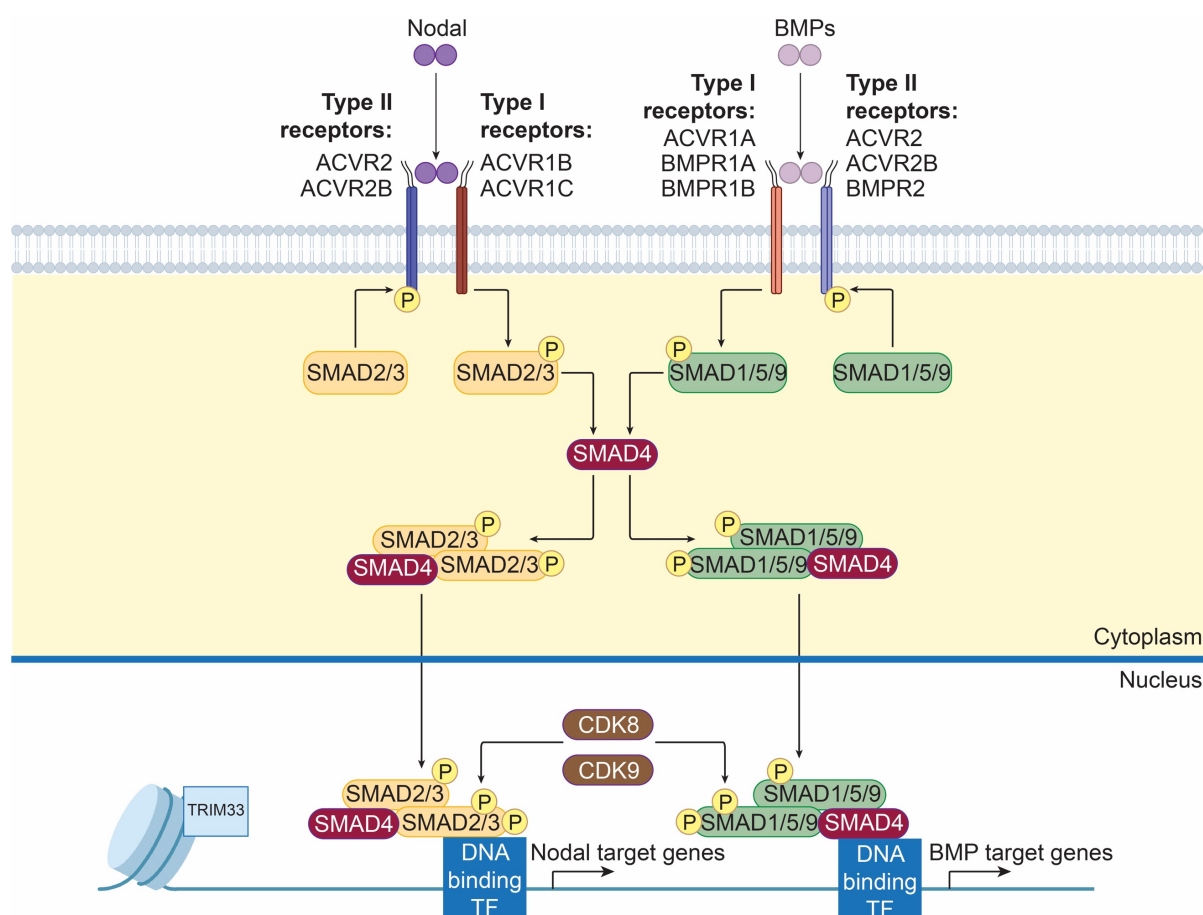


Figure 1.6 Illustration of the TGFβ signalling pathways

The TGFβ superfamily can be subdivided into two main subfamilies, TGFβ subfamily including Nodal and BMP subfamily including BMP2/4, which activate SMAD2/3 (in citrine) and SMAD1/5/9 (in green), respectively, both of which are called R-SMADs. The activated R-SMADs interact with SMAD4 (in burgundy) to form trimeric R-SMAD-SMAD4 complexes that translocate into the nucleus to regulate gene expression. R-SMADs are also associated with histone-bound TRIM33 which regulates gene expression epigenetically. Phosphorylation of R-SMADs by CDK8 and CDK9 (in brown) facilitates transcription. ACVR, Activin receptor; BMPR, Bone morphogenic protein receptor; CDK, Cyclin-dependent kinase; R-SMAD, Regulatory SMAD; TF, Transcription factor.

ii. *WNT signalling*

WNT signalling is a key pathway for embryonic patterning and development and starts from the synthesis of the WNT ligands in the cell. There are 19 WNT genes in humans encoding 19 WNT proteins which require post-translational palmitoylation by the ER-bound acyltransferase Porcupine for their secretion and receptor recognition (Biechele et al., 2011; Gao and Hannoush, 2014; Kadowaki et al., 1996; Takada et al., 2006; Willert et al., 2003). Extracellularly secreted WNT proteins bind and activate the FZD-LRP5/6 (Frizzled- LDL receptor-related protein 5 or 6) receptor complex, which triggers transduction of the downstream signalling (Fig 1.7 right panel) (Bhanot et al., 1996; Cong et al., 2004; Pinson et al., 2000; Tamai et al., 2000). Depending on whether the downstream signalling involves β -catenin degradation and translocation, WNT signalling is divided into the canonical and non-canonical WNT signalling.

In the canonical WNT signalling, the FZD-LRP5/6 receptor complex leads to phosphorylation of LRP5/6 and recruitment of the signal transducers DVL (Dishvelled) and the AXIN/GSK3 (axis inhibition protein/glycogen synthase kinase 3) complex to the plasma membrane (Fig 1.7 right panel) (Gammons et al., 2016a; Gammons et al., 2016b; Habib et al., 2013; Kim et al., 2013b; Piao et al., 2008; Zeng et al., 2005). Phosphorylated LRP5/6 prevents β -catenin degradation by directly inhibiting GSK3 (Piao et al., 2008; Stamos et al., 2014; Zeng et al., 2005), while DVL has a high binding affinity towards AXIN which dissociates from the destruction complex, thereby stabilising β -catenin (Cliffe et al., 2003; Kishida et al., 1999; Yamanishi et al., 2019). Subsequently, as cytoplasmic β -catenin accumulates, it is translocated into the nucleus and functions as a TF by interacting with DNA-binding proteins mainly from the T cell factor (TCF)/lymphoid enhancer-binding factor (LEF) family (Behrens et al., 1996; Cadigan and Waterman, 2012; Molenaar et al., 1996). β -catenin then induces transcription of TCF/LEF-bound sites. In the absence of a ligand, the central effector β -catenin is constantly ubiquitinated and degraded through phosphorylation by casein kinase 1 α (CK1 α) and GSK3 with the assistance from AXIN and adenomatous polyposis coli (APC), four of which together form the destruction complex (Fig 1.7 left panel) (Nusse and Clevers, 2017; Rim et al., 2022). E3 ubiquitin ligase β TRCP recognises phosphorylated β -catenin and induces ubiquitylation and subsequent proteasomal degradation of β -catenin. In the nucleus, Groucho family transcription repressors, instead, bind to TFC/LEF and repress WNT-responsive enhancers.

The noncanonical β -catenin-independent WNT signalling pathway is divided into two main branches: the planar cell polarity (PCP) pathway and the Ca²⁺-dependent pathway, although

these two pathways are far less studied (Akoumianakis et al., 2022). In both pathways, WNT ligands bind not only to FZD but also to tyrosine-protein kinase receptors ROR and RYK (Akoumianakis et al., 2022). In the PCP pathway, this leads to DVL-mediated activation of small GTPases, such as Ras homolog family member A (RHOA) and Rac Family Small GTPase 1 (RAC1) (James et al., 2008; Marinou et al., 2012). Nonetheless, Ca^{2+} -dependent pathway does not require DVL but instead works synergistically with phospholipase C (PLC) to increase intracellular Ca^{2+} concentration (Slusarski et al., 1997).

Several mechanisms are present to regulate the canonical and noncanonical WNT signalling at various cellular levels. Small molecule inhibitors such as IWP and C59 suppress Porcupine, preventing WNTs from secretion (Chen et al., 2009; Proffitt et al., 2013). In the extracellular space, Notum functions as a proteolytic enzyme that reduces WNT activity by removing its palmitoleic acid moiety (Kakugawa et al., 2015; Zhang et al., 2015). In addition, while secreted antagonists such as secreted Frizzled-related proteins (sFRPs) and Wnt inhibitory factor 1 (WIF1) bind and inhibit WNTs, Dickkopf (DKK) serves as a negative regulator by competing with WNTs for LRP5/6 binding (Bafico et al., 2001; Hsieh et al., 1999; Leyns et al., 1997; Rattner et al., 1997; Semenov et al., 2001; Wang et al., 1997). On the cell surface, the ubiquitin ligases, ring finger 43 (RNF43) and zinc and ring finger 3 (ZNRF3) which are also the target genes of the WNT pathway, facilitate ubiquitylation and degradation of the WNT receptors FZD and LRP, thereby inhibiting the WNT signalling (Hao et al., 2012; Koo et al., 2012). However, R-spondin reverses downregulation of the WNT signalling mediated by the two ubiquitin ligases by internalisation (Chen et al., 2013a; Zebisch et al., 2013). XAV939 and IWR inhibits WNT signalling by stabilising AXIN as a part of the destruction complex (Huang et al., 2009; Kulak et al., 2015), whereas CHIR99021 promotes WNT signalling by suppressing GSK3 activity (Ying et al., 2008). Finally, the inhibitor of β -catenin and T cell factor (ICAT) directly binds and inhibits β -catenin in the nucleus, so transcription of the WNT targets genes is not induced (Sato et al., 2004).

It has been shown that in mouse embryos, BMP4 secreted from extra-embryonic tissues is able to activate expression of the canonical WNT genes, such as *Wnt3*, but the exact mechanisms remain unclear (Ben-Haim et al., 2006; Martyn et al., 2018). The canonical pathway controls cell renewal, proliferation, and differentiation, while the non-canonical pathways modulate cell migration and polarity through regulation of RHO GTPases (Rim et al., 2022; Steinhart and

Angers, 2018). Therefore, the WNT signalling pathways play a pivotal role during early embryonic development.

Even though the canonical and noncanonical WNT signalling negatively regulate each other, they act in synergy to promote embryonic development, even at the early stage of gastrulation (Akoumianakis et al., 2022; James et al., 2008; Wang and Chen, 2016). Expression of a few WNT ligands is already observed in the ICM and the trophectoderm (TE) of a blastocyst in mouse embryos, but nuclear β -catenin is only present in the ICM, suggesting the role of the canonical WNT signalling in the maintenance of pluripotency in mouse ESCs (mESCs) (Seto and Bellen, 2004; ten Berge et al., 2011; Wang et al., 2004). The importance of the canonical WNT signalling in later gastrulation and PS formation is supported by the fact that WNT/ β -catenin remains its expression at the posterior side of the embryo throughout the PS and that *Wnt3* deletion results in failure of PS formation (Currier et al., 2010; Liu et al., 1999; Maretto et al., 2003; Rivera-Pérez and Magnuson, 2005). Mice that lack *Wnt3* fail to establish posterior gene expression and to form the PS, mesoderm, or node, suggesting the essential role of WNT3 in the posterior epiblast and PS formation (Liu et al., 1999). At the onset of gastrulation, WNT/ β -catenin induces transcription of a wide range of its target genes that establish anterior-posterior axis and ME differentiation, such as *DKK1* and *MIXL1* as well as *BRA*, respectively (Funa et al., 2015; Kimura-Yoshida et al., 2005; Mendjan et al., 2014). WNT3 is also crucial in later embryonic development, such as brain development, vascular development, and limb formation (Veerapathiran et al., 2020). While the canonical WNT signalling plays an important role in gastrulation, the noncanonical WNT signalling seems to be involved to a lesser extent. Activated RHOA and RAC1 regulate cytoskeletal rearrangement, cell polarity and motility, thereby facilitating EMT (James et al., 2008; Semenov et al., 2007). An increase of intracellular Ca^{2+} concentration mediated by PLC stimulates activation of PKC and calcium/calmodulin-dependent protein kinase II (CaMKII) as well as the nuclear factor of activated T cells (NFAT) (James et al., 2008). Although PKC, CaMKII, and NFAT have an extensive range of downstream targets in the Ca^{2+} -dependent WNT signalling pathway that regulate many important biological processes, it is worth noting that this pathway plays an essential role in embryonic development (James et al., 2008). Furthermore, the noncanonical ligand WNT11 is required for convergent extension movements in which cells elongate and exchange their positions during mouse gastrulation (Lickert et al., 2001). In hESCs, WNT5A and WNT11 are associated with later stage development, such as haematopoietic differentiation and cardiac development (Mazzotta et al., 2016; Vijayaragavan et al., 2009).

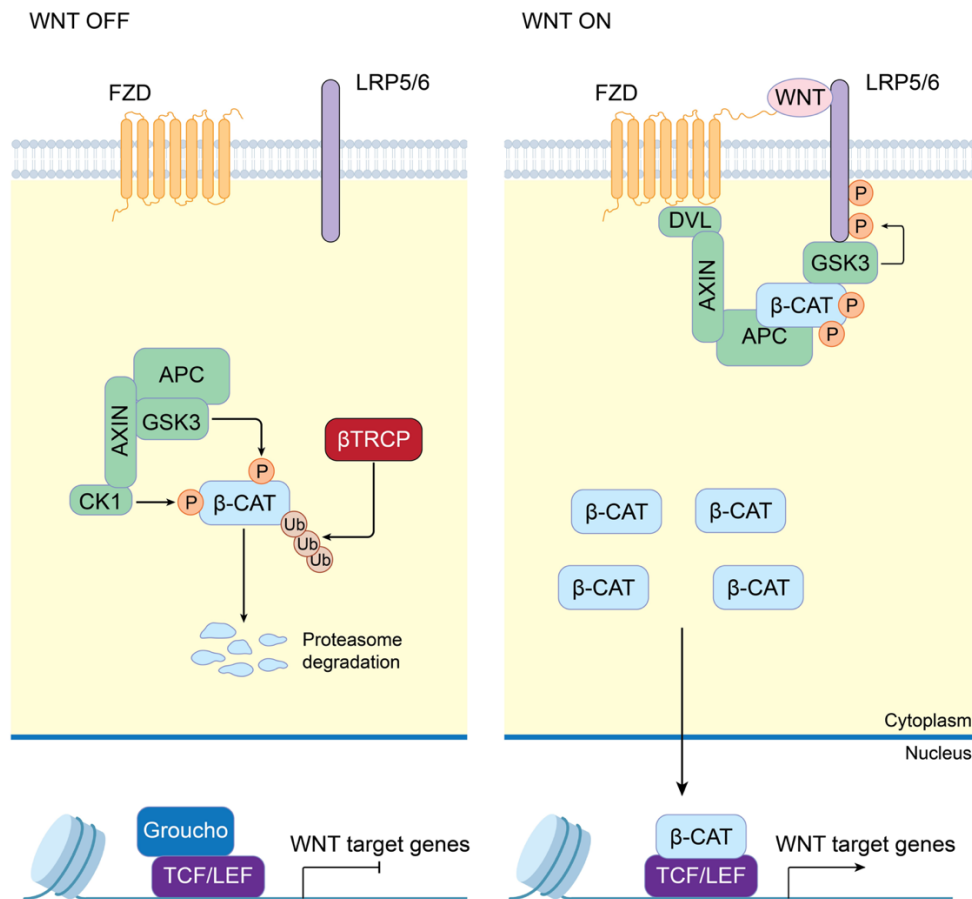


Figure 1.7 Simplified representation of the canonical WNT signalling pathway

Illustration of the canonical WNT signalling cascade in the presence or absence of WNT ligands. The degradation complex (in green) consists of AXIN, GSK3, APC and CK1 and acts to target β -CAT for degradation by the E3 ubiquitin ligase β TRCP (in red). The Groucho family transcriptional repressors inhibit TCF/LEF-mediated gene expression and are replaced by β -CAT upon its translocation.

iii. Nodal signalling

Nodal belongs to the TGF β subfamily of ligands. Like BMPs, Nodal becomes active after being cleaved and binds to the type I and type II receptors (Fig 1.6). Membrane co-receptors cripto and cryptic facilitate this binding process (David and Massagué, 2018). Then the type I receptors phosphorylate another R-SMADs, SMAD2 and SMAD3 (SMAD2/3) in Nodal signalling. SMAD4 forms heterotrimers with SMAD2/3 which then translocate to the nucleus and function as a TF.

Similar to BMP signalling, Nodal is also controlled at various levels (David and Massagué, 2018). Nodal shares the same ligand traps with BMPs, thereby preventing it from activating the signalling cascade. Nodal specifically binds to the type I receptors ACVR1B and ACVR1C but shares the type II receptors ACVR2 and ACVR2B with BMPs (Fig 1.6) (David and Massagué, 2018). Thus, small molecule inhibitors like SB431542 and SB505124 were introduced to specifically target Nodal type I receptors (DaCosta Byfield et al., 2004; Inman et al., 2002). Co-receptors cripto and cryptic assist in the binding of Nodal to receptors (Massagué, 2012). Antagonist ligands Lefty1/2 compete with Nodal for binding the receptors. In the cytoplasm, SMAD6 and SMAD7 still function as inhibitory SMADs. Likewise, phosphorylation of SMAD2/3 by CDK8 and CDK9 in the nucleus results in ubiquitylation and degradation (Fig 1.6).

Nodal signalling plays an essential role in gastrulation and ME differentiation through activation of specific DNA binding TFs. *Nodal* expression is initially present in epiblast cells in the ICM and then is confined to the posterior epiblast, where the primitive streak is formed (Conlon et al., 1994). Disruption of *Nodal* in the mouse embryo results in failure of PS formation (Brennan et al., 2001) and deletion of *Smad2* contributes to similar phenotypes (Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998). In mouse and human PSCs, SMAD2/3 in Nodal signalling induces ME differentiation through increased expression of TFs, including BRA, MIXL1 (Mix-like homeodomain protein 1), EOMES, GSC (Goosecoid) and FOXA2 (Forkhead box protein A2) (David and Massagué, 2018; Genander et al., 2014). Forkhead box protein H1 (FOXH1) already marks and occupies the region of these genes prior to SMAD activation by Nodal and allows binding of SMAD2/3 to initiate ME differentiation (Charney et al., 2017; Kim et al., 2011c). These TFs are also upregulated by the action of SMAD2/3 binding to TRIM33, which relieves the repressive chromatin state on the promoter region of these genes (Xi et al., 2011). EOMES and GSC then bind to SMAD2/3 to promote endoderm differentiation (Faial et al., 2015; Izzi et al., 2007; Teo et al., 2011). More importantly, activated SMAD2/3 promotes EMT by activating expression of EMT transcription factors (EMT-TFs), such as *SNAIL* and *SLUG*, which repress the PSC adhesion molecule E-cadherin (Dongre and Weinberg, 2019). Therefore, SMAD complexes in both BMP and Nodal signalling serve as primary developmental TFs, facilitating the recruitment of chromatin modifiers with lineage-specific factors, which eventually orchestrates the regulation of early embryonic development.

1.2.3 Intrinsic factors of the mesendoderm fate

Several genes have been demonstrated to be specifically expressed in the PS and play a crucial role in PS formation. In particular, WNT and NODAL signalling directly induce the expression of these genes which act in synergy to form the PS and to specify the mesoderm or DE fate.

i. BRACHYURY

BRACHYURY (BRA) is a T-box TF which is characterised by a highly conserved DNA-binding domain (Herrmann et al., 1990; Showell et al., 2004). BRA is commonly used as a ME marker, and BRA⁺ mouse and human PSCs are able to differentiate into mesoderm and definitive endoderm lineages (Gouon-Evans et al., 2006; Kubo et al., 2004; Lolas et al., 2014; Mazzotta et al., 2016). In mouse, this gene is initially expressed in the ExE and restricted to the posterior epiblast (Rivera-Pérez and Magnuson, 2005). During gastrulation *Bra* expression appears at the PS and posterior mesoderm and later on, its expression is confined to the developing notochord and tail bud (Chen et al., 2020; Showell et al., 2004). Disruption of *Bra* in mouse embryos leads to embryonic death shortly after gastrulation due to defective development of the PS and mesoderm (Herrmann et al., 1990; Showell et al., 2004). BRA expression is activated by several signalling pathways, such as WNT and NODAL in mouse and human ESCs (Arnold and Robertson, 2009; Mazzotta et al., 2016). BRA then directs mesoderm specification by targeting the promoter regions of genes such as *AXIN2*, *WNT3A*, *TBX6*, and *FGF8* to initiate their transcription (Evans et al., 2012; Lolas et al., 2014), forming a positive feedback loop with WNT signalling. In addition, BRA contains an enhancer-binding domain which facilitates transcription of genes related to EMT and embryonic development (Chen et al., 2020).

ii. MIXL1

Mix-like homeodomain protein 1 (MIXL1), the founding member of Mix/Bix family of Paired-like homeobox genes, is a TF that is responsible for the specification of the ME, endoderm, and later haematopoiesis in vertebrates. In *Xenopus*, Mixl1 is the downstream target of the growth factor Nodal in the transforming growth factor β (TGF β) pathway (Hart et al., 2002; Nelson et al., 2017). In mouse embryos, *Mixl1* expression is in its prime in the PS and nascent mesoderm and later restricted to the posterior PS (Pearce and Evans, 1999; Robb et al., 2000). *Mixl1*-null mouse embryos die around E9.0 at the gastrulation stage due to the defective PS and node, thus implicating the importance of MIXL1 in PS patterning (Hart et al., 2002). *In*

vitro studies have demonstrated that MIXL1 interacts closely with BRA and other members from the TBX family to induce mesoderm patterning, while it also activates GSC expression to direct endoderm differentiation (Pereira et al., 2011; Zhang et al., 2009).

iii. *EOMES*

The T-box TF Eomesdermin (EOMES) was originally recognised as a mesoderm marker in *Xenopus*, but emerging evidence suggests its role in the ME formation since EOMES is also crucial for DE specification in mouse embryos and in hPSCs (Arnold et al., 2008; Teo et al., 2011). In mouse, *Eomes* mRNA firstly appears in the TE at pre-implantation and its expression is subsequently restricted to the posterior epiblast before PS formation (Russ et al., 2000). During gastrulation, *Eomes* expression is co-localised with *Mixl1*, which is confined within the PS and nascent mesoderm, but later becomes confined to the anterior PS before being downregulated, suggesting a dual role of EOMES in ME and later DE specification (Russ et al., 2000). EOMES acts downstream of NODAL and functions as an activator of EMT by suppressing the expression of E-cadherin to facilitate mesoderm migration (Arnold et al., 2008). In hPSCs, EOMES cooperates with SMAD2/3 to direct ME differentiation with concomitant expression of BRA, EOMES itself and GSC, and subsequently induce DE differentiation while inhibiting the expression of mesoderm TFs (Teo et al., 2011; Yoney et al., 2022).

1.3 Human Pluripotent Stem Cells (hPSCs) – a cell model for studying early human development

1.3.1 Human Pluripotent Stem Cells

Laboratory animal models, especially mice, have been exploited as a model to study embryonic development for a long time and played an instrumental role to facilitate our understanding in embryonic development. However, using animal models has its own limitations for understanding embryonic development, particularly in humans. Firstly, *in vivo* animal models pose challenges to further study the molecular mechanisms underlying the resulted phenotypes. In addition, embryonic lethality is in conflict with animal welfare and genetic modification is labour-intensive and time-consuming. Thirdly, increasing evidence shows that regulatory mechanisms in mouse and human embryonic development are not the same. These limitations make pluripotent stem cells (PSCs), especially human PSCs (hPSCs), a great illustrative model to investigate molecular mechanisms during early embryonic development.

hPSCs constitute two main types based on their derivation materials, human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). hESCs are derived from the ICM of pre-implantation human embryos, while iPSCs are reprogrammed from somatic cells by introducing key pluripotent transcription factors with various methods (Takahashi et al., 2007; Thomson et al., 1998). Both hESCs and iPSCs have the capacity to self-renew indefinitely and meanwhile, retain their ability to give rise to all the cell types within the human body. Therefore, hPSCs have marked a new era in the study of human embryonic development and become a great source of cells for disease modelling as well as regenerative medicine.

Studies in mouse embryos have shown that PSCs are derived from not only the ICM of pre-implantation embryos but also the post-implantation epiblast, which are named as mouse ESCs and mouse epiblast stem cells (mEpiSCs), respectively (Tesar et al., 2007). They require different culture conditions and exhibit different colony morphology. mESCs *in vitro* organise into three-dimensional (3D) dome-shaped colonies, while mEpiSCs form 2D flat colonies. Despite both being pluripotent, mEpiSCs have downregulated expression of most of the pluripotent transcription factors but upregulated expression of lineage-specific factors (Buecker et al., 2014; Hackett and Surani, 2014). mESCs and mEpiSCs also possess different epigenetic characteristics. The latter inactivates the X chromosome in females and dramatically increases global DNA methylation levels (Gafni et al., 2013; Marks et al., 2012). Moreover,

mESCs can form a chimaera with high efficiency after being injected into blastocysts, but mEpiSCs cannot, possibly due to different epigenetic settings (Brons et al., 2007; Tesar et al., 2007). Therefore, mESCs and mEpiSCs are considered to be in different states of pluripotency, namely the naïve and primed state, respectively (Nichols and Smith, 2009; Weinberger et al., 2016). Interestingly, hESCs, which are derived from the ICM of pre-implantation embryos, exhibit morphological, molecular, and epigenetic hallmarks that are more similar to those of mEpiSCs than of mESCs, and both hESCs and mEpiSCs have the capability of teratoma formation to give rise to all three germ layers (Hanna et al., 2010; Pera and Tam, 2010). Thus, hESCs are considered to be in the primed state.

1.3.2 Control of pluripotency

Pluripotency of PSCs in culture is governed by two sets of factors, namely intrinsic and extrinsic factors (Pera and Tam, 2010; Weinberger et al., 2016; Young, 2011). Intrinsic factors are transcription factors that directly bind to regulatory elements of genes to control their expression, while extrinsic factors include growth factors, cytokines, and extracellular matrix (ECM) that have a great impact on the expression of intrinsic factors. Adequate application of extrinsic factors can keep the expression of intrinsic factors in balance to maintain self-renewal and pluripotency of PSCs.

1.3.2.1 Intrinsic factors

The intrinsic factors are represented mainly by the three core transcription factors OCT4, SOX2 and NANOG (collectively OSN). They work collaboratively to promote the expression of pluripotent genes and repress lineage-specific genes, thus maintaining the pluripotent state of PSCs (Boyer et al., 2005; De Los Angeles et al., 2015; Loh et al., 2006; Young, 2011).

i. OCT4

OCT4 is a member of the POU family proteins and is the most important and indispensable regulator of pluripotency. OCT4 interacts with SOX2 to form a protein complex that binds and activates a large number of pluripotency-related genes, including *OCT4* and *SOX2* themselves, *NANOG*, *FGF4* and others (Ambrosetti et al., 1997; Chew et al., 2005; Rodda et al., 2005; Shi and Jin, 2010). *Oct4* expression is first found in the blastomeres and later restricted to the

epiblast in the ICM as well as primordial germ cells in mouse embryos (Kehler et al., 2004; Palmieri et al., 1994). Deletion of *Oct4* in mouse embryos results in embryonic death at implantation due to a lack of pluripotent cells in the ICM, and these cells differentiate to the trophoblast lineage (Nichols et al., 1998), while overexpression of *Oct4* in mESCs leads to cell differentiation into primitive endoderm and mesoderm (Niwa et al., 2000). These findings suggest the function of OCT4 in inhibiting TE differentiation but promoting endoderm and mesoderm specification. Indeed, more findings demonstrate that an increase of OCT4 expression in ESCs could promote cell differentiation predominantly into the mesendoderm lineage while suppressing the ectoderm and extra-embryonic lineages (Thomson et al., 2011; Wang et al., 2012; Zeineddine et al., 2006). Therefore, OCT4 is not only crucial in the establishment and maintenance of pluripotency but is also involved in lineage specification in ESCs.

ii. SOX2

SOX2 belongs to the SRY homology box family proteins and is another key factor of retaining pluripotency. Similar to *Oct4*, *Sox2* expression is progressively restricted to the ICM of the pre-implantation blastocyst and the loss of its expression in mouse embryos results in malformation of the ICM (Avilion et al., 2003). Consistently, depletion of *Sox2* in mESCs compromises the pluripotent state and drives them into TE differentiation (Ivanova et al., 2006; Masui et al., 2007). The strikingly resembling phenotypes of *Oct4*-null and *Sox2*-null mESCs suggest that both SOX2 and OCT4 have a redundant role of regulating the same set of genes important in maintaining pluripotency, as described earlier. Nevertheless, silencing *SOX2* in both mouse and human ESCs results in downregulation of key pluripotent factors, such as OCT4 and NANOG (Fong et al., 2008; Masui et al., 2007), indicating that the triad cannot form the circuitry with one missing. Interestingly, SOX2 has also been found to regulate germ layer differentiation in ESCs. It promotes neuroectoderm differentiation but represses mesendoderm differentiation (Thomson et al., 2011; Wang et al., 2012). Therefore, SOX2 works synergistically with OCT4 to maintain pluripotency while mutually regulating each other to prevent spontaneous differentiation.

iii. NANOG

NANOG is a homeobox protein and is the third core factor that contributes to the maintenance of pluripotency. *Nanog* mRNA is initially detected in the morula and later confined to the ICM

in vivo; in the later stage, its expression is only restricted to the epiblast but not to the primitive endoderm (Mitsui et al., 2003). *Nanog*-null mouse embryos are arrested at the blastocyst stage due to a lack of epiblast cells (Mitsui et al., 2003). *In vitro* studies have demonstrated that high levels of Nanog in mESCs can sustain pluripotency in the absence of leukaemia inhibitory factor (LIF) which is an important extrinsic factor that prevents mESC from differentiation (Chambers et al., 2003; Mitsui et al., 2003). By contrast, a decrease in NANOG expression promotes both mouse and human ESC differentiation into extra-embryonic lineages (Hough et al., 2006; Hyslop et al., 2005; Ivanova et al., 2006), although mutant mESCs still retain the self-renewal and pluripotent property and contribute to somatic chimaeras (Chambers et al., 2007). In addition, NANOG was found to repress ectoderm differentiation in hESCs (Wang et al., 2012). Although NANOG is activated by OCT4/SOX2 and may be dispensable for maintaining pluripotency (Kuroda et al., 2005; Rodda et al., 2005), its expression definitely consolidates the pluripotent state of ESCs. Studies that demonstrate successful generation of iPSCs also confirm that OCT4 and SOX2 are the most important factors for the regulation of pluripotency (Takahashi et al., 2007; Takahashi and Yamanaka, 2006).

1.3.2.2 Extrinsic factors

Signalling that regulates and defines the pluripotent state in mESCs has been extensively studied. The initial culture condition for mESCs is to use mitotically inactivated MEFs as a feeder layer with foetal bovine serum (FBS), which provides the external cues required for maintaining the pluripotent state (Evans and Kaufman, 1981; Martin, 1981). Then, it was found that leukaemia inhibitor factor (LIF) could replace the MEF feeder (Smith et al., 1988; Williams et al., 1988). Furthermore, it was identified that BMP2/4 in the FBS is the main factors supporting mESCs in the presence of LIF. Thus, LIF and BMP4, which activate LIF-STAT3 and BMP signalling, respectively, were discovered to be the important ingredients that could replace the MEFs and serum to maintain mESC in a chemically defined environment (Ying et al., 2003). Later, a combination of three inhibitors for MEK, FGF, and GSK3 (termed 3i), which mimic blocking of MEK-ERK signalling and FGF signalling and activation of WNT/ β -catenin signalling, respectively, was reported to grow mESCs without other exogenous factors (Ying et al., 2008), suggesting the compensatory role of the three signalling for LIF-STAT3 signalling. However, mESCs in two inhibitors for MEK and GSK3, supplemented with LIF (termed 2i/LIF) conditions enhances proliferation and displays a more naïve compared to

in 3i conditions (Silva et al., 2008). Collectively, these findings reveal that activation of LIF-STAT3, BMP, and WNT signalling while inhibition of MEK-ERK and FGF signalling is essential to maintain the naive pluripotent state and growth in mESCs.

By contrast, primed mEpiSCs retain their pluripotent state *in vitro* by a different set of signalling. mEpiSCs can be maintained and propagated in widely used fibroblast growth factor 2/Activin A (FGF2/AA) defined growth conditions (Brons et al., 2007; Tesar et al., 2007). AA is an effective alternative to Nodal in the *in vitro* culture conditions as it is more stable while still stimulating the same downstream signalling cascade as Nodal without the need of co-receptor binding. FGF inhibits neuroectodermal differentiation and prevents reversion to a naïve state (Greber et al., 2010). Similarly, Nodal/Activin prevent differentiation into neuroectoderm (Greber et al., 2010). In addition, Nodal/Activin were found to activate the expression of *Oct4* and *Nanog* in mEpiSCs (Greber et al., 2010; Shen, 2007). Alternative growth conditions that use two inhibitors GSK3i and IWR (AXIN activator) simultaneously to inhibit the canonical WNT signalling are used to culture mEpiSCs without FGF2/AA supplementation (Kim et al., 2013a). Although the mechanisms by which the retention of cytoplasmic β -catenin regulates mEpiSC pluripotency remain unclear, cytoplasmic β -catenin increases E-cadherin stability and prevents induction of mesodermal gene expression, thereby possibly inhibiting differentiation (Chen et al., 2013b; Faunes et al., 2013). Interestingly, mEpiSCs can be converted to naïve mESCs by simple exposure to LIF, BMP4, and a feeder layer (Bao et al., 2009) or by reprogramming (Guo et al., 2009; Hanna et al., 2009). Thus, these findings suggest the essential role of MEK-ERK and Nodal/Activin signalling in the primed pluripotent state.

