UNDERSTANDING THE ROLE OF MECHANOSENSORS YAP AND TAZ IN DIFFERENTIATING HUMAN PLURIPOTENT STEM CELLS

DOCTORAL DISSERTATION

FOR ATTAINMENT OF THE DEGREE OF **DOCTOR OF PHILOSOPHY**

IN **BIOLOGICAL SCIENCES**

SUBMITTED TO

SVKM'S NMIMS (DEEMED-TO-BE) UNIVERSITY

SUBMITTED

BY

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UNDER THE GUIDANCE OF **DR. PRASAD PETHE**

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This is to certify that the work represented in this thesis entitled '**UNDERSTANDING THE ROLE OF MECHANOSENSORS YAP AND TAZ IN DIFFERENTIATING HUMAN PLURIPOTENT STEM CELLS**' for the award of the Degree of Doctor of Philosophy in Biological Science, is my own contribution and the research work carried out under the supervision of Dr Prasad Pethe. The work has NOT been submitted for the award of any degree or to any other University. Wherever a reference has been made to the earlier findings, it has been cited in the thesis. The thesis fulfils the requirement of the ordinance relating to the award of the Ph.D. degree at SVKM's Narsee Monjee Institute of Management Studies (Deemed-to-be) University.

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Dedicated to my parents

AMRIT KAUR and **AMARJEET SINGH VIRDI**

and my brother

JASPREET SINGH VIRDI

For their endless love, support and encouragement

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This work was initially conceived and conceptualized by P.P. All the experiments were performed by JKV. The analysis of the data and interpretations were performed by P.P. and J.K.V. The published manuscripts were written by J.K.V. and reviewed by P.P.

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ABSTRACT

Background: Human embryonic stem cells (hESCs) are pluripotent cells that have unique ability to self-renew and differentiate into specialized cell types. Due to these qualities, they have greater potential in stem-cell based regenerative medicine therapies, where hESCs are coaxed to differentiate into specific cell type. Multiple pre-clinical and clinical trials using stem-cell based therapies are on-going, however, critical aspect of cell survival and differentiation after transplantation still remains a challenge. Growth factors and chemical cues are well known regulators of stem cells proliferation and differentiation, but recent studies demonstrate a crucial role of biophysical signals in regulating stem cell proliferation and differentiation through mechanotransduction pathways. More importantly, data on mesenchymal stem cells (MSCs) have shown that the stiffness of the substrate on which the stem cells are cultured can regulate stem cell via Hippo pathway transcriptional coactivator, Yes-associated protein (YAP). However, how YAP regulates lineage specification in hESCs in response to mechanical signals still remains a mystery. Our study aims to understand the expression of YAP in pluripotent hESCs and in differentiated cells cultured on substrates of different stiffnesses.

Methodology: The hESCs line, KIND1 cells were first cultured on traditional plastic culture plates (TCP) in pluripotency sustaining medium, and were characterized for their pluripotency. Next, we cultured hESCs on soft substrates (0.2kPa, 0.5kPa, 2.0kPa, 8kPa, 16kpa, 32kPa, 64kPa) firstly in serum-free medium that supports pluripotency and later in medium with minimum serum which would encourages differentiation but without adding lineage-specific growth factors; followed by characterization of markers that define pluripotency and lineage specification. Further, we differentiated hESCs on TCP and soft substrates towards definitive endoderm lineage by using high concentrations of ACTIVIN A. The differentiated cells were characterized at transcript levels by qRT-PCR and at protein levels by immunofluorescence and immunoblot. Importantly, we investigated the levels of YAP, pYAP and other Hippo core proteins to understand how changing substrate stiffness affected YAP, and how this correlated with pluripotency or differentiation. Lastly, we modulated YAP expression by using pharmacological inhibitor Verteporfin and activator Lysophosphatidic acid in differentiated cells cultured on

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various substrates and determined the effect of YAP on hESCs differentiation using qRT-PCR and immunoblot.

Results: Pluripotency for hESCs cultured on TCP in pluripotency sustaining medium was checked by expression of *OCT4*, *NANOG*, *SOX2, SOX17, PAX6, BRACHYURY* transcripts, OCT4 protein expression was observed using immunofluorescence and immunoblotting. hESCs cultured on varying stiffness in pluripotency maintaining medium did not undergo substrate-induced differentiation apparent from the protein levels of OCT4 and NANOG, and absence of the *SOX17*, *PAX6*, and *BRACHYURY* expressions. However, hESCs cultured on the same stiffnesses in differentiation inducing medium without lineage-specific growth factors showed expression of lineage specific markers. YAP expression was observed in hESCs cultured on TCP and soft substrates and maintained in pluripotency sustaining medium and differentiation inducing medium. In presence of high levels of ACTIVIN A, hESCs cultured on soft substrates differentiated as efficiently as hESCs cultured on TCP as observed by the transcript levels of *SOX17*, *FOXA2*, *CXCR4*, and *BRACHYURY*. Surprisingly, equivalent expression of YAP and pYAP was observed in the hESCs harvested from all the substrate stiffness. YAP inhibition and stimulation did not show major changes in the differentiation potential of the hESCs present on varying stiffness as indicated by the expression of lineage specific markers.

Conclusion: The results of our study show that hESCs retain their stemness profile on soft substrates in presence of pluripotency sustaining factors, and undergoes differentiation when supplemented with low levels of morphogens. During directed differentiation of hESCs on varying stiffness, we observed that YAP expression was not effected by the differentiation or by substrate stiffness. Our findings revealed that hESCs maintain a minimum basal level of YAP expression for cell survival and proliferation, but YAP might not corelate directly with pluripotency. We further demonstrate the effect of YAP inhibition by using a pharmacological inhibitor on hESCs proliferation and differentiation when cultured on substrate with varying stiffness. Our novel finding clearly show that biochemical cues and substrate stiffness are interdependent and play an important role in cell differentiation.

Keywords: Human Embryonic Stem Cell, Pluripotency, Differentiation, Mechanobiology, HIPPO pathway

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HIGHLIGHTS

- Following a prolonged culture on soft substrate, hESCs maintained pluripotency only in pluripotency maintaining medium and but differentiate in media that does not support pluripotency
- Directed differentiation of hESCs towards definitive endoderm was not affected by substrate stiffness
- Compared to the undifferentiated hESCs and differentiated cells cultured on TCP, no significant change in YAP protein levels were seen in endoderm differentiated cells grown on soft stiffness
- YAP expression in human embryonic stem cells was not affected by the substrate stiffness. On soft substrate, YAP and pYAP protein expression were almost similar in YAP inhibited and stimulated cells during differentiation
- Stimulation of YAP protein increased the differentiation potential of hESCs

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ABBREVIATIONS

APTMS	3-Aminopropyl Trimethoxy Silane
BSA	Bovine Serum Albumin
CS	CytoSoft® Substrates
CXCR4	C-X-C Chemokine Receptor type 4
DAPI	4',6-Diamidino-2-Phenylindole
DE	Definitive Endoderm
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra Acetic Acid
EMT	Epithelial to Mesenchymal Transition
ESCs	Embryonic Stem Cells
EtBr	Ethidium Bromide
FBS	Fetal Bovine Serum
Fgf	Fibroblast Growth Factor
FOXA2	Forkhead Box A2
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GelMA	Gelatin Methacryloyl
GPa	Giga Pascal
hESCs	Human Embryonic Stem Cells
hMSCs	Human Mesenchymal Stem Cells
hPMSCs	Human Placental Mesenchymal Stem Cells
hPSCs	human Pluripotent Stem Cells
HT29	Human Colorectal Adenocarcinoma Cell Line

IMDM	Iscove's Modified Dulbecco's Medium
ITS	Insulin Transferrin Selenite
kPa	Kilo Pascal
LATS 1/2	Large Tumor Suppressor Kinase 1
LPA	Lysophosphatidic Acid
MAPK	Mitogen-Activated Protein Kinases
mESCs	Mouse Embryonic Stem Cells
MOB 1	Monopolar Spindle-One-Binder Proteins
MPA	Mega Pascal
MST 1/2	Mammalian Sterile 20-Like Kinase 1/2
mTOR1	Mechanistic Target of Rapamycin Kinase 1
NANOG	Nanog Homebox
OCT4	Octamer-Binding Transcription Factor 4
Pa	Pascal
PA	Polyacrylamide hydrogels
Pa-aPKC	Protease-Activated Receptor-Atypical Protein Kinase C
Pax6	Paired Homeobox 6
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide 3-Kinase
PIC	Protease Inhibitor Cocktail
PMSF	Phenylmethylsulfonyl Fluoride
PureCol®	Pure Collagen solution
PVDF	Polyvinylidene Difluoride Membrane
pYAP	phosphorylated Yes-associated protein
RPMI-1640	Rosewell Park Memorial Institute 1640 medium
RUNX	Runt-Related Transcription Factor 1
SAV1	Salvador
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel electrophoresis
shRNA	Short Hairpin RNA

siRNA	Small Interfering RNA
SMAD	Suppressor of Mothers against Decapentaplegic
Snail1	Snail Family Transcriptional Repressor 1
SOX1	SRY (Sex Determining Region Y)-Box 1
SOX17	SRY (Sex Determining Region Y) Box 17
SS/SulfoSANPAH	Sulfosuccinimidyl 6-(4′-azido-2′-nitrophenylamino) hexanoate
STAT3	Signal Transducer and Activator of Transcription 3
TAZ	Tafazzin Family Protein
TBST	Tris Buffered Saline-Tween 20
TCP	Traditional plastic culture plates
TGF-β	Transforming Growth Factor Beta
Twist2	Twist Family BHLH Transcription Factor 2
ULAD	Ultra-Low Attachment dishes
VP	Verteporfin
Wnt	Wingless-Related Integrated Site
YAP	Yes-Associated Protein

Note: Following the HUGO gene nomenclature committee (HGNC) guidelines, gene and proteins use the same abbreviations. For mice, gene symbols are italicized, with only the first letter in upper-case while protein symbols are not italicized with only the first letter in uppercase. For humans, gene symbols are italicized, with all letters in upper-case while protein symbols are not italicized with all letters in upper-case.

Chapter One

Introduction

Mammalian pluripotency involves a continuum of several discrete stages of embryonic development, each stage having its own set of molecular and functional attributes. *In vitro* these stages are interconvertible under appropriate conditions and stimuli. Embryonic development is usually understood through chemical pathways, expression of specific genes, and hormones. Continued intense research elucidated the importance of mechanical and biophysical forces in the embryogenesis and tissue morphogenesis (Davidson, 2017).