The signalling that regulates self-renewal and pluripotency in hESCs is far less studied compared to mESCs and mEpiSCs. Consistent with mEpiSC, primed hESCs also require FGF2 and Nodal/Activin signalling to maintain pluripotency (Brons et al., 2007; Tesar et al., 2007). FGF upstream of MEK-ERK, is necessary for the maintenance and enhanced proliferation of hESCs (Amit et al., 2000; Dvorak et al., 2005), while inhibition of ERK results in cell differentiation (Kang et al., 2005; Li et al., 2007). Nodal/Activin have been shown to inhibit hESC differentiation by activating SMAD2/3 which, in turn, promote the expression of *OCT4* and *NANOG* (James et al., 2005; Vallier et al., 2005; Vallier et al., 2009a; Vallier et al., 2004; Xu et al., 2008). In addition, FGF2 and Nodal/Activin work in synergy to activate PI3K/AKT signalling to promote proliferation and growth (Greber et al., 2007; Vallier et al., 2005), and to

suppress *BMP4* expression to prevent spontaneous differentiation in hESCs (Greber et al., 2007). Unlike BMP signalling in mESCs, its presence drives hESC differentiation, while the addition of the BMP4 antagonist Noggin in culture media retains pluripotency (Xu et al., 2005). Given that long-term propagation of naïve hESCs can be achieved after they are converted from primed cells (Gafni et al., 2013; Guo et al., 2021; Pastor et al., 2016; Warrier et al., 2017; Weinberger et al., 2016), it is evident that distinct states of pluripotency in both mouse and human ESCs are determined by their culture conditions and external cues. However, exactly what exogenous factors or signalling defines hESC pluripotency needs further investigation.

1.3.3 hPSCs in primed pluripotency with epithelial cell characteristics

hPSCs as primed PSCs exhibit similar morphology as post-implantation epiblast with epithelial cell characteristics having a structural polarity of apical, basal, and lateral regions (Eastham et al., 2007; Krtolica et al., 2007; Lamouille et al., 2014). hPSC colonies are flat and individual cells are tightly aligned by anchoring cell-cell junctions as most of epithelial cells (Pieters and van Roy, 2014).

1.3.3.1 Cell junctions

Cell-cell junctions are constructed by adhesion protein complexes and are crucial for intercellular communication and epithelial integrity. From lateral surfaces of the cells, there are four types of junctions: tight junctions (TJs), adherens junctions (AJs), desmosomes, and gap junctions (GJs) (Fig 1.8) (Huang et al., 2012).

i. Tight junctions

TJs are the most apically localised junctions and contain rows of transmembrane proteins as well as junctional plaque components. Transmembrane proteins are mainly tetraspan proteins, such as claudin and occludin, from the claudin family and the junctional MARVEL domain proteins, respectively, as well as single-span proteins such as junctional adhesion molecules (JAMs) and Crumbs (Fig 1.8) (Zihni et al., 2016). The junctional plaque is a cytosolic protein network comprised of cytoplasmic proteins that link the transmembrane proteins to actin filaments (Otani and Furuse, 2020). The main structural parts in the plaque are adaptor proteins, most notably, zonula occludens 1 (ZO-1) (Van Itallie and Anderson, 2014). Adaptor proteins,

especially ZO-1 and ZO-2, are required for TJ assembly (Otani et al., 2019; Umeda et al., 2006). The plaque also contains signalling proteins, such as PKCs and RHO GTPases, which are recruited by binding to adaptors or to transmembrane proteins (Fredriksson et al., 2015; Steed et al., 2014). Polarity signalling proteins promote apical polarisation and TJ maturation (Elbediwy et al., 2012; Liu et al., 2004; Wells et al., 2006). They regulate TJ formation and function by interacting with actin filaments (Itoh et al., 2012; Terry et al., 2011). The function of TJs is to mediate cell adhesion and therefore, they act as a barrier in two ways: (1) as the paracellular barrier to regulate the passive transport of molecules across the tissue, and (2) as the intracellular barrier to restrict the exchange of membrane substances between the apical and basolateral sides of the cell surface (Zihni et al., 2016). Moreover, TJs play a crucial role in epithelial polarity by stabilising polarity signalling proteins the PAR3/PAR6/aPKC complex and Crumbs/PALS1 (protein associated with Lin-7 1)/PATJ (PALS1-associated tight junction) complex (Roh et al., 2003; Roh et al., 2002; Suzuki et al., 2002; Suzuki et al., 2001). Although TJs seem to be dispensable for maintaining pluripotency in hPSCs, disrupting TJs enhances hPSC propensity for differentiation into specific lineages (Krtolica et al., 2007; Vasic et al., 2023).

ii. Adherens junctions and desmosomes

AJs and desmosomes lie below TJs, three of which together are called the apical junctional complex (Takeichi, 2014). Both AJs and desmosomes are cadherin-based junctions, but they differ in cadherin density (Fig 1.8) (Harris and Tepass, 2010). Desmosomes are formed by a single large cluster of cadherins which are packed densely but mediate strong cell adhesion; however, AJs have hundreds of clusters of cadherins disseminated through a larger junctional area (Harris and Tepass, 2010).

AJs are formed by three main protein complexes: cadherins, catenins, and actin filaments (Fig 1.8). E-cadherin is a highly expressed cadherin protein in epithelial cells that contains an extracellular domain, a transmembrane domain, and a cytoplasmic domain (Takeichi, 2014). The extracellular domain is required for Ca^{2+} -dependent homophilic interactions between cadherin molecules expressed from the neighbouring cells (Boggon et al., 2002). The cytoplasmic tail of E-cadherin is bound by p120 catenin and β -catenin. Catenins act as the key bridges that link cadherins to actin filaments. p120 catenin positively regulates E-cadherin by inhibiting its endocytosis and degradation (Davis et al., 2003; Ireton et al., 2002; Ishiyama et al., 2010). β -catenin interacts with the actin-binding protein α -catenin which acts as a linker

between the cadherin-catenin complex and actin filaments (Mège and Ishiyama, 2017). AJs have a mutual relationship with actin and microtubules, in that AJs modulate actin and microtubules and they promote AJ formation and stability (Harris and Tepass, 2010). AJ stability is strengthened by α -catenin directly binding to F-actin, a part of actin filaments, under stress (Buckley et al., 2014). Similar to TJs, the polymerisation and stability of actin filaments at AJs are predominantly modulated by small GTPases, including RHO GTPases, such as RHO, RAC and CDC42, and the RAS GTPase, RAP1 (repressor/activator protein 1) (Takeichi, 2014).

The main functions of AJs are cell-cell adhesion and tissue morphogenesis (Harris and Tepass, 2010; Mège and Ishiyama, 2017; Takeichi, 2014). As an important protein in AJs, E-cadherin is indispensable for pluripotency and survival of hPSCs (Li et al., 2012). Interfering with E-cadherin-mediated adhesion causes hPSC blebbing and apoptosis (Chen et al., 2010; Li et al., 2010a; Ohgushi et al., 2010), while stabilising E-cadherin and consequent reestablishment of cell-cell adhesion are necessary for hPSC survival (Xu et al., 2010). Inhibition of E-cadherin results in downregulation of the core pluripotency factors OCT4, SOX2 and NANOG (Li et al., 2010a; Li et al., 2010b). Mechanistically, both RHO and RAP1 play an important role in mediating cell adhesion by regulating E-cadherin stability in hPSCs (Li et al., 2010b; Xu et al., 2010).

iii. Gap junctions

GJs are localised at the basal side of the cell surface and they are formed by transmembrane proteins from the connexin protein family (Fig 1.8) (Aasen et al., 2016). Connexins assemble as hexamers and form the full channels with the neighbouring cells, which enables cell-cell communication through the passage of ions, particularly calcium ions, and small metabolic molecules, such as cAMP and NAD (Kanaporis et al., 2008; Stauffer, 1995). Because of cell-cell communication, GJs are essential for spread of electrical impulses of excitable tissues, such as the heart (Simon et al., 1998). GJs not only promote cell-cell adhesion but also regulate cytoskeleton organisation through interaction with actin filaments (Kotini and Mayor, 2015). Because of the importance of GJ integrity, connexins have been reported to be associated with cell migration and tissue morphogenesis (Aasen et al., 2016; Kotini and Mayor, 2015). GJs are essential for the establishment and maintenance of pluripotency in human PSCs, since Connexin 43 (CX43) and CX45 are required for retaining a pluripotent and proliferative state in hPSCs (Boyer et al., 2005; Ke et al., 2013; Ke et al., 2017; Sharovskaya et al., 2012; Todorova et al., 2008). The core pluripotent transcription factors OCT4, SOX2, NANOG target

the promoters of connexin family genes, further proving their critical role in pluripotency (Boyer et al., 2005). CX43 also participates in ESC differentiation as its downregulation in GJs impairs cardiomyocyte differentiation in mESCs (Zheng et al., 2017b).

1.3.3.2 Basement membrane

The basement membrane is a thin layer of ECM located at the bottom of tissues with which it strongly interacts (Sekiguchi and Yamada, 2018). The basement membrane contains a complex protein network and the core proteins are collagen IV, laminin, nidogen, and perlecan (Bonnans et al., 2014; Sekiguchi and Yamada, 2018). It forms a structural barrier and has important roles in cell attachment, migration, differentiation, polarisation, and tissue shaping (Sekiguchi and Yamada, 2018). During early embryonic development, the basement membrane lies at the boundary of the epiblast and hypoblast and it has a role in establishing and directing the epiblast in early morphogenesis (Kyprianou et al., 2020; Xiang et al., 2020). Proteins such as laminins and collagen IV mediate cell attachment by binding to the cell surface ECM receptors, integrins (Fig 1.8) (Yurchenco, 2011). Disruption of laminins, collagen IV, or their respective surface receptors results in early embryonic lethality in mice (Miner et al., 2004; Smyth et al., 1999; Williamson et al., 1997). Suppressing the activity of RHO and its downstream effector Rho-associated kinase (ROCK) promotes cell attachment to ECM and cell survival in hPSCs (Watanabe et al., 2007; Xu et al., 2010). In addition, the basement membrane sequesters and releases growth factors, including FGFs, BMPs, and NODAL, which stimulate hPSCs and regulate their pluripotency, migration, differentiation and survival (Jayadev and Sherwood, 2017; Rodin et al., 2014). Therefore, the basement membrane is crucial for survival, proliferation, and differentiation of hPSCs.

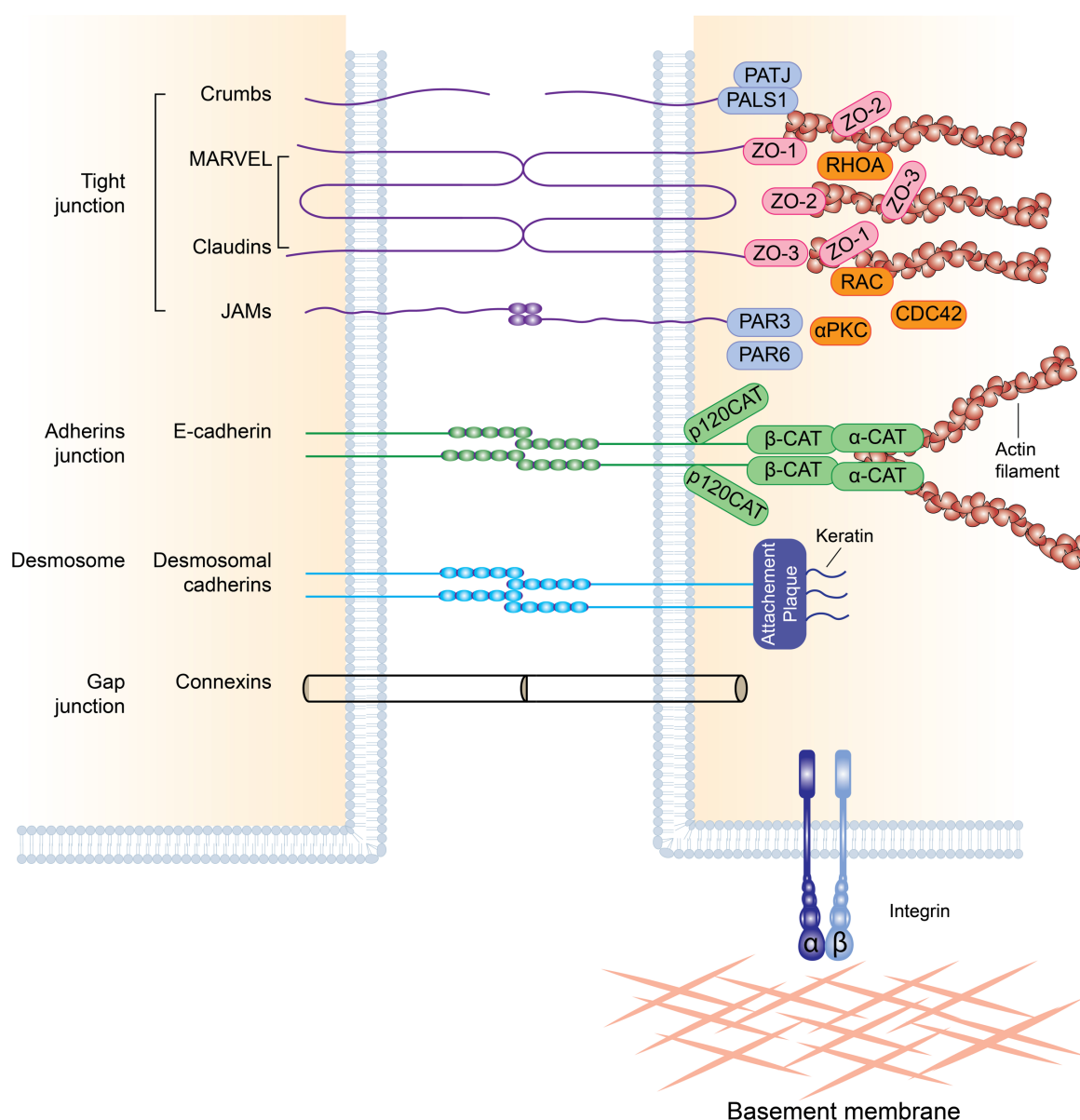


Figure 1.8 Simplified representation of cell junctions and adhesion protein complexes

Illustration of cell junctions and their associated adhesion protein complexes from lateral sides of hPSCs. From apical to basal sides of the cell, there are tight junctions (in purple), adherens junctions (in green), desmosomes (in light blue) and gap junctions (in black). Tight junctions and adherens junctions connect to actin filaments with ZO proteins (in pink) and cadherin-catenin complex (in green), respectively, linking in between. The bottom right side showing the cell attaches to the basement membrane through integrins (in cerulean).

1.3.4 hPSCs – a cell model for studying mesoderm/endoderm differentiation

As outlined earlier, gastrulation is initiated by growth factors and morphogens like BMPs, WNTs, Nodal and FGFs, and as a result, the PS is formed. However, the exact molecular mechanisms of how these signalling pathways orchestrate the formation of PS in humans remain unclear. hPSCs provide us with a valuable cell model for investigation into the molecular mechanisms as these cells can differentiate into mesoderm and endoderm. During mesoderm differentiation, hPSCs are initially cultured with BMP4, a WNT agonist, AA and FGF2 (Cheung et al., 2012; Loh et al., 2016; Matsuda et al., 2020; Mendjan et al., 2014), and subsequently specified to lateral mesoderm by stimulating BMP and WNT signalling (Burridge et al., 2014; Lian et al., 2012; Loh et al., 2016; Mendjan et al., 2014), while to paraxial mesoderm by inhibiting BMP signalling (Cheung et al., 2012; Loh et al., 2016; Matsuda et al., 2020; Umeda et al., 2012). Similarly, hPSCs, at first, are subjected to temporal treatment of BMP, WNT, AA and FGF2 for later DE differentiation (Green et al., 2011; Loh et al., 2014; Nostro et al., 2011; Touboul et al., 2010). Then a high dosage of AA supplemented with FGF2 induces DE specification and results in a great number of SOX17-positive cells (D'Amour et al., 2005; McLean et al., 2007). The striking similarity of the initial step between both mesoderm and endoderm differentiation suggests that the two lineages come from a common precursor ME (Tada et al., 2005; Vallier et al., 2009b). Extensive studies have demonstrated that hPSCs can induce BRA-positive (BRA+) PS-like ME cells by manipulating these signalling pathways *in vitro* and that different combinatorial treatments of extrinsic factors with varying concentrations all lead to the generation of BRA+ ME cells characterised by distinct regions along the anterior-posterior axis of the PS, which suggests that dynamic signalling gradients are formed along the axis (Bernardo et al., 2011; Faial et al., 2015; Loh et al., 2016; Matsuda et al., 2020; Mendjan et al., 2014; Vallier et al., 2009b).

Pioneering work has demonstrated that culturing hPSCs on the microcircular-patterned matrix-coated surface with BMP signalling leads to the generation of concentric ring patterns of germ layer derivatives reminiscent of those in the PS during gastrulation (Warmflash et al., 2014). Despite the homogenous exposure of BMP4, germ layers are organised in an ordered pattern that CDX2+ cells of an extraembryonic fate are localised at the colony edge, followed by SOX17+ endoderm, BRA+ mesoderm, and SOX2+ ectoderm cells towards the colony centre (Minn et al., 2020; Warmflash et al., 2014). Similar to the hierarchy of BMP/WNT/Nodal

signalling during mouse gastrulation, this signalling hierarchy is also observed in this human micropattern model which is termed human gastruloids (Martyn et al., 2018). Thus, this two-dimensional (2D) human gastruloid promises to be a robust model that helps analyse how cell fates are defined by the signal gradients from the colony edge through computational modelling (Chhabra et al., 2019; Shahbazi et al., 2019; Tewary et al., 2017).

1.4 mTORC2 signalling in PSCs

1.4.1 mTORC2 signalling in the regulation of PSCs

Due to a lack of mTORC2-specific inhibitors and the presence of intertwined signalling, the functions and underlying mechanisms of mTORC2 in cells and during embryonic development remain largely unknown. Investigation solely has recourse to gene manipulation of mTORC2 key component, RICTOR or mSIN1. Although several studies have been done using such approaches in animals as described in section 1.1.3, it has its limitations as described in section 1.3.1. Thus, these limitations make pluripotent stem cells (PSCs), especially human PSCs (hPSCs), a great illustrative model to investigate the functions and molecular mechanism of mTORC2 signalling in human embryonic development.

Previous studies have demonstrated the importance of mTOR signalling in the maintenance of self-renewal and pluripotency (Alhasan et al., 2021; Correia et al., 2020; Meng et al., 2018; Yu and Cui, 2016). Inhibition of mTOR by rapamycin promotes hESC differentiation into mesoderm and endoderm (Nazareth et al., 2016; Zhou et al., 2009), which suggests the essential role of mTOR in regulation of the undifferentiated state. Consistently, chemokine receptor 2 (CXCR2) inhibition-mediated downregulation of mTOR enhances mesoderm and endoderm differentiation in hPSCs (Jung et al., 2016). However, because mTOR signalling has been often studied collectively by combining two mTOR complexes together, the specific role of mTORC2 in PSCs, especially in human PSCs, is still poorly understood.

Nevertheless, recent studies have gained some insights into the role of mTORC2 in PSCs. Disturbing plasma membrane localisation of mTORC2 in mESCs impairs mesoderm specification via inactivation of AKT/GSK3/ β -catenin signalling pathway (Gómez-Salineró et al., 2016). This notion is further supported by two similar studies demonstrating that disruption of *Raptor* facilitates cardiomyocyte differentiation and that deletion of *Rictor* prevents cardiac differentiation in mESCs (Zheng et al., 2017a; Zheng et al., 2017b). Similarly, knockout of *RICTOR* in hESCs shows the loss of self-renewal and pluripotency, promoting their differentiation into neural lineages (Chu et al., 2022). Together these findings indicate that mTORC2 might play a role in the regulation of PSC pluripotency and differentiation. However, it is still largely unclear how mTORC2 regulates hPSC self-renewal and lineage specification.

1.4.2 Knockout of *RICTOR* in hESCs - Preliminary data from our lab

To investigate the role of mTORC2 in self-renewal and cell fate determination in hPSCs, our group have generated *RICTOR*-knockout (RIC-KO) hESCs by CRISPR/Cas9 and single-guided RNA (sgRNA) targeting technique (Chiu, 2019). *RICTOR* gene was disrupted in H1 hESCs using two sgRNAs that were designed to target exons 4 and 5 simultaneously, while *RICTOR* gene remained intact in wild-type (WT) controls that were transfected with the targeting vector containing no sgRNAs (Fig 1.9A) (Chiu, 2019). After clonal selection and validation of the absence of *RICTOR* expression, two RIC-KO clones (K1 & K5) were expanded.

Followed by the successful generation of H1 RIC-KO hESCs, they were subjected to further characterisation. RIC-KO hESCs expressed the core pluripotent transcription factors OCT4, SOX2, and NANOG at similar levels compared to their WT counterparts (Fig 1.9B) (Batool, 2022; Chiu, 2019). However, RIC-KO hESCs showed lower expression of E-cadherin and more cytoplasmic expression of β -catenin (β -CAT) (Fig 1.9C) (Batool, 2022). In addition, RIC-KO hESCs showed reduced expression of ZO-1 and disorganised assembly of actin filaments (Fig 1.9D). These data demonstrate that *RICTOR*/mTORC2 deficiency affects AJ stability and hinders TJ assembly in hESCs. When passaged on Matrigel-coated plates by mechanical dissection, RIC-KO hESCs exhibited lower cell attachment ability than WT controls and application of 10 μ M ROCKi clearly mitigated this effect (Fig 1.9E). To assess pluripotency of RIC-KO hESCs, they were subjected to an embryoid body (EB) differentiation assay. Unlike WT hESCs that only relied on ROCKi on the 1st day to assist the formation of aggregates, RIC-KO cells required ROCKi throughout EB formation, which, otherwise, caused the aggregates to gradually break down into small clusters (Fig 1.9F). To prevent the off-target effect of sgRNAs and eliminate cell line bias, *RICTOR*-knockdown (RIC-KD) cell line mediated by shRNA was generated from H7 hESCs and similar results were also observed in these KD hESCs (Batool, 2022). These results demonstrate that *RICTOR*/mTORC2 deficiency in hESCs has no detrimental effect on self-renewal and the expression of pluripotent markers, but that it prevents both cell-ECM and cell-cell interactions.

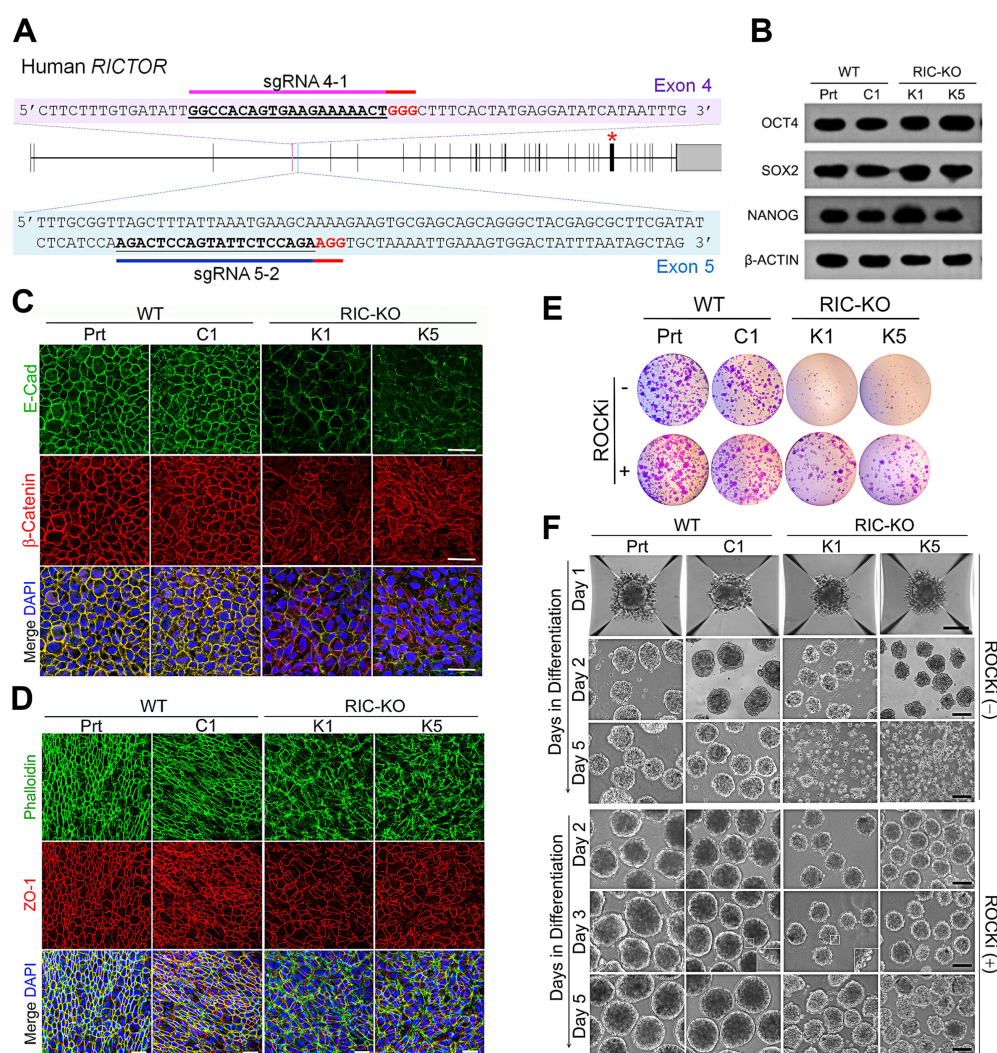


Figure 1.9 RICTOR-knockout hESCs retain the expression of the core pluripotent markers but exhibit reduced cell adhesion

(A) Schematic diagram depicting human *RICTOR* gene and sgRNA targeting sequences in exon 4 and 5 for generation of RICTOR-knockout (RIC-KO) hESCs. sgRNA sequences are underlined with the protospacer adjacent motif (PAM) highlighted in red. (B) Immunoblots show the protein expression of the core pluripotent transcription factors in RICTOR-wildtype (RIC-WT) and RIC-KO hESCs. Prt, parental hESCs; C1, WT control clones; K1 & K5, RIC-KO clones. (C) Immunostaining of WT and RIC-KO hESCs with E-cadherin (E-CAD) and β -catenin (β -CAT) antibodies. Scale bar, 30 μ m. (D) Immunostaining of WT and RIC-KO hESCs with ZO-1 antibody and Phalloidin (F-ACTIN) stain. Scale bar, 50 μ m. (E) Representative images of violet staining of hESC colonies 24 hours after plating in the presence or absence of Y-27632, ROCK inhibitor (ROCKi). (F) Representative phase-contrast images of two independent embryoid body (EB) formation experiments with WT and RIC-KO hESCs in the absence or presence of ROCKi. Scale bar, 100 μ m. All images were obtained and adapted from Batool and Chiu (Batool, 2022; Chiu, 2019).

After EB differentiation in the presence of ROCKi, both WT and KO hESCs were able to generate cells from three germ layers, even though KO cells revealed much less differentiation efficiency towards mesoderm and endoderm lineages. The proportions of mesodermal (Brachyury⁺) and endodermal (AFP⁺) cells were less in RIC-KO EBs (Fig 1.10A), and mesoderm (*BRACHYURY*, *TBX6* and *FLK1*) as well as endoderm (*FOXA2*, *GATA6* and *SOX17*) markers were expressed at much lower levels (Fig 1.10B) (Batoool, 2022). Therefore, *RICTOR*-deficiency in hESCs hinders their differentiation into both mesoderm and endoderm lineages via EB formation, which indicates that mTORC2 signalling might have a role in the regulation of ME differentiation. However, the underlying mechanisms are yet to be discovered.

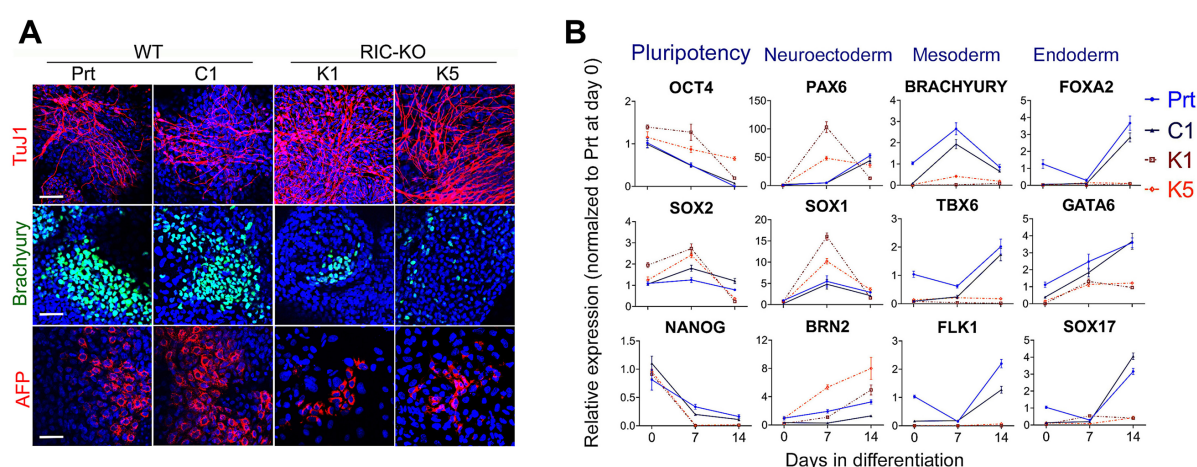


Figure 1.10 RICTOR/mTORC2 deficiency impedes mesoderm and endoderm differentiation in hESCs

(A) Immunostaining of three germ layer-specific markers, *TUJ1* (neuroectoderm marker), *Brachyury* (mesoderm marker), and *AFP* (Alpha-Fetoprotein, endoderm marker), in WT and RIC-KO hESCs after 14 days of embryoid body (EB) differentiation. Scale bar, 50 μ m. (B) Dynamic mRNA expression during EB differentiation by qRT-PCR. Data are presented as mean $\pm$ SD of 6 measurements of 2 independent experiments. All images were obtained and adapted from Batoool (Batoool, 2022).

1.5 Aims and objectives

My PhD project aims to elucidate the underlying mechanisms of mTORC2 in early embryonic development and cell fate determination. Based on our preliminary results, I posit that RICTOR/mTORC2 plays an important role in ME differentiation in hESCs through regulating genes or signalling pathways that are required for this process. To achieve the overall aim and test my hypothesis, I have the following objectives:

1. To explore if there is any transcriptomic difference between WT and RIC-KO hESCs that would have an impact on self-renewal and lineage specification;
2. To identify what gene(s) or signalling pathway(s) involved in driving ME differentiation are regulated by RICTOR/mTORC2 signalling;
3. To investigate how RICTOR/mTORC2 regulates such gene(s) or signalling pathway(s) in ME differentiation;
4. To examine if overexpression of the gene(s) or upregulation of the signalling pathway(s) will restore the efficiency of ME differentiation in RIC-KO hESCs.

Chapter Two

Materials and Methods

2.1 Cell Culture

2.1.1 Materials

2.1.1.1 Cell Lines

All cells were grown at 37 °C with 5 % CO₂.

Cell lines	Source
H1 hESCs (WA-01)	Male human ES cell line derived by (Thomson et al., 1998), distributed by WiCell.
H1 <i>RICTOR</i> -knockout hESCs	H1 hESCs transfected with the CRISPR-Cas9 vectors containing sgRNAs targeting exon 4 and 5 of <i>RICTOR</i> gene simultaneously (Chiu, 2019).
H1 <i>RICTOR</i> -control hESCs	H1 hESCs transfected with the CRISPR-Cas9 vector without <i>RICTOR</i> sgRNAs (Chiu, 2019).
Mouse Embryonic Fibroblasts (MEFs)	MEFS isolated from E13.5 CD-1 mouse embryos

2.1.1.2 Growth Factors and Inhibitors

Reagents	Supplier, Catalogue #	Working concentration
Activin A (AA)	Peprtech, 120-14E	50 -100 ng/ml
Basic Fibroblast Growth Factor (bFGF)	Peprtech, 100-18B	6 - 10 ng/ml
Bone Morphogenetic Protein 4 (BMP4)	R&D, 314-BP	50 ng/ml
CHIR-99021 (CHIR)	Strattech, A3011	6 µM
Y27632-Rho Kinase Inhibitor (ROCKi)	Cell guidance systems, SM-02	10 µM

2.1.1.3 Components of Culture Media

Component	Supplier, Catalogue #
200 mM L-Glutamine	Thermo Scientific, 25030024
100x Penicillin-Streptomycin (P/S)	Merck, P0781
30% Bovine Serum Albumin (BSA)	Merck, A9576
Dulbecco's Modified Eagle (DMEM) - High Glucose	Merck, D0819
Chemically Defined Lipid Concentrate (CDL)	Thermo Fisher, 11905031
Ham's F-12 Nutrient Mix (F12)	Thermo Fisher, 31765035
Heat-inactivated Foetal Bovine Serum (FBS)	Merck, F9665
Iscove's Modified Dulbecco's Medium (IMDM)	Thermo Fisher, 31980022
Insulin, Human Recombinant	Merck, 91077C
Knockout DMEM	Thermo Fisher, 10829018
Knockout Serum Replacement (KSR)	Thermo Fisher, 10828028
50 mM β -mercaptoethanol	Thermo Fisher, 31350010
Monothioglycerol	Merck, M6145
100x Non-essential Amino Acids (NEAA)	Merck, M7145
Apo-transferrin	Merck, T5391

2.1.1.4 Dissociation Enzymes

Enzyme	Supplier, Catalogue #
Accutase	Merck, A6964
Collagenase IV	Thermo Fisher, 17104019
Trypsin-EDTA solution	Merck, T3924

2.1.1.5 Chemicals, Reagents, and Cell Culture Matrix

Reagent	Supplier, Catalogue #
2% Gelatin Solution in H ₂ O	Merck, G1393
Dimethyl Sulphoxide (DMSO)	Merck, D2650
Dulbecco's Phosphate Buffered Saline (DPBS)	Merck, D8537
Matrigel® Matrix Growth Factor Reduced	Corning, 354230

2.1.1.6 Culture Media Recipes

Medium	Component
Chemically Defined Medium 2 (CDM2)	50% F12 and 50% IMDM supplemented with: 5 mg/ml BSA 1% CDL 0.7 µg/mL Insulin 450 µM Monothioglycerol 15 µg/mL Apo-transferrin 1x Penicillin-Streptomycin
MEF medium	DMEM - High Glucose supplemented with: 10% FBS 1x Penicillin-Streptomycin
Knockout Serum Replacement Medium (KSR-M) for MEF conditioning	KO-DMEM supplemented with: 6 ng/ml bFGF (freshly added) 20% KSR 1 mM L-glutamine 1x NEAA 0.1 M β-mercaptoethanol 1x Penicillin-Streptomycin
MEF-conditioned medium (MEF-CM) for hESCs	MEF conditioned KSR-M supplemented with: 10 ng/ml bFGF (freshly added) 1 mM L-glutamine 1x Penicillin-Streptomycin

2.1.2 Methods

2.1.2.1 Culture and Maintenance of hESCs

i. Isolation of MEFs from mouse embryos

Uterine horns from terminated pregnant CD-1 mice (E13.5) were washed three times with DPBS containing 2x P/S. Each embryo was taken out from the uterine horns, dissected to remove the viscera, finely minced, and dissociated by Trypsin into single cells at 37 °C in a bijoux tube. Tissues were subjected to an incubation and vortex cycle for several times to break any large clumps. MEFs in suspension were then isolated and transferred to a T75 flask on one bijoux tube per flask basis after trypsin neutralisation with MEF medium by allowing large undissociated clumps to settle. Cells were allowed to grow overnight and replenished with fresh medium on the following day after they were checked for contamination. After another 2-3 days of culture, MEFs in all flasks were trypsinised and combined. Then they were frozen down at 1×10^7 cells per cryovial in MEF medium supplemented with 10% DMSO and labelled as P0 (passage 0)

ii. Generation of MEF-CM

Frozen MEFs were thawed and propagated for 3 to 4 passages depending on how long the cells had been stored in liquid nitrogen in order to obtain a sufficient number for seeding. After MEFs were at 90% confluency, they were trypsinised, collected, and irradiated at 40 grays, 4000 rads using an IBL 637 Cell irradiator (CIS-Bio International) to prevent cell proliferation. Irradiated MEFs were counted and seeded into T225 flasks that were already coated with 0.5% gelatine at a density of 2.5×10^7 cells per flask. On the next day, MEFs were washed with DPBS and MEF medium was replaced with 150 ml KSR-M supplemented with 6 ng/ml bFGF per flask. After 24 hours, KSR-M conditioned with MEFs became MEF-CM and the medium was collected, labelled as Day 1, and stored at -80 °C for further use. Fresh KSR-M with bFGF was replenished into the flasks again as Day 2 medium and the entire collection process was repeated for up to 7 days. Contamination was checked on the final day of medium collection. Before use, defrosted MEF-CM was filtered to remove cell debris and 2 mM L-glutamine was freshly added. MEF-CM was stored at 4 °C and used within one week.

iii. Preparation of Matrigel[®] coated plates

Matrigel[®] was slowly thawed overnight on ice at 4 °C and diluted 1 in 2 with chilled KO-DMEM before being transferred into pre-chilled tubes as 1 ml aliquots. Aliquots were stored at -20 °C for further use. When needed, an aliquot was thawed at 4 °C for at least 2 hours before being further diluted 1 in 25 with chilled KO-DMEM. 6-well plates were normally used for routine hESC culture and coated with 1 ml of the diluted solution per well. Coated plates were allowed to set and stored at 4 °C for a maximum of 10 days and warmed to room temperature prior to use. Matrigel[®] solution was aspirated off right before adding fresh medium. In an emergency, coated plates could be used on the same day if they were set for at least 2 hours at room temperature.

iv. Routine culture and maintenance of hESCs

WT hESCs were cultured in Matrigel[®]-coated 6 well plates in MEF-CM supplemented with 10 ng/ml bFGF with medium refreshed daily. They were passaged every 4-6 days as smaller clusters by using 200 U/ml collagenase IV at 37 °C for 5-8 min to remove spontaneously differentiated cells followed by mechanical dissociation with 1ml MEF-CM. hESCs were regularly split at a 1:2-1:3 ratio depending on the confluency. For freezing hESCs, a confluent well (at least > 80%) was treated with collagenase IV and mechanically scraped in 1 ml chilled KSR. Cells were then transferred into cryovials and 10% v/v DMSO was added dropwise into the cryovials. Cells were stored at -80 °C overnight before being transferred into a liquid nitrogen tank for long term storage. RIC-KO hESCs were cultured the same way as WT hESCs, except that they were split at a 1:3-1:4 ratio and seeded with 10 µM ROCKi in the medium.

2.1.2.2 Directed hESC Mesendoderm Differentiation in Single Cells

Undifferentiated hESC cultures which were determined by cell morphology in colonies were chosen for mesendoderm differentiation. Normally, a 75-80% confluent well of a 6-well plate containing about 1.5 million hESCs was used for directed mesendoderm differentiation, the method of which was adapted from (Loh et al., 2016). Cells were washed once with DPBS and dissociated as single cells by Accutase at 37 °C for 5 min. MEF-CM supplemented with 10 ng/ml bFGF and 10 μ M ROCKi was used to neutralise Accutase followed by centrifugation at 800 rpm for 5 min. After resuspension, hESCs were seeded at 1×10^5 cells per well in a 24-well plate which was pre-coated with Matrigel[®]. The seeding number was adjusted accordingly with different cell culture plates. On the following day, hESCs in single cells were washed once with DPBS and subjected to differentiation in MEF-CM supplemented with 50 ng/ml AA, 50 ng/ml BMP4, 6 μ M Chir, and 10 ng/ml bFGF. After 24 hours, cells were then ready for further analysis.

2.1.2.3 BMP4-induced Differentiation in Colony

60-70% confluent wells of hESCs were treated with collagenase IV to remove spontaneously differentiated cells followed by fine mechanical dissociation with 1 ml MEF-CM per well. Colonies suspended in MEF-CM were subjected to several rounds of vigorous pipetting to break them into even smaller clumps. WT and RIC-KO hESCs were split in a 1:4 ratio and a 1:5 ratio, respectively, with MEF-CM supplemented with 10 ng/ml bFGF and 10 μ M ROCKi. Next day, hESCs were observed under the microscope for their colony size. Due to the nature of mechanical dissociation, colonies inevitably varied in size; however, the majority of the colonies should be approximately 150 – 500 μ m in diameter initially. Cells were then washed once with DPBS and refreshed with MEF-CM without ROCKi. On the following day, cells were washed again with DPBS and treated with the following ligands or small molecules: 50 ng/ml BMP4, 100 ng/ml AA, or 6 μ M Chir in MEF-CM supplemented with 10 ng/ml bFGF for 48 hours. Cells were then ready for further analysis.

2.2 Molecular Biology – DNA/RNA techniques

2.2.1 Materials

2.2.1.1 Reagents, Chemicals, and Kits

Reagent	Supplier, Catalogue #
1 kb Plus DNA ladder	NEB, N3200
50 bp DNA ladder	NEB, N3236
Acetic acid	Merck, A6283
Agarose (molecular biology grade)	Merck, A9539
Boric acid	Merck, B6768
Chloroform:Isoamyl alcohol 24:1	Merck, 25666
Ethylenediaminetetraacetic acid (EDTA)	Merck, E9884
Ethanol	VWR, 20821.310
Isopropanol	Thermo Fisher, BP2618212
KiCqStart [®] SYBR [®] Green qPCR ReadyMix [™]	Merck, KCQS02
Methanol	VWR, 20847.307
Nuclease-free water (molecular biology grade)	Merck, W4502
Oligo(dT) ₁₂₋₁₈	Thermo Fisher, 10753741
RiboLock RNase inhibitor	Thermo Fisher, EO0381
SafeGreen nucleic acid stain	NBS Biologicals, NBS-SG1
TRI Reagent [®]	Merck, T9424
Trizma [®] base (Tris base)	Merck, T1503
TRIzol [™] Reagent Plus RNA Purification Kit	Thermo Fisher, 12183555

2.2.1.2 Buffers and Solutions

Buffer	Component
50X Tris-Acetate-EDTA Buffer (TAE)	242 g Tris Base 57.1 ml Acetic Acid 18.6 g EDTA Autoclaved water to 1 L
10X Tris-Borate-EDTA Buffer (TBE)	108 g Tris Base 55 g Boric Acid 9.3 g EDTA Autoclaved water to 1 L

2.2.1.3 DNA Modifying Enzymes

Enzyme	Supplier, Catalogue #
DNase I	Thermo Fisher, 10753741
ProtoScript II Reverse Transcriptase	NEB, M0368
PureLink™ DNase	Thermo Fisher, 12185010
Q5® High Fidelity Polymerase	NEB, M0491

2.2.1.4 Primers for qRT-PCR

All primers were reconstituted to 100 μ M in nuclease-free water and stored at -20 °C as a stock. They may be further diluted to 10 μ M.

Gene	Forward strand	Reverse strand
<i>AXIN2</i>	GTGAGGTCCACGGAAACTGT	TGGCTGGTGCAAAGACATAG
<i>BRACHYURY</i>	TGCTTCCCTGAGACCCAGTT	GATCACTTCTTTCCTTTGCATCAAG
<i>BRN2</i>	ATCGGAGAGAGTTGAAGCCA	CCTCGTAAGGGGAATGTGAA

<i>CDH1</i>	CCCGCCTTATGATTCTCTGCTCGTG	TCCGTACATGTCAGCCAGCTTCTTG
<i>CDH2</i>	TCCAGACCCCAATTCAATTAATATTAC	AAAATCACCATTAAGCCGAGTGA
<i>CDX2</i>	GGGCTCTCTGAGAGGCAGGT	CCTTTGCTCTGCGGTTCTG
<i>CER1</i>	CTTGCCCATCAAAAGCCATG	CCAGGAAAATGAACAGACCCG
<i>DVL2</i>	TGAGCAACGATGACGCTGTG	GCAGGGTCAATTGGCTGGA
<i>EOMES</i>	AGGAATTCTTGCTTTGCTAATTCTG	CGAAGAAACAGCAAGAGCAGC
<i>FZD2</i>	CTTCTCACAGGAGGAGACGC	AAATGATAGGCCGCTCTGGG
<i>FZD7</i>	CGCCTCTGTTCTGCTACCTC	TCATGATGGTGCGGATACGG
<i>ID1</i>	CAGCCAGTCGCCAAGAAT	ACAGACAGCGCACCACCT
<i>LEF1</i>	AATGAGAGCGAATGTCGTTGC	GCTGTCTTTCTTTCCGTGCTA
<i>LRP6</i>	GACTGGGTGTCACGAAATCT	CGGGGTTCTCTAAGTCCTC
<i>MIXL1</i>	CCGAGTCCAGGATCCAGGTA	CTCTGACGCCGAGACTTGG
<i>NANOG</i>	TGATTTGTGGGCCTGAAGAAAA	GAGGCATCTCAGCAGAAGACA
<i>NOGGIN</i>	GCCAGCACTATCTCCACATCCG	AGCAGCGTCTCGTTCAGATCCT
<i>OCT4</i>	TCGAGAACCGAGTGAGAGGC	CACACTCGGACCACATCCTTC
<i>RPL22</i>	TCGCTCACCTCCCTTTCTAA	TCACGGTGATCTTGCTCTTG
<i>SOX1</i>	AACACTTGAAGCCCAGATGGA	GCAGGCTGAATTCGGTTCTC
<i>SOX2</i>	GCCGAGTGGAACCTTTTGTCG	GCAGCGTGTAATTATCCTTCTT
<i>TFAP2A</i>	GAGGTCCCGCATGTAGAA	CCGAAGAGGTTGTCCTTGT
<i>WNT3</i>	CCTTTGCTCTGCGGTTCTG	GAGGCGCTGTCATACTTGTC
<i>WNT3A</i>	TGTTGGGCCACAGTATTCCT	GGGCATGATCTCCACGTAGT
<i>WNT5A</i>	CCACATGCAGTACATCGGAG	CACTCTCGTAGGAGCCCTTG
<i>WNT5B</i>	CGGGAGCGAGAGAAGAACT	CGTCTGCCATCTTATACACAGC
<i>WNT8</i>	TGTGATGGGTCAAACAATGG	TCCTTCCCCTTCTCCAAACT
<i>β-ACTIN</i>	TGTCTGGCGGCACCACCATG	AGGATGGAGCCGCCGATCCA

2.2.2 Methods

2.2.2.1 Gene expression analysis

i. RNA isolation and extraction

Generally, about 10^6 cells were lysed in 1 ml of TRI Reagent[®] and were stored at -80 °C until further use. To obtain RNA, samples were thawed at room temperature and left to stand for 5 min to homogenize. For every 1 ml of TRI Reagent[®] used, 200 µl of chloroform:isoamyl alcohol was added in each tube followed by vigorous shaking, after which the samples were allowed to stand at room temperature for 15 min for phase separation. After centrifugation at 12000 xg for 15 min at 4 °C, the aqueous layer at the top containing RNA was transferred into a new tube and approximately an equal volume of isopropanol was added to precipitate DNA and RNA. The tubes were allowed to stand for 10 min at room temperature and subsequently were centrifuged 12000 xg for 15 min at 4 °C to get the pelleted DNA/RNA mixture by removing the supernatant. The pellet mixture was washed once with 1 ml of 75% ethanol followed by a final centrifugation at the maximum speed for 5 min at 4 °C. After ethanol was removed, the DNA/RNA mixture was allowed to air-dry for about 15 min and then reconstituted in 16 µl of nuclease-free water. Samples were further treated with DNase I in order to remove any genomic DNA in the samples. 2 U of DNase I and 2 µl of 10X reaction buffer were added into each sample which was incubated at 37 °C for 40 min in a Bio-Rad Dyad DNA Engine thermocycler. The DNase activity was fully inactivated by the addition of 2 µl of 50 mM EDTA at 65 °C for 20 min. The RNA concentration was measured by NanoDrop[™] Spectrophotometer 1000. If the quality of RNA was desired, 1 µg to 1.5 µg of RNA was used to generate cDNA.

ii. cDNA synthesis

Typically, 1.5 µg of total RNA was used as a template to synthesise cDNA for gene expression analysis. RNA was incubated with 1 µl of 50 µM Oligo(dT)₁₂₋₁₈ and 1 µl of 10 mM dNTP in a total volume of 10 µl in a thermocycler at 65 °C for 5 min to anneal the Oligo(dT)₁₂₋₁₈ primers to the RNA template. After the annealing step, each RNA sample reaction was added with a pre-mixed cocktail containing 4 µl of 5X ProtoScript II RT reaction buffer, 2 µl of 0.1 M DTT, 8 U of RiboLock RNase inhibitor and 200 U of ProtoScript II reverse transcriptase to a final

volume of 20 μ l. In the meanwhile, a “RT minus” cocktail where reverse transcriptase was not added was also prepared for each RNA sample to serve as the negative control for the detection of possible genomic contamination. Reactions were incubated at 42 °C for 1 hour followed by 20 min at 65 °C to inactivate the enzyme. The generated cDNA was diluted 1 in 10 with the addition of 180 μ l of nuclease-free water and stored at -20 °C until further use.

iii. Semi-quantitative Polymerase Chain Reaction (PCR)

Before quantitative real-time analysis of gene expression using cDNA, samples and their “RT minus” counterpart were checked by standard PCR with known housekeeping genes such as *RPL22* or *β -ACTIN* or genes that have several their own pseudogenes such as *OCT4* (Liedtke et al., 2007) to ensure that cDNA was synthesised successfully and not contaminated. In addition, newly designed primers were also tested to ensure their specificity for use in quantitative analysis. Generally, 2 μ l of diluted cDNA solution was used as a template, and 5 μ l of 5X Q5 reaction buffer, 200 μ M dNTP, 500 nM forward and reverse primer, and 0.5 U of Q5[®] high-fidelity DNA polymerase was added to a final volume of 25 μ l. Mixed reactions were incubated in the thermocycler with the following cycling conditions:

Step	Temperature	Time
1: Initial denaturation	98 °C	30 sec
2: Cycling denaturation	98 °C	10 sec
Primer annealing (T_a)	60 °C	30 sec
Extension	72 °C	30 sec
Repeat Step 2 for 25-35 cycles		
3: Final extension	72 °C	2 min
4: Hold	4 °C	Forever

Primers should be designed with a melting temperature (T_m) in a range of 60-65 °C. Usually, the T_a in the annealing step was set about 3-5 °C lower than the T_m of the primers. Reactions were terminated upon the addition of 6X SafeGreen nucleic acid stain and were analysed via agarose gel electrophoresis. Samples along with the 50 bp DNA ladder were loaded into a 1.5% agarose gel dissolved in 1X TAE buffer and were run at 100V for 30 min. PCR products should appear as a clean single band around 100-200 bp in size.

iv. Quantitative real-time Polymerase Chain Reaction (qRT-PCR)

After validation by standard PCR, cDNA samples were used in qRT-PCR to measure gene expression. 1 µl of the cDNA template was used per reaction to mix with 5 µl of KiCqStart® SYBR® Green qPCR ReadyMix™, 500 nM forward and reverse primer was added to a final volume of 10 µl per reaction. Each sample reaction was made at least in duplicate for each gene. Reactions were run on an Applied Biosystems StepOnePlus™ Real-Time PCR System with the following cycling conditions:

Step	Temperature	Time
1: Initial denaturation	95 °C	2 min
2: Cycling denaturation	95 °C	15 sec
Primer annealing (T _a)	60 °C	30 sec
Extension and data collection	72 °C	30 sec
Repeat Step 2 for 40 cycles		
3: Melt curve read/sec	67-91°C	+ 0.3 °C/sec

Production of the PCR product was recorded in real-time by measuring the intensity of SYBR® green fluorescence. Melt curve step was carried out for each pair of primers to ensure a single PCR product at the end. Results were analysed by software provided by the manufacturer and calculated by Comparative Ct ($2^{-\Delta\Delta C_t}$) method. The difference of raw Ct values in duplicate should be within 0.8 cycle, which was considered to be acceptable. Raw Ct values were normalised to the Ct values of endogenous housekeeping genes such as *RPL22* or *β-ACTIN* by subtraction to generate the ΔC_t value. $\Delta\Delta C_t$ values were calculated by normalisation to control samples, and then the relative gene expression was obtained by taking the $-\Delta\Delta C_t$ values as the exponential function of 2.

2.2.2.2 RNA-sequencing (RNA-seq) analysis

i. RNA isolation and extraction

Because RNA-seq requires high-quality RNA from samples, RNA extraction was performed by TRIzol™ Plus RNA Purification Kit according to the manufacturer's instruction. In brief, one 75-80% confluent well containing about 1.5×10^6 high-quality hESCs was lysed in 1 ml of TRIzol™ Reagent and were stored at -80 °C until further use. To extract RNA, samples were transferred to the homogenised Phasemaker™ Tubes followed by the addition of 200 µl of chloroform:isoamyl alcohol. The Phasemaker™ Tubes form a barrier between the upper and lower phases, which facilitates the transfer of the aqueous phase. After centrifugation at 12000 xg for 5 min at 4 °C, the aqueous layer was transferred into a new tube and an equal volume of 70% ethanol was added to precipitate DNA and RNA. In order to obtain high-quality RNA, each sample was transferred to a spin cartridge and centrifuged at 12000 xg for 15 sec to remove impurities. After the flow-through was discarded, 350 µl of Wash Buffer I was added and the samples were centrifuged again at 12000 xg for 15 sec. The samples were subjected to on-column DNase treatment for which 8 µl of 10X DNase I Reaction Buffer and 30 U of PureLink® DNase was added to a final volume of 80 µl per sample. The samples were incubated for 40 min at room temperature followed by the addition of another 350 µl of Wash Buffer I and centrifugation at 12000 xg for 15 sec. The membrane-bound RNA was washed twice with 500 µl of Wash Buffer II and centrifuged as mentioned above. After flow-through was discarded, the membrane-bound RNA in the spin cartridge was dried by centrifugation at the maximum speed, after which purified RNA samples were spun down at the maximum speed and eluted in collection tubes by adding 30 µl of nuclease-free water. The RNA concentration and quality was measured by NanoDrop.

ii. RNA-seq

The library preparation, sequencing, and initial quality check were performed by Imperial BRC Genomics Facility (IGF). Libraries were prepared as strand-specific and sequenced on Illumina HiSeq 4000 (2 x 75 bp) to a depth of 30 million reads.

iii. RNA-seq data analysis

After the raw data were sent back, quality filtering of the paired-end reads was performed by FastQC v0.11.9 (Andrews, 2010) followed by combining analysis results in a summary report by MultiQC v1.9 (Ewels et al., 2016). Data trimming was carried out by Fastp v0.23.2 (Chen et al., 2018) to remove sequencing adaptors and low-quality bases (< Q15). The human genome FASTA file (https://ftp.ensembl.org/pub/release104/fasta/homo_sapiens/dna/Homo_sapiens.GRCh38.dna.primary_assembly.fa.gz) and gene annotation GTF file (https://ftp.ensembl.org/pub/release104/gtf/homo_sapiens/Homo_sapiens.GRCh38.104.gtf.gz) were obtained from Ensembl. STAR v2.7.10a (Dobin et al., 2013) was then used to create genome mapping indices using the two files above as input followed by mapping the trimmed reads to the human genome by the indices. Quality of mapping such as uniquely mapped rates was checked by SAMtools v1.6 (Li et al., 2009), after which the expression of genes and transcripts was quantified by RSEM v1.3.3 (Li and Dewey, 2011). All downstream analyses were carried out in R v4.1.2 using Bioconductor packages. After genes with no reads across all the samples were removed, *variance stabilising transformation* (VST) was used to correct for variance differences caused by sequencing depth across the samples and *limma* v3.50.3 (Ritchie et al., 2015) was used to remove the batch effect between biological replicates so that principal component analysis (PCA) would best reflect samples' actual clustering. Differential expression and statistical analysis were performed by DESeq2 v1.34.0 (Love et al., 2014) with a false discovery rate (FDR) threshold of 0.05, which implied that there was only 5% chance of false positives among all significantly expressed genes. P-values were adjusted using the Benjamini-Hochberg method in which the formula is:

$$p\text{-adj} = p\text{-value} \times \frac{m}{k}$$
 where m is the number of independent tests (in this case, the number of non-zero genes) and k is the rank of a p-value sorted from the smallest to the largest (Benjamini and Hochberg, 1995; Jafari and Ansari-Pour, 2019).

Genes with a p-adj value < 0.05 and a log2 fold change (FC) > 1 (*i.e.*, FC > 2) were considered to be differentially expressed. Gene z-score values of the 50 most differentially expressed genes were used for heatmap and hierarchical clustering visualisation.

iv. Gene ontology (GO) and pathway analyses

GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed with the over-representation analysis using *clusterProfiler* v4.2.2 in R (Yu et al., 2012). Differentially expressed genes with p-adj value < 0.05 and $\log_2FC > 1$ were first classified into either the upregulated or downregulated group after which the two groups were individually run in the program to obtain enriched GO terms and pathways. P-values were again adjusted using the Benjamini-Hochberg method with a cut-off at 0.05.

2.3 Molecular Biology – Protein techniques

2.3.1 Materials

2.3.1.1 Reagents, Chemicals, and Kits

Reagent	Supplier, Catalogue #
30% Acrylamide/Bis-acrylamide	Merck, A3699
4',6 diamidino-2-phenylindole dihydrochloride (DAPI)	Merck, D9542
Ammonium persulphate (APS)	Merck, A3678
Pierce™ Bicinchoninic Acid Assay (BCA) Protein Assay Kit	Thermo Fisher, 23227
Bovine Serum Albumin (BSA)	Merck, A9647
Bromophenol blue	Merck, B0126
Clarity Western Enhanced chemiluminescence (ECL) substrate	BioRad, 1705061
Donkey serum	Merck, D9663
Ethylenediaminetetraacetic acid (EDTA)	Merck, E9884
Pierce™ 16% Formaldehyde (w/v)	Thermo Fisher, 28908
Glycine	Merck, G8898
Goat serum	Merck, G9023
Immobilon® Forte Western HRP substrate	Millipore, WBLUF
Immobilon®-P polyvinylidene fluoride (PVDF) Membrane	Millipore, IPVH00010
Isopropanol	Thermo Fisher, BP2618212
Methanol	VWR, 20847.307
Mowiol 4-88	Millipore, 475904
Nonidet® P-40 substitute (NP-40)	VWR, E109
Phalloidin-iFluor 488 reagent	Abcam, ab176753
Phenylmethanesulfonyl fluoride (PMSF) solution	Merck, 93482
Phosphatase inhibitor cocktail 2	Merck, P5726
Prestained protein ladder	Abcam, ab116028
Protease inhibitor cocktail	Merck, P8340

Skim milk powder	VWR, 84615.05
Sodium azide (NaN_3)	Merck, S2002
Sodium chloride	Merck, S9888
Sodium deoxycholate	Merck, D6750
Sodium orthovanadate (Na_3VO_4)	Merck, S6508
Sodium dodecyl sulfate (SDS)	Merck, L3771
20% SDS solution	Fisher BioReagents, BP13111
Sodium fluoride (NaF)	Merck, 450022
Tetramethylethylenediamine (TEMED)	Merck, T7024
Triton X-100	Merck, T8787
Trizma [®] base (Tris base)	Merck, T1503
Tween-20	Merck, P1379
X-Ray film	SLS, MOL7016
β -mercaptoethanol	Merck, M6250

2.3.1.2 Buffers and Solutions

Immunoblotting Buffer/Solution	Component
Radio Immunoprecipitation Assay (RIPA) Buffer	25 ml 1 M Tris-HCl pH 8.0 (50 mM) 5 ml NP-40 (1% v/v) 4.383 g NaCl (150 mM) 2.5 g Sodium deoxycholate (0.5% w/v) 0.5 g SDS (0.1% w/v) Autoclaved water to 500 ml
Sodium fluoride stock	420 mg NaF Dissolved in 50 ml autoclaved water (200 mM)
Sodium orthovanadate stock	183.9 mg Na_3VO_4 Dissolved in 10 ml autoclaved water (100 mM) Adjust pH to 10.0

	Repeat boil/cool cycles until solution remains colourless at pH 10.0
5X Sample buffer	1.25 ml 0.5 M Tris-HCl pH 6.8 (50 mM) 2.5 ml glycerol (25 % v/v) 2 ml 10% SDS (2% w/v) 400 µl 0.5% bromophenol blue (0.02 % w/v) 500 µl β-mercaptoethanol (715 mM) Autoclaved water to 10 ml
4% Polyacrylamide Stacking Gel	2.5 ml 0.5 M Tris-HCl pH 6.8 (125 mM) 1.3 ml 30% Acrylamide/Bis (4% v/v) 100 µl 10% SDS (0.1% w/v) 50 µl 12% APS (0.06% w/v) 12 µl TEMED (0.12% v/v) Autoclaved water to 10 ml
8% Polyacrylamide Running Gel	2.5 ml 0.5 M Tris-HCl pH 6.8 (125 mM) 2.7 ml 30% Acrylamide/Bis (6% v/v) 100 µl 10% SDS (0.1 % w/v) 75 µl 12% APS (0.075 % w/v) 18 µl TEMED (0.18 % v/v) Autoclaved water to 10 ml
10X SDS Running Buffer	30.3 g Tris base (250 mM) 144.2 g Glycine (1.9 M) 50 ml 20% SDS (1% w/v) Autoclaved water to 1 L
10X Transfer Buffer	30.3 g Tris base (250 mM) 144.2 g Glycine (1.9 M) Autoclaved water to 1 L
1X Transfer Buffer	100 ml 10X Transfer Buffer 200 ml methanol (20% v/v) Autoclaved water to 1 L
Tris Buffered Saline with Tween-20 (TBS-T)	20 ml Tris-HCl pH 7.6 (20 mM) 7.597 g NaCl (130 mM) 1 ml Tween-20 (0.1 % v/v) Autoclaved water to 1 L
Blocking buffer	10 g BSA or Skim milk (5% w/v) 200 ml 1X TBS-T

Immunoblotting Buffer/Solution	Component
Fixation buffer	10 ml Pierce™ 16% Formaldehyde PBS to 40 ml
Phosphate buffered saline with Tween-20 (PBS-T)	1L PBS 1 ml Tween-20 (0.1 % v/v)
Blocking/permeabilising buffer	1 ml goat/donkey serum (10% v/v) 0.25 g BSA (2.5% w/v) 30 µl Triton X-100 (0.3% v/v) PBS-T to 10 ml
Mowiol mounting solution	12 ml 200 mM Tris-HCl pH 8.5 (133 mM) 6 g glycerol 2.4 g Mowiol 4-88 6 ml autoclaved water Heat tubes at 50 °C to dissolve Mowiol

Flow Cytometry Buffer	Component
Staining Buffer	1 ml FBS (2% v/v) 29 mg EDTA (2 mM) PBS to 50 ml
Blocking Buffer	1 ml goat serum (40% v/v) 1.5 ml Staining Buffer (60% v/v)

2.3.1.3 Antibodies

Antigen	Supplier, Catalogue #	Application and Dilution
AKT	Cell Signaling, 9272	IB: 1:1000
AKT-pS473	Cell Signalling, 4060	IB: 1:2000
α-TUBULIN	Cell Signalling, 3873	IB: 1:4000
β-ACTIN	Proteintech, 66009	IB: 1:50000

BRACHYURY	Cell Signaling, 81694	IF: 1:250
E-CADHERIN	Thermo Fisher, 13-1700	IB: 1:2000, IF: 1:250
FOXO1-pS256	Cell Signalling, 2880	IB: 1:2000
FOXO1	Cell Signalling, 84192	IB: 1:1000
GSK3 α / β -pS21/9	Cell Signaling, 9331	IB: 1:1000
GSK3 α / β	Santa Cruz, sc-7291	IB: 1:800
LEF1	Cell Signaling, 2230	IB: 1:500, IF: 1:200
mLST8 (GBL)	Thermo Fisher, MA5-14993	IB: 1:1000
mTOR	Cell Signaling, 2983	IB: 1:500
NANOG	Cell Signaling, 4903	IB: 1:2000
OCT4	Cell Signaling, 75463	IB: 1:2000
p70-S6K-pT389	Cell Signaling, 9234	IB: 1:1000
p70-S6K	Cell Signaling, 9202	IB: 1:1000
PKC α / β II-pT638/641	Cell Signaling, 9375	IB: 1:1000
PKC α	Cell Signaling, 59754	IB: 1:1000
PKC ζ -pT560	Abcam, ab62372	IB: 1:2000
PKC ζ	Thermo Fisher, PA5-17589	IB: 1:2000
RAPTOR	Cell Signaling, 2280	IB: 1:800
RICTOR	Bethyl Laboratories, A300-459A	IB: 1:1000
SMAD1/5/9-pS463/465/467	Cell Signaling, 13820	IB: 1:500, IF: 1:800
SMAD1	Cell Signaling, 6944	IB: 1:1000
SOX2	R&D Systems, AF2018	IB: 1:2000, IF: 1:300
ZO-1	Thermo Fisher, 33-9100	IB: 1:2000, IF: 1:200
SSEA1	Thermo Fisher, MA1-022	Flow: 1:100
SSEA4	Thermo Fisher, MA1-021	Flow: 1:50
Tra-1-81	Thermo Fisher, MA1-024	Flow: 1:50
Goat IgG-HRP	Santa Cruz, sc-2354	IB: 1:10000
Mouse IgG (H+L)-HRP	Thermo Fisher, G-21040	IB: 1:5000-1:20000

Rabbit IgG (H+L)-HRP	Thermo Fisher, 31462	IB: 1:2000-1:10000
Mouse IgG3 Isotype Control	Thermo Fisher, MG300	Flow: 1:100
Mouse IgM Isotype Control	Thermo Fisher, 02-6800	Flow: 1:100
Mouse IgG3-FITC	Santa Cruz, sc-2858	Flow: 1:100
Mouse IgM-PE	Santa Cruz, sc-2870	Flow: 1:100
Goat IgG-Alexa Fluor™ 488	Thermo Fisher, A-11055	IF: 1:500
Mouse IgG-Alexa Fluor™ 488	Thermo Fisher, A-11001	IF: 1:500
Mouse IgG-Alexa Fluor™ 568	Thermo Fisher, A-11004	IF: 1:500
Rabbit IgG-Alexa Fluor™ 488	Thermo Fisher, A-11008	IF: 1:500
Rabbit IgG-Alexa Fluor™ 568	Thermo Fisher, A-11011	IF: 1:500

2.3.2 Methods

2.3.2.1 Protein extraction and quantification

Typically, cells were refreshed with appropriate medium for 1 hr prior to protein extraction. After cells were washed with ice-cold PBS, every 10^6 cells were lysed in 100 μ l ice-cold RIPA buffer supplemented with phosphatase (Na_3VO_4 , 1:100 and NaF, 1:100, or phosphatase inhibitor cocktail 2, 1:100) and protease (PMSF, 1:100, and protease inhibitor cocktail, 1:100) inhibitors followed by passing through a 21-gauge syringe needle several times to shear DNA. Cell lysates were centrifuged at 14000 rpm for 20 min at 4 °C to remove debris, after which the supernatant containing proteins was aliquoted in a desired volume and stored at -80 °C until further use. Protein concentration was determined by Pierce™ BCA Assay Protein Quantification Kit according to manufacturer's instructions. The protein absorbance reads were measured at a wavelength of 562 nm.

2.3.2.2 Immunoblotting

10-25 μ g of proteins diluted in 5X sample buffer were denatured at 95 °C for 5 min before being loaded in the stacking gel. Protein samples were resolved at 120 V for 90 min through the stacking and running polyacrylamide gels filled with SDS running buffer. Proteins were then transferred to PVDF membranes using the classical wet transfer at 100 V for 60 min at 4 °C. Successful transfer of the proteins would be indicated by coloured blots of the prestained protein ladder. Blots were probed with desired primary antibodies at 4°C overnight after incubation in blocking buffer for 1 hr. The following day, membranes were washed 3 times with 1X TBS-T followed by 1-hour incubation with the appropriate horse radish peroxidase (HRP)-conjugated secondary antibody diluted in blocking buffer. Membranes were washed 3 times with 1X TBS-T again to wash off excess antibody. Blots were then able to be detected by enhanced chemiluminescent (ECL) substrate and exposed onto X-ray films using an Optimax X- ray film processor (Thermo Fisher). The intensity of the blots, which was proportional to the levels of protein expression, was quantified using ImageJ. The expression of target proteins was normalised to that of loading-control proteins, such as α -TUBULIN and β -ACTIN, while the expression of phospho-proteins was first normalised to that of their respective total version and then to that of the loading controls.

2.3.2.3 Immunostaining of hESCs and their derivatives

hESCs were seeded and cultured on Matrigel® coated Thermanox™ plastic coverslips (Thermo Fisher, 174950) until the appropriate confluency or treatment time was reached. Cells were washed with ice-cold PBS and fixed for 15 min with 4% formaldehyde. Excess formaldehyde was removed by washing 2 times with PBS before coverslips were incubated with blocking/permeabilising buffer for 1 hr followed by overnight incubation with primary antibodies in the appropriate dilution at 4 °C with rocking. Next day, cells were washed 3 times with PBS-T followed by 1-hour incubation with the appropriate fluorophore-conjugated secondary antibody in the dark. Coverslips were washed once with PBS-T followed by a PBS-T + DAPI or PBS-T + DAPI + Phalloidin wash with DAPI at a final concentration of 1 μ g/mL

to stain the nuclei and Phalloidin at the dilution of 1:1000 to stain actin filaments and a final wash with PBS-T in the dark. Coverslips were placed onto microscope slides using Mowiol 4-88 solution to dry overnight. Stained cells were visualised under a Leica SP5 II confocal fluorescent microscope.