A single, fertilized ovum goes through innumerous differentiation to form mature cells which make up a living organism. Throughout life, differentiation continues in mature tissues, wherein stem cells divide and differentiate during regeneration for example during wound healing or to maintain homeostasis (Snippert and Clevers, 2011). In the past decade, we have developed a better understanding of the interaction between the molecular signalling pathways and extracellular stimuli in regulating differentiation. Advances in the pluripotent stem cells (PSCs) research have revealed a complex and dynamic interaction between multiple signalling pathways such as Fgf/MAPK, TGFβ/SMAD2,3 and insulin/PI3K, and transcriptional factors such as Oct4, Nanog and Sox2 in maintaining their undifferentiated state (Pan and Thomson, 2007; Shi and Jin, 2010; Dalton, 2013), promoting its self-renewal (Niwa *et al.*, 1998; Fong *et al.*, 2008), and numerous other transcription factors in inducing lineage-specific differentiation (Chamber *et al.*, 2009; Van *et al.*, 2009; Pang *et al.*, 2011; Inamura *et al.*, 2011; Albini *et al.*, 2013; Oh and Jang, 2019). Simultaneous studies using adult stem cells, such as mesenchymal stem cells (MSCs), have shown that the mechanical signals generated from the extracellular microenvironment activates biochemical signals to induce differentiation (McBeth *et al.*, 2004; Chaudhuri and Mooney, 2012; Perestrelo *et al.*, 2018). For instance, in a breakthrough study, Engler and his colleagues showed that MSCs cultured on a hydrogel with stiffness mimicking the biological tissue stiffness commits towards that specific lineage compared (Engler *et al.*, 2006).

Additionally, apart from the traditional two-dimensional (2D) culture, three-dimensional (3D) organoid models have also contributed in understanding the roles of mechanical forces in regulating stem cell behaviour. For example, in a chemically defined 3D culture system where the matrix stiffness mimics the physiological stiffness of the liver, hepatic progenitor cells differentiate into hepatic organoids, whereas matrices

softer than liver prevented organoid formation (Sorrentino *et al.*, 2020). Such reports in both the 2D and 3D culture systems have piqued the interest of the researchers in defining the roles of mechanical cues in regulating pluripotent differentiation. Although, we have tried to replicate the regulatory mechanisms that control proliferation and differentiation of embryonic cells, the approaches mentioned above elucidates the complexities of the embryonic microenvironment.

In this chapter, I have touched upon the role of mechanical forces and biophysical signalling pathways in early embryogenesis. Next, I have described in detail the effect of substrate stiffness on stem cell behaviour. The chapter concludes with the importance of mechanical signals in tissue engineering. Throughout the chapter, I will introduce and focus on the role of one particular transcriptional factor, Yes-associated protein (YAP), which plays a crucial role during early embryo development and has been labelled as a mechanotransducer. The early embryo development studies mentioned in this chapter are based on the mouse model, unless stated otherwise.

1.1 Embryogenesis and Mechanical signals:

Embryogenesis begins when a single fertilized ovum undergoes series of cell divisions to form a round cellular aggregate called morula. The blastomeres have equal geometry and differentiation potential. In human embryo, first polarization occurs between 8-cell stage to 12-cell stage and is triggered by apical polarization of F-actin and Par-aPKC (protease-activated receptor-atypical protein kinase C) complex along with embryo compaction (Zhu *et al.*, 2020). The mouse embryo polarizes in the similar manner, but the entire compaction process occurs at 8-cell stage of the development (Zhu *et al.*, 2017). The daughter cells of the 8-cell stage embryo undergo asymmetric division due to myosin-mediated contraction; this results in a 16-cell stage embryo that contain a core with cells having higher cortical tension surrounded by a layer of cells with lower cortical tension, i.e., cells with apicobasal polarity (Li and Gundersen, 2008; Samarage *et al.*, 2015; Maître *et al.*, 2016; Lim and Plachta, 2021). This apico-basal polarization of cells is crucial for the subsequent segregation of the cell lineages. Although, polarization is largely driven by molecular and genetic factors, it is notable that the mechanical forces resulting from cortical tension plays a significant role mammalian embryo development **(FIGURE 1.1)**.

The cells eventually organize to form blastocyst, which consists of inner cell mass (ICM) formed from non-polar cells and a blastocyst cavity, surrounded by a layer of epithelial trophectoderm (TE) formed from polar cells. This specification is mainly attributed to the mechanical signals generated from the changes in extracellular matrix (ECM) and neighbouring cells, which activates Hippo signalling pathway (Nishioka *et al.*, 2009; Sasaki, 2017). The nuclear localization of one of the Hippo core protein, YAP, distinguishes the two fates of cells (Gu *et al.*, 2022), which I have discussed in Section 1.3.1. The pluripotent stem cells from the ICM undergoes the process of gastrulation, a critical step where three distinct germ layers: ectoderm, endoderm and mesoderm are formed. In mouse embryogenesis, mechanical signals from the cell movement, extracellular basement membrane along with increase in tissue fluidity has been known to promote gastrulation (Srinivas *et al.*, 2004; Trichas *et al.*, 2012; Shioi *et al.*, 2017; Kyprianou *et al.*, 2020).

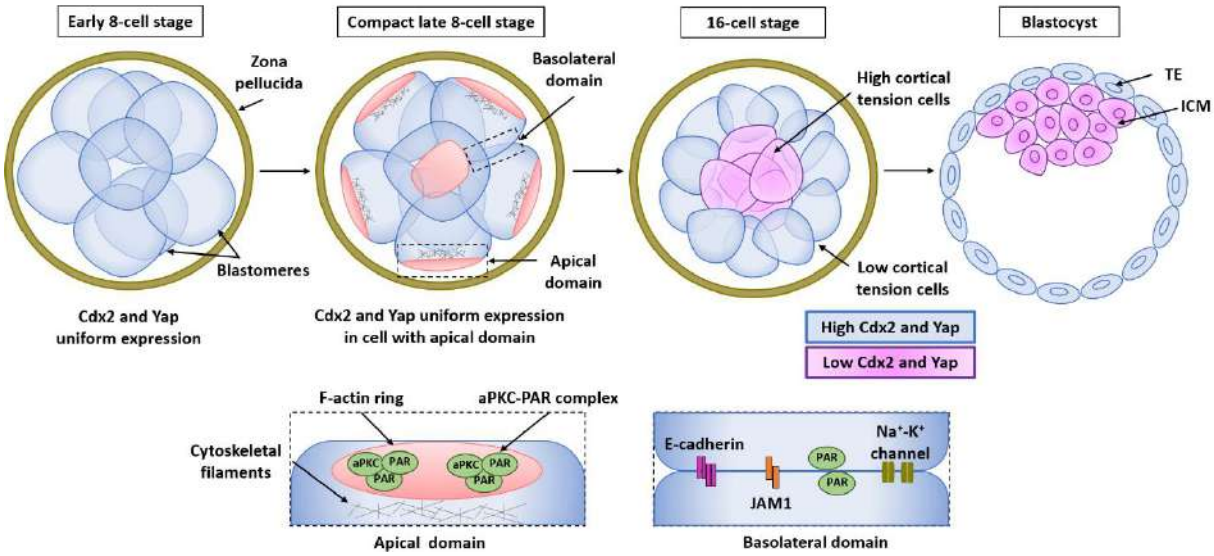


FIGURE 1.1: Overview of cell fate decision in pre-implantation embryo due to mechanical signal. At 8-cell stage, the cell-cell contact between the blastomeres increases and due to compaction prominent apical and basal domains are formed. The embryo is polarised by the formation of PAR-aPKC complex and accumulation of F-actin at the apical domain, and localization of adherens junction protein E-cadherin, tight junction protein JAM1, PAR and ion channel to the basolateral domain. At the 16-cell stage, cells divide asymmetrically to generate apolar and polar cells. The apolar cells have high cortical tension and polar cells have low cortical tension. This difference between the tensions causes apolar cells to internalize to form the ICM and polar cells to become the TE. (Adapted from Lim and Plachta, 2021).

Earlier, cell fate decisions were explained based on the expression of proteins; however, it is now important to note that cell-fate decisions can also be due to changes in

cell morphology, migration, or polarity. For instance, as mentioned above the cells surrounded by other cells experiences completely different mechanical forces than the cells with apicobasal polarity. And these mechanical forces regulate certain proteins, or mechanosensor, such as YAP, thereby directing differentiation.

1.1.1 Pluripotent Stem Cells:

Pluripotency is defined by the ability of the cell to self-renew and, upon receiving appreciate signal, differentiate into the cells of the three-germ layers. Pluripotent stem cells include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs).

ESCs are derived from the inner cell mass of the embryo at the blastocyst stage, which is 4 to 5 days after fertilization (Evans and Kaufman, 1981; Thomson *et al.*, 1998; Bongso *et al.*, 1994). In contrast to the adult stem cells, such as MSCs which are multipotent and have restricted differentiation potential, ESCs are pluripotent and can differentiate into cells of any germ layer. ESCs also do not undergo senescence during long-term culture *in vitro* (Zeng, 2007; Koch *et al.*, 2013). Because of this, they have many potential applications in regenerative medicine, such as the treatment of degenerative diseases like Parkinson's disease, diabetes, and heart disease. ESCs, specifically mouse ESCs (mESCs), are used as models to study early embryonic processes and the effect of mechanical forces during embryogenesis. The derivation of human ESCs (hESCs) from the inner cell mass of the developing embryo raises many ethical concerns which limits their use in research and therapies (Lo and Parham, 2009).

iPSCs are generated by reprogramming adult cells, such as skin cells, into pluripotent stem cells. They were first derived from adult fibroblast cells by the reprogramming of four transcriptional factors Oct4, Sox2, Klf4 and Myc (Takahashi and Yamanaka, 2006). One of the several advantages of iPSCs is that they can be generated from a patient's own adult cells, thereby reducing the risk of immune rejection when used for regenerative therapies. iPSCs have many potential applications in medicine, including the development of personalized cell therapies, disease modelling, and drug discovery. Additionally, use of hiPSCs for research and therapeutic does not raises any ethical issues.

A major challenge associated with the use of ESCs and iPSCs is the difficulty to control their differentiation into specific cell types. Although significant advances have been made in developing protocols for direct differentiation which reply solely on the

biochemical signals. With the knowledge of the influence of mechanical signals in embryogenesis, it has become necessary to explore the effect of mechanical signals generated from the extracellular microenvironment in the differentiation of PSCs.

1.2 Mechanical Signals in Cultured Cells:

Embryonic stem cell culture system represents a simple method to study how stem cells differentiate into cells of specific lineage. The culture system is easily accessible and allows easy manipulation, but in terms of physiological context, it differs from the *in vivo* system. *In vivo*, stem cells are surrounded by dynamic microenvironment consisting of different cellular components, secreted factors, extracellular membrane, physical parameters, immunological components and metabolic control (Gattazzo *et al.*, 2014). Whereas, *in vitro* cells are cultured under controlled conditions on plastic or glass plates with defined medium. It is therefore possible to gain a better understanding of stem cell behaviour by changing the physiological conditions as well as the biochemical components.