2.3.2.4 Imaging analysis

Quantification of % BRACHYURY⁺ (BRA⁺) cells in immunofluorescent images was performed in ImageJ. The brightness of the BRA, SOX2 and DAPI stained images was adjusted by subtracting background using the Rolling Ball method with a radius of 50 pixels. The adjusted images were then split into three individual channels followed by threshold adjustment to fine tune the signal in each channel. Then individual channels were converted into binarized images before a built-in watershed algorithm was applied to separate overlying nuclei. Finally, the number of nuclei was counted using “analyze particles” in which the size of a particle greater than 25 pixel² would be recognised as a nucleus. The % of BRA⁺ cells in each image would be calculated as the ratio of the number of BRACH cells relative to the number of DAPI cells. Results were plotted as mean $\pm$ 95% CI in each cell line for each condition.

2.3.2.5 Flow cytometry for cell surface antigens of hESCs

Typically, a 70-80% confluent well of good-quality hESCs in one 6-well plate was used to analyse one antigen. hESCs were washed once with DPBS followed by dissociation into single cells using Accutase which was then neutralised by adding MEF-CM. After pelleted by centrifugation at 1300 rpm for 5 min, cells were resuspended in 150 μ l blocking buffer and incubated on ice for 15 min. Blocking buffer was removed by centrifugation at 1300 rpm for 5 min and staining buffer was used to wash cells two times via centrifugation. Cells resuspended

in staining buffer were transferred into two tubes in which an appropriate primary antibody and its isotype control (IgM or IgG) were added. After incubation on ice for 30 min, cells were centrifuged at 1300 rpm for 5 min to remove primary antibody and resuspended in staining buffer. Cells were then incubated with the appropriate fluorescent dye-conjugated secondary antibody diluted in staining buffer on ice for 30 min in the dark. Cells were washed 3 times with PBS via centrifugation at 1300 rpm for 5 min followed by pellet resuspension in PBS. Cells were loaded on a FACSCalibur flow cytometer (BD Biosciences), and data were acquired and analysed using CellQuest software.

2.4 Software and Online Tools

Computer Software

Adobe Photoshop 2023

Adobe Illustrator 2023

CellQuest

GraphPad Prism 9

IGV: Integrative Genomics Viewer

Leica LAS AF Lite

Image J (FIJI)

Serial Cloner

Step One Plus RT-PCR

RStudio

Online Tool	Website link
Anaconda	https://anaconda.org/anaconda/conda
Basic Local Alignment Search Tool (BLAST)	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Bioconductor	https://www.bioconductor.org/
ClusterProfiler	https://github.com/YuLab-SMU/clusterProfiler
DESeq2	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
Ensembl	https://useast.ensembl.org/Homo_sapiens/Info/Index

Fastp	https://github.com/OpenGene/fastp
FastQC	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
GeneCards	https://www.genecards.org/
Gene Ontology	http://geneontology.org/
KEGG pathway	https://www.genome.jp/kegg/pathway.html
Limma	http://bioconductor.org/packages/release/bioc/html/limma.html
MultiQC	https://multiqc.info/
SAMtools	http://www.htslib.org/
STAR	https://github.com/alexdobin/STAR
R	https://www.r-project.org/
RSEM	https://github.com/deweylab/RSEM
UniProt Protein Database	http://www.uniprot.org/

Chapter Three

**RICTOR/mTORC2 is not essential for the
maintenance of pluripotency marker expression in
hESCs**

3.1 Introduction

mTOR signalling has an essential role in regulating cell metabolism, differentiation, growth, and survival (Battaglioni et al., 2022; Liu and Sabatini, 2020). However, the role of mTOR in the regulation of PSCs remains unclear. Early work demonstrates that deletion of *Mtor* in mESCs leads to cell shrinkage and defective proliferation (Gangloff et al., 2004; Murakami et al., 2004). More recent studies have reported that inhibition of mTOR signalling induces diapause, a state of paused pluripotency and suspended embryonic development, in both mouse ESCs and embryos (Bulut-Karslioglu et al., 2016; Hussein et al., 2020), highlighting the importance of mTOR in maintaining proper self-renewal of mESCs as well as in mouse embryonic development. Similarly, inhibition of mTOR by rapamycin is reported to impair pluripotency and prevent cell proliferation in hESCs (Nazareth et al., 2016; Zhou et al., 2009). Mechanistically, mTOR integrates signals from extrinsic factors to stabilise the core pluripotency markers and promote cell proliferation while suppressing transcription of differentiation-associated genes (Zhou et al., 2009). Chemokine receptor 2 (CXCR2) inhibition-mediated downregulation of mTOR is also reported to result in a loss of pluripotency in hPSCs (Jung et al., 2016). By contrast, DEPTOR, an mTOR inhibitor is found to reduce its expression in mESCs after neural differentiation and knockdown of DEPTOR in mESCs results in loss of pluripotency (Agrawal et al., 2014). Furthermore, transient inhibition of mTOR by Torin promotes conversion of primed hPSCs to the naïve state (Hu et al., 2020). These data suggest that mTOR inhibition is important in maintaining pluripotency. Intriguingly, mTOR signalling is upregulated in mESCs after LIF withdrawal from the culture medium, which leads to mESC differentiation (Cherepkova et al., 2016), but it remains ambiguous whether this increased mTOR activity is a direct consequence or a compensatory effect of LIF withdrawal. Thus, although mTOR signalling is essential for the maintenance of pluripotency in PSCs, the underlying mechanisms need to be further elucidated.

Additionally, all the above experiments mainly focus on the catalytic subunit mTOR which affects both mTORC1 and mTORC2. Although rapamycin only directly inhibits mTORC1, prolonged treatment also suppresses mTORC2 (Sarbassov et al., 2006). Therefore, little is known about the complex-specific role of mTOR in PSCs, especially in human PSCs. It has been thought that mTOR might have different functions in mouse and human ESCs (Alhasan et al., 2021). However, it is unclear whether this is due to different pluripotent states of mESCs (naïve) and hESCs (primed) or species variation.

In order to delve into mTORC2 signalling in the regulation of self-renewal and pluripotency of hESCs and utilise them as a model for studying mTORC2 in human embryonic development, *RICTOR* has been disrupted in H1 hESCs by CRISPR/Cas9 gene editing technique (see section 1.4.2) (Chiu, 2019). Interestingly, *RICTOR*-knockout (RIC-KO) in hESCs does not alter the expression of pluripotency markers, but significantly impairs cell attachment and cell-cell interactions. Upon differentiation via embryoid body (EB) formation, RIC-KO cells showed low differentiation efficiency towards mesoderm and endoderm lineages (Batoool, 2022).

To further explore the effect of RIC-KO in hESCs, I compared the transcriptomic profiles of RIC-KO hESCs with their corresponding wildtype (WT) controls using bulk RNA-seq in this chapter. I anticipated that this analysis would provide us with more information on the role of mTORC2 in hESCs.

3.2 Results

3.2.1 RICTOR deficiency specifically disrupts mTORC2 signalling in hESCs

I firstly validated the previous findings in two RIC-KO clonal lines (K1 & K5) together with two wildtype (WT) control lines (C1 & C3) and H1 parental (Prt) hESCs (Batool, 2022; Chiu, 2019). K1 and K5 clones were obtained by expressing CRISPR/Cas9 with *RICTOR*-specific sgRNAs in H1 hESCs, while C1 and C3 clones were selected after expressing CRISPR/Cas9 without sgRNAs. Using the antibody which recognises the C-terminal region of RICTOR protein, I found that the complete RICTOR protein was not able to be detected in both K1 and K5 clones by immunoblotting (Fig 3.1A, Appendix Fig A-1A). This also caused the reduced expression of mSIN1, while the expression of other core components of mTORC2, mLST8 and mTOR, was not affected (Fig 3.1A, Appendix Fig A-1A). Correspondingly, mTORC2 activity was not detected in RIC-KO hESCs, as phosphorylation of its substrates, AKT1-Ser473 at the AKT HM and PKC α / β II-Thr638/641 at the PKC TM showed no signals (Fig 3.1B, Appendix Fig A-1B). In addition, a drastic decrease in PKC α protein expression was observed in these cells, which is caused by protein misfolding due to the absence of phosphorylation at the TM (Facchinetti et al., 2008). To explore the effect of defective HM phosphorylation of AKT on its activity, I also examined its downstream effectors GSK3 and FOXO1. There were hardly any changes detected on phosphorylation of GSK3 α and GSK3 β on Ser21 and Ser9, respectively, and they both exhibited similar phosphorylation in RIC-KO hESCs as in WT controls (Fig 3.1C, Appendix Fig A-1C), indicating that phosphorylation of AKT at the HM has no clear effect on its effector, GSK3. Similarly, phosphorylation of FOXO1-Ser256, another substrate of AKT, was also not affected in RIC-KO hESCs (Fig 3.1C, Appendix Fig A-1C). AKT phosphorylates FOXO1 and prevents its translocation in the nucleus, thereby suppressing transcription of its target genes which regulate cell death (Eijkelenboom and Burgering, 2013). Taken together, my results show that mTORC2 deficiency in hESCs has no clear effect on AKT activity.

Moreover, I also inspected whether RIC-KO has any effect on mTORC1. mTORC1-specific component RAPTOR was expressed at the similar levels in RIC-KO and WT hESCs (Fig 3.1D, Appendix Fig A-1D). Consistently, mTORC1 signalling was functional, as p70S6K, the direct target of mTORC1, was phosphorylated on Thr389 (Fig 3.1D, Appendix Fig A-1D). These

results confirm that *RICTOR* is disrupted in RIC-KO hESCs and that RICTOR deficiency results in defective mTORC2 signalling but with no clear effect on mTORC1 signalling.

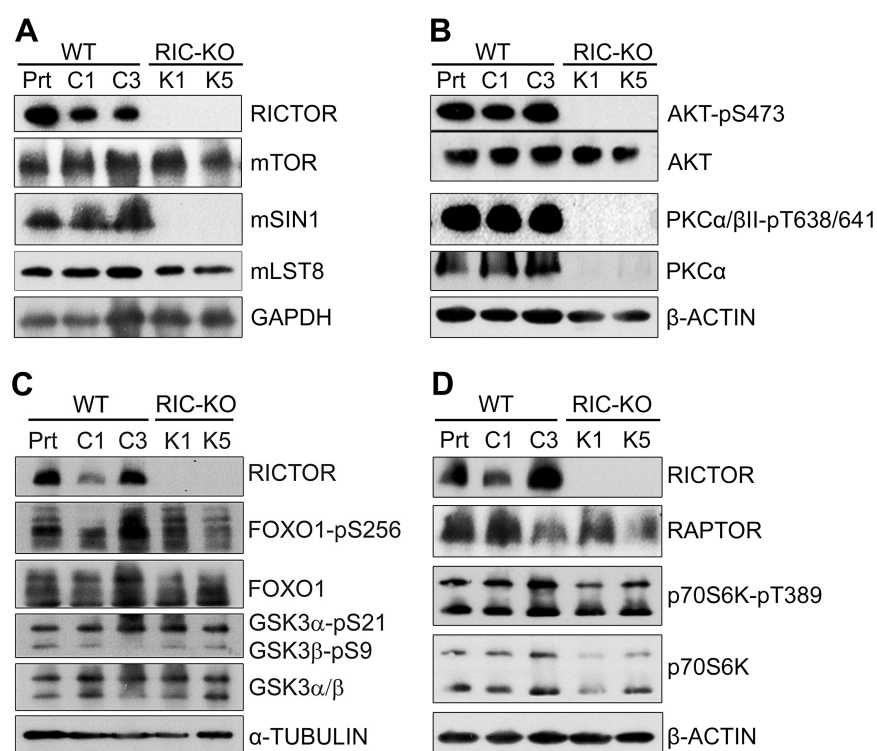


Figure 3.1 Effect of RICTOR deficiency on key mTOR signalling in hESCs

(A) Immunoblots showing the protein expression of mTORC2 key components in WT and RIC-KO hESCs. Prt, parental hESCs; C1 & C3, WT control clones; K1 & K5, RIC-KO clones. (B-C) Immunoblots of the above lysates with indicated antibodies against direct targets of mTORC2 (B) and AKT (C). (D) Immunoblots showing the protein expression of the mTORC1- and mTORC2-specific component as well as the mTORC1 substrate in WT and RIC-KO hESCs. Representative images depicted from three independent experiments. Images of replicate immunoblots included in Appendix Figure A-1.

3.2.2 RICTOR/mTORC2 does not affect the expression of core pluripotency factors

In our routine cultures, hESCs were maintained in MEF-CM supplemented with bFGF and were propagated by collagenase-assisted mechanical dissociation (Noisa et al., 2011). The resulting small clumps of hESCs after dissection were directly seeded onto Matrigel-coated culture plates and the cells would attach to Matrigel within 1 hour. However, RIC-KO hESCs showed poor attachment and only very few clumps of cells attached to the plates 24 hours after seeding. Notably, application of ROCKi clearly improved the attachment (see section 1.4.2) (Batool, 2022). Therefore, ROCKi was applied in the routine mechanical propagation of RIC-KO cells to assist their attachment.

Under MEF-CM culture conditions, RIC-KO hESCs were able to expand and form colonies. RIC-KO colonies displayed typical hESC morphology, including a large nucleus-to-cytoplasm ratio and multiple visible nucleoli in the nucleus (Fig 3.2A). However, their colonies were less compact than those of WT counterparts (Fig 3.2A). Then I sought to inspect the expression of cellular junction markers and cytoskeleton structure. In agreement with their morphological phenotypes, RIC-KO hESCs showed reduced expression of E-cadherin (E-CAD), an adherens junction (AJ) protein (Fig 3.2B, C, Appendix Fig A-2). Zonula occludens-1 (ZO-1) is a tight junction protein and immunostaining with antibody against ZO-1 showed that its expression was at lower levels in RIC-KO hESCs and displayed a discontinuous pattern throughout the whole colony, unlike the pattern in WT hESCs which shows disconnected staining only on the colony periphery (Fig 3.2D). Immunofluorescent images illustrate that TJs are also affected in RIC-KO hESCs. Moreover, F-ACTIN revealed disorganised cytoskeleton in both the edges and centre of RIC-KO colonies, whereas this disorganised cytoskeleton was only observed in the cells on the periphery of WT colonies. F-ACTIN in WT hESCs was clearly organised in a regular pattern which co-localised with ZO-1, especially in the colony centre (Fig 3.2D). These results together indicate that RICTOR/mTORC2 deficiency hinders cell attachment and cell-cell interactions in hESCs through altering, at least partly, the RHO-ROCK signalling, which also leads to a change in cytoskeleton.

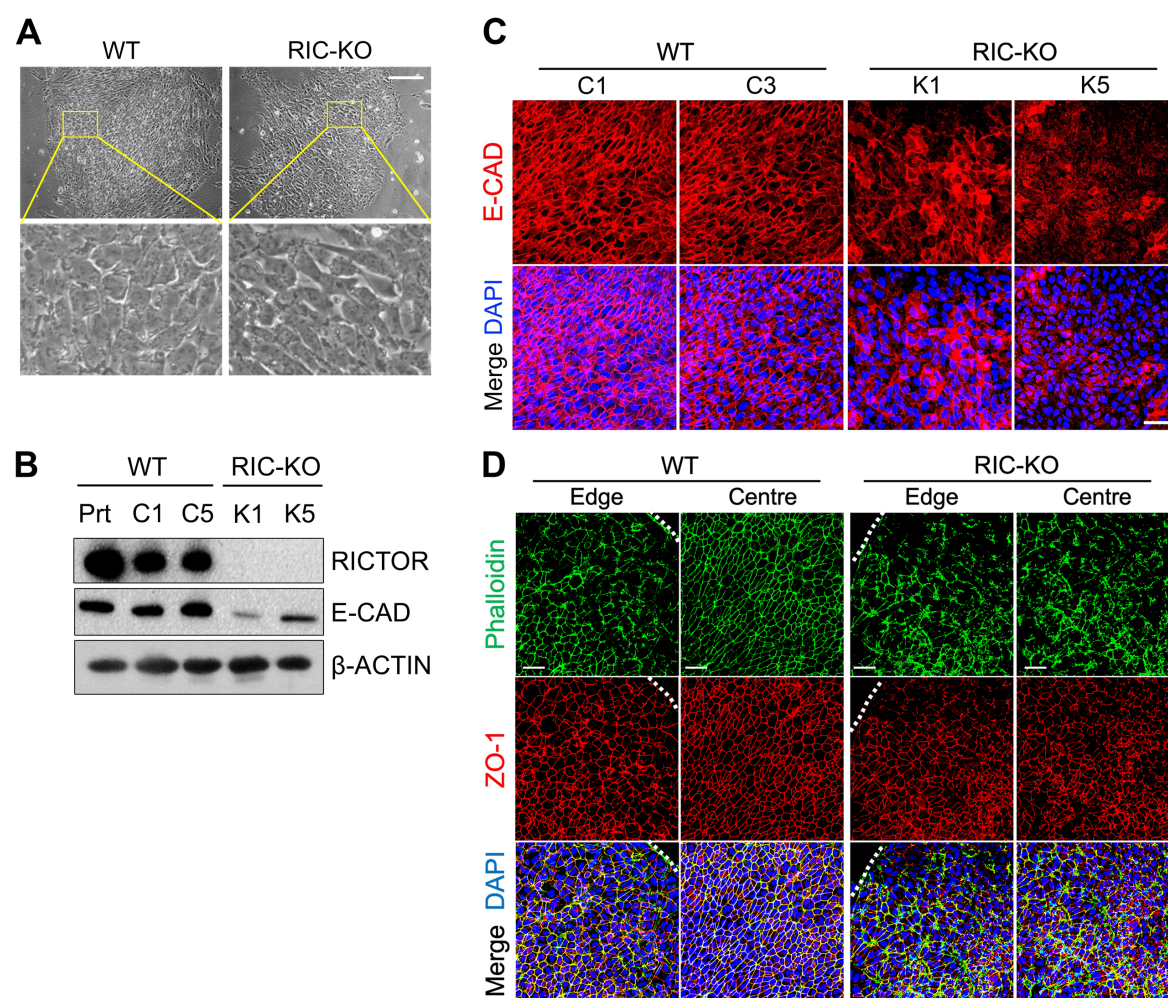


Figure 3.2 RIC-KO hESCs show reduced cell-cell interaction and E-cadherin expression
(A) Representative phase contrast images of WT and RIC-KO hESCs. Zoomed images show reduced cell-cell interaction in RIC-KO colonies. Scale bar, 200 μ m. **(B, C)** Immunoblotting **(B)** and immunostaining **(C)** analysis of WT and RIC-KO hESCs with indicated antibodies. Scale bar, 75 μ m. E-CAD, E-CADHERIN. Images of replicate immunoblots included in Appendix Figure A-2. **(D)** Immunostaining of WT and RIC-KO hESC colonies at the edges and centre with Zonula occludens-1 (ZO-1) antibody and Phalloidin (F-ACTIN) stain as indicated. Dashed lines delineate the border of colonies. Scale bar, 50 μ m. Representative images depicted from three independent experiments.

Next, I investigated if RICTOR deficiency would affect pluripotent markers in hESCs. The core pluripotent transcription factors OCT4, SOX2 and NANOG had similar expression on both mRNA and protein levels in WT and RIC-KO hESCs (Fig 3.3A, B, Appendix Fig A-3). Consistently, pluripotency-associated hESC surface antigens Tra-1-81 and SSEA4 also showed

positive in over 86% of cells in both WT and RIC-KO hESCs, and in fact, RIC-KO hESCs even expressed Tra-1-81 at higher levels (Fig 3.3C). On the other hand, the differentiation-associated marker, SSEA1 was negative in more than 95% of RIC-KO hESCs as well as of WT hESCs (Fig 3.3C). Therefore, my data confirm the previous observation that RIC-KO hESCs are able to self-renew and meanwhile, retain the expression of pluripotent markers in our culture system.

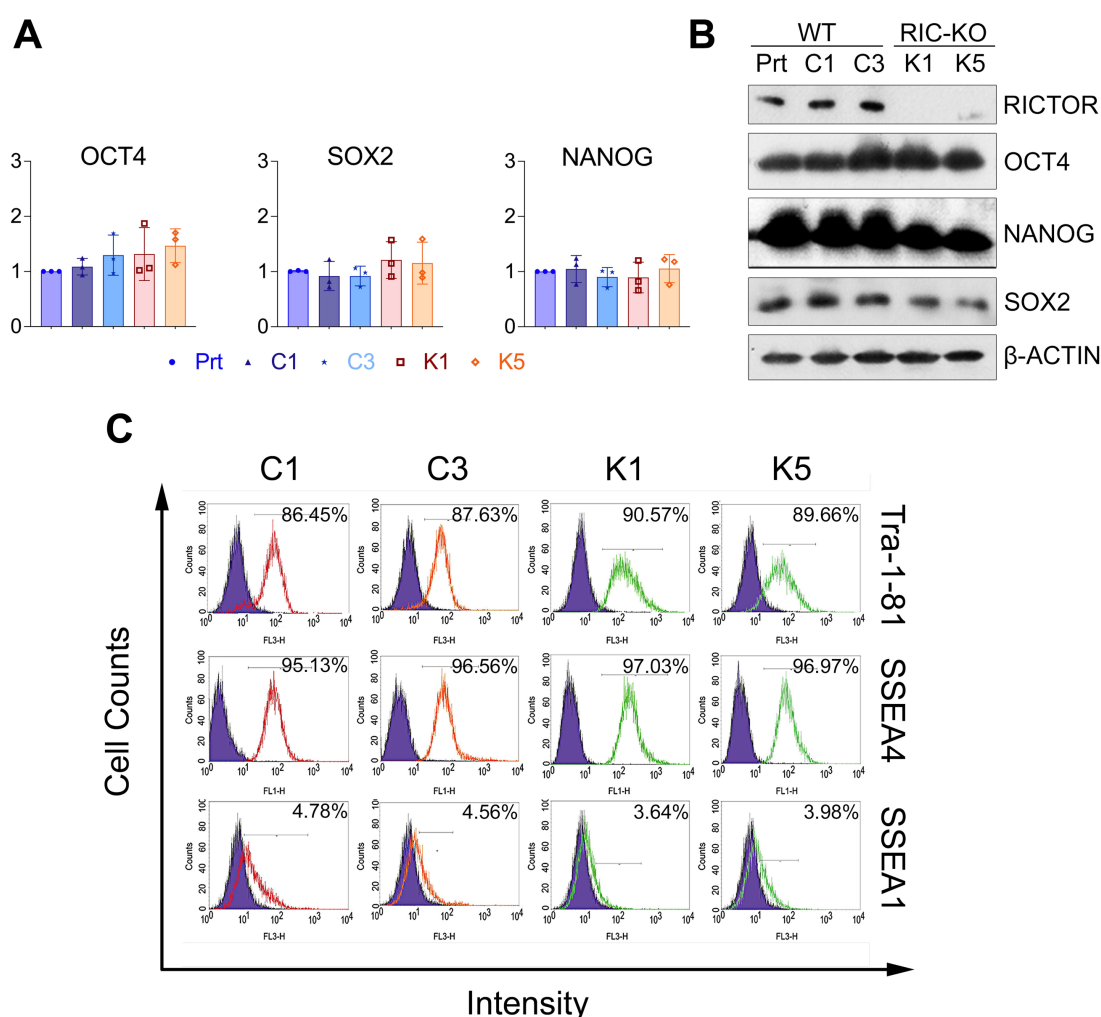


Figure 3.3 Effect of RIC-KO on pluripotency factors in hESCs

(A) qRT-PCR analysis of the mRNA expression of the core pluripotent transcription factors in WT and RIC-KO hESCs. Data are presented as the means $\pm$ SD ($n = 3$) relative to Prt hESC line. (B) Representative images of immunoblots showing the protein expression with indicated antibodies in WT and RIC-KO hESCs ($n = 3$). Images of replicate immunoblots included in Appendix Figure A-3. (C) Flow cytometry analysis of indicated cell surface antigens in WT controls (C1 & C3) and RIC-KO (K1 & K5) hESCs ($n = 3$).

3.2.3 RIC-KO hESCs have similar transcriptomic profiles as WT controls

To further explore the function of RICTOR/mTORC2 in hESCs, I performed RNA-seq in RIC-KO hESCs and their corresponding controls, including two WT (C1 & C3) and two RIC-KO (K1 & K5) hESC clonal lines. Since our RIC-KO hESCs showed a gradual loss of self-renewal and decreased expression of pluripotent markers in chemically defined, commercial mTeSR™ medium (Batoool, 2022), I performed RNA-seq with all four hESC clonal lines cultured in MEF-CM. WT and RIC-KO hESCs were simultaneously cultured in MEF-CM from the same batch to minimise batch variability and other environmental effects. Total RNAs were extracted from the cells for two consecutive passages as two biological replicates for each clonal line. Extracted RNAs were analysed for quality, and the RNA integrity number (RIN) for all samples was 10 (Table 3.1), which suggests that the samples were in a good quality without any degradation. After quality control and filtering, paired-end reads from a minimum of 20 million to almost 50 million were obtained in which over 88% of bases had a quality score over 30 (bases ≥ 30) from each cell line ($n = 2$) (Table 3.1). Overall, 72% - 92% of reads were uniquely mapped to the human genome (GRCh38, release 104) (Fig 3.4A). Finally, 32,673 genes were mapped by at least one read in one of the samples.

Table 3.1 Summary of quality control and filtering of RNA-seq samples

Sample*	RIN	Before filter # Reads	After filter # Reads	% Bases ≥ 30
C1_1	10.0	28,604,914	27,544,535	88.5
C1_2	10.0	49,631,090	49,394,339	95.8
C3_1	10.0	24,911,363	24,133,204	89.6
C3_2	10.0	36,246,376	36,017,850	95.7
K1_1	10.0	36,597,190	36,183,282	94.5
K1_2	10.0	46,152,682	45,963,856	96.0
K5_1	10.0	21,223,069	20,940,449	94.4
K5_2	10.0	38,704,800	38,545,619	96.3

*_1 and _2 represent two biological replicates in each cell line.

The analysis showed that the majority of the 32,673 genes detected by RNA-seq exhibited a similar expression pattern between WT and RIC-KO hESCs, while only 0.6% of the genes (195 genes) were differentially expressed between the two groups (false discovery rate [FDR] < 0.05 , fold change [FC] > 2). Surprisingly, *RICTOR* gene did not show clear differential mRNA expression between WT and RIC-KO groups. However, close inspection on *RICTOR*

expression by mapping the RNA-seq data to the reference genome revealed that the two RIC-KO clones, K1 and K5 had deletion mutations at exon 4 but that the WT controls remained intact (Fig 3.4B). In K1, most of the cells show a one-base deletion of guanine (G) at position 245 in the cDNA sequence, followed by three substitution mutations occurring from positions 256 to 258 (Fig 3.4B). The deletion caused a frameshift which changed the amino acid sequence of RICTOR protein from Glu to Lys at 75 and generated an early stop codon 9 amino acids after Lys75, resulting in the disruption of RICTOR at the N-terminal Armadillo domain (Fig 3.4C). In K5, most of the cells show a deletion of 23 bases at the beginning of exon 4 (Fig 3.4B), which also led to a frameshift and an early termination of RICTOR at the N-terminal Armadillo domain (Fig 3.4C). Together, it confirms that RICTOR protein is mutated and terminated at position close to the N-terminus and explains why RICTOR could not be detected by the antibody in the immunoblots (Fig 3.1A). Interestingly, reads in the RIC-KO clones were still able to be mapped to *RICTOR* gene after exon 5 in the reference genome with no clear reduction in expression compared to WT control clones, which suggests that CRISPR/Cas9-mediated mutation/deletion in *RICTOR* does not lead to its mRNA decay and explains why no differential expression was detected in it.

Notably, the core pluripotent transcription factors, including *OCT4* (also known as *POU5F1*), *SOX2*, and *NANOG* showed similar expression between WT and RIC-KO hESCs (Fig 3.5A), which was in agreement with my qRT-PCR result (Fig 3.3A), ascertaining that RICTOR/mTORC2 deficiency does not have an impact on the expression of pluripotency makers. Since adhesion proteins E-CAD and ZO-1 showed lower expression on the protein level in RIC-KO hESCs (Fig 1.9D, 3.2C), I inspected their mRNA expression along with expression of other adhesion associated proteins required for constructing adherens junctions (AJs), tight junctions (TJs), and gap junctions (GJs) which are crucial for intercellular communication and epithelial integrity and have been reported to have a pivotal role in regulation of pluripotency and differentiation in hPSCs (Boyer et al., 2005; Ke et al., 2017; Li et al., 2012; Vasic et al., 2023). Interestingly, genes that encode these adhesion-associated proteins revealed no significant difference in their transcript expression between the WT and RIC-KO group (Fig 3.5B), suggesting that changes in E-CAD and ZO-1 in RIC-KO hESCs are post-transcriptionally regulated.

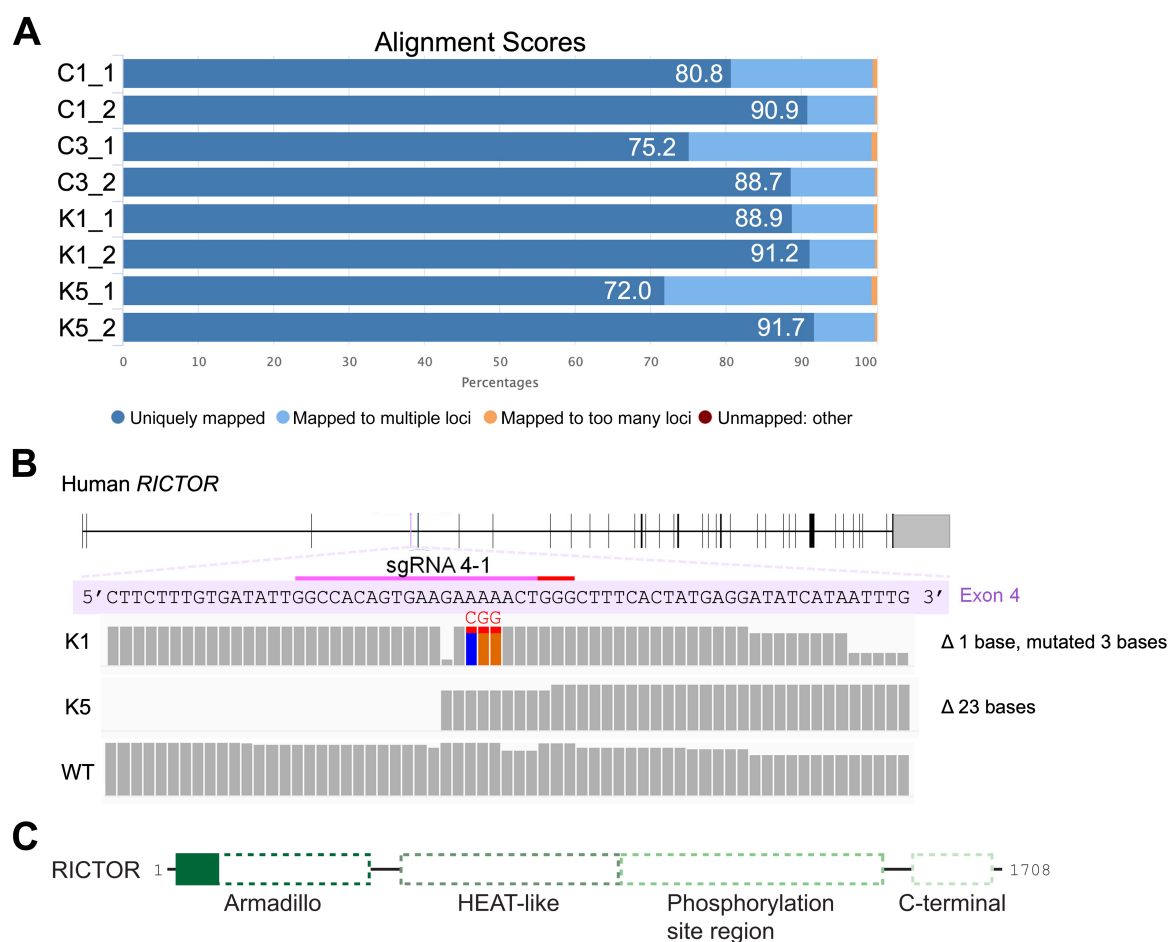


Figure 3.4 RNA-seq analysis identifies mutations of *RICTOR* gene and their associated effect on the protein in RIC-KO hESCs

(A) Bar chart depicting the percentage of mapped reads in each WT and RIC-KO hESC sample. The numbers in white indicate the percentage of uniquely mapped reads in each sample. (B) Top: schematic diagram showing the cDNA sequence of exon 4 of *RICTOR* in the human reference genome with sgRNA indicated. Bottom: coverage plots visualising coverage of sequenced reads mapped to this region in RIC-KO and WT cells from the RNA-seq data. The height of each peak represents the coverage of each base. Three bases labelled in red are substitution mutations occurred in K1. Summary of mutations in each RIC-KO clone (K1 and K5) is indicated. (C) Linear representation of domains of disrupted *RICTOR* protein in RIC-KO hESCs. Domains with dashed lines represent the missing parts of normal *RICTOR* protein.