Stem cells, like any other cell, constantly senses their microenvironment through adhering, protruding, and spatially interacting with surrounding cells and extracellular matrix. In cultured cells, mechanical signals are generated by changing the substrate's stiffness (Pelham & Wang, 1997; Li *et al.*, 2011) or topology (Ankam *et al.*, 2013), modulating the fluid's shear stress (Huang *et al.*, 2021), or stretching the cells (Fang *et al.*, 2019; Muncie *et al.*, 2020).

1.2.1 ECM as a Substrate:

ECM is a complex network of proteins and carbohydrates which provides structural support to the tissues and organs and also acts as a substrate for cell adhesion and migration. It is mainly composed of fibrous proteins such as collagen, fibronectin, laminin, vitronectin, fibronectin; and proteoglycans, with many ECM-binding cross-linking proteins. The ratio of the fibrous proteins and proteoglycans vary between the tissue; therefore, the stiffness of each tissue is different. Stiffness is measured as the elastic modulus of the material, which describes how much stress a material can sustain per unit strain. The higher the elastic modulus, the stiffer the material is. Stiffness is defined as Young's modulus (E) and has unit's pascal (Pa). For example, brain ECM is composed of mainly of proteoglycans and has low content of fibrous proteins, therefore

it has stiffness (*E*) of approximately 1-2 kilopascal (kPa) (Budday *et al.*, 2015). Conversely, bone is majorly composed of collagen which makes it stiffest tissue of the body with *E* of 100kPa – 1 gigapascal (GPa) (Ruoslahti, 1996; Wells *et al.*, 2008). The Young's modulus of muscles is between 11-45 kPa (Collinsworth *et al.*, 2002; Zhu *et al.*, 2009), blood vessels which is composed largely of elastin collagen and muscles cells have *E* of 1.16-860 MPa (Gauvin *et al.*, 2011; Awad *et al.*, 2018) and tendons have stiffness of 136 MPa-1 GPa (Ker *et al.*, 1988; Maganaris and Paul, 1999; Brennan *et al.*, 2018). Numerous lab groups have tried to synthesis substrates that mimics the physiological tissue stiffness to study tissue development and its pathology. The different types of natural and synthetic ECM commonly used for studying the effect of mechanical signals in stem cells is described in detail in Chapter 2.

1.2.2 Concept of Mechanobiology:

A basic question arises about how does cell sense the change in ECM? The cells respond to the substrate stiffness in three steps: (1) Mechano-sensation, (2) Mechano-transducing, and (3) Mechano-response. Briefly, mechano-sensation refers to when various protein receptors and adherent molecules, present along the cell membrane, such as integrins, E-cadherins, focal adhesion kinases (FAKs), stretch-activated ion channels such as transient receptor potential channels (TRP channels), communicates with the extracellular microenvironment and sense the mechanical forces (Li *et al.*, 2012; Vitillo *et al.*, 2016). These membrane molecules, also known as mechanosensors, undergo conformational changes which leads to activation of transducing molecules such as actin binding proteins, β-catenin, talin, vinculin, Src, members of MAPK family, YAP and Rho family GTPases present in the cytoplasm (Dupont *et al.*, 2011; Holle *et al.*, 2013; Liu and Lee, 2014). These proteins act as a mediator between surface receptors and the cytoskeletal filaments. The cytoskeletal components include actin filaments, microtubules and non-muscle myosin, which undergo conformational changes such as stretching or relaxing, and shortening or elongation, thereby controlling the cytoplasm/nuclear localization of transcriptional factors, for example YAP. These transcriptional factors are known as mechanotransducers (Bissell and Aggeler, 1987; Humphrey *et al.*, 2014). The mechanotransducers either localises into the nucleus to coordinate morphological organization and signalling events through gene expression, or is retained in the cytoplasm where it undergoes degradation. The shuttling of these

transcriptional factors between cytoplasm and nucleus is gated by linker of nucleoskeleton and cytoskeleton (LINC) complex present on the nucleus envelope. The tensed actin cytoskeleton causes stretching of actin binding LINC complex component Nesprin1, resulting in stiffening of nuclear envelope and localization of transcriptional factors (Guilluy *et al.*, 2014; Driscoll *et al.*, 2015) as illustrated in **FIGURE 1.2**.

It is crucial to note that a cell will never experience the mechanical signals alone, there are other microenvironment components present as well. Therefore, mechanical signal-regulated signalling pathways are complex, because these transcriptional factors have been known to be regulated by other biochemical molecules and specific signalling pathways.

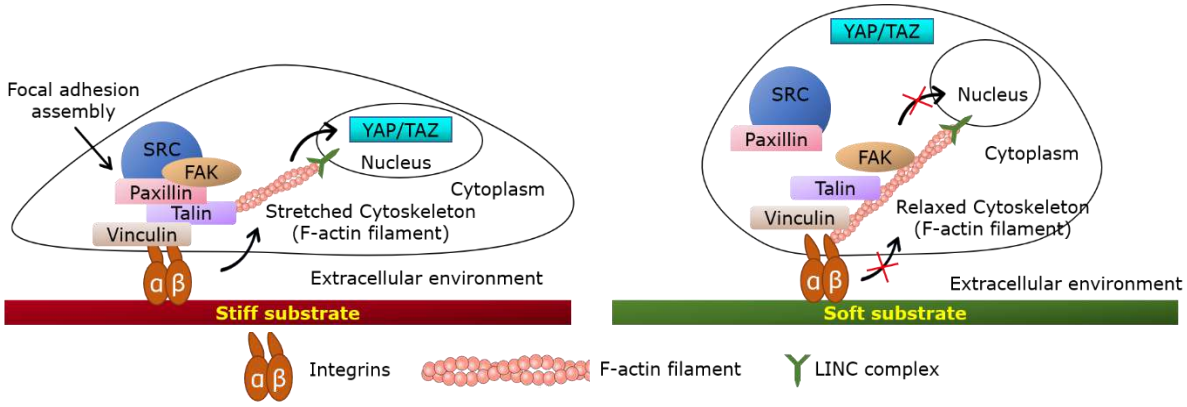


FIGURE 1.2: Mechanical transduction due to variation in substrate stiffness. Integrin binds to its respective receptor on the stiff substrate leading to FA assembly and polymerization of F-actin filaments. The increase in cytoskeletal tension activates the formation of LINC complex on the nuclear envelope, thus allowing nuclear translocation of transcriptional factors (e.g., YAP). Conversely, soft substrate prevents integrin-mediated assembly of FA proteins. Additionally, F-actin filaments are depolymerized and substrate-mediated translocation of transcriptional factors is prevented.

1.2.3 Substrate stiffness controls cell behavior:

The effects of the substrate stiffness on the cellular behaviour have been studied extensively. Pelham and Wang (1997) showed that the kidney epithelial and fibroblast cells cultured on polyacrylamide gel respond to the difference in the substrate flexibility by altering their adhesion structures and motile behaviour. A now classic study by Engler *et al.* (2004, 2006) reported that MSCs cultured on substrates of varying stiffness commit to phenotype corresponding to their biological tissue. Soft stiffness that mimics brain induces cells to commit towards neurogenic lineage, stiff substrates that mimic muscle

induces cells to commit towards myogenic lineage, and comparatively rigid stiffness that mimic bone commit towards osteogenic lineage. Adding to this, Evans *et al.* (2009) demonstrated that the cell spreading, growth rate, gene expression and differentiation of ESCs is influenced by the change in the substrate stiffness. Transcriptional co-activators: Yes-associated proteins (YAP) has emerged as mechanosensor which respond to the substrate stiffness and cell morphology (Dupont *et al.,* 2011; Aragona *et al.*, 2013; Brusatin *et al.*, 2018; Lee *et al.*, 2019).

1.3 YAP – Yes Associated Protein:

The Hippo/YAP pathway was first identified in Drosophila (Sudol, 1994) for regulating organ size; now it is recognised as a well-conserved pathway in mammals (Pan, 2010; Yu *et al.*, 2015), and also as potential therapeutic target in cancer (Cunningham and Hansen, 2022). YAP is the primary effector of the Hippo pathway, but it also interacts with TGFβ signalling pathway, WNT pathway, biophysical pathways and several others (Morgan *et al.*, 2013; Pocaterra *et al.,* 2020). These upstream signalling pathways have shown to regulate YAP activity with a broad range of extracellular microenvironment factors, such as (i) soluble bioactive ligands (Yu *et al.*, 2012; Ohgushi *et al.*, 2015), (ii) mechanical cues (Dupont *et al.*, 2011; Oliver-De La Cruz *et al.*, 2019), (iii) osmotic pressure and hypoxia (Ma *et al.*, 2015; Hong *et al.*, 2017), and (iv) tissue repair (Juan and Hong, 2016; Wang *et al.*, 2017).

In mammals, the core kinases include MST1/2 (mammalian STE20 like kinase 1/2) and LATS1/2 (large tumour suppressor kinase 1/2), while the downstream effector includes YAP (also known as YAP1), WWTR1 (WW domain containing transcription regulator 1, also known as TAZ) and a DNA binding protein TEAD1-4 (TEA domain transcription factor 1-4) (Holden and Cunningham, 2018). When stimulated by the upstream signals from cell-cell and cell-matrix contact, MST1/2 is phosphorylated and activated. The active MST1/2 phosphorylates its regulatory subunit SAV1 (Salvador family WW domain containing protein 1) (Callus *et al.*, 2006). The MST1/2-SAV1 complex further phosphorylates MOB1A/B (MOB kinase activator 1A/B), regulatory subunit of LATS, which then phosphorylates and activates LATS1/2 (Hergovich *et al.*, 2006). The LATS1/2-MOB1A/B complex then phosphorylates YAP/TAZ. The phosphorylated YAP/TAZ is inactive and sequestrated into the cytoplasm by 14-3-3 protein (Zhao *et al.*,

2007) and can further undergo ubiquitination and degradation (Zhao *et al.*, 2010; Low *et al.*, 2014). In absence of the upstream signals, MST1/2 and LATS1/2 are unphosphorylated, causing YAP to translocate into the nucleus and bind TEAD1-4. YAP and TAZ also bind to other DNA-binding transcription factors, such as RUNX2 (runt-related transcription factor 2), p73 (tumor protein 73) and the ErbB4 (Erb-B2 Receptor Tyrosine Kinase 4), to activate certain genes (Hong *et al.*, 2005; Lei *et al.*, 2008; Kim *et al.*, 2018). It is important to note that YAP expression can also be regulated by many non-Hippo signalling pathways as well (Piccolo *et al.*, 2014; Heng *et al.*, 2021), therefore, the expression of YAP is partially nuclear and partially cytoplasmic. Thus, this balance between unphosphorylated (nuclear) and phosphorylated (cytoplasmic) YAP plays an important role in lineage determination and in regulating stem cells during regeneration process.

1.3.1 YAP in Developing Mammalian Embryo:

From the above studies, it has been established that unlike other signalling pathways, Hippo pathways works as an integrator for biophysical and mechanical signals as well, and plays a crucial role in early embryo development, namely in maternal to zygote transition, in maintaining zygote pluripotency, and TE/ICM differentiation. Maternal RNA and protein predominate the very early stage of development. Maternal YAP knockout mice embryos showed prolonged 2-cell stage, and slower progression into 4-cell stage than the wild-type embryos. Additionally, the genome analysis of 4-cell stage knockout and wild-type embryo showed significant differences in thousands of genes, and downregulation of many genes targeted by YAP (Yu *et al.*, 2016). Furthermore, zygotes and blastomeres without maternal YAP were reported to perish before the blastocyst stage (Frum *et al.*, 2019).

During early blastocyst stage, Hippo pathway is active in the non-polar cells, which will form the ICM, and is inactive in the polar cells, which will from the TE. But how does cell-cell adhesion and polarity controls Hippo signalling? As discussed in Section 1.1, cell polarity is determined by evolutionarily conserved proteins, PAR-aPKC protein complex (Alarcon, 2010; Gerri *et al.*, 2020). It has been reported that the cell polarity and junction-associate scaffolding protein angiomotin (AMOT) together regulate YAP expression. In apolar cells, AMOT is phosphorylated at the adherens junctions and interacts with LATS

kinase, thereby facilitating phosphorylation and cytoplasmic retention of YAP. Whereas, in polar cells of TE, AMOT is sequestered from the adherens junctions to the apical domains by Par-aPKC complex, where AMOT interacts with F-actin. This supresses Hippo signalling and causes nuclear localization of YAP (Hirate *et al.,* 2013; Leung and Zernicka-Goetz, 2013) as illustrated in **FIGURE 1.3**.

The involvement of Hippo signalling pathway in TE/ICM specification was first identified in *Tead4* mutant mouse embryos. CDX2 (caudal type homeobox 2) expression is important for TE formation, and in Tead4 null embryos, *Cdx2* expression is downregulated; therefore, a functional TE is not formed (Yagi *et al.*, 2007; Nishioka *et al.*, 2008). A subsequent study demonstrated that in TE, TE-specific genes such as *Cdx2* and *Gata3* are upregulated due to the activation and nuclear localization of YAP, whereas in ICM, YAP is phosphorylated by LATS kinases leading to cytoplasmic sequestration of YAP (**FIGURE 1.3**) (Nishioka *et al.*, 2009; Ralston *et al.*, 2010, Gu *et al.*, 2022). Interestingly, YAP not only regulates TE-specific gene expression, it also regulates the expression of SOX2, one of the earliest pluripotency markers. SOX2 is expressed in ICM, but prevention of YAP nuclear localization in TE cells, have shown to induce SOX2 expression. This shows that YAP activates a repression mechanism in TE cells to regulate SOX2 expression (Wicklow *et al.*, 2014).

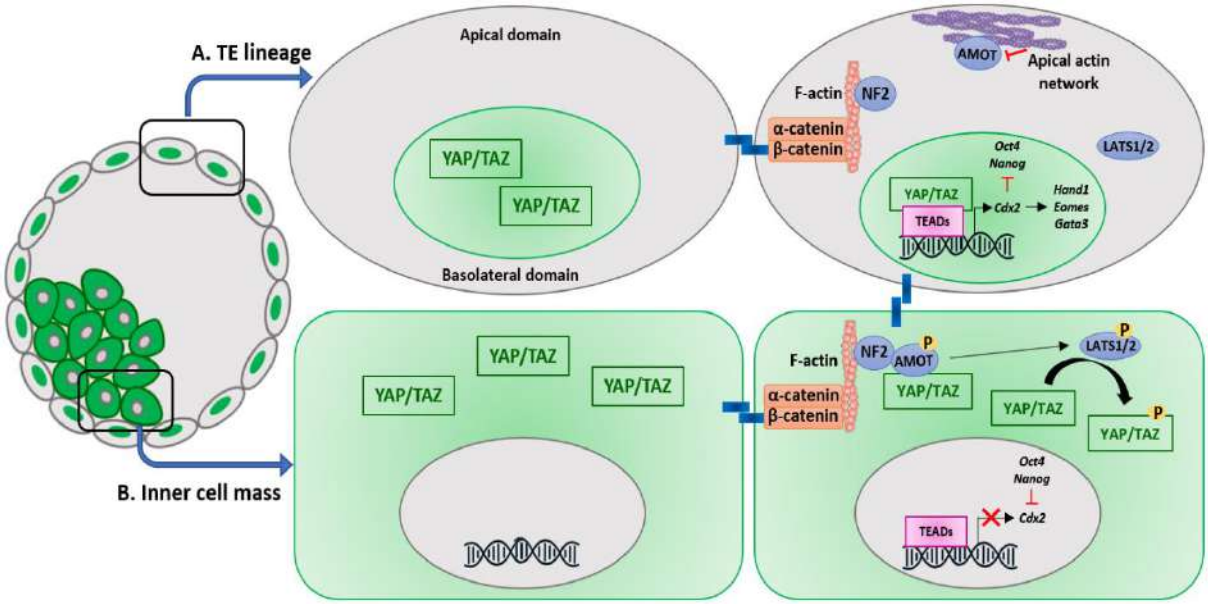


FIGURE 1.3: Schematic representation of the subcellular localization of lineage-specific markers regulated by Hippo pathway at blastocyst stage. The outer polar cells generate trophectoderm encloses the inner apolar cells which forms inner cell mass. (A) In polar cells, the apical actin network sequestered AMOT at the network thus preventing its interaction with NF2 (neurofibroma 2 or merlin), one of the upstream proteins of Hippo pathway. YAP translocates

into the nucleus and interacts with TEAD to active expression of TE-specific markers like Cdx2, Gata3, Eomes, Hand1. (B) In apolar cells, in the absence of apical actin network, AMOT binds to NF2 at the cell-cell adhesion junction. Phosphorylated AMOT activates LATS phosphorylation, thereby sequestering YAP into the cytoplasm and limiting its interaction with TEAD and expression of TE-specific genes. Expression of Oct4 and Nanog in apolar cells maintains pluripotency and generates the ICM. The yellow 'P' represents phosphorylation of the proteins. (Adapted from Mo *et al.*, 2014)

1.3.2 YAP in Pluripotent Stem Cells

Several studies in mouse and human ESCs (mESCs and hESCs) have shown elevated YAP/TAZ expressed under normal culturing conditions (Ramalho-Santos *et al.*, 2002; Varelas *et al.*, 2008; Ohgushi *et al.*, 2015). Previous YAP knockout studies have demonstrated that YAP promotes stem cell self-renewal and pluripotency, and that loss of YAP leads to the loss of pluripotency in both the human and mouse ESCs (Lian *et al.*, 2010; Qin *et al.*, 2016; Papaspyropoulos *et al.*, 2018; Wang *et al.*, 2021). In addition, it was reported that overexpression of YAP suppresses hESCs and mESCs differentiation (Lian *et al.*, 2010). Conversely, another study in mESCs reported that YAP is dispensable for self-renewal, depletion of YAP inhibits differentiation, whereas, overexpression of YAP stimulates differentiation (Chung *et al.*, 2016). It has also been shown that YAP depletion does not affect any of the normal stem cell characteristics in hiPSCs (Lorthongpanich *et al.*, 2020). These results suggest that the function of YAP is context specific and its role during human pluripotent stem cell differentiation needs has not been uncovered.

1.3.3 YAP as Mechanotransducer in Stem Cells

Duport *et al.* (2011) identified YAP and TAZ as nuclear transducers of mechanical signals exerted by the ECM rigidity and cell shape. They reported that stiff substrates, large adhesive areas and in cells with high contractile forces, the unphosphorylated YAP shuttles into the nucleus where it promotes proliferation of primary mammary epithelial cells (MECs) and MSCs differentiation towards osteogenic lineage. Conversely, YAP phosphorylated and bound by 14-3-3 and localized in the cytoplasm on soft substrate, small adhesive area and in cells with low contractile forces, cause MEC apoptosis and differentiate MSCs towards adipogenic lineage. Piccolo's laboratory reported that the subcellular localization and activity of YAP is regulated by actin cytoskeleton remodelling, cell substrate rigidity and topography, and cell stretching. The stiff substrate and high filamentous actin (F actin) levels have been shown to result in their nuclear

translocation (Aragona *et al.*, 2013). Thus, confirming that substrate stiffness regulates the YAP activity in MSCs and hPSCs.

1.4 Current Challenges :

From the studies mentioned above, it is evident that mechanobiological processes have crucial impact on early embryogenesis and in stem cells fate. Therefore, it can be said that processes in mechanobiology will impact the development of innovative therapeutic methods for tissue engineering and, eventually, regenerative medicine applications. The successful outcome of any stem cell-based regenerative medicine critically depends on cell survival after transplantation and to maintain tissue homeostasis mainly by differentiating into the respective lineage. To attain this, it is crucial to maintain optimal physiologically similar culture conditions *in vitro* for stem cell maintenance, proliferation, and quick differentiation when required. The field of bioengineering and material science has made it possible to mimic natural ECM for studying the mechanical signals.

The stiffness of the substrate has shown to regulate MSCs differentiation, with the stiffest substrate leading to osteogenic differentiation while less stiff substrate led to adipogenic differentiation (Engler *et al.*, 2006; Dupont *et al.*, 2011). In hESCs, substrate stiffness in combination with soluble molecules help maintain self-renewal on stiff substrate and caused neurogenic differentiation on soft substrate (Maldonado *et al.*, 2015). Similar studies have reported that hPSCs on soft substrate differentiate into neuroectoderm (Hindley *et al.*, 2016). However, no study has shown the interaction between hPSCs and substrate stiffness in absence of differentiation inducing medium. Understanding the interaction between hPSCs and stiffness is important because hPSCs are being used in many stem-cell based therapy especially in the light of new technologies such as 3D bio printing or tissue engineering biomaterials.

Chapter Two

Review of Literature

2.1 How do cells communicate?

Metazoans have unique organs that enables them to sense their environment through the five senses, similarly individual cells perceive their environment through a variety of subcellular structures and mechanisms, which allows the cells to detect and respond to the signals from soluble molecules, electric current and mechanical signals.

The relationship between the bioelectricity and muscle contraction through nerve stimulation was first reported by Luigi Galvani in the 18th century (Piccolino, 1998), and since then the bioelectrical signalling, which is now a unique property of neurons, cardiac muscles and skeletal muscles, has been extensively studied and understood (Levin, 2012; Harris, 2021). It involves flow of charged ions: Na^{+}, K^{+}, Ca^{+2} and Cl^{-} across the cell membrane. Earlier, it was known that cells communicate with each other by means of soluble molecules and hormones released from glands and other tissues, however, the process was unclear. The discoveries made by Earl Sutherland, Alfred. G. Gilman and Martin Rodbell in the late 20th century opened the area of rapidly expanding research on biochemical signal transduction. This mode of signalling usually involves binding of a small soluble molecule, known as ligand, to a cell surface receptor, thereby initiating a cascade of kinases activation or inhibitions (Robinson *et al.*, 1968; Sutherland, 1972; Rodbell, 1980; Gilman, 1987).