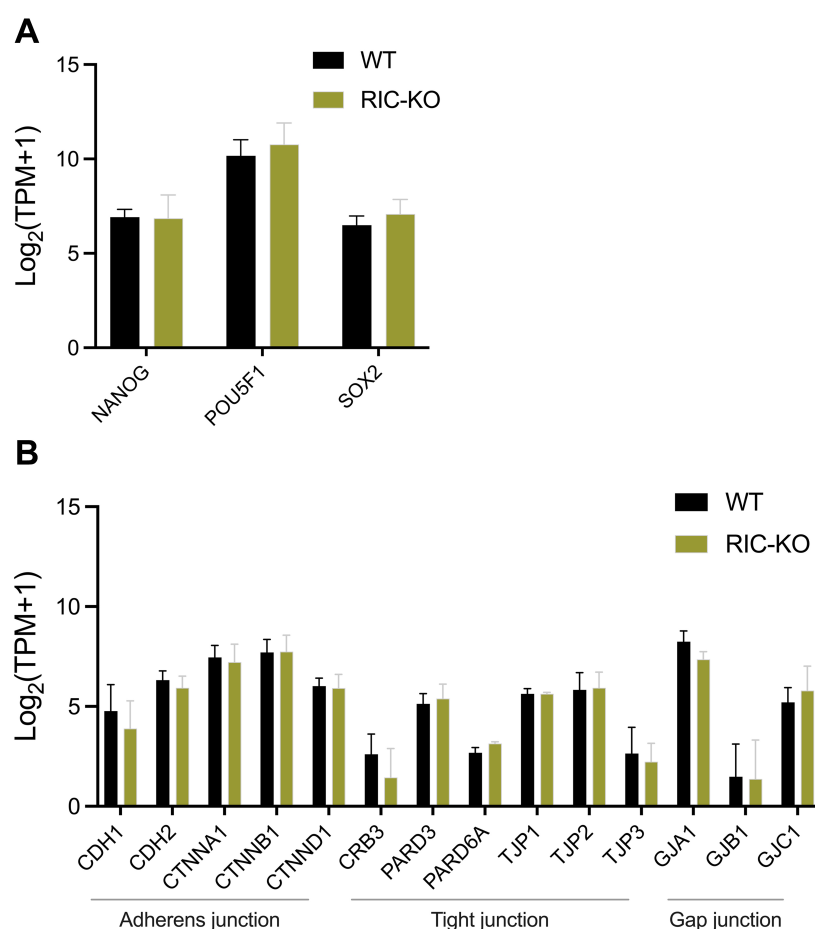


Figure 3.5 RNA-seq analysis reveals similar transcriptomic profiles between WT and RIC-KO hESCs

(A-B) RNA-seq data showing the normalised expression of the core pluripotency factors (A) and representative adhesion associated genes (B) between WT and RIC-KO hESCs. Data are presented as mean $\pm$ SD from 4 biological samples. TPM, transcripts per million.

3.2.4 Downregulated genes in RIC-KO hESCs associate with mesoderm and endoderm differentiation

The hierarchical clustering and heatmap of all differentially expressed genes (DEGs) showed two distinct clusters of samples with 55 genes upregulated and 140 genes downregulated in RIC-KO hESCs compared to WT controls (Fig 3.6A). The downregulated genes were composed of 114 protein coding genes, 23 long noncoding RNAs (lncRNAs), 2 novel genes and 1 pseudogene, while upregulated genes contain 45 protein coding genes, 9 lncRNAs and 1 novel gene (Fig 3.6B).

Interestingly, of the 114 downregulated protein-coding genes in RIC-KO hESCs, the most significantly ones were related to embryonic development, particularly mesoderm and endoderm formation. For example, *BMP2*, *DKK1*, *GATA4*, *LHX1*, and *SP5* promote mesoderm differentiation (Fig 3.6C, D), in which *GATA4*, *LHX1* and *SP5* are transcription factors (Costello et al., 2015; Rojas et al., 2005; Weidinger et al., 2005), while *BMP2* and *DKK1* are cytokines responsible for activating BMP signalling and inhibiting WNT signalling, respectively (Guo et al., 2019; Rogers et al., 2015). In addition, *CER1*, *CXCR4*, *FOXA2*, *GATA6* and *SOX17* have a critical role in endoderm specification (Fig 3.6A, B). *FOXA2*, *GATA6*, and *SOX17* are important transcription factors (Mukherjee et al., 2020; Scheibner et al., 2021), and *CXCR4* is an important cell surface marker (D'Amour et al., 2005).

However, the functions of upregulated genes were more diverse, although some of them were also essential in cell differentiation (Fig 3.6D). *ERVH48-1* encodes a protein which plays an essential role in placental development (Sugimoto et al., 2022). *EMX1* is a TF involved in brain development and neuron differentiation (Kobeissy et al., 2016). Overall, these data suggest that despite similar transcriptomic profiles between the WT and RIC-KO group, downregulated genes mainly include important transcription factors that regulate lineage specification, particularly mesoderm and endoderm.

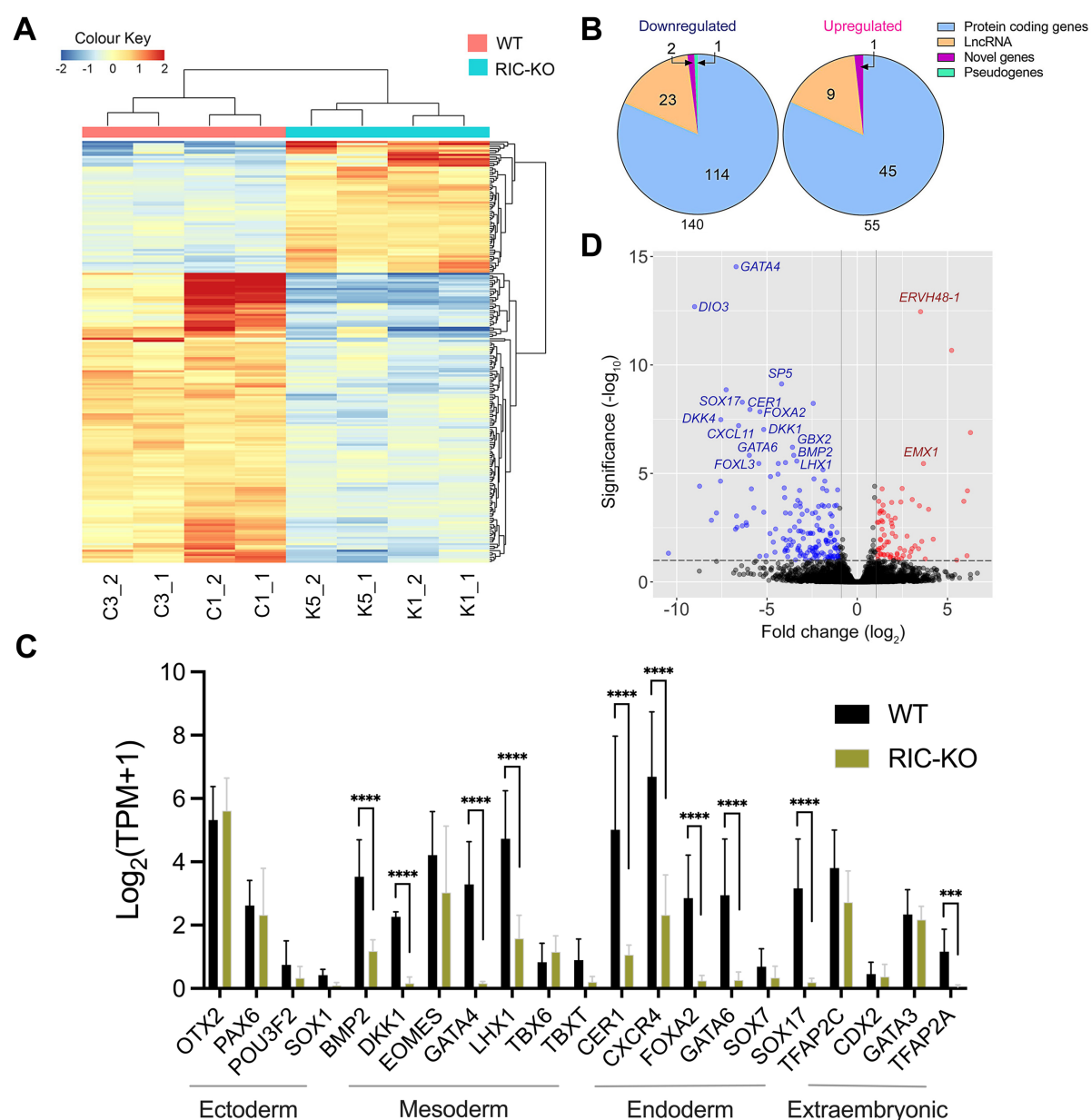


Figure 3.6 RICTOR deficiency causes downregulation of lineage-specific genes in hESCs (A) Hierarchical clustering and heatmap of differentially expressed genes (DEGs) (adjusted p value (p_{adj}) < 0.05 , $\log_2$ (fold change) > 1 or -1) between WT and RIC-KO hESCs. (B) Pie chart showing the number of DEGs with different types indicated by colours in RIC-KO hESCs relative to WT controls. (C) RNA-seq data showing the normalised expression of representative lineage-specific markers between WT and RIC-KO hESCs. Data are presented as mean $\pm$ SD from 4 biological samples. TPM, transcripts per million. *** & **** represent $p_{\text{adj}} < 0.001$ and 0.0001 , respectively. (D) Volcano plot showing DEGs in RIC-KO hESCs compared to WT controls. Significance is calculated as $-\log_{10}(p_{\text{adj}})$. Blue and red dots represent individual downregulated and upregulated genes, respectively. Dashed and straight lines indicate the cut-offs (significance > 1.30 and $\log_2$ (fold change) > 1 or -1 , respectively) for DEGs.

3.2.5 Gene ontology enrichment analysis reveals RIC-KO might affect mesoderm/endoderm differentiation

To investigate the molecular and cellular mechanisms represented by the DEGs in response to RICTOR/mTORC2 deficiency, gene ontology (GO) enrichment analysis was carried out on the 114 downregulated protein-coding genes. It revealed that these genes were predominantly involved in biological process related to embryonic development, embryo patterning, and muscle development (Fig 3.7A). The top 5 biological process terms on the list were 1) “muscle tissue development” ($-\log_{10}p_{\text{adj}} = 5.54$), 2) “skeletal muscle organ” ($-\log_{10}p_{\text{adj}} = 5.49$), 3) “anterior/posterior pattern specification” ($-\log_{10}p_{\text{adj}} = 5.49$), 4) “regionalization” ($-\log_{10}p_{\text{adj}} = 5.17$), and 5) “skeletal muscle tissue” ($-\log_{10}p_{\text{adj}} = 4.93$). It was worth noting that the term “endoderm development” was also enriched in the top 20 list (Fig 3.7A). Of the 114 downregulated genes, there were 35 transcription factors, accounting for 30.7% of the downregulated genes, and 14 growth factors/cytokines, accounting for 12.3% of the downregulated genes. The top 5 molecular function terms were “receptor ligand/activator activity” ($-\log_{10}p_{\text{adj}} = 3.17$), “DNA-binding transcription activator activity” ($-\log_{10}p_{\text{adj}} = 3.17$), “bacterial-type RNA polymerase transcriptional activator activity” ($-\log_{10}p_{\text{adj}} = 3.17$), “co-receptor binding” ($-\log_{10}p_{\text{adj}} = 2.55$), and “cytokine activity” ($-\log_{10}p_{\text{adj}} = 1.86$) (Fig 3.7B). These data suggest that the biological processes enriched by the downregulated genes probably function through the regulation of transcription activity and growth factor/cytokine activity. However, no statistically significant GO terms could be generated from the upregulated genes.

To explore what signalling pathways would be represented by these DEGs, the KEGG pathway analysis showed that there were 6 pathway terms enriched from the downregulated genes ($p_{\text{adj}} < 0.05$) (Fig 3.7C). Consistent with the GO terms related to cytokine activity in the molecular function category, the top KEGG pathway enriched term was “cytokine-cytokine receptor interaction”. Pathways related to embryonic development including “maturity onset diabetes of the young” and “WNT signalling pathway” were significantly downregulated in RIC-KO hESCs. Finally, pathways related to cell metabolism such as “folate biosynthesis” and “thiamine metabolism” were among the enriched terms (Fig 3.7C). By contrast, the upregulated genes did not have any statistically significant KEGG pathways when the cut-off was $p_{\text{adj}} < 0.05$. These data suggest that RICTOR/mTORC2 deficiency in hESCs might affect their cell fate specification, particularly mesoderm lineage.

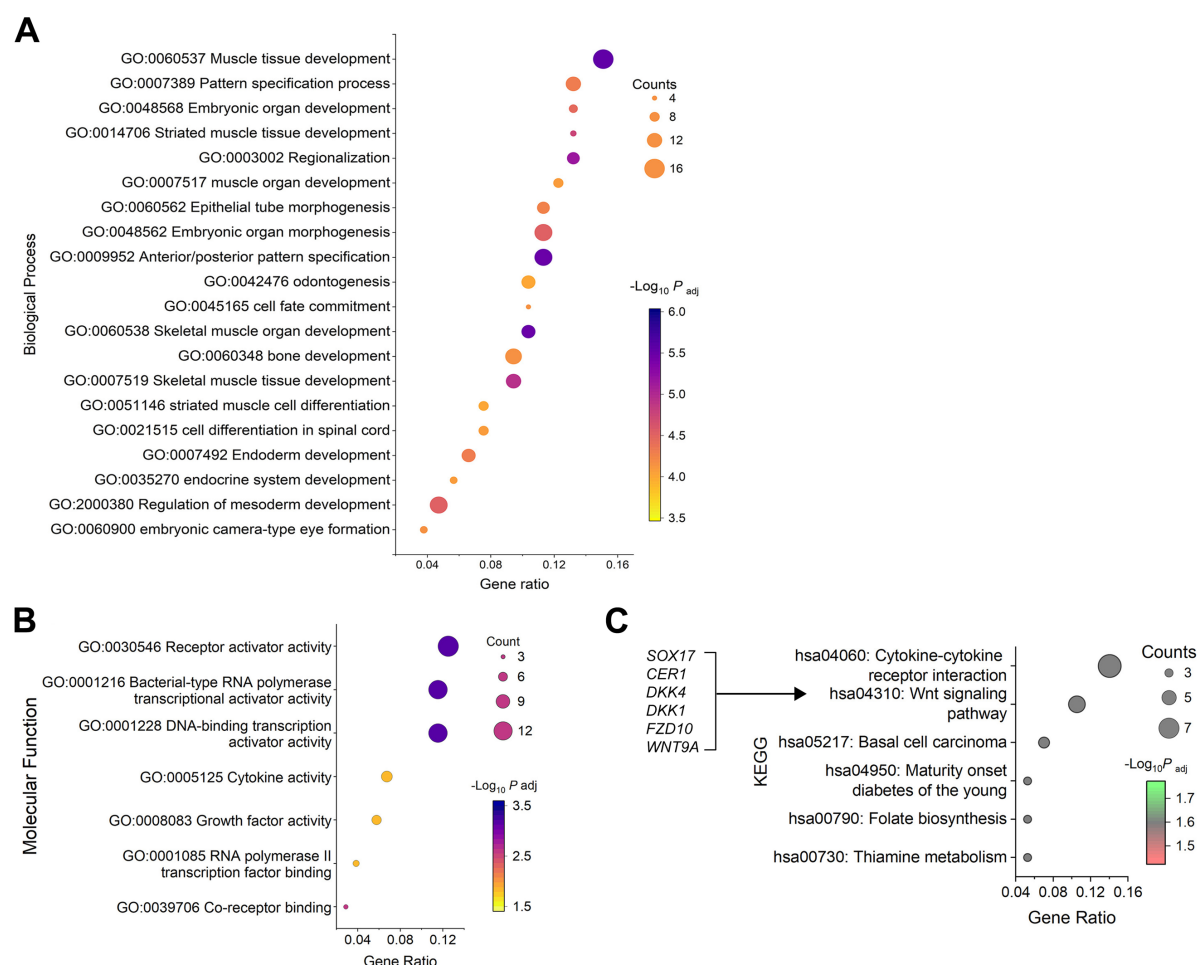


Figure 3.7 Changes in development-associated gene expression in RIC-KO hESCs
 (A) Gene ontology enrichment ($p_{adj} < 0.05$) of downregulated genes in RIC-KO hESCs showing terms of biological process predominantly involved in embryonic development. (B) Gene ontology enrichment ($p_{adj} < 0.05$) of downregulated genes in RIC-KO hESCs for terms related to molecular function. (C) KEGG pathway enrichment ($p_{adj} < 0.05$) of downregulated genes in RIC-KO hESCs. Genes which account for downregulation of the Wnt signalling pathway are listed.

3.3 Discussion

In this chapter, I have validated that RICTOR deficiency abolishes mTORC2 signalling with little effects on mTORC1 signalling in hESCs. In addition, even though phosphorylation of AKT1 on Ser473 is missing, the phosphorylation level of GSK3 α/β on Ser21/9 is similar between WT and RIC-KO hESCs, which shows that HM phosphorylation of AKT is not responsible for phosphorylation of GSK3 α/β . This finding is consistent with the previous report in *Rictor*-null MEFs (Guertin et al., 2006; Shiota et al., 2006), suggesting that mTORC2 does not control all AKT activity. In fact, phosphorylation of AKT1 on Thr308 at the activation loop by PDK1 is sufficient to activate AKT and phosphorylate GSK3 α/β on Ser21/9 (Facchinetti et al., 2008; Jacinto et al., 2006; Yang et al., 2015). Interestingly, it has been shown that phosphorylation of AKT1 on Ser473 at the HM is required to phosphorylate FOXO1-Ser256 in *Rictor*-null MEFs (Guertin et al., 2006; Jacinto et al., 2006). However, the phosphorylation level of FOXO1-Ser256 in RIC-KO hESCs does not change much. This could arise from the ability of hESCs in rewiring the signalling network like cancer cells, as phosphorylation of FOXO1-Ser256 was lower in RIC-KO hESCs than WT controls at early passages (Chiu, 2019). Likewise, my data show that the phosphorylation level of p70S6K does not change much between WT and RIC-KO hESCs, as opposed to the higher phosphorylation level in early-passage RIC-KO cells (Chiu, 2019). I reason that these differences between early-passage and later-passage RIC-KO cells might be attributed to cell adaptation and signalling rewiring, indicating that other signalling pathways might also regulate FOXO1 to compensate for the loss of mTORC2 activity. The rewiring ability has been reported in many cancers, and hESCs also have the tumorigenic ability, capable of forming teratoma when injected into immunodeficient mice, whereas MEFs are not tumorigenic. This might account for the difference observed in hESCs and MEFs (Chiu, 2019).

Furthermore, my results confirm our previous data that RICTOR/mTORC2 signalling is dispensable for the expression of pluripotent markers in hESCs. This appears to be contradictory to a recent report that ablation of *RICTOR* in H9 hESCs impairs self-renewal and the expression of pluripotent transcription factors, OCT4, SOX2 and NANOG (Chu et al., 2022). This discrepancy between the results might arise from different culture conditions. Our hESCs are cultured in MEF-CM, while their hESCs are maintained in chemically defined, commercial mTeSR medium. Our unpublished data showed that our RIC-KO hESCs did show gradually decreased expression of pluripotency markers and eventually became differentiated

into the neuroectoderm lineage after 10 passages in mTeSR medium (Batool, 2022). Unlike defined medium, MEF-CM relies on the secretion of beneficial and supportive factors from MEFs, which reduce spontaneous differentiation and promote self-renewal in hESCs (Dakhore et al., 2018). It is still not completely clear what exactly these beneficial and supportive factors are. Besides growth factors (e.g. bFGF, Activin, TGF β) which also exist in mTeSR, it is likely that these factors also include ECM proteins and extracellular vesicles (EVs). ECM and EVs in MEF-CM probably help retain hESC pluripotency in the absence of RICTOR/mTORC2 signalling (Dakhore et al., 2018; Kalluri and McAndrews, 2023). The exact mechanisms that cause this disparity between the two culture conditions require further investigation.

The main result in this chapter is the transcriptomic profiling in WT and RIC-KO hESCs by RNA-Seq. Using RNA-seq analysis, I gain some insights into the molecular mechanisms by which mTORC2 signalling regulates self-renewal and cell fate determination in hESCs. Of note, *RICTOR* ($p_{\text{adj}} = 0.998$) is not picked up as a downregulated gene by RNA-seq even though it is disrupted by CRISPR/cas9 and sgRNAs. This implies that the mutated *RICTOR* mRNA might not undergo nonsense-mediated decay (NMD), but instead, might survive and be translated to other proteins through multiple mechanisms (Supek et al., 2021; Tuladhar et al., 2019). The analysis also shows that genes associated with cell adhesion proteins are expressed at similar levels between WT and RIC-KO hESCs. Contradictory to lower expression of the adhesion proteins E-CAD and ZO-1 in RIC-KO hESCs, mRNA expression of *CDH1* and *TJPI* does not have significant difference between the WT and RIC-KO group, suggesting that regulation of these proteins occurs at the post-transcriptional stage or post-translational stage. Our unpublished data show the imbalance of the two RHO GTPases and reveal significantly higher RHOA activity but lower RAC1 activity in RIC-KO hESCs (Batool, 2022). Since RHO-ROCK signalling negatively regulates stability of E-CAD in hESCs (Xu et al., 2010), while RAC1 stabilises E-CAD and contributes to the formation of AJs (Nakagawa et al., 2001), this might be the reason why E-CAD expression is lower in RIC-KO hESCs. Furthermore, the formation of the mature AJs affects the formation of TJs (Zihni et al., 2016), which likely explains why ZO-1 expression is also lower. Indeed, application of ROCKi could recover, at least partly, cell-cell interactions, as evidenced by the formation of aggregates in RIC-KO hESCs (see section 1.4.2) (Batool, 2022). Culturing RIC-KO hESCs with MEF-CM may also partially compensate for reduced cell-cell interactions.

Although WT and RIC-KO hESCs exhibit similar transcriptomic profiles, the most downregulated genes in response to RICTOR deficiency are associated with embryonic development, particularly mesoderm and endoderm formation. Interestingly, ectoderm markers show similar expression, which partly explains why RIC-KO hESCs promote neuroectoderm differentiation through EB formation and why they gradually differentiate into this lineage in mTeSR medium (Batoool, 2022). This downregulation of lineage-specific markers in RIC-KO hESCs corresponds to slightly higher expression of *OCT4* and *SOX2*, which might partly result from less spontaneous differentiation observed during routine culture. This is also evidenced by marginally higher expression of the pluripotency-associated surface marker Tra-1-81 in RIC-KO hESCs, given its high sensitivity as the pluripotent marker (Noisa et al., 2012).

In addition, GO enrichment analysis shows that downregulated genes are mainly related to embryonic development, particularly mesoderm differentiation, which is in line with the findings that RICTOR deficiency impairs PSC differentiation into mesoderm lineage (Chu et al., 2022; Zheng et al., 2017). By contrast, previous RNA-seq analysis of H9 hESCs and their RIC-KO counterparts reveals that downregulated genes are related to extracellular structure organisation and stem cell maintenance (Chu et al., 2022). This discrepancy is possibly due to different culture conditions as discussed earlier. Intriguingly, KEGG pathway analysis pinpointed that downregulated genes in RIC-KO hESCs are linked to downregulation of WNT signalling. Extensive studies demonstrate that the WNT signalling plays an important role in hPSC differentiation into ME and subsequent mesoderm lineage (Funa et al., 2015; Lian et al., 2012; Martyn et al., 2018; Mendjan et al., 2014; Yoney et al., 2022). Thus, downregulation of WNT signalling might be the reason why mesoderm and endoderm differentiation were hindered in RIC-KO hESCs upon EB formation (see section 1.4.2). Taken together, my data strongly suggest that RICTOR/mTORC2 deficiency has no detrimental effect on the expression of pluripotent markers in hESCs and that mTORC2 is dispensable for maintaining hESCs.

Chapter Four

**RICTOR/mTORC2 regulates mesendoderm
differentiation in hESCs through activation of
canonical WNT genes**

4.1 Introduction

Given that mTORC2 is crucial for cell survival and cytoskeleton remodelling, it is expected that mTORC2 might play an important role in embryonic development, particularly at the gastrulation stage, when epiblast cells undergo massive cytoskeleton remodelling and migration. Surprisingly, loss-of-function studies show that mice deficient in either Rictor or mSin1 progress through gastrulation but die at E10.5-11.5 due to vasculature defects (Guertin et al., 2006; Jacinto et al., 2006; Shiota et al., 2006). Interestingly, the phenotypes of tissue-specific mTORC2 deficiency in mice, including in endothelial cells, are very mild, showing no developmental defects or lethality, unless the disruption occurs around gastrulation, which leads to defective development and embryonic death at mid-gestation, similar to the phenotypes of germline deletion (Aimi et al., 2015; Bentzinger et al., 2008; Cybulski et al., 2009; Lamming et al., 2012; Zhao et al., 2014). These findings suggest that mTORC2 may be crucial for early embryonic development, particularly at the gastrulation stage.

Consistent with *in vivo* mouse embryos, recent studies have gained some insights into the role of mTORC2 in PSCs. Disturbing plasma membrane localisation of mTORC2 in mESCs impairs mesoderm specification (Gómez-Salineró et al., 2016). This notion is further supported by the study demonstrating that knockdown of *Rictor* hinders cardiac differentiation and function in mESCs (Wang et al., 2021; Zheng et al., 2017). Similarly, knockout of *RICTOR* in hESCs shows impaired mesoderm differentiation while promoting their differentiation into the neural lineage (Chu et al., 2022). These findings indicate that mTORC2 is important for mesoderm differentiation in PSCs, but the detailed mechanisms underlying the role of mTORC2 remain unclear.

Although RIC-KO hESCs exhibit similar expression of the core pluripotent transcription factors and pluripotency-associated cell surface markers as WT controls (Fig 3.4), their EB-mediated differentiation reveals impaired generation of both mesoderm and endoderm lineages (Fig 1.10). These data are consistent with previous findings and suggest that mTORC2 signalling might have a role in the regulation of ME formation, a common precursor of mesoderm and endoderm that is similar to the cells in the PS during gastrulation *in vivo* (Wang and Chen, 2016).

The signalling pathways involved in regulation of the PS have been well-studied in mouse gastrulation. BMP4 secreted from the ExE (extraembryonic ectoderm) activates WNT3 in the

posterior epiblast, and this activated WNT signalling maintains levels of NODAL expression, together both of which activate the expression of genes for ME differentiation (Arnold and Robertson, 2009; Morgani and Hadjantonakis, 2020; Tam and Loebel, 2007). Therefore, the three key signalling pathways, BMP-WNT-NODAL are identified to be essential for driving epiblast cells into the ME fate (Fig 4.1A). Based on this, several *in vitro* models have been developed in which hESCs can be efficiently differentiated into ME in either unconfined colonies or the micropatterned form by BMP4 stimulation (Bernardo et al., 2011; Warmflash et al., 2014). This further confirms that BMP4 can induce hESC differentiation into ME.

Given that RIC-KO hESCs exhibit impeded mesoderm/endoderm formation in EB-mediated differentiation (Fig 1.10) and a reduced level of WNT signalling in RNA-seq analysis (Fig 3.7A, C), I focused my investigation on ME differentiation in WT and RIC-KO hESCs using the BMP4-induction protocol in this chapter (Bernardo et al., 2011). By modulating BMP/WNT/NODAL signalling pathways, I aimed to further explore the possible link of mTORC2 with these signalling pathways.

4.2 Results

4.2.1 RICTOR deficiency considerably hinders BMP4-induced mesendoderm differentiation of hESCs

I firstly adapted a BMP4-induced ME differentiation approach in 2D colony cultures to compare the differentiation between WT and RIC-KO hESCs (Bernardo et al., 2011; Deglincerti et al., 2016). In this approach, hESCs were mechanically dissociated and small cell clumps were seeded onto the Matrigel-coated plates in MEF-CM supplemented with ROCKi (Fig 4.1B). Next day, ROCKi was removed and these unconfined small colonies were allowed to grow for one more day in hESC culture medium. When most of the colonies reached about 500 μm in diameter, they were then treated with 50 ng/ml BMP4 for 48 hours, as previously described (Deglincerti et al., 2016). After 48-hour BMP4 induction, immunostaining was used to verify the ME differentiation using BRACHYURY (BRA) as a marker of ME (Bernardo et al., 2011; Vallier et al., 2009) and SOX2 as a marker of pluripotency and neuroectoderm (Thomson et al., 2011; Wang et al., 2012). Symmetry breaking occurred in the majority of WT control hESC colonies in which BRA⁺ cells were predominantly at one side while SOX2⁺ cells at the opposing side (Fig 4.1C, Appendix Fig A-4A), accounting for 51.7% of the total colonies (Appendix Fig A-4B). The pattern of concentric rings, mostly found in micropatterned colonies in which SOX2⁺ cells at the colony centre were surrounded by BRA⁺ cells, was also observed in few WT colonies, accounting for 17.2% of the total colonies (Appendix Fig A-4B). In contrast, RIC-KO colonies revealed much fewer BRA⁺ cells (Fig 4.1C). By quantifying BRA⁺ cells, it was confirmed that there was a significantly higher percentage of BRA⁺ cells in WT colonies than in RIC-KO colonies (Fig 4.1D) and that this difference was independent of the colony size (Fig 4.1E, Appendix Fig A-4A). These results demonstrate that ME differentiation induced by BMP4 is considerably hindered in RIC-KO hESCs, indicating that RICTOR/mTORC2 plays an important role in ME differentiation.

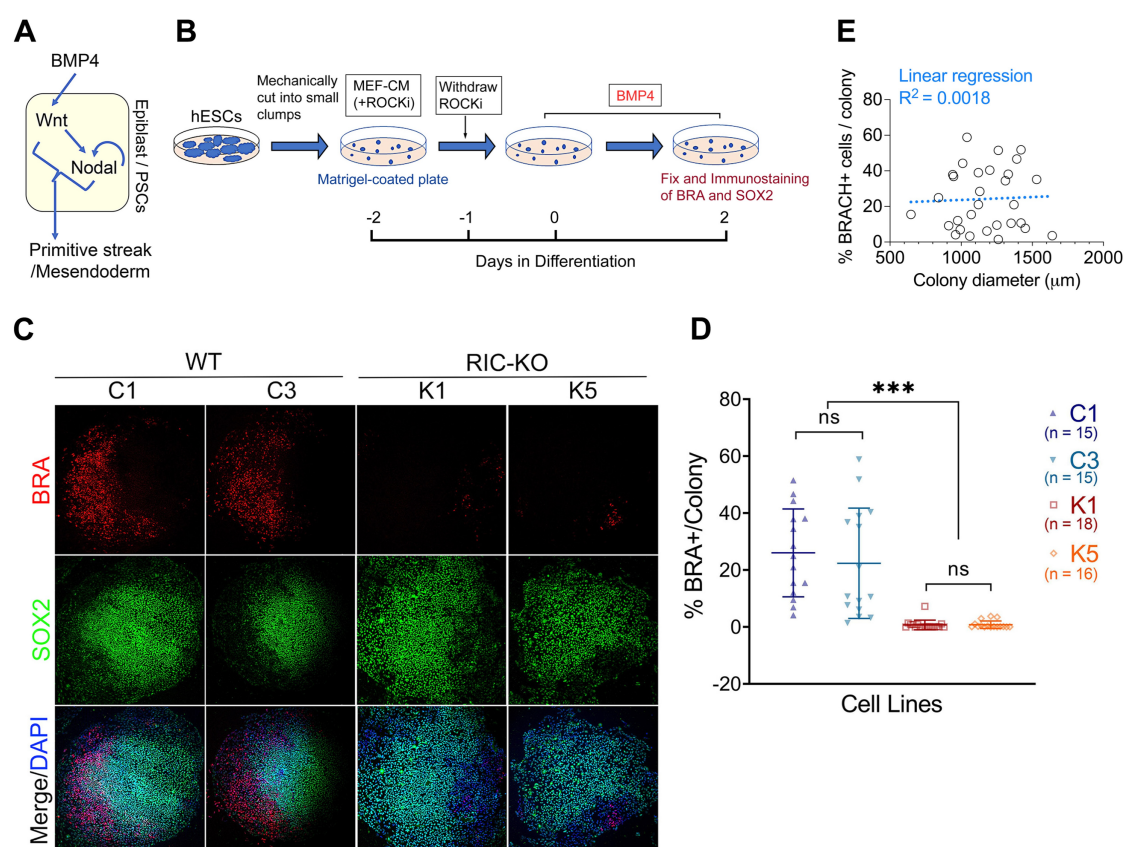


Figure 4.1 Reduced mesendoderm lineage in RIC-KO hESCs via BMP4-induced differentiation

(A) Schematic diagram illustrating signalling pathways involved in the regulation of gastrulation and mesendoderm (ME) differentiation. (B) Schematic diagram depicting the experimental procedure of BMP4-induced differentiation. (C) Representative images of immunostaining showing BRACHYURY (BRA) and SOX2 expression after 48-hr differentiation shown in (B). Scale bar, 250 μ m. (D) Dot plot showing the percentage of BRA+ cells in each colony with median and 95% CI (confidence interval) indicated. Each dot represents a single colony and the numbers of colonies analysed from three independent experiments are also indicated. ***, $p < 0.001$ by one-way ANOVA. ns, not significant. (E) Scatter plot showing the correlation between % BRA+ cells and colony size in WT cells after differentiation. Each round circle represents a single colony. Linear regression R^2 is indicated.

Given that cells of all three germ layers as well as the extraembryonic lineage might be produced in hESC colonies in response to BMP4 stimulation (Gunne-Braden et al., 2020; Vasic et al., 2023), analogous to circular micropatterning (Minn et al., 2020; Warmflash et al., 2014), I asked what different lineage derivatives RIC-KO hESCs would have become after BMP4 stimulation if ME lineage was considerably hindered. qRT-PCR analysis showed that WT and

RIC-KO cells expressed pluripotent markers (*OCT4*, *SOX2* and *NANOG*) at similar levels before the BMP4 treatment as previously found and that the expression of *OCT4* and *SOX2* was clearly decreased to ~25% after 48-hour BMP4 treatment in both cell types (Fig 4.2), suggesting that these cells became differentiated. *NANOG* expression in WT cells was upregulated at 24 hr and then decreased by 48 hr, whereas in RIC-KO cells its expression was continuously downregulated after BMP4 induction. A transient increase of *NANOG* expression in WT cells might be associated with ME differentiation (Teo et al., 2011; Yu et al., 2011). ME markers (*BRA*, *EOMES* and *MIXL1*) revealed increased expression after BMP4 induction but their upregulation was significantly lower in RIC-KO cells than in WT controls (Fig 4.2), which was consistent with fewer BRA⁺ cells in RIC-KO cells by immunostaining (Fig 4.1C, D). In addition, extraembryonic markers (*CDX2* and *TFAP2A*) were also expressed at lower levels in RIC-KO cells (Fig 4.2), which are recently reported to be specific markers of amnion (Bergmann et al., 2022; Munger et al., 2022; Weatherbee et al., 2023). By contrast, ectoderm markers (*BRN2* and *SOX1*) showed significantly higher expression in RIC-KO cells than WT controls at 24 hr (Fig 4.2), indicating that RIC-KO hESCs might differentiate to neuroectoderm in response to BMP4 treatment. However, while *BRN2* increased its expression from 24 hr to 48 hr, *SOX1* exhibited downregulation in both WT and KO cells. One possible reason for this different expression pattern between *SOX1* and *BRN2* is that *SOX1* is a relatively early neural progenitor marker, particularly responsible for neuronal differentiation, which could be inhibited by extended BMP4 treatment (Gerrard et al., 2005), while *BRN2* is not only a neural progenitor marker but is also found to contribute to cerebrovascular development in primates (Zhu et al., 2022). Further characterisation is required to reveal the cell types at different hours of BMP4 treatment by single cell RNA-seq. Overall, these findings suggest that ME and amnion differentiation are impaired in RIC-KO hESCs in response to BMP4 stimulation.

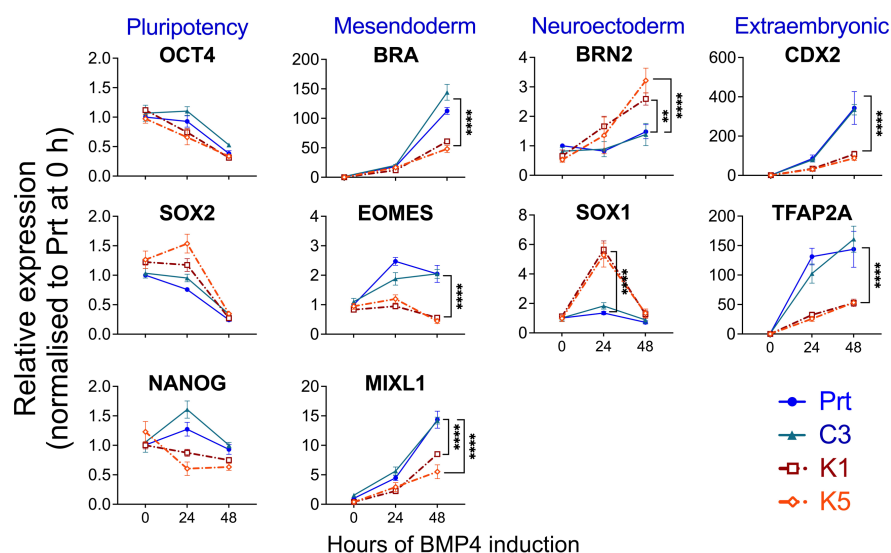


Figure 4.2 Enhanced neuroectoderm but reduced extraembryonic lineage in RIC-KO hESCs via BMP4-induced differentiation

*qRT-PCR analysis of the dynamic mRNA expression of pluripotent and lineage-specific markers in WT and RIC-KO hESCs. Data are presented as mean $\pm$ SD of 6 measurements from 3 independent differentiation experiments. ** and ****, $p < 0.01$ and $p < 0.0001$, respectively, by two-way ANOVA.*

4.2.2 Inhibition of GSK3 rescues BMP4-induced mesendoderm differentiation in RIC-KO hESCs

Next, I sought to explore the underlying mechanisms by which mTORC2 regulates ME differentiation in hESCs. I asked whether RICTOR/mTORC2 was directly involved in activating the expression of ME-specific transcription factors or regulating the signalling pathways that are crucial for ME differentiation. Given that the three key signalling pathways, BMP, WNT and NODAL work in synergy to promote the formation of PS and ME across species (Arnold and Robertson, 2009; Cui et al., 2022; Martyn et al., 2018; Tam and Loebel, 2007) (Fig 4.1A), I first activated all three pathways simultaneously by treating hESCs with BMP4 together with the GSK3 inhibitor CHIR99021 (CHIR) to activate WNT signalling and Activin A (AA) to simulate the activation of NODAL signalling for 48 hours (see section 2.1.2.3 for details). This combined treatment dramatically increased BRA⁺ cells in RIC-KO colonies, even producing a significantly higher proportion of BRA⁺ cells than that in WT

controls (Fig 4.3A). These results suggest that RIC-KO hESCs are capable of generating BRA⁺ cells and mTORC2 is not directly responsible for the activation of ME transcription factors.

Thus, I anticipated that mTORC2 deficiency possibly affects the activation of WNT and/or NODAL signalling in response to BMP4 stimulation, resulting in defective ME differentiation. To test this hypothesis, I then activated WNT or NODAL signalling individually with BMP4 by treating WT and RIC-KO hESCs with BMP4 in combination with either CHIR or AA. Remarkably, the addition of AA with BMP4 did not alter the overall outcome and produced a similar pattern as BMP4 alone, showing a significantly lower percentage of BRA⁺ cells per colony in RIC-KO cells than WT controls (Fig 4.3B), whereas the addition of CHIR with BMP4 massively increased BRA⁺ cells in both WT and RIC-KO hESCs and the percentage of BRA⁺ cells was similar in WT and RIC-KO cells (Fig 4.3C). These results indicate that mTORC2 deficiency may hinder the activation of WNT signalling by BMP4. In supporting this, treating hESCs with CHIR alone showed similar ME differentiation as shown by the similar percentage of BRA⁺ cells between WT and RIC-KO hESCs (Fig 4.3D).

Interestingly, when comparing various treatments in the same cell line, I noticed that BMP4 plus CHIR treatment induced a higher fraction of BRA⁺ cells than CHIR alone treatment in both WT and RIC-KO colonies (Fig 4.4). This was probably because BMP4-activated SMAD1/5/9 cooperates with BRA to enhance mesoderm differentiation but repress endoderm differentiation (Faial et al., 2015). In addition, the extra addition of AA in BMP4 plus CHIR treatment reduced production of BRA⁺ cells in WT colonies, but not in RIC-KO colonies (Fig 4.4), suggesting that the combined treatment further differentiated BRA⁺ ME cells into the definitive endoderm lineage after WNT priming in WT cells, while lower WNT signalling in RIC-KO cells was unable to do so at 48 hr of BMP4 induction (Yoney et al., 2022). This also explains why the extra addition of AA in BMP plus CHIR treatment produced the similar percentage of BRA⁺ cells as BMP plus CHIR treatment in RIC-KO cells (Fig 4.4). Taken together, mTORC2 deficiency impedes ME differentiation probably by preventing BMP4-induced WNT activation and that WNT signalling is essential for driving ME differentiation in hESCs.

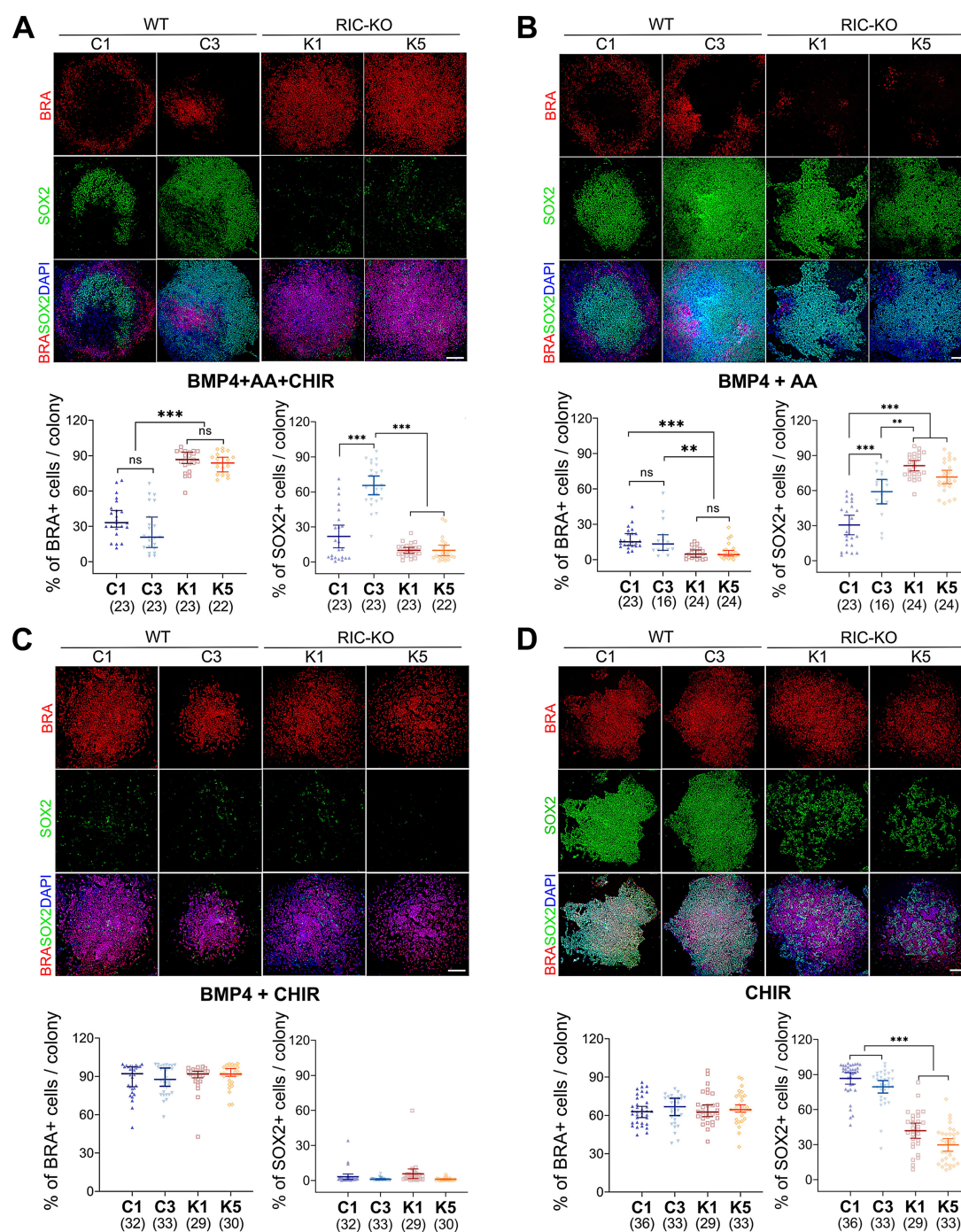


Figure 4.3 Highly efficient mesendoderm differentiation in RIC-KO hESCs after inhibition of GSK3 β

Both WT and RIC-KO hESCs were treated with BMP4, Activin A (AA) and CHIR99021 (CHIR) (A), BMP4 and AA (B), BMP4 and CHIR (C), or CHIR alone (D) for 48 hours before immunostaining with BRA and SOX2 antibodies. Top panels: representative images. Scale bar, 250 μ m. Bottom panels: quantitative analyses of % BRA+ cells (left) and SOX2+ cells (right) are presented as median with 95% CI. Each symbol represents a colony and the total number of colonies analysed for each cell line is indicated in brackets from three independent experiments.

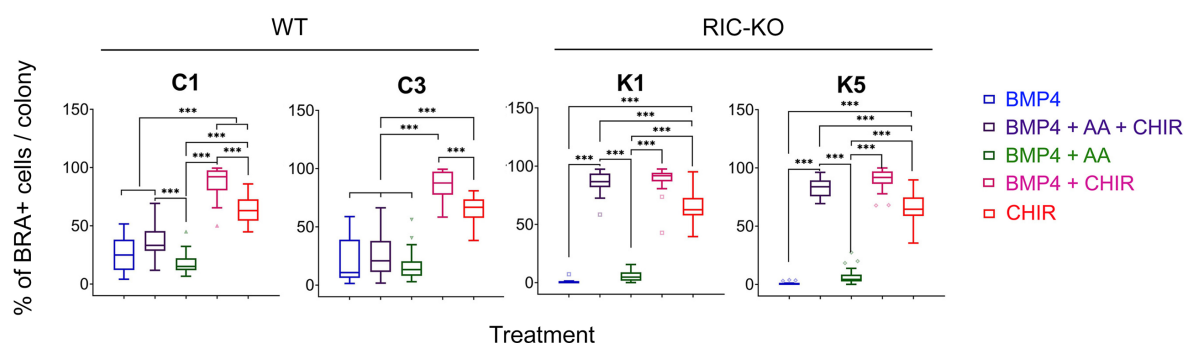


Figure 4.4 Effect of different treatments on generating BRA+ cells in each cell line

Comparing the effect of activating indicated pathways on generating BRA+ cells within the same cell line. Data are presented as the Tukey box plot with the numbers of colonies in the dot plots shown above. ** and ***, $p < 0.01$ and $p < 0.001$, respectively, by one-way ANOV. Ns, not significant.

4.2.3 BMP4-induced expression of canonical WNT genes is impeded in RIC-KO hESC colonies

Since ME differentiation was completely rescued by the activation of WNT/ β -catenin signalling by CHIR in RIC-KO hESCs, I reasoned that mTORC2 deficiency in these cells might affect the activation of WNT signalling in response to BMP4, possibly upstream of GSK3.

To explore how mTORC2 deficiency affects BMP4-induced activation of WNT signalling in hESCs, I examined the dynamic changes in GSK3 activity and canonical WNT signalling during BMP4-induced differentiation by immunoblotting. As expected, the immunoblots showed that mTORC2 signalling remained inactive in RIC-KO cells throughout the differentiation as manifested by the absence of RICTOR expression and PKC ζ protein that is stabilised by mTORC2 through phosphorylation at its TM (turn motif) (Fig 4.5A). In addition, as previously observed in undifferentiated hESCs, E-CAD was still expressed at much lower levels in RIC-KO cells throughout the differentiation (Fig 4.5A), demonstrating their reduced cell-cell interactions. Interestingly, although AKT has been reported to have an inhibitory effect on GSK3 via phosphorylation on Ser9 and Ser21 of GSK3 α and β , respectively, and mTORC2 is responsible for phosphorylation of AKT at the HM (hydrophobic motif) to maximise AKT activity (Manning and Toker, 2017), phosphorylation of GSK3 revealed no clear change in RIC-KO cells compared to WT controls throughout the differentiation (Fig 4.5A). By contrast,

expression of the transcription factor LEF1, a direct target of the canonical WNT signalling (Cadigan and Waterman, 2012, Estarás et al., 2015), was undetectable in undifferentiated hESCs but became clearly visible at 48 hours of BMP4 stimulation, indicating that BMP4 stimulation indeed activates the canonical WNT signalling as previously reported in the epiblast (Morgani and Hadjantonakis, 2020). Notably, this upregulation of LEF1 appeared considerably higher in WT than RIC-KO cells (Fig. 4.5A), demonstrating higher levels of the WNT signalling in WT than RIC-KO cells after BMP4 stimulation. All the immunoblots have been performed with lysates from two independent differentiation experiments and showed the similar expression pattern. Therefore, these results suggest that mTORC2 deficiency attenuates the BMP4-induced WNT signalling, which is independent of GSK3 activity.

Next, I asked whether any components of the upstream WNT signalling were affected in RIC-KO hESCs after BMP4 stimulation. Since application of GSK3 inhibitor can rescue ME differentiation in RIC-KO hESCs, it is plausible that the defects in the WNT signalling occur at the upper parts of the signalling in RIC-KO hESCs. Thus, I compared the expression of genes encoding WNT ligands, receptors, and associated proteins during BMP4-induced differentiation by qRT-PCR. The results showed that the canonical WNT genes *WNT3*, *WNT3A* and *WNT8A* were dynamically upregulated upon BMP4 stimulation and that the levels of upregulation were significantly higher in WT than RIC-KO cells (Fig 4.5B). Correspondingly, canonical WNT target genes, *AXIN2* and *LEF1* also revealed higher expression in WT cells, which was consistent with the protein expression of LEF1 (Fig 4.5A). Although the expression of the non-canonical WNT genes *WNT5A* and *WNT5B* appeared higher in WT cells, no significant difference was observed (Fig 4.5B), suggesting that the non-canonical WNT genes might not greatly account for impaired ME differentiation in RIC-KO cells during BMP4-induced differentiation. Surprisingly, the expression of WNT receptors *FZD2* and *FZD7* and the coreceptor *LRP6* exhibited a transient increase in RIC-KO hESCs at 24 hours of BMP4 stimulation, and then reduced to the similar levels as WT cells (Fig 4.5B). It is unclear whether this transient upregulation of WNT receptors in RIC-KO hESCs is due to a lack of negative feedback as a result of reduced expression of WNT genes. Nonetheless, this increase in receptor expression did not seem to activate the WNT signalling. The WNT agonist *DVL2* did not show clear difference in expression between WT and RIC-KO cells (Fig 4.5B). Taken together, these findings demonstrate that BMP4-induced activation of WNT genes is hindered in RIC-KO hESCs, which likely accounts for the attenuated WNT signalling and impaired ME differentiation in these cells.

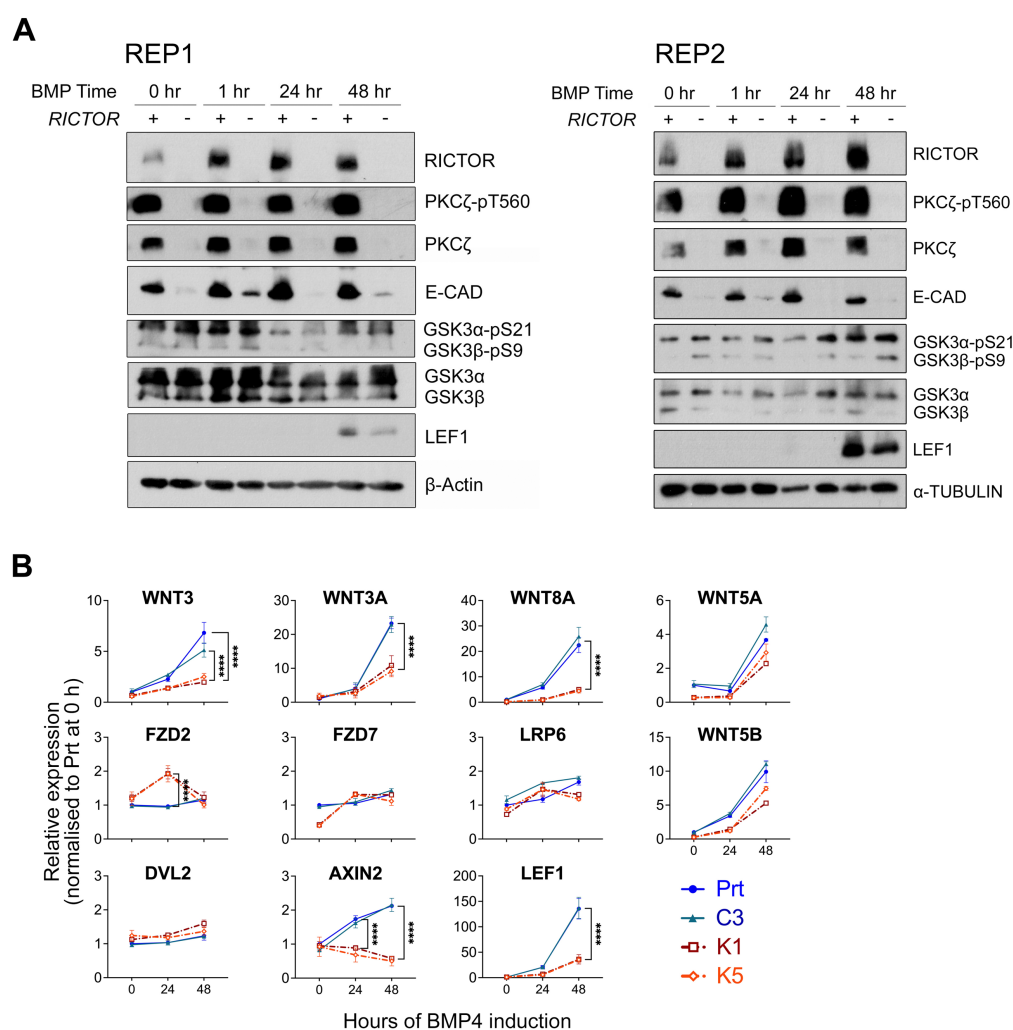


Figure 4.5 Impaired mesendoderm differentiation due to poor canonical WNT activation in RIC-KO hESCs with BMP4 stimulation

(A) Immunoblots showing similar protein expression as indicated in WT and RIC-KO hESCs at timepoints as depicted during BMP4-induced differentiation from two independent differentiation experiments ($n = 2$). (B) Dynamic expression of genes associated with WNT signalling during BMP4-induced differentiation by qRT-PCR in WT and RIC-KO hESCs. Data are presented as mean $\pm$ SD of 6 measurements from 3 independent differentiation experiments.

****, $p < 0.0001$ by two-way ANOVA.

4.3 Discussion

By utilising a BMP4-induced ME differentiation approach, I clearly show that ME differentiation is considerably hindered in RIC-KO hESCs, further corroborating our preliminary data that mTORC2 deficiency appreciably impedes hESC differentiation into mesoderm and endoderm lineages. I also demonstrate that defective ME differentiation in RIC-KO hESCs is mainly attributed to an insufficient upregulation of canonical WNT genes induced by BMP4, which results in impeded WNT signalling.

BMP4-induced differentiation approaches have been used by several laboratories for modelling gastrulation and investigating mesoderm differentiation in hPSCs, which are performed either in colonies up to 500 μm in diameter as outlined in my study (Vasic et al., 2023) or in the micropatterned model smaller than 1000 μm in diameter (Deglincerti et al., 2016; Warmflash et al., 2014), all showing reproducible patterning and differentiation. Although the colony size has been reported to influence the proportion of ME-fate cells during differentiation (Bauwens et al., 2008), I notice that there is no appreciable correlation between the proportion of BRA⁺ cells per colony and its diameter if a colony is approximately 150 – 500 μm in diameter at the start of the induction day, thereby proving that the colony size within this range is not a confounding factor in my study. Interestingly, several differentiation experiments using such approaches reveal the radial patterning of differentiation in which SOX2 expressing cells are located in the centre of the colonies while BRA⁺ cells form a concentric ring surrounding the SOX2⁺ cells (Gunne-Braden et al., 2020; Vasic et al., 2023). However, my differentiation experiments exhibit symmetry breaking, where one side contains mostly BRA⁺ cells and the other side has SOX2⁺ cells. This symmetry breaking is previously reported in the 3D BMP4 differentiation model in hPSCs (Simunovic et al., 2019). One possibility may contribute to the different patterning is that hESCs in both my study and the 3D human epiblast model (Simunovic et al., 2019) that reveals symmetry breaking in BMP4-induced differentiation were cultured in MEF-CM before and during differentiation, while hPSCs showing radial patterning upon BMP4-induced differentiation were maintained in chemically defined commercial mTeSR1 medium before and during differentiation (Gunne-Braden et al., 2020; Vasic et al., 2023). The different culture conditions may affect hPSC ability to pattern. The exact reasons for these two different patterns await further investigation.

One of my main findings in this chapter is that mTORC2 deficiency does not affect the intrinsic competence of hESCs to generate mesoderm and endoderm; rather it impedes activation of the

WNT signalling by BMP4. Therefore, forced activation of the WNT/ β -catenin signalling pathway by CHIR completely rescues ME differentiation in RIC-KO cells. As CHIR acts to inhibit GSK3, newly synthesised β -catenin is no longer phosphorylated for proteasome degradation. This leads to accumulation of β -catenin in the cytoplasm that is subsequently translocated into the nucleus and participates in the activation of genes involved in ME differentiation, such as *BRA*, *EOMES*, *MIXL1* and *NODAL* (Funa et al., 2015; Mazzotta et al., 2016; Yoney et al., 2022). This finding corroborates the essential role of the canonical WNT signalling in ME differentiation and gastrulation (Steinhart and Angers, 2018). By contrast, BMP4 alone or with AA does not efficiently promote ME differentiation in RIC-KO cells. These results suggest that the main role of BMP4 in ME differentiation is to activate the WNT signalling as CHIR alone can efficiently induce ME differentiation in hESCs without extra BMP4 and that the NODAL signalling is mainly responsible for endoderm specification (Faial et al., 2015; Loh et al., 2014; Martyn et al., 2018).

Furthermore, my results also suggest that mTORC2 deficiency does not affect the WNT signalling downstream of GSK3 in hESCs. Instead, activation of canonical WNT genes by BMP4 stimulation is impeded, which directly leads to defective ME differentiation in RIC-KO hESCs. In contrast, expression of the noncanonical WNT ligands does not show significant difference between WT and RIC-KO cells at 48 hr of BMP4 treatment. Although the noncanonical WNT ligands such as WNT5A and WNT5B are also essential for mesoderm specification, they are required at a later stage than the canonical ligands (Mazzotta et al., 2016). However, it still remains unclear how mTORC2 affects the expression of the WNT genes in PSCs and I will explore more in the next chapter. In summary, my data so far suggest that RICTOR/mTORC2 regulates ME differentiation in hESCs through BMP4-induced activation of canonical WNT genes.

Chapter Five

**Cell-cell interactions in hESCs are important for
BMP4-induced expression of WNT genes**

5.1 Introduction

During implantation, pluripotent epiblast cells undergo epithelialisation after which they become further polarised with distinct apical and basal compartments and form a tightly aligned epithelial layer by anchoring cell-cell junctions in both mouse and human embryos (Krtolica et al., 2007; Lamouille et al., 2014; Pieters and van Roy, 2014). Cell-cell junctions constructed by adhesion protein complexes are essential for intercellular communication and epithelial integrity. hPSCs resemble post-implantation epiblast cells referred as primed PSCs that also exhibit epithelial cell characteristics (Rossant and Tam, 2022; Shahbazi, 2020; Zhai et al., 2022). From lateral surfaces of PSCs, there are four types of junctions: tight junctions (TJs), adherens junctions (AJs), desmosomes, and gap junctions (GJs) (Fig 1.8) (Huang et al., 2012; Lamouille et al., 2014). Each junction has its specific and irreplaceable roles to mediate cell-cell adhesion and maintain epithelial integrity as previously discussed (section 1.3.3.1), which highlights the importance of junctions in the maintenance of hPSCs and indicates that the junctions may also have a function in cell fate determination in response to extracellular stimulations.

The epithelialisation event is considered as a crucial process for gastrulation (Shahbazi, 2020; Sheng, 2015), as intercellular communication mediated by cell-cell junctions is required to trigger spatial organisation and patterning of epiblast cells, which leads to the formation of lineage-specific populations after gastrulation (Rossant and Tam, 2022; Shahbazi, 2020; Shahbazi et al., 2019). *In vivo* studies reveal that a lack of ZO-2 leads to embryonic lethality shortly after implantation due to increased apoptosis in early gastrulation (Xu et al., 2008), and mouse embryos deficient in ZO-1 die at mid-gestation due to defective vasculature in the yolk sac (Katsuno et al., 2008), demonstrating the importance of cell-cell interactions during gastrulation in mouse embryos. Consistently, *in vitro* 3D aggregates of PSCs are able to self-organise into elongated gastruloids, which generate derivatives of all three germ layers (Turner et al., 2017; van den Brink et al., 2020; van den Brink et al., 2014). Interestingly, this self-organisation is feasible only when appropriate cell aggregates are formed (Cermola et al., 2021; Ohtsuka et al., 2012), indicative of robust cell-cell interactions required for self-organisation. Moreover, hPSCs in 2D monolayer that are seeded in single cells need to achieve a certain high cell density to acquire sufficient intercellular communication before they can be efficiently induced for ME differentiation (Etoc et al., 2016; Matsuda et al., 2020). However, the reasons why cell-cell interactions are required for the formation of germ layers, particularly the ME

lineage, in PSCs remain to be identified. Recent studies using micropatterned discs reveal that the homogenous presence of BMP4 stimulates generation of the ME lineage in hPSC colonies (Minn et al., 2020; Warmflash et al., 2014). As opposed to this, sparsely seeded hPSCs or hPSC colonies consisting of only 1 – 8 cells are unable to differentiate into the ME lineage after BMP4 treatment, but instead become cells of mainly the extraembryonic origin (Etoc et al., 2016, Nemashkalo et al., 2017). These phenotypes indicate that activation of BMP signalling alone does not induce the ME fate and that cell-cell interactions are required for the BMP4-induced ME differentiation.

Mechanistic studies show that BMP receptors are localised at the basolateral sides of polarised hPSCs and hence the majority of the cells in compact hPSC colonies are not accessible to BMP4 supplied in the medium due to tight cell-cell interactions mediated by cell junctions except cells at the colony edges (Etoc et al., 2016). Upon BMP4 induction, expression of the BMP4 effector, phosphorylated SMAD1/5/9 (briefed as pSMAD1, hereafter), predominantly appears on the colony periphery. However, high concentration of BMP4 overcomes the edge-to-centre difference in sensitivity at the start of the treatment and induces the pSMAD1 signal throughout the colony, which upregulates the expression of *NOGGIN*, a BMP target as well as inhibitor. *NOGGIN* is secreted and diffused to the colony centre and acts to organise pSMAD1 expression into the signalling gradient from the colony edge the strongest to the centre the weakest (Etoc et al., 2016). The differential BMP4 signalling in the colonies may result in radial fate patterning in micropatterned colonies in which extraembryonic cells are localised at the colony edge, followed by ME, and ectodermal cells towards the colony centre (Minn et al., 2020; Warmflash et al., 2014). Diminished cell-cell interactions by disrupting cell-cell junctions, such as TJs or AJs, alter cellular responsiveness to morphogens and abolishes the signalling gradients, thereby overriding the normal patterning in hPSC colonies (Legier et al., 2023; Libby et al., 2018; Martyn et al., 2019; Vasic et al., 2023). These studies highlight an essential role of AJs and TJs as cellular junctions to mediate intercellular interactions required for the patterning and formation of germ layers in PSCs.

Previous work in our lab (Chapter 1) and my current work (Chapter 3) have shown that RIC-KO considerably reduces cell adhesion and interactions and that BMP4-induced activation of canonical WNT genes is impeded, which directly leads to defective ME differentiation in RIC-KO hESCs (Chapter 4). Taken these together, I reason that this insufficient activation of canonical WNT genes in response to BMP4 might be associated with poor cell-cell interactions

in RIC-KO hESCs. Therefore, in this chapter, I further investigated this issue and addressed whether a reduction in cell-cell interactions in RIC-KO hESCs would affect the BMP signalling during BMP4-induced differentiation and whether decreased cell-cell contact in WT hESCs would affect BMP4-induced WNT signalling, leading to diverse cell fate specification.

5.2 Results

5.2.1 Cellular interaction-mediated pSMAD1 patterning is abolished in RIC-KO hESC colonies in BMP4-induced differentiation

To investigate whether a reduction in cell-cell interactions in RIC-KO hESCs would affect the dynamics of BMP signalling in RIC-KO hESCs, I examined expression of proteins and genes associated with the BMP signalling at different time points during BMP4-induced differentiation by immunoblotting and qRT-PCR. It was clear that E-CAD was continuously expressed at lower levels in RIC-KO cells throughout the differentiation. BMP4 signalling was inactive in both undifferentiated WT and RIC-KO hESCs as no pSMAD1 signal, the effector of the BMP signalling, was detected at 0 hr (Fig 5.1A). Upon BMP4 stimulation, there was an appreciable increase in the pSMAD1 signal at 1 hr post treatment in both WT and RIC-KO hESCs and the level of pSMAD1 expression was higher in RIC-KO cells (Fig 5.1A), indicative of augmented BMP signalling. This was probably due to basolateral localisation of BMP receptors in hPSCs on the colony periphery, which allowed RIC-KO hESCs to get more access to BMP4 in the media because of their weak cell-cell junctions (Etoc et al., 2016, Zhang et al., 2019). Surprisingly, pSMAD1 as well as total SMAD1 expression were dramatically reduced in RIC-KO cells at 24 hr after BMP4 stimulation and kept at a low level at 48 hr, while their expression was maintained in WT controls (Fig 5.1A). This could be attributed to a stronger upregulation of the BMP inhibitor as well as the BMP4 signalling target gene, *NOGGIN* in RIC-KO hESCs (Fig 5.1B). Similarly, another BMP inhibitor *CER1* also showed a higher upregulation in RIC-KO cells than WT controls at 24 hours post-BMP4 treatment (Fig 5.1B). Together, they generated a strong negative feedback loop to the BMP4 signalling in RIC-KO cells. As this negative feedback persisted, the BMP4 signalling target, *ID1* revealed an initial upregulation at 24 hr followed by reduced expression at 48 hr post BMP4 treatment in hESCs, particularly in RIC-KO cells (Fig 5.1B). Surprisingly, expression of *NOGGIN* still increased in RIC-KO cells from 24 hr to 48 hr (Fig 5.1B), which suggests that other signalling might be

involved in regulating *NOGGIN* expression. Overall, these data demonstrate that WT and RIC-KO hESCs exhibit differential dynamic patterns in the BMP4 signalling.

Next, I examined the BMP4 signalling in hESC colonies using immunostaining and found BMP4 signalling was not active in both WT and RIC-KO hESCs before the treatment (Fig 5.1C), which is consistent with the immunoblot results. However, at 24 hr post BMP4-induction, the BMP4 signalling showed different patterns between WT and RIC-KO cells. In WT colonies, pSMAD1 expression was mainly restricted to strong E-CAD⁺ area at the edges, while pSMAD1 showed ubiquitous expression and E-CAD expression was overall low in RIC-KO colonies (Fig 5.1C, Appendix Fig A-5), suggesting that the pSMAD1 pattern is associated with cell-cell interactions. At 48 hr post BMP4 treatment, pSMAD1 expression was still restricted to the edges but slightly moved inward to the E-CAD⁺ area near the centre of WT colonies, and the cells on the periphery gradually lost E-CAD expression, indicating that these cells underwent EMT (Fig 5.1C, Appendix Fig A-5). However, pSMAD1 expression was still ubiquitous in RIC-KO colonies. Therefore, pSMAD1 reveals distinct patterning in WT and RIC-KO hESCs with BMP4 stimulation and poor cell-cell interactions affect the formation of the BMP signalling gradient in RIC-KO hESCs.

Taken together, although RICTOR/mTORC2 deficiency does not inhibit the BMP signalling in hESCs in response to BMP4 stimulation, reduced cell-cell interactions make more cells in colonies responsive to BMP4 initially, thereby abolishing the normal BMP signalling gradient observed.