Researchers have long recognized that the cells can interpret the mechanical signals and respond accordingly (Pelham and Wang, 1997; Lehoux and Tedgui, 1998). However, only recently the molecular mechanisms through which cells perceive and transform the mechanics of the ECM has been elucidated. Mechanical signals are classified as those arising from: (i) the forces applied by the neighbouring cells or gravity or shear fluid flow, and (ii) the mechanical resistance generated by a cell in response to the intracellular force. In either case, the glycocalyx; a protective gel-like layer surrounding the cells; and the ECM surrounding a cell resists these forces (Buffone and Weaver *et al.*, 2019). The molecular processes that transform these physical signals into biological responses is collectively referred to as mechanotransduction.

2.2 Mediators of Mechanotransduction

Many small molecules, cellular components, and extracellular structures have been shown to contribute to mechanotransduction. These include ECM, cell-ECM adhesions, cell-cell adhesions, membrane receptors and ion channels, cytoskeletal filaments and nuclear components as illustrated in **FIGURE 2.1**. In this section, I have tried to provide the reader a comprehensive overview of numerous molecules and subcellular structures that respond to mechanical signalling.

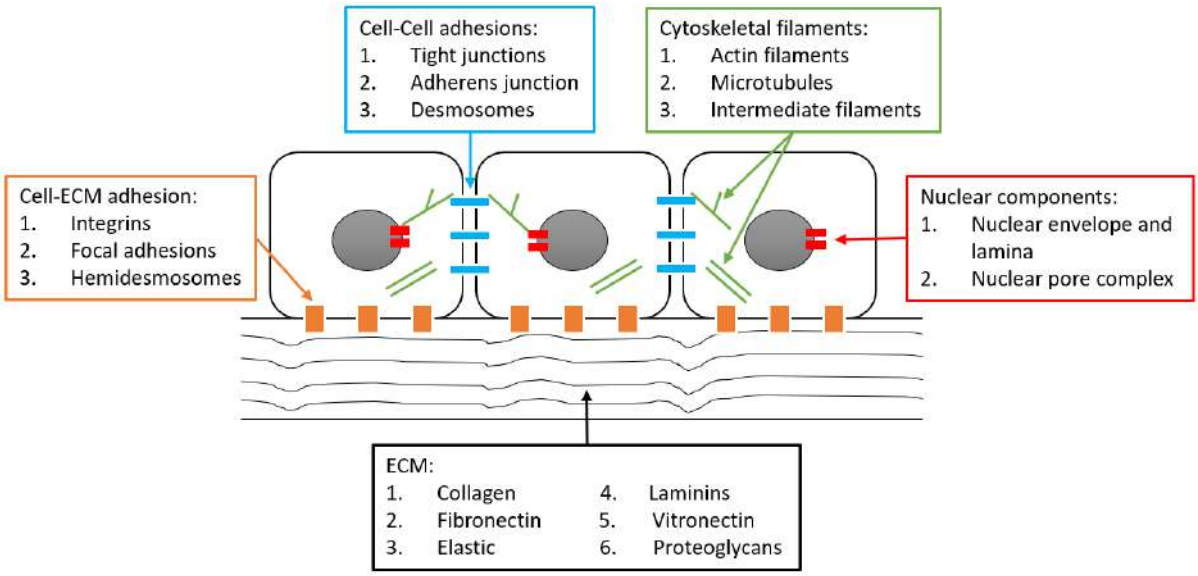


FIGURE 2.1: Mediators of Mechanotransduction. (clockwise) The composition, stiffness and topology of the ECM is first sensed by the cell-ECM adhesion receptors present on the cell surface. The cells communicate with the neighbouring cells through cell-cell adhesion junctions. Cytoskeletal filaments link the cell surface to the nucleus thus relaying the mechanical stimuli into the cytoplasm. The nuclear components convey the stimuli from the cytoplasm into the nucleus, finally resulting in targeted-gene expression.

2.2.1 Extracellular Matrix (ECM)

As introduced in Section 1.2.1, the extracellular matrix is an essential component of the cell microenvironment. Fibrous proteins and proteoglycans are the two main classes of macromolecules present in the ECM. The proteins collagens, fibronectin, elastin, laminins, and vitronectin provide structural support to the cells by forming an interconnected network. Proteoglycans and cell-binding glycoproteins on the other hand fill up most of the tissue ECM. Each component has its own distinct physical and biochemical properties, and the composition of these macromolecules gives the ECM its unique mechanical property.

Collagen, is the most abundant fibrous protein in most of the tissues ECM. In vertebrates, 28 subtypes of collagens have been identified which are made up of 46 collagen chains assembly (Gordon and Hahn, 2010). They are classified as fibril forming collagens (type I, II, III, most abundant type of collagen), network forming collagens (type IV, which form the basement membrane), and other collagen (for example, collagen type VI). Collagens are widely used as a natural substrate in *in vitro* culture system (Yang and Nandi, 1983; Somaiah *et al.*, 2015; Parmar *et al.*, 2016 and 2017). Collagen scaffolds are used clinically to promote wound healing (Gould, 2016). The composition, stiffness and porosity of the collagen scaffold has also been used to culture and differentiate mESCs and hiPSCs towards neural lineage (Kothapalli and Kamm, 2013; Macri-Pellizzeri *et al.*, 2015), and MSCs towards osteogenic lineage (Rico-Llanos *et al.*, 2021). Collagen alone has a low mechanical property, but when present in abundance, it is the major contributor to high ECM stiffness.

Elastin, is another major protein present in the ECM of soft tissues, such as skin, ligaments, arterial walls, and lungs. Elastin provide recoil to tissues which undergo repeated stretch. The integrity of the elastin fibers depends on its associates with collagen fibrils and glycoproteins (Wise *et al.*, 2009). Elastin alone or in combination with other ECM proteins has been used as substrate for *in vitro* myogenesis (D'Andrea *et al.*, 2015), and osteoblast proliferation and differentiation (Amruthwar and Janorkar, 2013). Interestingly, MSCs cultured on elastin-based biomaterials efficiently differentiate towards skin (Rnjak-Kovacina *et al.*, 2012; Ozsvar *et al.*, 2015) and cartilage cells (Betre *et al.*, 2006; Haider *et al.*, 2008).

Fibronectin, is the third most abundant protein present in the ECM. It functions as 'biological glue' because of the presence of ligand Arg-Gly-Asp (RGD) which is essential for cell attachment and migration. Therefore, fibronectin has been used as a coating on traditional plastic/glass plates and on synthetic substrates (Hunt *et al.*, 2012; Silva *et al.*, 2020), or as a 3D construct to induce differentiation (Linsley *et al.*, 2013). In addition to its resting length, fibronectin can be stretched several times by the cellular traction forces. The unfolding of fibronectin exposes several integrin-binding sites that results in changes in cellular behaviour. This implicates fibronectin as mechano-regulator (Smith *et al.*, 2007).

Laminins, are a group of 20 glycoproteins that are interwoven with collagen type IV to form the basement membrane. Laminins closely associate with cells through cell surface receptors and are key ECM regulators of cell adhesion, migration, differentiation and proliferation (Durbeej, 2010). Laminin has been reported to form the ECM niche for trophoblast stem cells *in vivo* (Kiyozumi *et al.*, 2020). Laminin surface coatings have been found to enhance neuronal stem cell migration, expansion and differentiation (Flanagan *et al.*, 2006).

Vitronectin is a multifunctional glycoprotein that binds to glycosaminoglycans (GAGs), collagen, and plasminogen. Vitronectin binds to four specific receptors: αvβ1, αvβ3, αvβ5 and αIIbβ3 (Felding-Habermann and Cheresh, 1993). It is widely recognised as an adhesive substrate for *in vitro* cell culture for cells expressing either of the vitronectin receptors (Yap *et al.*, 2011). Other than attachment, it supports cell spreading, migration, proliferation and differentiation (Schvartz *et al.*, 1999).

Proteoglycans are proteins which are glycosylated. Glycosylated proteins have a core protein and one or more anionic GAGs covalently attached. Almost all extracellular matrixes of connective tissue contain proteoglycans in various forms. Proteoglycans are highly diverse in terms of the core protein and GAG chains, namely chondroitin sulfate, keratan sulfate, dermatan sulfate and heparan sulfate. Secreted proteoglycans are classified as large proteoglycans (aggrecan and versican), small proteoglycans (decorin and lumican), and basement membrane proteoglycan (perlecan). The ubiquitous nature of PGs makes them capable of modulating cellular proliferation, differentiation, and gene expression. Each proteoglycan has a distinct function, for example, aggrecan present in cartilage generates elasticity and high biomechanical resistance to pressure; decorin regulates collagen fibril formation in connective tissues (Nguyen and Panitch, 2022).

These components of the ECM associate with each other to form a structurally stable network, thereby contributing to the mechanical properties of the tissues. However, under certain conditions, like injury, repair or disease, these components of the ECM are remodelled either enzymatically or non-enzymatically. This constant remodelling of the ECM determines the mechanical properties, such as tensile strength, stiffness and elasticity of each tissue.

2.2.2 Cell-ECM adhesions

The interaction of the cell with the ECM is mediated by cell surface receptors, which upon binding to ECM ligands gets activated and undergo conformational changes, thereby recruiting adhesion proteins complex, namely focal adhesions and hemidesmosomes to the receptor-ligand binding site. These adhesions complexes interact with actin cytoskeleton and intermediate microfilament respectively present in the cell cytoplasm. This entire assembly is known as cell-matrix adhesion complexes (CMACs) (Lock *et al.*, 2008). The CMACs enables cells to sense the changes in extracellular microenvironment such as substrate type, change in its chemical composition (Reilly and Engler, 2010; Gattazzo *et al.*, 2014), stiffness (Discher *et al.*, 2005; Engler *et al.*, 2006) or surface topology (Ankam *et al.*, 2013; Deglincerti *et al.*, 2016; Abagnale *et al.*, 2017), and convey the information through subsequent mechanotransduction pathways and biochemical signalling pathway into the nucleus, thereby influencing diverse cellular processes such as cell shape and polarity, self-renewal and differentiation, motility, etc. (Discher *et al.*, 2005; Gupton and Waterman-Storer, 2006; Cavalcanti-Adam *et al.*, 2007; Li and Gundersen, 2008; Geiger *et al.*, 2009).

Cell-ECM adhesions receptors are classified based on the ligand they bind . Various cell-ECM receptors exist, of which integrins predominantly interacts with most of the major ECM proteins such as collagen, fibronectin, laminin, and vitronectin. Fibronectin receptor binds to transmembrane proteoglycans family, collagen receptors interact with tyrosine kinases and glycoproteins, whereas laminin receptor interacts with dystrophin glycoprotein complex, lutheran and basal cell adhesion molecule.

Integrins are transmembrane receptors, and in mammals, it consists of eighteen α-subunits and eight β-subunits, which generate 24 different integrin receptors (Hynes, 2002). This heterodimeric receptor function as a mechanical link between the ECM and the cytoskeleton (Sun *et al.*, 2016). The integrin receptors are broadly grouped into four categories based on the ECM ligands they bind to: RGD (arginine-glycine-aspartic acid) receptors, collagen receptors, laminin receptors and leukocyte-specific receptors (Takada *et al.*, 2007). Some specific heterodimer receptors play an important role in stem cell maintenance. For example, integrin α2 is upregulated in hMSCs cultured on stiff substrates and regulate osteogenic differentiation (Shih *et al.*, 2011). hESCs express several integrin heterodimers as it interacts with many ECM proteins. For example, hESCs

cultured on laminin-rich ECM protein mixture (commercially known as Matrigel®) adhere and proliferate through α2β1, α6β1, and αVβ3; hESCs cultured on fibronectin bind to α5β1, while on vitronectin hESCs attach to αVβ5 (Meng *et al.*, 2010; Braam *et al.*, 2008). hiPSCs adhere and proliferate on Matrigel via β1 integrins and on vitronectin via αVβ5 and β1 integrins (Rowland *et al.*, 2010). Although integrin receptors are well established as mechanosensors, mechanosensing via integrins in regulating stem cell behaviour is still an active area of research.

Focal adhesions (FA) contain heterodimers of α and β type integrins. The extracellular part of the integrins binds to the ECM, and the intracellular region interacts with intracellular focal adhesion proteins: talin, vinculin, actinin, zyxin, paxillin, myosin, tensin, kindlin2, vasodilator-stimulated phosphoprotein (VASP), and focal adhesion kinases (FAK). These FA proteins activate and gathers integrins to a site, connects integrins to actin filament and transduce the signals. The mechanical properties of talin have been extensively studies. Talin associates with vinculin, an intracellular protein that binds to actin filaments and α-actin, thereby enhancing the formation of actin polymers (Yan *et al.*, 2015). Application of tensile forces, cell stretching and substrate stiffness has shown to facilitate binding of vinculin to talin (Del Rio *et al.*, 2009; Ciobanasu *et al.*, 2014; Elosegui-Artola *et al.*, 2016). These findings indicate that talin functions as mechanosensor that senses mechanical signals and therefore, plays an important role in mechanotransduction.

Similarly, *hemidesmosomes* which are formed in epithelial cells contribute in mechanotransduction. Hemidesmosomes consists of integrins and plectin where integrin facilitates in anchoring cells to the basement membrane by binding to laminin, and plectin forms a bridge of intermediate filaments network in the cytoplasm in response to cell adhesion, proliferation, differentiation and migration (Walko *et al.*, 2015; Osmani and Labouesse, 2015).

2.2.3 Cell-Cell adhesions

Cell-cell adhesion enables cells to communicate with each other through chemical, electrical or mechanical signals, facilitated by special junctions. In mammals, three main types of cell-cell adhesive junctions have been detected to be involved in mechanotransduction: tight junctions, adherens junctions and desmosomes.

Tight junctions (TJ) are present between the epithelial and endothelial monolayer cells. They are formed of two main protein complexes: transmembrane proteins (occludin, claudins and junctional adhesion molecules (JAMs)) and cytoplasmic proteins (zonula occludens (ZO) 1-3 and cingulin family). The actin cytoskeleton and microtubules are connected to transmembrane proteins via ZO1-3. TJs play a role in mechanotransduction through ZO1. Mechanical tension generated due to extracellular stiffness and JAMs regulates ZO1 which modulates actomyosin contractility thereby acting as mechanosensor (Haas *et al.*, 2020; Angulo-Urarte *et al.*, 2020).

Adherens junction (AJ), like tight junctions, consists of transmembrane protein and intracellular proteins. Transmembrane proteins consist of cadherins and calcium-dependent ion channels, whereas intracellular protein complex is made up of catenin, namely p120, α and β, and vinculin. AJs connect neighbouring cells via actin filaments. Actin stress fibres generated due to the mechanical forces affect AJ maturation, thereby affecting motility, and morphogenesis of epithelial cells and tissues (Maki *et al.*, 2016). Additionally excessive tensional force from the microenvironment exposes vinculin binding sites in α-catenin which leads to accumulation of vinculin at AJs. This complex enables anchoring of actin filaments to the AJs and formation of actomyosin bundles (Yonemura *et al.*, 2010). These finding established the mechanosensory function of catenin.

Desmosomes consists of desmosome cadherins, namely desmoglein and desmocolin which binds to the extracellular domains, and intracellular cytoplasmic proteins: plakoglobin, plakophilin and desmoplakin. Desmosomes connect intermediate filaments between two cells through desmoplankin. It provides mechanical resistance to tissues such as the epidermis and heart against external forces (Garrod and Chidgey, 2008). A study reported that in presence of the external force, desmosomes accumulate on the site of force application thus revealing its function in mechanosensing (Weber *et al.*, 2012). Further investigations could help in understanding the role of desmosomes during mechanotransduction.

2.2.4 Cytoskeletal filaments

The cytoplasm of eukaryotic cells consists of a dynamic network of interlinking protein filaments known as cytoskeleton. The cytoskeleton consists of three types of

filaments: actin filaments, intermediate filaments (IF) and microtubules (MTs). Apart from maintaining cell shape and internal structure, the cytoskeletal filaments also provide mechanical support thereby enabling cells to migrate or divide. Cytoskeleton enables cells to adjust to their microenvironment, communicate with neighbouring cells and also plays an important role in integrating several signals thereby regulating cell behaviour.

Actin filaments are composed of filamentous (F) actin molecules and many actin-binding proteins which are arranged in a helix. As mentioned in above sections, actin filaments are linked to FAs and AJs at cell-ECM and cell-cell adhesion junctions respectively. Numerous studies have proved that actin filaments play a crucial role in mechanotransduction mediated by FAs and AJs. Actin filaments responds to tensile forces by increasing the length of helical F-actin and orienting parallel to the direction of the force (McGough *et al.*, 1997). This arrangement reduces the affinity of F-actin severing protein, cofilin, and increases the affinity of myosin II to actin filaments. The formation of actin-myosin II complex generates contractile forces which stabilizes actin filaments and facilitates the formation of stress fibres (Uyeda *et al.*, 2011). In contrast, in the absence of tensile forces actin filaments are relaxed, and their length is decreased. This leads to cofilin binding and ultimately severing of actin filaments (Hayakawa *et al.*, 2011). This assembling and disassembling of actin filaments has been linked to cell proliferation, differentiation and other gene expression for example Yes-associated protein (YAP)-, myocardial-related transcriptional factor (MRTF)- and serum response factor (SRF)-targeted genes which includes genes related to cytoskeletal and cell adhesion components (Olson and Nordheim, 2010; Dupont *et al.*, 2011; Halder *et al.*, 2012; Finch-Edmondson and Sudol, 2016).

Microtubules are longer filaments and play an essential role in the formation of mitotic spindle, cilium and cell polarity. It is documented that mechanosensors talin and actomyosin detect the substrate stiffness and control the acetylation (post-translational modification) of microtubules (Seetharaman *et al.*, 2022). Several studies have shown that MTs contribute in the stress-mediated functioning of cilia and positioning of mitotic spindle fibers (Kaverina *et al.*, 2002; Fink *et al.*, 2011; Prasad *et al.*, 2014), but so far, the direct role of MT as mechanosensor has not been reported.

Intermediate filaments, in contrast to the actin filaments and microtubules, are very stable. In human, approximately 70 distinct genes encode for IF proteins (Szeverenyi *et al.*, 2008). Depending on the signals, the IF proteins can form homodimers, heterodimers or antiparallel tetramers, which assemble to form rope-like structures (Chernyatina *et al.*, 2015). IFs are anchored to desmosomes and hemidesmosomes, therefore any mechanical stress sensed by these junctions results in assembling and disassembling of IFs (Weber *et al.*, 2012; Wang and Pelling, 2012). Therefore, IFs are most likely involved in mechanotransduction, but their direct role as mechanosensor has not been demonstrated.

2.2.5 Nuclear Components

So far, we have seen that mechanical signals generated from the extracellular microenvironment are sensed by the mechanosensors present on the cell surface. These signals are relayed into the cell by various proteins complexes and cytoskeletal filaments. To effectuate proper gene expression, these signals are relayed into the nucleus via nuclear components: nuclear envelope proteins (NE), nuclear lamina and nuclear pore complex (NPC).

Nuclear envelope and nuclear lamina mediate the transmission of mechanical signals from the cytoskeleton into the nucleus. NE acts as a barrier between the cytoplasm and nucleoplasm. It also consists of transmembrane proteins which connect the cytoskeleton to the nuclear components (Janota *et al.*, 2017). NE comprises of outer nuclear membrane and inner nuclear membrane which are connected together by the LINC complex (linker of the nucleoskeleton and cytoskeleton). LINC is formed by two transmembrane proteins: nesprins on the outer nuclear membrane, which interacts with the cytoskeletal filaments, and SUN (Sad1 and UNC-84) on the inner nuclear membrane attached to a dense network of fibrillar proteins nuclear lamina (Starr and Fridolfsson, 2010). In presence of a mechanical tension, the actin stress fibers regulate the formation of LINC complexes, and transfer the mechanical forces to the nesprin, thereby leading to stiffening of the nuclear lamina (Arsenovic *et al.*, 2016). Nuclear lamina is connected to the chromatin; therefore, any upstream mechanical stress leads to change in nuclear morphology and chromatin regulation via nuclear lamina. However, the exact mechanism of how cytoskeletal forces assemble LINC complexes and remodel nuclear lamina needs to be investigated further.

Nuclear pore complex, is composed of more than 30 different proteins and spans the nuclear envelope. They are bilateral pathways mediating the transport of macromolecules between the cytoplasm and the nucleus (Strambio-De-Castillia *et al.*, 2010). NPC binds to cytoskeleton in the cytoplasm, LINC complex in the NE and chromatin in the nucleus (Goldberg, 2017), therefore it could be regulated by mechanical forces. The NPC allows mechanotransduction through two proposed hypotheses (Matsuda and Mofrad, 2022). The first theory proposes that under mechanical stretch conditions, the NPC pore dilates, thereby promoting entry of transcriptional factors into the nucleus. This was experimentally proved when force was applied on mouse fibroblast cells cultured on stiff microenvironment promoted translocation of the mechanotransducers, where the mechanotransducers bind to their respective target and instigate desired gene expression (Elosegui-Artola *et al.*, 2017). The second hypothesis, which was proven experimentally, suggested that mechanical signals lead to conformational changes in the transcription factors and thus, altering their affinity towards nuclear transport receptors. For instance, MRTF-A binds to the importin α and β which enables it to diffuse through the NPC (Pawłowski *et al.*, 2010; Mouilleron *et al.*, 2011). These results, however, raises several questions on the role of NPC and its link with mechanical forces and whether NPCs can be mechano-activated through a different process other than stretching.