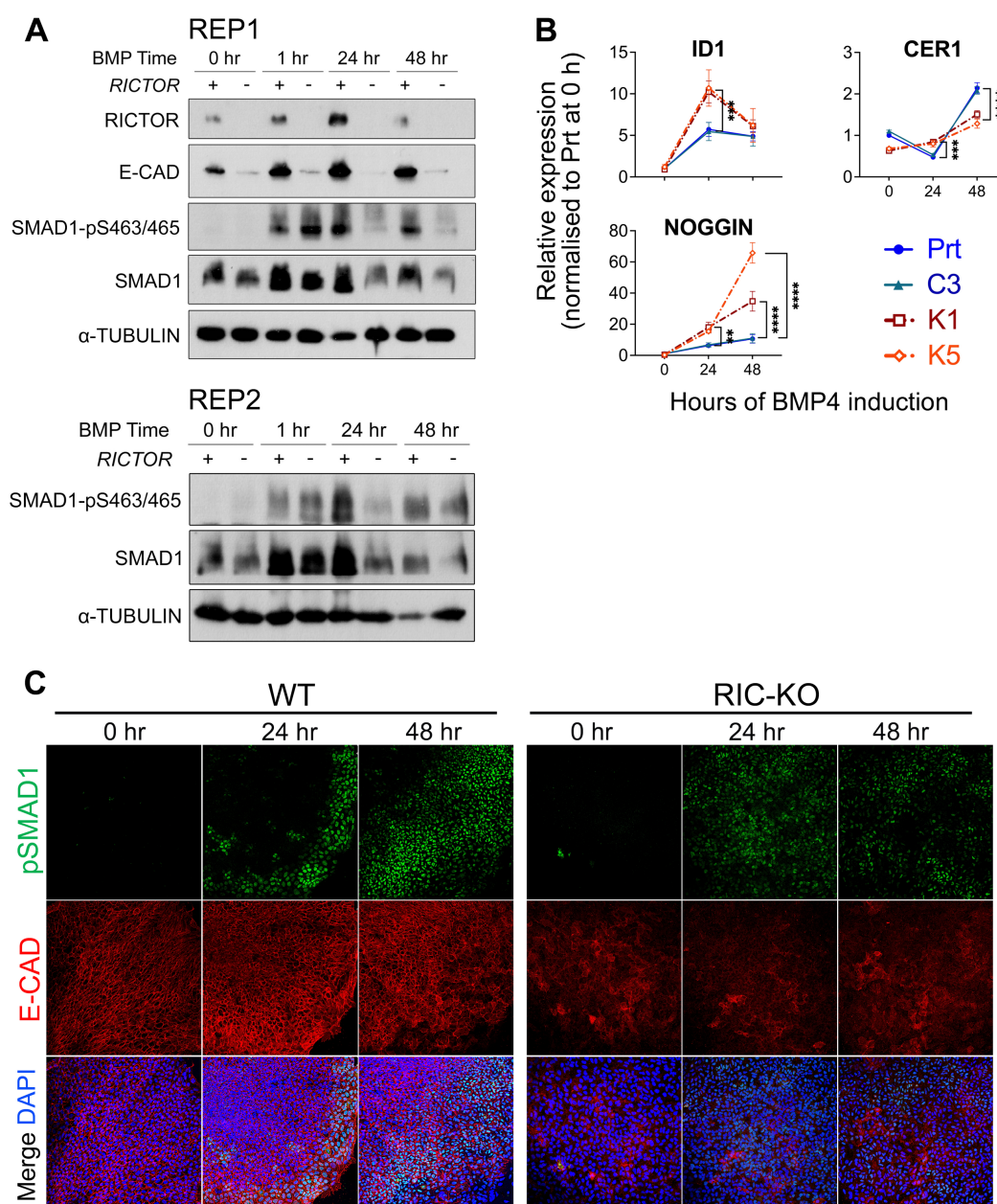


Figure 5.1 Abolished pSMAD1 pattern due to reduced cell-cell interactions in RIC-KO hESC colonies

(A) Immunoblots showing similar protein expression as indicated in WT and RIC-KO hESCs during BMP4-induced differentiation from two independent experiments ($n = 2$). (B) Dynamic expression of genes associated with BMP signalling during BMP4-induced differentiation by qRT-PCR in WT and RIC-KO hESCs. Data are presented as mean $\pm$ SD of 6 measurements from 3 independent differentiation experiments. **, *** and ****, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively, by two-way ANOVA. (C) Immunostaining showing the dynamic pattern of phospho-SMAD1/5/9 (briefed as pSMAD1) and E-CADHERIN (E-CAD) during BMP4-induced differentiation in WT and RIC-KO hESCs. Scale bar, 100 μ m. Timepoints analysed during differentiation are indicated.

Interestingly, the expression of WNT signalling target, LEF1, also exhibited a pattern associated with E-CAD expression. Before BMP4 treatment, there was no LEF1 expression in WT and RIC-KO hESC colonies before the BMP4 treatment (Fig 5.2). As the BMP4 treatment progressed to 24 hr, a considerable number of cells in WT colonies started to express LEF1 and its expression was restricted to the strong E-CAD⁺ area at the colony edge (Fig 5.2, Appendix Fig A-6). Interestingly, this was the same area where WT cells expressed pSMAD1 in colonies at 24 hr (Fig 5.1C, Appendix Fig A-5). However, LEF1 expression was hardly detectable in RIC-KO colonies and E-CAD expression maintained low at 24 hr post BMP4 induction (Fig 5.2), suggesting that the emergence of LEF1 expression is strongly correlated with the pSMAD1 pattern mediated by cell-cell interactions. At 48 hr post BMP4 treatment, there was a stark difference of LEF1 expression between WT and RIC-KO colonies. Its expression expanded inward to the E-CAD⁺ area closer to the centre of WT colonies, while there were only few cells expressing LEF1 in RIC-KO colonies (Fig 5.2, Appendix Fig A-6). This is consistent with the immunoblotting result that LEF1 expression was much lower at 48 hr (Fig 4.5A).

Overall, these data suggest that the BMP signalling pattern may play a role in the activation of canonical WNT genes and that cell-cell interactions are important for the formation of the BMP4 signalling pattern.

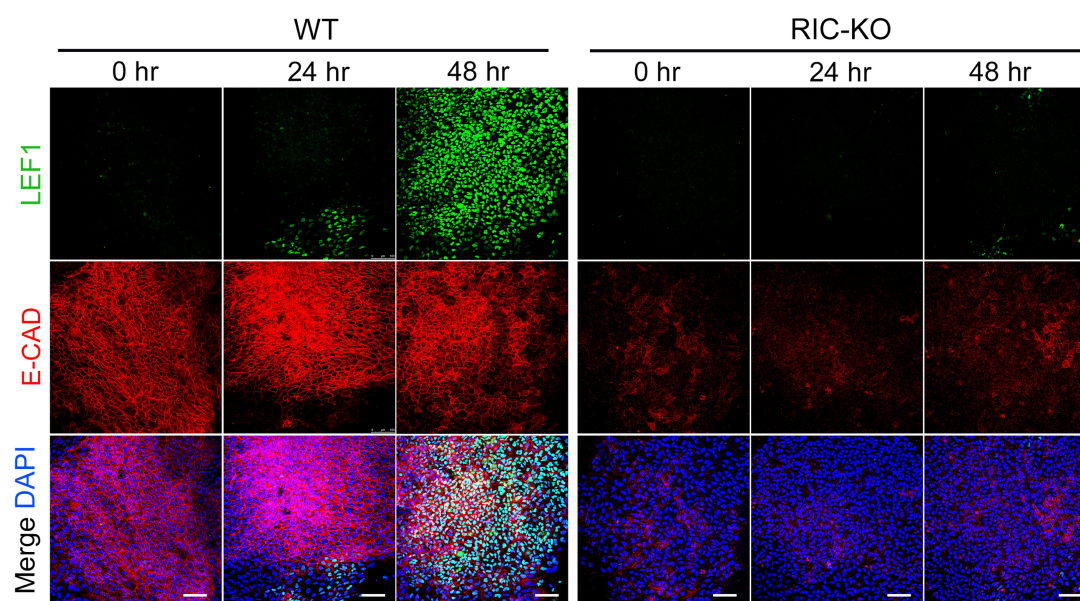


Figure 5.2 Differential levels of canonical WNT signalling between WT and RIC-KO hESC colonies

Immunostaining showing the expression pattern of LEF1 and E-CAD during BMP4-induced differentiation in WT and RIC-KO hESCs. Timepoints analysed during differentiation are indicated. Scale bar, 100 μ m.

5.2.2 Limited cell-cell interactions alter pSMAD1 patterning observed in hESC colonies in response to BMP4

To further investigate that cell-cell interactions indeed play an important role in the formation of the BMP signalling gradient in hESCs, I performed the same BMP4-induced differentiation in WT hESCs that were seeded either in colonies (CL) or in single cells (SC) (Fig 5.3A). For the SC cultures, WT hESCs were dissociated into single cells and seeded at a density of 5.3×10^4 cells/cm² to effectively limit cellular interactions. As manifested by the immunoblots, E-CAD showed lower expression in SC-plated hESCs (Fig 5.3B, Appendix Fig A-7). The BMP4 signalling was inactive in both SC and CL cultures as there was hardly any pSMAD1 expression before BMP4 induction (Fig 5.3C). Consistent with the immunoblotting result, immunostaining also showed obviously lower E-CAD in SC than CL cultures (Fig 5.3C), suggesting low cell-cell interactions in SC cultured hESCs. Remarkably, both cultures revealed differential responses to BMP4 treatment. In CL cultures, pSMAD1 expression was restricted at the edges of colonies at 24 hr post BMP4 treatment as previously observed, while its expression was relatively uniform in SC cultures, similar to that appeared in RIC-KO hESCs

(Fig 5.3C). By 48 hours post-BMP4 stimulation, the pSMAD1 signals spread inward in CL cultures, while its pattern did not show clear changes in SC cultures (Fig 5.3C). Therefore, these results confirm that cell-cell interactions are required for pSMAD1 patterning in hESCs in response to BMP4 stimulation.

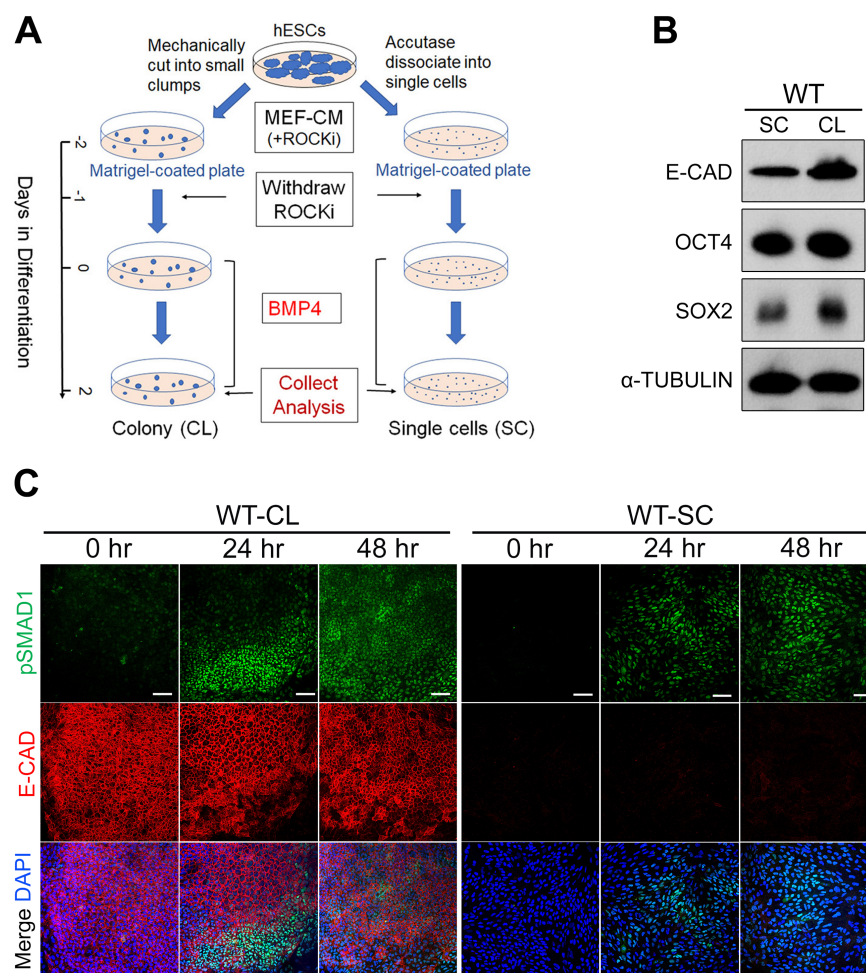


Figure 5.3 Distinct pSMAD1 pattern between WT hESCs in single-cell and colony cultures in response to BMP4

(A) Schematic diagram illustrating the experimental procedure in colony (CL)- and single cell (SC)-plated hESCs. (B) Immunoblotting showing protein expression as indicated in WT hESCs in SC and CL cultures before BMP4 treatment. Representative images depicted from three independent experiments. Images of replicate immunoblots included in Appendix Figure A-7. (C) Immunostaining showing the expression pattern of pSMAD1 and E-CAD during BMP4-induced differentiation in CL- and SC-plated WT hESCs. Timepoints analysed during differentiation are indicated. Scale bar, 100 μ m.

5.2.3 Single-cell seeded hESCs do not differentiate into mesendoderm efficiently due to poor canonical WNT activation

Next, I validated if cellular interactions are crucial for BMP4-induced activation of WNT genes and ME differentiation in hESCs. I stained the cells in CL and SC cultures after 48 hours BMP4 treatment with BRA and SOX2 antibodies. As expected, asymmetrically distributed BRA⁺ cells were detected in CL cultures after 48 hours of BMP4 treatment, whereas very few BRA⁺ cells were observed in SC cultures (Fig 5.4A), demonstrating that ME differentiation was impeded in SC-plated hESCs, similar to CL-plated RIC-KO hESCs. To explore whether impaired ME differentiation was also associated with defective activation of WNT or NODAL in SC-plated hESCs in response to BMP4, I treated these cells with AA and CHIR in addition to BMP4. As expected, this combined treatment was able to efficiently direct differentiation into the ME lineage even at 24 hours of the treatment, as evidenced by a great number of BRA⁺ cells (Fig 5.4B). Therefore, the results ascertain that a lack of cell-cell interactions in hESCs impede BMP4-induced activation of WNT and/or Nodal signalling, which leads to impaired ME differentiation.

This is further supported by the dynamic expression of genes associated with WNT signalling during BMP4-induced differentiation in these two cultures. Both canonical WNT ligands (*WNT3*, *WNT3A* and *WNT8A*) and noncanonical WNT ligands (*WNT5A* and *WNT5B*) showed significantly lower expression in SC cultures than in CL cultures throughout the differentiation after BMP4 stimulation (Fig 5.4C). The dynamic expression of WNT receptors (*FZD2* and *FZD7*), the coreceptor *LRP6*, and the agonist *DVL2* shared a similar pattern. They were all expressed at higher levels in CL than SC cultures before differentiation; however, after BMP4 induction, they showed an upregulation at 24 hr followed by a decrease at 48 hr in SC cultures while a downregulation at 24 hr followed by an increase at 48 hr in CL cultures. At 48 hr post BMP4 treatment, these genes showed significantly lower expression in SC cultures than in CL cultures (Fig 5.4C). Consistently, the canonical WNT target genes, *LEF1* and *AXIN2* were expressed at lower levels in SC cultures at 48 hr (Fig 5.4C). These data support the notion that BMP4-induced ME differentiation requires close cell-cell contact and that cellular interactions are vital for the activation of WNT genes by BMP4 induction in hESCs.

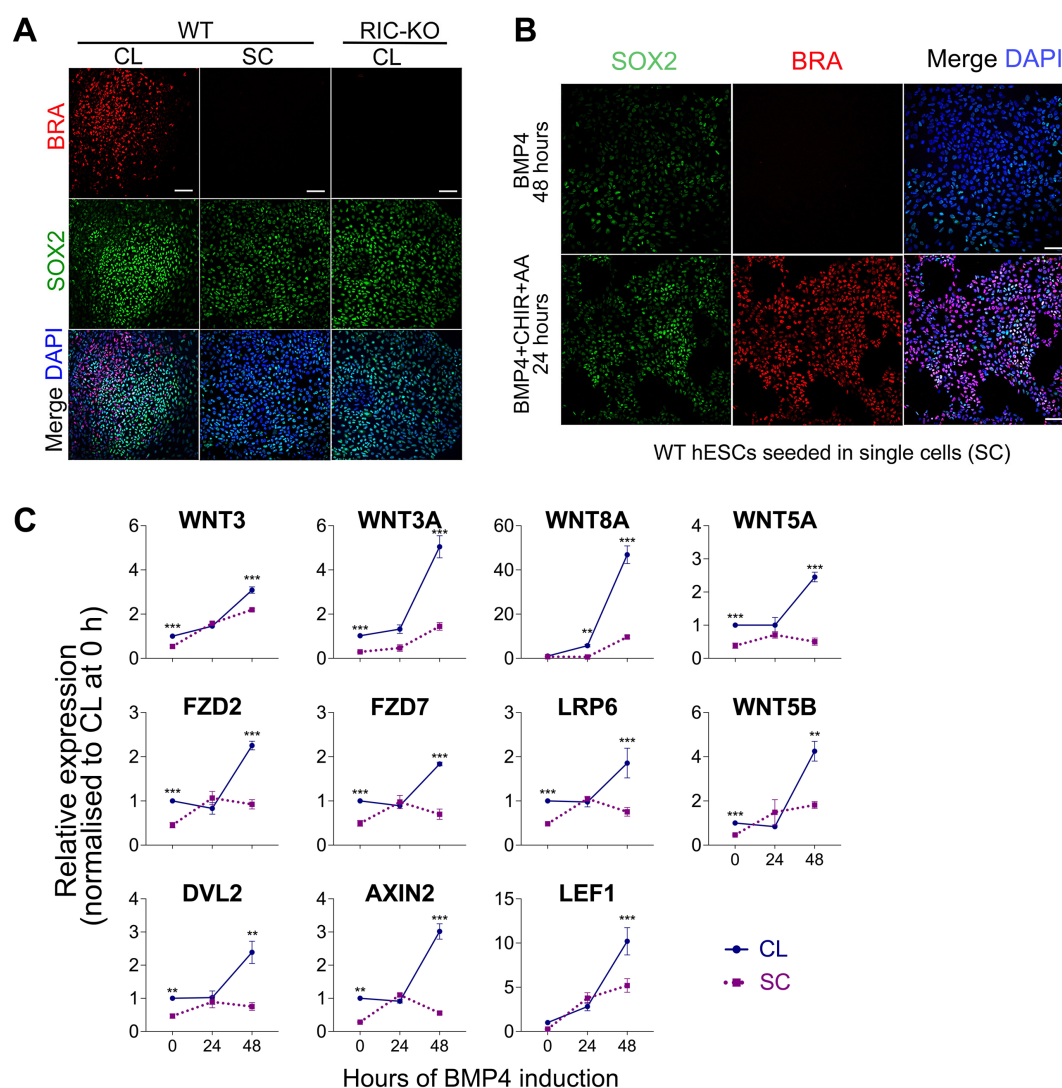


Figure 5.4 Impaired mesendoderm differentiation due to poor canonical WNT activation in single-cell seeded hESCs with BMP4 stimulation

(A) Immunostaining with BRA and SOX2 antibodies in CL- and SC-plated WT hESCs and in CL-plated RIC-KO hESCs for 48-hour BMP4-induced differentiation ($n = 3$). Scale bar, 250 μm . (B) Immunostaining with BRA and SOX2 antibodies in WT hESCs plated in SC after treatment with either BMP4 or BMP4 combined with CHIR and AA ($n = 3$). Treatment and differentiation duration are indicated. Scale bar, 100 μm . (C) Dynamic expression of genes associated with the WNT signalling during BMP4-induced differentiation in CL vs SC WT hESCs by qRT-PCR. Data are presented as mean $\pm$ SD of 9 measurements from 3 independent differentiation experiments. ** and ***, $p < 0.01$ and < 0.001 by two-way ANOVA.

Since hESCs in SC cultures partially lost SOX2 expression after 48-hr BMP4 induction (Fig 5.4A, B), indicative of cells being differentiated, I sought to examine what lineage derivatives these cells would differentiate into, given that they did not give rise to ME efficiently. mRNA

analysis confirmed that hESCs in SC cultures expressed the core pluripotent markers (*OCT4*, *SOX2* and *NANOG*) at the similar levels as those in CL cultures before BMP4 treatment, and their expression decreased after 48-hr BMP4 treatment, especially *SOX2* in SC cultures (Fig 5.5). In agreement with few *BRA*⁺ cells in SC cultures (Fig 5.4A), ME markers (*BRA*, *EOMES* and *MIXL1*) were expressed at much lower levels in SC than CL cultures at 48 hr post BMP4 induction (Fig 5.5). By contrast, extraembryonic markers (*CDX2* and *TFAP2A*) were expressed at significantly higher levels in SC than in CL cultures at 48 hr, again implying their amnion-like fate (Bergmann et al., 2022; Munger et al., 2022; Weatherbee et al., 2023). Interestingly, ectoderm markers (*BRN2* and *SOX1*) showed significantly lower expression in SC cultures compared to CL cultures (Fig 5.5), which were opposite to RIC-KO hESCs that revealed higher expression of neural markers (Fig 4.2). Taken together, my data suggest that hESCs in SC cultures promote differentiation into the extraembryonic lineage, possibly amnion in response to BMP4 stimulation.

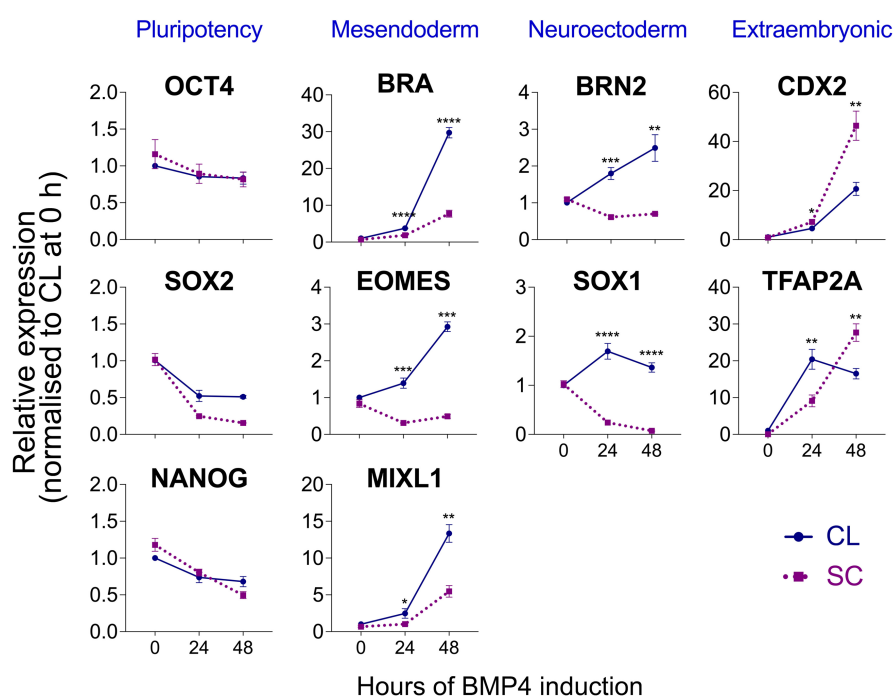


Figure 5.5 hESCs seeded in colony and single cells exhibit different fate outcome after BMP4-induced differentiation

*qRT-PCR analysis of dynamic mRNA expression of pluripotent and lineage-specific markers in WT hESCs plated in CL or SC. Data are presented as mean $\pm$ SD of 9 measurements from 3 independent differentiation experiments. *, **, *** and ****, $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively, by two-way ANOVA.*

5.2 Discussion

In this chapter, I demonstrate that a reduction in cell-cell interactions in RIC-KO hESCs disrupts the BMP signalling gradient with BMP4 stimulation and that activation of the canonical WNT signalling seems to be associated with this BMP signalling pattern, which accounts for impaired ME differentiation in RIC-KO hESCs. Manipulating WT hESCs to recapitulate this poor cell-cell contact also results in significantly lower expression of E-CAD, the altered BMP signalling pattern and inadequate activation of WNT genes in BMP4-induced ME differentiation. These results support the notion that cellular interactions are integral to BMP4-induced activation of WNT genes in hESCs, which is necessary for ME differentiation.

In my study, RIC-KO cells in CL are similar to WT cells in SC regarding cell-cell interactions and BMP4-pSMAD1 signals. Both exhibit reduced cell-cell interactions and more uniform pSMAD1 signals after 24-hr BMP4 induction, whereas in WT CL cultures, the BMP4-pSMAD1 signals are restricted to the edges of the colonies at 24 hr and gradually spread inward to the centre, suggesting that cell-cell interactions affect the BMP signalling gradient in hESCs. BMP4 receptors are localised at the basolateral sides of hPSCs, so that only the cells at the edges of a colony are accessible to BMP4 in the media due to tight cell-cell junctions (Etoc et al., 2016; Zhang et al., 2019). Because CL-plated RIC-KO cells and SC-plated WT cells exhibit reduced cell-cell interactions, BMP4 can get access to nearly all cells, hence explaining the more uniform pSMAD1 expression in these cells. A recent study demonstrates that ZO-1 knockdown (KD) hPSC colonies exhibit ubiquitous expression of pSMAD1 after BMP4 treatment, resembling the pSMAD1 pattern in RIC-KO colonies (Vasic et al., 2023), which supports the notion that TJs play a crucial role in forming this BMP4-pSMAD1 expression pattern. As a result of the ubiquitous BMP4-pSMAD1 signalling in the colonies, ZO-1 KD cells also show higher expression of BMP target genes *ID1* and *NOGGIN* than WT controls (Vasic et al., 2023), reminiscent of the BMP signalling in RIC-KO colonies. The strikingly similar pSMAD1 patterns in RIC-KO and ZO-1 KD colonies and their respective WT colonies suggest that TJs play a pivotal role in the perception of the BMP4 signal in hPSC colonies to establish the initial pSMAD1 pattern.

Since the pSMAD1 pattern induced in WT colonies is different from the ubiquitous pSMAD1 expression in RIC-KO colonies with BMP4 stimulation and the BMP signalling gradient is predicted to dictate the subsequent spatiotemporal fate patterning (Etoc et al., 2016; Tewary et al., 2017), it is reasonable to think that this signalling gradient triggers secretion of the

secondary signal of WNT ligands, such as WNT3 to activate the WNT/ β -catenin signalling to fulfil this BMP-WNT-NODAL signalling cascade (Martyn et al., 2018). WNT ligands are reported to be transferred in short distance from the source cells, only affecting neighbouring cells (Farin et al., 2016). Thus, LEF1 expression is observed and only restricted to the E-CAD⁺ area at the periphery of colonies where WT cells co-express pSMAD1 at 24 hr post BMP4 induction, while LEF1 expression is barely discernible in RIC-KO colonies due to the ubiquitous expression of pSMAD1. This suggests that this edge-resided pSMAD1 expression mediated by tight cell-cell interactions might be required to stimulate the activation of canonical WNT genes. Then, as the cells at the edge of WT colonies lose E-CAD expression and undergo EMT, cells closer to the colony centre have their BMP receptors exposed and get access to BMP4 either through its presence in the media or through paracrine signalling (Etoc et al., 2016; Zhang et al., 2019), thereby showing both pSMAD1 and LEF1 expression moving inward to E-CAD⁺ area closer to the centre from 24 hr to 48 hr. Analogous to the finding that activation of the canonical WNT genes is suppressed in RIC-KO colonies with BMP4 stimulation, this is also confirmed in SC-plated hESCs. It further proves that cell-cell interactions is associated with the activation of the canonical WNT genes in hESCs. However, the exact molecular mechanisms by which cell-cell interaction regulate WNT gene expression in response to BMP4 stimulation remain unclear.

Actually, in the ZO-1 KD study, expression of canonical WNT genes induced by BMP4 stimulation is higher in ZO-1 KD colonies than WT controls, which leads to more efficient, wide-spread ME differentiation (Vasic et al., 2023). This differs with my data that show impaired ME differentiation induced by BMP4 in RIC-KO colonies due to insufficient activation of canonical WNT genes. However, it is not reported in their study whether disruption of ZO-1 affects other cellular junctions, such as AJs and GJs, nor is it shown whether cell-cell interactions are reduced in ZO-1 KD colonies (Vasic et al., 2023). In RIC-KO hESCs, both AJs and TJs are affected as manifested by reduced expression of E-CAD and ZO-1, respectively (see section 3.2.2). If KD of ZO-1 does not affect AJs and other cell-cell junctions, this difference between our RIC-KO and their ZO-1 KD hPSCs might account for the discrepancy in BMP4-induced WNT expression and ME differentiation between the two studies. The contrasting data also suggest that the BMP4-pSMAD1 signalling gradient alone does not regulate the expression of WNT genes. The question of how WNT genes are activated is more complicated and requires more studies in the future.

Chapter Six

General Discussion

6.1 RICTOR/mTORC2 is dispensable for the maintenance of hESCs

One of the findings in my study is that RICTOR/mTORC2 is not necessarily required for maintaining self-renewal of hESCs as RIC-KO hESCs show no detrimental effect on their proliferation and pluripotent marker expression. Although mTORC2 signalling is abolished in RIC-KO hESCs and its downstream effector AKT is not phosphorylated on the HM, AKT and mTORC1 activity show no clear alterations. Therefore, it is not surprising that RIC-KO hESCs still retain the property of self-renewal since AKT is a central effector of PI3K signalling which promotes cell survival and proliferation (Liu and Sabatini, 2020; Manning and Toker, 2017).

By contrast, a recent study on RIC-KO H9 hESCs reports that these cells cannot maintain self-renewal and pluripotency and become gradually differentiated (Chu et al., 2022). The possibility of cell line bias is excluded because RIC-KD (knockdown) in H7 hESCs in our lab also reveals similar results as RIC-KO H1 hESCs in my study (Batool, 2022). One explanation may lie in the different hESC culture media used between the two laboratories: chemically defined mTeSR medium in Chu's study and MEF-CM in our studies. Opposed to the defined medium which contains recombinant growth factors (e.g. bFGF, Activin, TGF β) and other formulated proteins (e.g. insulin, transferrin, cholesterol) to ensure batch-to-batch consistency, MEF-CM relies on the secretion of beneficial and supportive factors from MEFs to reduce spontaneous differentiation and promote self-renewal in hESCs (Dakhore et al., 2018). Although it is still not completely clear what exactly these beneficial and supportive factors are in MEF-CM, it is likely that these factors are similar to those in mTeSR medium but also include ECM (e.g. laminin, laminin-bound integrins, collagen), proteins and other components (e.g. RNAs and small peptides) (Dakhore et al., 2018). They help promote cell adhesion, cell survival and self-renewal of hPSCs in the absence of RICTOR/mTORC2 signalling (Rodin et al., 2014; Watanabe et al., 2007; Xu et al., 2010). In addition, I speculate that plasma membrane- or endosome-localised Rictor/mTORC2 may be secreted from cells through extracellular vesicles (EVs), so that MEF-CM might contain certain EVs in addition to beneficial and supportive factors secreted by MEFs (Ebner et al., 2017; Kalluri and McAndrews, 2023; Liu et al., 2019). These EVs likely contain murine Rictor/mTORC2 which could partly help retain the maintenance of RIC-KO hESCs, given the highly conserved structure and function of these proteins between mice and humans (Battaglioni et al., 2022; Scaiola et al., 2020). Therefore, it would be interesting to perform following experiments in the future to clarify the difference between the two media: 1) to investigate if MEF-CM

contains a trace of murine mTORC2; 2) to culture RIC-KO hESCs with mTeSR medium on MEFs as feeder cells to see if they could retain hESC properties.

6.2 mTORC2 deficiency alters differentiation propensity of hESCs

As indicated by RNA-seq analysis at the undifferentiated stage, GO enrichment shows that downregulated genes in RIC-KO hESCs are mainly associated with embryonic development, specifically mesoderm and endoderm differentiation, suggesting that they are less primed to differentiate into these two lineages. Consistently, my study clearly demonstrates that the ME lineage, the precursor of mesoderm and endoderm, is considerably hindered in RIC-KO hESCs by utilising a BMP4-induced differentiation approach. A previous report supports the notion that RIC-KO impairs hESC differentiation to mesoderm (Chu et al., 2022). I also demonstrate that defective ME differentiation in RIC-KO hESCs primarily arises from insufficient activation of the WNT signalling. Forced activation of the WNT signalling by using CHIR alone to inhibit GSK3 is enough to efficiently rescue defective ME differentiation in RIC-KO hESCs, while activation of the NODAL signalling by application of AA plus BMP4 does not show any improvement of ME differentiation. This is consistent with the notions that the WNT signalling is crucial for the activation of genes involved in ME differentiation (Funa et al., 2015; Mazzotta et al., 2016; Yoney et al., 2022), while the Activin/NODAL signalling acts to direct endoderm specification in hESCs (Martyn et al., 2018). Finally, I identify that the impeded WNT signalling in RIC-KO cells results from attenuated upregulation of canonical WNT genes induced by BMP4. This is consistent with the RNA-seq analysis in which KEGG pathway reveals that the WNT signalling is downregulated in RIC-KO hESCs at the undifferentiated stage, confirming that these cells are less primed to differentiate into mesoderm and endoderm.

Although mTORC2-deficient hESCs exhibit defects in ME differentiation, mTORC2-null mouse embryos undergo successful gastrulation to generate three germ layers (Guertin et al., 2006; Jacinto et al., 2006; Shiota et al., 2006). Given that altered TJ distribution as well as disrupted cell polarity promote ME differentiation in micropatterned colonies in response to BMP4 (Legier et al., 2023), it is possible that rapid proliferation of epiblast and limited space in post-implantation embryos may mechanically force the physical contacts between epiblast cells so that gastrulation can proceed. Cells in geometry-confined micropatterned colonies are forced to have tight cell-cell contact, thereby possibly activating WNT gene expression with

BMP4 stimulation. This makes me wonder whether ME lineage would be restored in RIC-KO hESCs if they are seeded at a high density in micropatterned colonies after BMP4 treatment.

Seminal work has reported that BMP4 alone treatment in hESCs leads to the extraembryonic lineage (Etoc et al., 2016; Li et al., 2013; Nemashkalo et al., 2017; Xu et al., 2002). In agreement, WT hESCs in SC cultures preferentially differentiate into the extraembryonic lineage after 48-hr BMP4 induction. However, it is unexpected that this lineage is impaired in RIC-KO hESCs in CL cultures after BMP4 treatment. This extraembryonic lineage with expression of *CDX2* and *TFAP2A* was considered to be TE (Chhabra et al., 2019; Krendl et al., 2017). However, more recent studies compare these differentiated datasets with *in vivo* embryonic single-cell RNA-seq data and demonstrate them an amnionic fate (Minn et al., 2020; Weatherbee et al., 2023). Although it remains unclear about the molecular mechanism of amnion specification in humans, the amnion of cynomolgus monkeys is a source of BMP4 and WNT3A (Sasaki et al., 2016), suggesting that the formation of amnionic cells might require the BMP and WNT signalling. Consistently, hPSCs with BMP stimulation directs differentiation into the amnionic fate (Guo et al., 2021; Minn et al., 2020; Weatherbee et al., 2023; Zheng et al., 2019). Therefore, the formation of amnionic lineage is inhibited in CL-plated RIC-KO cells possibly due to the following reasons: 1) the BMP inhibitor *NOGGIN* is expressed at higher levels; 2) the canonical WNT genes are not sufficiently upregulated throughout BMP4 induction. In contrast, WT hESCs in SC cultures show significantly lower *NOGGIN* expression than in CL cultures at 48 hr post BMP4 induction (data not shown), suggesting that the amnion specification is favoured in SC-plated WT hESCs. It will be interesting to investigate how mTORC2 affects the extraembryonic lineage, particularly the amnionic fate during human development.