It should be noted here that all the studies have used *in vitro* model to uncover the role of these mediators in mechanotransduction, how these mediators behave *in vivo* cannot be correlated with the *in vitro* results. Therefore, it will be interesting to understand how cells orchestrate these mediators *in vivo*, especially in pre-implantation embryos where mechanical forces are known to play a crucial role, to respond to the mechanical stimulation.

2.3 Mechano-transducing Proteins

In the above sections we have seen that mechanical signals generated by the extracellular microenvironment are sensed and transmitted into the nucleus by cell surface receptors, integrin-mediated cell-ECM adhesion, stretch-activated ion channels, cadherin-mediated cell-cell adhesions, cytoskeletal filaments, and nucleus components. Another mechanism through which cell respond to the mechanical stimuli is through activation of protein kinases or secondary messengers, and their subsequent signalling

pathways, which requires phosphorylation/dephosphorylation of regulatory proteins present in the cytoplasm. These proteins sense the state of actin cytoskeleton and are able to activate cascade of kinase activity, leading to gene expression or repression (Densham *et al.*, 2009; Hayakawa *et al.*, 2011). Till date, researchers are trying to undermine how actin polymerization / depolymerization trigger nuclear localization of transcriptional factors (TFs) and affect specific gene expression. These mechanically-regulated TFs are known as mechanotransducers. So far, the following TFs have been identified as mechanotransducers: MRTF (Speight *et al.*, 2016), YAP and its homologue transcriptional coactivator with PDZ-binding motif (TAZ) (Dupont *et al.*, 2011), transforming growth factor β (TGF-β) (Vermeulen *et al.*, 2020), RUNX-2 (Yang *et al.*, 2014; Yamazaki *et al.*, 2021), β-catenin (Warboys, 2018), nuclear factor-kappa-B (NF-κB) (Kumar and Boriek, 2003), nuclear factor erythroid-2-related factor 2 (Nrf2) (Owen and Dormer, 2021), and epigenetic regulator HDAC3 (Li *et al.*, 2011). It should be noted that these TFs are well known regulatory proteins which are activated by soluble signalling molecules and regulate various cellular processes and do not function as mechanotransducers alone. Also, there could be other TFs which functions as mechanotranducers but not yet identified.

2.4 Molecular Pathways involved in Mechanical Signalling

Over the years, it has become increasingly clear, that mechanical cues trigger biochemical signalling pathways to regulate fundamental cellular functions. Mechanical cues can often alter gene expression by activating or suppressing a given pathway. The key biochemical pathways involved in the transduction of mechanical signals are explained briefly below.

Ion channels, are one of the most rapid signalling pathways that responds to the mechanical signalling. In endothelial cells, mechanical strain sensed by the integrin receptors leads to activation of mechanosensitive ion channel, transient receptor potential cation channel subfamily V member 4 (TRPV4) within five milliseconds of the application of the mechanical force. This caused Ca^{2+} influx, activation of phosphatidyl inositol-3-kinase (PI3K), assembly of integrin receptors and finally remodelling of the cytoskeletal filaments, which guided the reorientation of endothelial cells (Thodeti *et al.*, 2009; Matthews *et al.*, 2010).

Mitogen-activated protein kinase (MAPK) pathways, are known to be activated by mechanical forces through focal adhesion kinases in osteoblasts, endothelial and fibroblasts cells. On activation by mechanical forces, the upstream kinases are phosphorylated, which results in nuclear translocation of all the three MAPK family proteins: extracellular signal regulated kinases (ERK1/2), jun amino terminal kinases (JNKs or MPK8) and stress activated protein kinases (SPKs or p38 or MAPK14). These mechanotransducers phosphorylate number of TFs such as activator protein-1 (AP-1), Elk-1, and serum response factor (SRF), thereby regulating the expression of collagen and osteopontin, the genes crucial in tissue remodelling (Ishida *et al.*, 1996; Hoffman *et al.*, 2017; Zhao *et al.*, 2020).

Transforming growth factor β (TGF-β) family includes TGF-βs (1-3), activin, nodal, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs) and mullerian inhibiting substances (MIS). TGF-β family is an evolutionary conserved signalling pathways in mature cells and during embryogenesis; where it mediates a diverse range of cell processes, such as cell proliferation, differentiation, apoptosis, migration, homeostasis (Huminiecki *et al.*, 2009; Etoc *et al.*, 2016). It regulates tissue homeostasis by regulating the expression of ECM genes, and thus, influences cell proliferation, differentiation and migration, in part, by modulating ECM proteins (Hocevar *et al.*, 1999; Verrecchia *et al.*, 2001; Verrecchia and Mauviel, 2002). Under normal culture conditions, TGF-β and TGF-β/Activin/Nodal signalling contribute in maintaining the undifferentiated state of hESCs by regulating pluripotency markers (James *et al.*, 2005; Ludwig *et al.*, 2006). Mechanical forces generated through cell stretching, substrate stiffness, and shear fluid stress, releases TGF-β from the ECM, which binds to its cell surface receptor, type 1 and type 2 (Maeda *et al.*, 2011). This leads to the phosphorylation of R-SMADs (Receptor-regulated Suppressor of Mothers Against Decapentaplegic 1/5 and 2/3), which forms a complex with co-SMAD protein (SMAD4), this complex translocates into the nucleus and initiate transcriptional process. SMAD1/5 and 2/3 are specific for BMP and TFG-β receptors respectively. For instance, in hESCs mechanical strain have shown to induce TGF-β/activin A/Nodal expression and phosphorylate SMAD2/3, thereby, repressing spontaneous differentiation of hESCs, moreover, inhibition of TGF-β-Activin-Nodal pathway using a pharmacological inhibitor promoted hESCs differentiation under mechanical strain (Saha *et al.*, 2008). The precise mechanism by which TGF-β signalling is involved in mechano-transduction is not yet fully

understood, but studies have shown that it can be mediated by a network of interactions between TGF-β and other signalling pathways, such as through integrins, Rho (Ras homologous) family GTPases and YAP. For example, in adipose tissue-derived MSCs, the mechanical force due to topographical changes on the substrate have shown to induce TGF-β signalling through actin reorganization and Rho/ROCK/SRF signalling pathway (Vermeulen *et al.*, 2020). Moreover, MSCs on stiff substrates exhibited stress fibers compared to the MSCs on soft substrates, and addition of TGF-β induced more thicker stress fibers in MSCs cultured on stiff substrates but not on soft substrates (Park *et al.*, 2011). TGF-β along with appropriate mechanical signal from the substrate has shown to differentiate MSCs into cartilage, chondrogenic cells or smooth muscles cells either through SMAD signalling or via actin cytoskeleton modelling (Williams *et al.*, 2003; Steward and Kelly, 2015; Moustakas and Heldin, 2008; Li *et al.*, 2010; Park *et al.*, 2011).

Wingless-related integration site (WNT) pathway is another well studied pathway regulating diverse set of cell functions such as cell proliferation, differentiation, it also plays an important role during embryogenesis, in stem cell differentiation, and in maintaining tissue homeostasis (Clevers and Nusse, 2012). The Wnt pathway is highly complex which is divided into: canonical pathway, which requires phosphorylation and activation of β-catenin, and non-canonical pathway, which is independent of β-catenin activity. β-catenin is a known mechanoregulator during gastrulation (Farge, 2003). The mechano-signalling through the Wnt pathway was first discovered in bone development and homeostasis. In response to mechanical loading, *in vivo* osteoblast cells showed an increase in the Wnt signalling and β-catenin activity (Robinson *et al.*, 2006), additionally, osteoblast cells cultured *in vitro* showed increased Wnt/β-catenin activity in response to strain (Case *et al.*, 2008; Hens *et al.*, 2005) or shear fluid stress (Norvell *et al.*, 2004; Santos *et al.*, 2009; Kamel *et al.*, 2010). In MSCs, acute exposure to oscillatory shear stress has shown to release β-catenin from the membrane bound N-cadherin along with increased expression of Wnt5A which also contributed to the β-catenin levels through RhoA and receptor tyrosine kinase-like orphan receptor-2 (Ror2). Phosphorylated β-catenin translocated into the nucleus and activated osteogenic gene expression, specifically RUNX2 (Arnsdorf *et al.*, 2009). Another study showed evidence of Wnt activation on application of force to MSCs, which caused activation of β-catenin and increased expression of osteogenic genes (Sen *et al.*, 2009). It has been found that hESCs cultured on a soft hydrogel-based substrate accumulate β-catenin at cell-cell adhesions

and undergo Wnt-dependent mesoderm differentiation, and conversely, on stiff substrate, hESCs showed β-catenin degradation and minimal differentiation (Przybyla *et al.*, 2016).

Hippo pathway, an evolutionarily conserved signalling pathway, was first identified in *Drosophila melanogaster* for regulating organ size and tumor progression (Sudol, 1994). The pathway is described in Section 1.3, but briefly, when Hippo signalling is off, that is in absence of upstream signal, the core kinases of Hippo pathway kinases MST1/2 and LATS1/2 along with their co-factors SAV1 and MOB1A/B respectively are unphosphorylated allowing the transcription co-activators YAP and TAZ to translocate into the nucleus and bind to the respective DNA binding protein to turn on the expression of target genes. Upon phosphorylation of Hippo core kinases by upstream signal, that is when Hippo is active, phosphorylated core kinases retain YAP/TAZ is into the cytoplasm. Over the past decade, extensive evidence has linked regulation of the Hippo pathway to mechanical forces through ECM modulation and cytoskeletal tension. Hippo is considered active under low mechanical stress, i.e., on soft substrates, high cellular density and on small adhesive area where cells are round; and Hippo is inactive under high mechanical stress, i.e., on stiff substrates, low cell density and on larger adhesive area which allows cell to spread.