RIC-KO hESCs mainly differentiate into the neuroectoderm lineage after EB differentiation and similarly, they also gradually differentiate into the neural lineage in mTeSR medium (Batoöl, 2022; Chu et al., 2022). Therefore, with mTORC2 deficiency, hESCs differentiate more into neuroectoderm upon exiting the pluripotent state. Even in BMP4-induced ME differentiation, my data show that neuroectoderm might be enhanced, at least in the first 24 hrs, in RIC-KO hESCs. Paradoxically, neural specification has been well studied and it is efficiently induced in hPSCs by inhibition of both BMP4-SMAD1/5/9 and TGF β -SMAD2/3 (Chambers et al., 2009). I reason that enhanced neural differentiation in RIC-KO hESCs upon BMP4 induction is probably due to much higher *NOGGIN* upregulation. This increased upregulation

is assumed to lead to an expansion of neuroectoderm cells in RIC-KO colonies as NOGGIN proteins are secreted apically to inhibit BMP4 both present in the media and secreted autonomously by the cells (Etoc et al., 2016; Phan-Everson et al., 2021; Tewary et al., 2017). Interestingly, two BMP4 target genes, *NOGGIN* and *ID1* show different expression patterns – *NOGGIN* expression was continuously upregulated and was at higher levels than in WT cells, while *ID1* expression increased at 24 hr followed by a great reduction at 48 hr post BMP4 treatment in RIC-KO hESCs (see section 5.2.1). This suggests that other mechanisms associated with mTORC2 activity might be involved in regulating *NOGGIN* expression in hPSCs, which requires further investigation. On the other hand, activation of the WNT signalling is suppressed in RIC-KO cells after BMP4 stimulation, which might affect the expression of SMAD2/3 target genes, since the expression of these genes requires WNT priming in hESCs (Yoney et al., 2022). Thus, it will be interesting to explore what the exact molecular mechanisms underlying the role of mTORC2 involved in regulation of neuroectoderm specification.

6.3 Cell-cell interactions regulate BMP4-induced activation of canonical WNT genes

The key finding in my study is that RIC-KO hESCs exhibit a significant reduction in ME differentiation and that forced activation of WNT signalling can rescue this defect. Since the main phenotype of undifferentiated RIC-KO hESCs is reduced cell-cell adhesion, I ask whether this affects BMP4-induced WNT activation in hESCs. I show that diminished cell-cell interactions in RIC-KO hESCs prevent the formation of the BMP signalling gradients with BMP4 stimulation. This results in ubiquitous pSMAD1 expression in RIC-KO colonies, which seems to be associated with impeded activation of the canonical WNT signalling. Comparing SL- and CL-cultured WT hESCs, I demonstrate that cell-cell interactions indeed play a crucial role in BMP4-induced upregulation of the canonical WNT genes as well as activation of the WNT signalling in hESCs, thereby leading to their ME differentiation.

Cell-adhesion tension has been identified as a key factor to regulate cell fate patterning, particularly ME specification (Przybyla et al., 2016b; Shahbazi, 2020). A recent study reveals that cell-adhesion tension is directly determined by cell-cell interactions in hESCs (Muncie et al., 2020), indicating that RIC-KO hESCs may have lower cell-adhesion tension than WT cells.

Interestingly, the domains with efficient ME differentiation in colonies with BMP4 stimulation correspond to areas of high cell-adhesion tension which facilitates the release of β -catenin from the AJs and its nuclear translocation, thereby promoting WNT gene expression (Muncie et al., 2020). On the other hand, KD of E-CAD in hESCs decreases cell-adhesion tension which reduces the level of ME specification due to β -catenin degradation before differentiation induction (Muncie et al., 2020; Przybyla et al., 2016a). Evidence from these studies supports the notion that mTORC2 deficiency might affect cell-adhesion tension in hESCs, which prevents ME differentiation through preventing WNT gene expression. Although preliminary findings demonstrate that RIC-KO cells show similar expression of β -catenin protein compared to WT controls, β -catenin protein is less membrane-bound (see section 1.4.2) and the expression of phosphorylated β -catenin by GSK3 is much higher in RIC-KO cells after proteasome inhibition by MG132 (Batool, 2022). This suggests that much more β -catenin proteins are degraded and that the proteasome might be saturated with β -catenin proteins in RIC-KO cells. Moreover, previous studies reveal that different dynamics of WNT signalling are induced by CHIR and WNT3A in hPSCs, in that CHIR results in a sustained nuclear β -catenin response while WNT3A leads to an adaptive nuclear β -catenin response in a timely manner (Massey et al., 2019). In my study, I demonstrate that inhibition of GSK3 by CHIR efficiently induces ME differentiation in RIC-KO cells. Thus, it will be interesting to examine if application of WNT3A or MG132 rescues defective ME differentiation in RIC-KO cells with BMP4 stimulation.

Surprisingly, in TJ-deficient hPSC colonies, BMP4 stimulates higher expression of canonical WNT genes and more efficient, wide-spread ME differentiation even though the BMP4-pSMAD1 signalling gradients are not formed (Vasic et al., 2023). This suggests that the BMP4-pSMAD1 signalling gradients alone in hPSC colonies do not dictate the expression of canonical WNT genes. The control of WNT expression is more complicated, likely involving other factors which are linked to cell-cell interactions.

For example, the Hippo pathway has also been reported to regulate WNT gene expression (Estarás et al., 2017). The Hippo pathway consists of a core kinase cascade comprised of MST1/2-SAV1 and subsequent LATS1/2-MOB1A/B, which regulate the nuclear effector YAP/TAZ (Yes-associated protein/transcriptional coactivator with PDZ-binding motif) by phosphorylation (Fig 6.1) (Kobayashi et al., 2023; Zheng and Pan, 2019). Phosphorylation of YAP/TAZ by LATS1/2 prevents them from entering the nucleus, thereby inactivating their

function as a transcriptional coactivator (Lei et al., 2008; Zhao et al., 2007). Nuclear localised YAP can bind to an intragenic enhancer of *WNT3* to prevent its activation (Fig 6.1) (Estarás et al., 2017). It has also been reported that the Hippo pathway is regulated by intercellular junctions as an upstream signal in epithelial cells (Fig 6.1) (Zheng and Pan, 2019). Cell adhesion proteins E-CAD and its associated cadherin-catenin complex activate NF2 and KIBRA (Kim et al., 2011b), while adhesion proteins at TJs activates AMOT (angiomotin) (Zhao et al., 2011). They are all Hippo signalling proteins and stimulate activity of the core kinase cascade, MST1/2-SAV1 and LATS1/2-MOB1A/B, in the Hippo signalling to inhibit nuclear localisation and transcriptional function of YAP/TAZ by phosphorylation (Kobayashi et al., 2023; Lei et al., 2008; Zhao et al., 2007; Zheng and Pan, 2019). Interestingly, RIC-KO hESCs exhibit disrupted AJs and TJs (see section 3.2.2), which likely reduces the Hippo signalling and allows nuclear localised YAP/TAZ to repress *WNT3* gene expression. Similarly, RIC-KO hESCs display loosely packed colonies and thus reduced cell-cell contact. This further supports the notion that YAP/TAZ is probably nuclear localised in RIC-KO hESCs, because studies have demonstrated that poor cell-cell contact, which alters cell geometry, results in nuclear localisation of YAP in epithelial cells (Aragona et al., 2013; Dupont et al., 2011; Zhao et al., 2007). Furthermore, RHO-ROCK signalling has been shown to antagonise the Hippo signalling, which leads to nuclear localisation of YAP (Dupont et al., 2011; Ohgushi et al., 2015). Therefore, since RIC-KO hESCs show higher RHOA activity than WT controls (see section 1.4.2) (Batool, 2022), it may further support nuclear localisation of YAP/TAZ in these cells. However, in mTORC2-deficient cardiomyocytes, the Hippo signalling has been found to be highly active, which inhibits YAP nuclear localisation and its transcriptional activity (Sciarretta et al., 2015), leading to increased *WNT3* expression. This result seems to be different from what we expected in RIC-KO hESCs. A possible explanation for this could be that the Hippo signalling in mTORC2-deficient hESCs might be overwhelmed by the phenotypic changes in intercellular junctions, cell-cell adhesion and cytoskeleton organisation which together mask the effect of mTORC2 itself on YAP localisation. In addition, different cell types may also account for the differences as hESCs are epithelial-like cells while cardiomyocytes are not. To further explore the molecular mechanisms that regulate WNT gene expression, it would be interesting to examine the localisation of YAP in mTORC2-deficient hESCs.

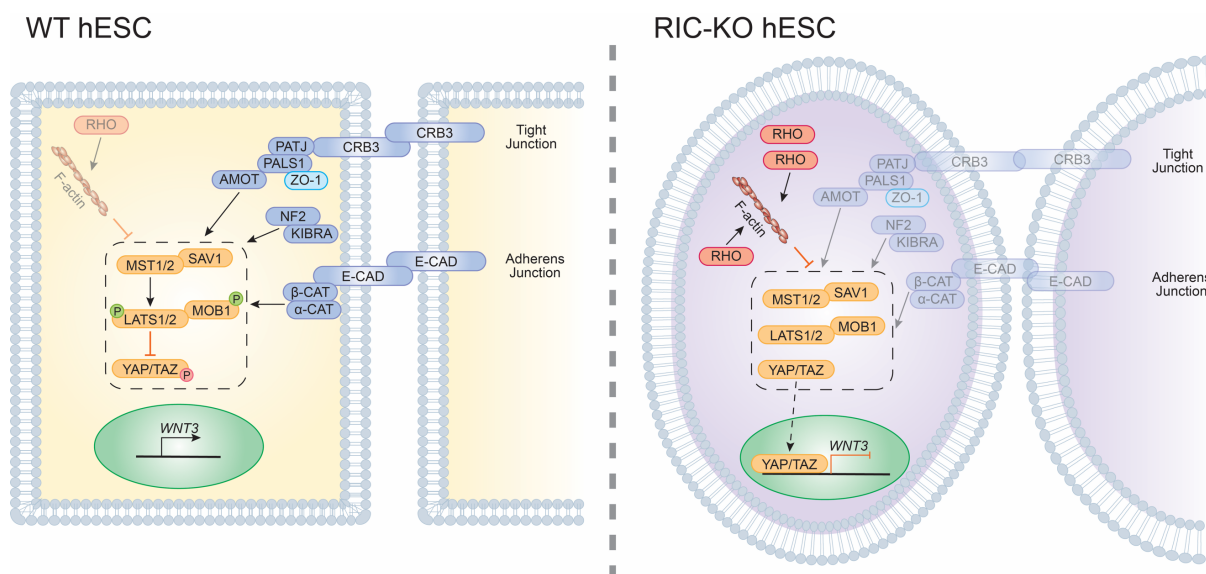


Figure 6.1 Predicted predominant localisation of YAP/TAZ and its association with *WNT3* expression in WT and RIC-KO hESCs

*Proposed schematic diagram illustrating upstream regulators of the Hippo pathway linked to phenotypic differences which possibly dictate different localisation of YAP/TAZ in WT and RIC-KO hESCs. In WT hESCs (left panel), the upstream regulators associated with robust cell junctions and cell-cell contact (proteins shown in opaque ovals) likely result in predominant cytoplasmic localisation of YAP/TAZ, which may not affect *WNT3* gene expression. In RIC-KO hESCs (right panel), due to reduced cell junctions and poor cell-cell contact (proteins shown in translucent ovals), the upstream regulators linked to higher RHO and F-actin activity (shown in opaque ovals) may lead to YAP/TAZ translocation (indicated with the dashed arrow) and their predominant nuclear localisation, which possibly suppresses *WNT3* expression. The core components of the Hippo kinase cascade (shown in the dashed line box) are indicated in yellow, while the upstream regulators that activate or inhibit Hippo signalling are highlighted in blue or red, respectively. Black arrow ends indicate activation and red blunt ends indicate inhibition. Green P represents activation through phosphorylation, while red P represents inhibition through phosphorylation.*

6.4 Other aspects

6.4.1 The function of RICTOR in mTORC2 activity during embryonic development

Although RICTOR is an important mTORC2-specific component responsible for mTORC2 assembly and function (Aimbetov et al., 2012; Glidden et al., 2012; Ruicci et al., 2019), it has been reported to have its own function independent of mTORC2 (see section 1.1.2.1). Loss-of-function studies have demonstrated that mouse embryos with a lack of the mTORC2-specific component Rictor or Sin1 succumb to death around the similar time, which also phenocopies *Mlst8*-deficient mice (Guertin et al., 2006; Jacinto et al., 2006; Shiota et al., 2006). This suggests that RICTOR possibly functions as a subunit of mTORC2 during early embryonic development. However, our preliminary results show that RIC-KO hESCs exhibit absent PKC ζ expression and reduced RAC1 activity which have been previously demonstrated to be associated with RICTOR, regardless of mTORC2 activity, in cancer cells (Agarwal et al., 2013; Zhang et al., 2010). Even though cancer cells may have aberrant regulation of signalling pathways that are not representative of those in normal cells, this still brings up a concern as to whether RICTOR deficiency faithfully reflects the effect of mTORC2 deficiency in hESCs. It will be intriguing to investigate if mSIN1 deficiency in hESCs would recapitulate the phenotypes observed in RIC-KO cells. In addition, since mouse embryos with a lack of either one of the direct effectors of mTORC2, *Pkc1* or *Sgk1* display the similar phenotypes as those with mTORC2 deficiency (see section 1.1.3.2) (Catela et al., 2010; Seidl et al., 2013; Soloff et al., 2004), and the proteins encoded by these two genes are directly regulated by mTORC2 but not RICTOR (Baffi and Newton, 2022), it is necessary to examine if overexpression of either gene in RIC-KO hESCs is able to rescue the phenotypes.

6.4.2 mTORC2 deficiency in cell-cell interactions

My data demonstrate that RICTOR/mTORC2 deficiency results in disorganised cytoskeleton and reduced cell-cell interactions in hESCs. The exact molecular mechanisms by which RICTOR/mTORC2 regulates cell-cell interactions remain unclear. Our preliminary findings show that RHOA/RAC1 activities are imbalanced – higher RHOA activity and lower RAC1 activity – in RIC-KO cells (Batoool, 2022). RHOA/RAC1 signalling and their precise balance

have been reported as key cytoskeletal regulators to establish cell-cell junctions, thereby mediating cell-cell interactions (Arnold et al., 2017). As the direct target of mTORC2, PKC α has been reported to inhibit RHOA activity through its negative regulator, p190ARHOGAP (Bass et al., 2008; Lévy et al., 2009). PKCs also directly regulate cytoskeletal architecture through inhibitory phosphorylation of RLCs (regulatory light chains) and of NMM II (non-muscle myosin II) (Vicente-Manzanares et al., 2009). In the meanwhile, cell-cell junctions are further disintegrated as RICTOR deficiency lowers RAC1 activity (Agarwal et al., 2013; Arnold et al., 2017; Lévy et al., 2009). Therefore, it is plausible that the absence of PKC α and PKC ζ , which results from structural instability and subsequent degradation in RIC-KO hESCs (see section 3.2.1), accounts for dysregulated RHOA/RAC1 activity and disorganised cytoskeleton. Thus, it is worth inspecting if expression of stable PKC α or PKC ζ in RIC-KO hESCs by genetic manipulations can rescue the RHOA activity and cytoskeletal disorganisation.

In addition, given that mTORC2 is present at the endoplasmic reticulum (ER) and mitochondria-associated ER membrane (MAM) (Battaglioni et al., 2022), it is not surprising that deletion of Rictor in mESCs prevents the release of calcium ions from the ER (Shao et al., 2019). Both extracellular and intracellular calcium ions have been reported to play an irreplaceable role in mediating cell-cell junctions, particularly AJs and TJs, the process of which is facilitated by RHO GTPases (Arnold et al., 2017; Clapham, 2007; Otani and Furuse, 2020). For example, followed by calcium switch of the cell media, RAC1 is activated to initiate junction assembly (Guillemot et al., 2008). Calcium-sensing receptor and RHOA along with other proteins form a complex in response to elevated levels of extracellular calcium ions, which improves AJ-mediated cell–cell adhesion (Tu and You, 2014). In addition, although both AJs and TJs are affected in RIC-KO hESCs, contributing to reduced cell-cell interactions, expression of other cell adhesion proteins, such as GJ-associated proteins, is not inspected in my study. Thus, it is necessary to elucidate if mTORC2 deficiency also affects GJs in hESCs. Also, it will be interesting to compare the concentration of extracellular and intracellular calcium ions in both WT and RIC-KO hESCs.

In summary, by comparing RIC-KO hESCs with WT controls, my data show that RICTOR/mTORC2 signalling is not necessarily required for the self-renewal and pluripotency of hESCs. However, RICTOR/mTORC2 deficiency weakens cell-cell interactions by affecting the formation of AJs and TJs and alters cytoskeleton organisation in hESCs. RNA-seq analysis reveals that downregulated genes in RIC-KO hESCs are mainly related to mesoderm and

endoderm differentiation, indicating that RICTOR/mTORC2 deficiency might affect differentiating propensity of hESCs. Results obtained from BMP4-induced differentiation demonstrate that ME specification is considerably hindered in RIC-KO cells, which suggests that mTORC2 signalling plays an important role in human embryonic development. Reduced cell-cell interactions in RIC-KO hESCs prevent the formation of the BMP signalling gradients with BMP4 stimulation, which is likely to be associated with insufficient activation of the canonical WNT genes, thereby leading to impaired ME differentiation. Further studies are required to unveil the exact molecular mechanism by which cell-cell interactions activates the expression of the canonical WNT genes in hESCs with BMP4 stimulation. This may allow researchers to understand more about early human development, fate determination, and related preterm diseases.

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Appendix

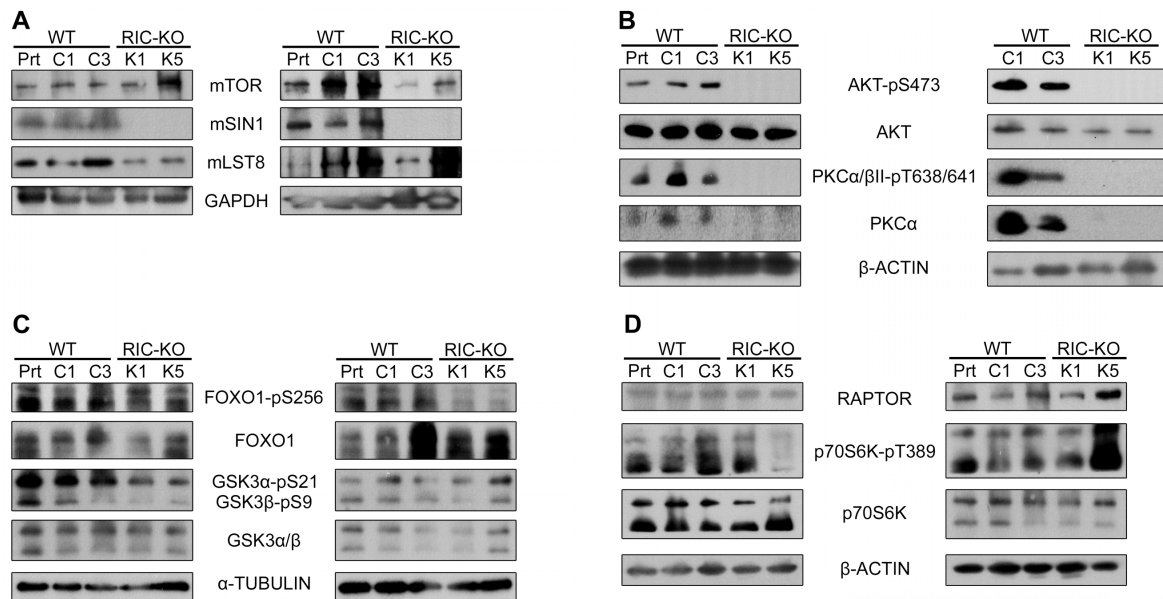


Figure A-1 Additional images associated with Fig 3.1

(A) Images of replicate immunoblots associated with Fig3.1A. (B) Images of replicate immunoblots associated with Fig3.1B. (C) Images of replicate immunoblots associated with Fig3.1C. (D) Images of replicate immunoblots associated with Fig3.1D.

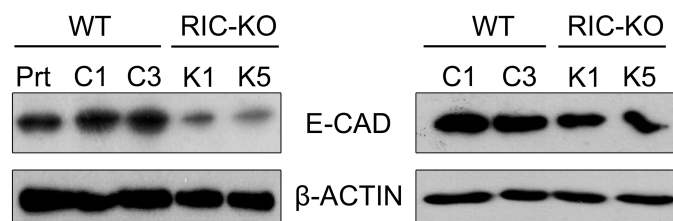


Figure A-2 Additional images associated with Fig. 3.2B

Images of replicate immunoblots with indicated antibodies corresponding to Fig 3.2B.

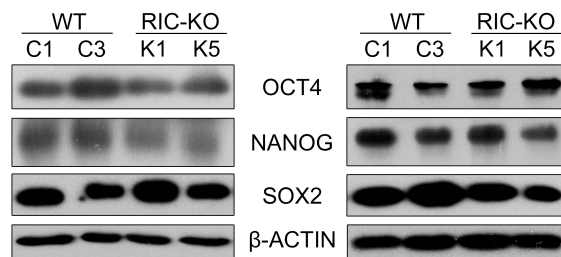


Figure A-3 Additional images associated with Fig. 3.3B
Images of replicate immunoblots corresponding to Fig 3.3B.

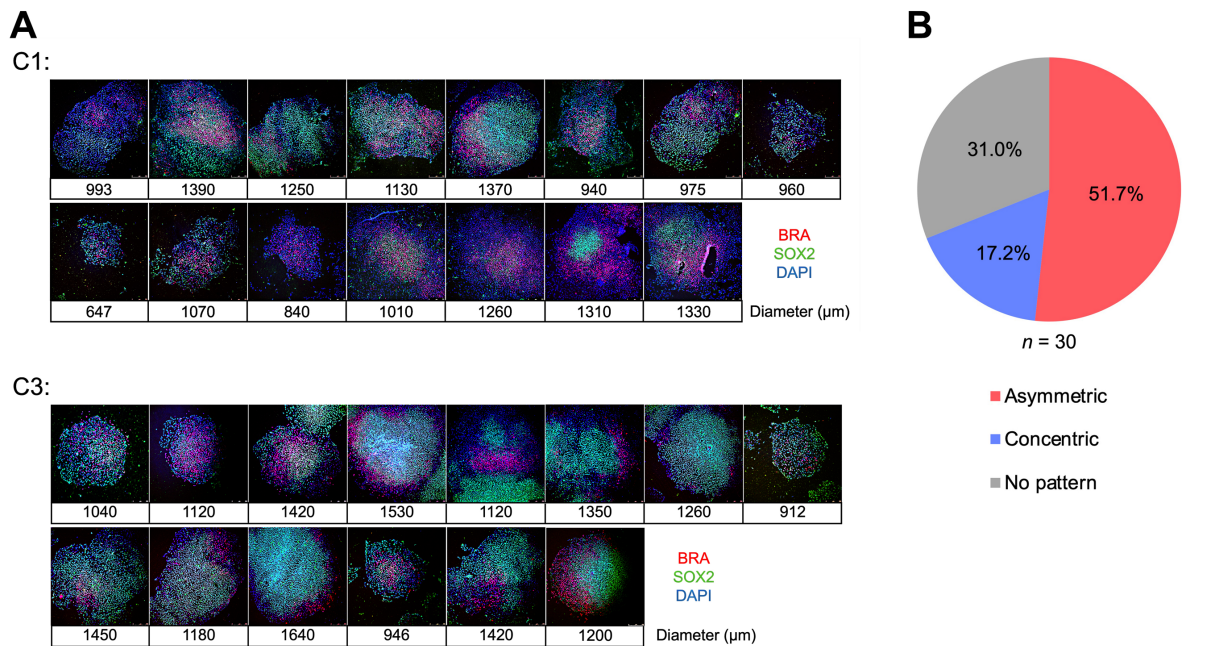


Figure A-4 Different patterning by BMP4 in WT colonies
(A) Images of immunostaining showing BRACHYURY (BRA) and SOX2 expression after 48-hr BMP4-induced differentiation in WT control clones, C1 and C3. The diameter of the colonies is illustrated at the bottom of each image. **(B)** Pie diagram showing the percentage of different patterning (asymmetric, concentric, or no pattern) observed in WT colonies. The number of colonies analysed from three independent experiments are indicated.

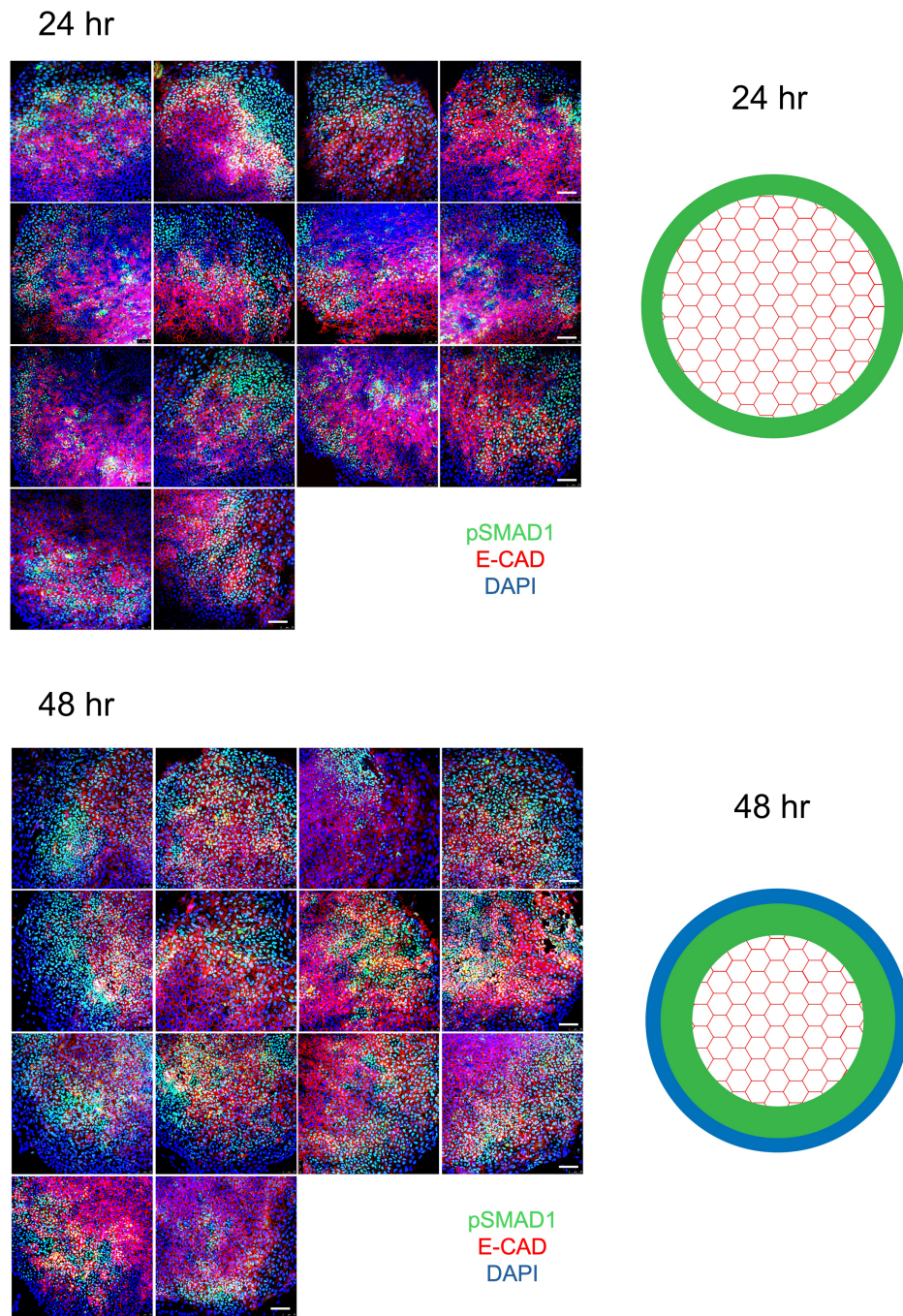


Figure A-5 Dynamic pattern of pSMAD1 expression induced by BMP4 during differentiation in WT hESCs

Left panel: images of immunostaining corresponding to Fig 5.1C, showing dynamic expression of proteins with indicated antibodies during BMP4-induced differentiation in WT colonies. Scale bar, 100 μ m. Timepoints analysed during differentiation are indicated.

Right panel: schematic diagram depicting the dynamic pattern of pSMAD1 and E-CAD in the WT colony as a whole at indicated times of differentiation based on the immunostaining images in the left panel.

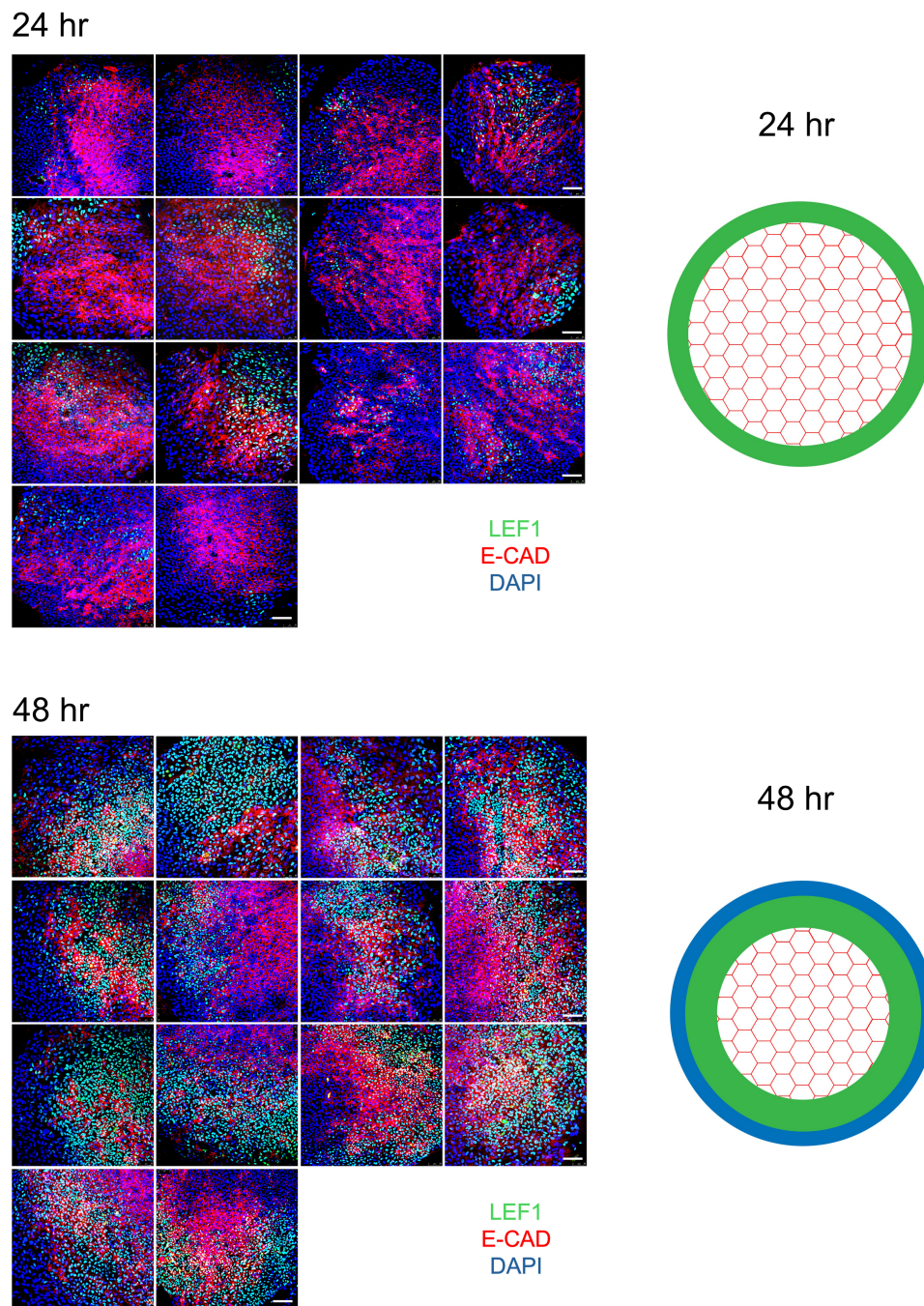


Figure A-6 Dynamic pattern of LEF1 expression induced by BMP4 during differentiation in WT hESCs

Left panel: images of immunostaining corresponding to Fig 5.2, showing dynamic expression of proteins with indicated antibodies during BMP4-induced differentiation in WT colonies. Scale bar, 100 μ m. Timepoints analysed during differentiation are indicated.

Right panel: schematic diagram depicting the dynamic pattern of LEF1 and E-CAD in the WT colony as a whole at indicated times of differentiation based on the immunostaining images in the left panel.

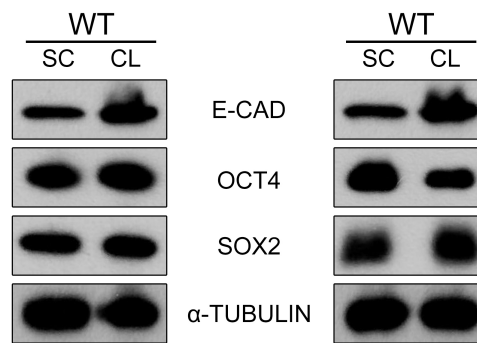


Figure A-7 Additional images associated with Fig 5.3B

Images of replicate immunoblots with indicated antibodies corresponding to Fig 5.3B.