One mechanism by which mechanical stimulus regulate Hippo pathway is through FAK-Src-PI3K pathway. Integrins bind to the fibronectin present in the ECM, which stimulate FAK, thereby activating Src and PI3K (phosphatidylinositol-3 kinase). Activated PI3K disrupts the core Hippo kinase proteins resulting in inhibition of Hippo signalling (Kim and Gumbiner, 2015; Fan *et al.*, 2013). Additionally, the influence of cytoskeleton on Hippo signalling was first observed in *Drosophila* where increased F-actin levels were associated with increased Yorkie activity (Sansores-Garcia *et al.*, 2011). A novel study in *Drosophila* has shown that the loss of actin capping proteins, which regulate actin polymerization, leads to accumulation of F-actin, reduced Hippo activity, and an increase in the expression of Yorkie target genes, which are involved in cell survival and proliferation (Fernández *et al.*, 2011). This relation between F-actin and Hippo signalling is also observed in mammalian cells (Aragona *et al.*, 2013). Disruption of actin stress fibers has shown to activate mammalian MST1/2, thereby preventing YAP nuclear localization (Densham *et al.*, 2009). Moreover, at low cell densities, cells appear

flat and spread over larger area, which promotes formation of F-actin stress fibers. The stress fibers inhibit YAP/TAZ phosphorylation by LATS, thereby promoting nuclear accumulation of YAP (Wada *et al.*, 2011).

Angiomotin (AMOT), a member of motin family of angiostatin binding proteins, has been identified as a link between F-actin and Hippo-YAP regulation. Unphosphorylated AMOT associates with F-actin, but upon phosphorylation by LATS, pAMOT binds to YAP/TAZ, thereby retaining YAP/TAZ in the nucleus (Mana-Capelli *et al.*, 2014). Remodelling of cytoskeleton by RhoGAPs, in response to extracellular signals, haven shown to regulate Hippo-YAP activity (Dupont *et al.*, 2011; Zhao *et al.*, 2012; Kim and Gumbiner, 2015; Mason *et al.*, 2019). Intriguingly, a study in MSCs reported that when cells are mechanically stretched, the force is transmitted to the nuclear envelope through the cytoskeleton, thereby directly regulating YAP translocation (Driscoll *et al.*, 2015). Hippo signalling is, therefore, highly sensitive to the changes in the extracellular microenvironment and cytoskeleton remodelling.

Importantly, the above described mechano-chemical conversions, and many more which are still unknown, take place simultaneously at several places within a cell or a tissue. It is, therefore, worthy to acknowledge that multiple signalling pathway are active during mechanotransduction and can converge onto a common mechanotransducer to induce gene expression and regulate cell fate.

2.5 Biomaterials as tool in Tissue Engineering

The ECM and the cells create a dynamic reciprocity, that is, through mechanical transduction the mechanical signal is converted to the biochemical signals, along with the changes brought by these biochemical signals for the cell to adapt to its physical microenvironment (Bissell and Aggeler, 1987; Thorne *et al.*, 2015). Dynamic reciprocity has been validated by development biology (von Dassow and Davidson, 2007; Mammoto and Ingber, 2010; Mammoto *et al.*, 2013; Biggins *et al.*, 2015), showing that mechanical force is as crucial to embryogenesis as biochemical signalling, transforming the view of the extracellular environment as a whole. *In vivo* mechanical signals due to stiffness variations in ECM is more relevant and widely studied because the biological stiffness of each tissue is unique **FIGURE 2.2**. However, with conventional cell culture methods, mimicking the interaction between the ECM and cells is challenging. Nonetheless,

researchers have tried to closely replicate the *in vivo* microenvironment by using various substrates instead of traditionally used plastic or glass plates.

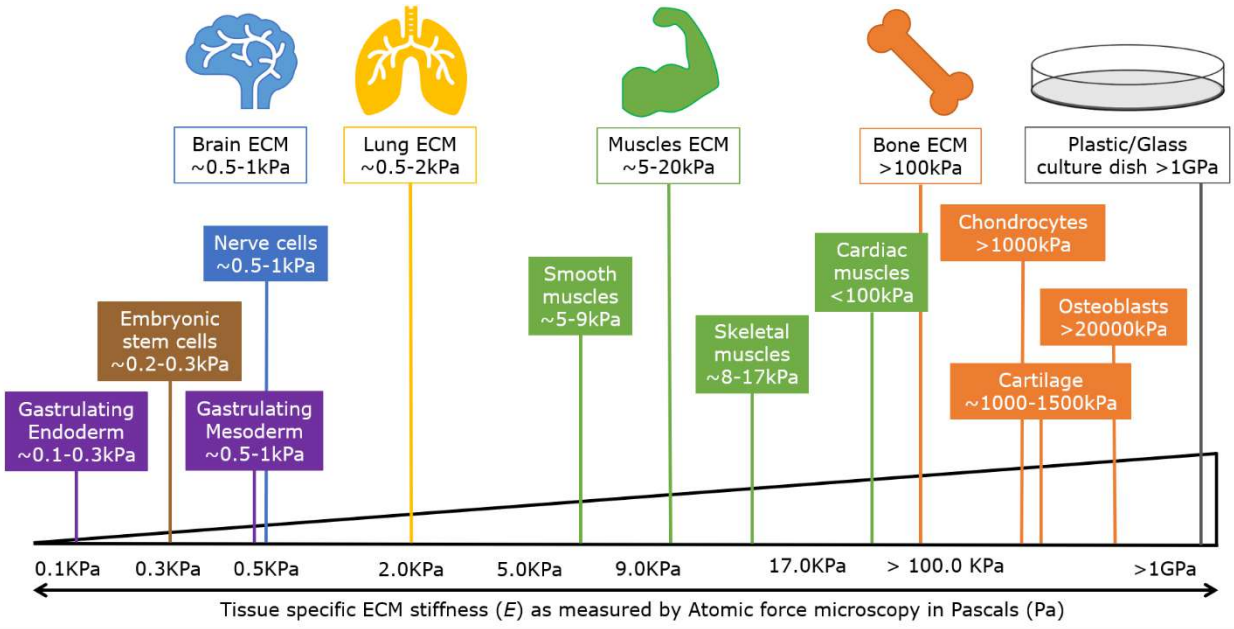


FIGURE 2.2: Diagrammatic representation of the range of biological stiffness of healthy tissues *in vivo.*

The traditionally used tissue culture treated plastic plates (TCP) or glass dishes have a stiffness of approximately 1 gigapascal (GPa), which is extremely stiff compared to the *in vivo* conditions. For sensitive cells, such as ESCs and iPSCs, TCP is coated with feeder cell layer or with ECM proteins such as: collagen, fibronectin, vitronectin or commercially available mixture of ECM proteins, Matrigel (Kleinman and Martin, 2005). However, these natural polymers do not mimic the biological stiffness of all the tissues. This disadvantage is overcome by using synthetic polymers as substrate such polyacrylamide (PA) gels, polydimethylsiloxane (PDMS), polyvinyl alcohol (PVA), polyethylene glycol (PEG) etc., in combination with a preferred adhesion ligand to provide attachment for the stem cells (Engler *et al.*, 2004; Goffin *et al.*, 2006; Muduli *et al.*, 2017; Gilbert *et al.*, 2010). Another approach is by using a mixture of nature and synthetic polymers, known as semi-synthetic polymers like gelatin methyl acrylate (GelMa), which combines biocompatibility of natural polymers and mechanical properties of synthetic substrates (Guilak *et al.*, 2009). The stiffness of synthetic substrates depends on the percentages of the polymers use and hence can be used to synthesis soft substrates (<1kPa), intermediate substrates (10-30kPa), and stiff substrate (>50kPa).

Pelham and Wang (1997) showed that the kidney epithelial and fibroblast cells cultured on PA gel substrate respond to the difference in the substrate flexibility by altering their adhesion structures and motile behaviour. In mESCs, soft synthetic substrates promoted expression of genes specific for endodermal lineage, *Sox17* and *Afp* (Jaramillo *et al.*, 2015), and stiff substrate promoted expression of mesodermal specific genes, specifically Brachyury (Evans *et al.*, 2009; Dado-Rosenfeld *et al.*, 2015). Also, soft hydrogel has shown to induce reprogramming of mouse fibroblast cells and hMSCs into iPSCs through activation of mesenchymal-to-epithelial transition (Choi *et al.*, 2016; Gerardo *et al.*, 2019).

hMSCs cultured on PA substrates of varying stiffness commit to phenotype corresponding to their biological tissue. hMSCs on soft substrates that mimics brain tissue's stiffness commits towards neurogenic lineage, substrates of intermediate stiffness mimicking muscle tissue commit towards myogenic lineage, and stiff substrates that mimic bone tissue commit towards osteogenic lineage (Engler *et al.*, 2004, 2006; Lanniel *et al.*, 2011; Sun *et al.*, 2018). Furthermore, muscle stem cells cultured on soft substrates that mimic the stiffness of muscle tissue, self-renew and efficiently differentiate into myocytes upon transplantation into mice (Gilbert *et al.*, 2010). Adult neural stem cells differentiate towards neurons when cultured on substrates favouring the physiological stiffness of brain tissue in presence of neuronal differentiation media. In contrast, relatively stiff substrates (~10kPa) promote glial-like cell culture (Saha *et al.*, 2008). The ability of bone marrow-derived hMSCs to self-renew and maintain multipotency is significantly enhanced when grown on a hydrogel that has an elastic modulus similar to bone marrow (Winer *et al.*, 2009).

Adding to this Zoldan *et al.* (2011) demonstrated that scaffolds engineered to mimic *in vivo* physiological stiffness can direct *in vitro* differentiation of hESCs into the three germ layers. Chen and colleagues reported that hiPSCs aggregate and differentiate towards cardiomyocytes best on surfaces containing flexible PDMS pillars of intermediate stiffness (E~9kPa) (Wang *et al.*, 2018). However, another research group have achieved long-term self-renewal of hPSCs cultured on relatively stiff PA-GAG substrate with stiffness of ~10kPa (Musah *et al.*, 2012), while, soft substrate (E ~ 0.7kPa) with differentiation-inducing media promoted neuronal-like phenotype and high expression of neuronal-specific protein marker tubulin β3 chain (TUJ1) (Musah *et al.*,

2014). Researchers found that hPSCs self-organized into spheres on electrospun nanofiber substrates rather than a flat colony as on TCP. They also maintained their stemness on stiff substrates but expressed more ectodermal markers, such as PAX6 and NEUROD1, on soft substrates (Maldonado *et al.*, 2015). Another study by the same group reported that neural induction of hPSCs is initially enhanced on soft substrate, but for further differentiation into neural progenitors and motor neurons stiff substrate is essential. Additionally, it was found that mesendodermal differentiation was enhanced on a stiff substrate, but further specification to the posterior foregut required a soft substrate based on the expression of MIXL1 and BRACHYURY (Maldonado *et al.*, 2017). According to Smith *et al.* (2017) the stiff PDMS substrate could modulate hiPSCs mesoderm differentiation kinetics. These contrary results in hPSCs indicates that dynamic changes of substrate stiffness have different effects on hPSCs than hMSCs and mESCs. Additionally, all studies report using differentiation-inducing media to culture stem cells on varying stiffnesses. It is, therefore, questionable whether stiffness or soluble molecules in the media facilitate differentiation.

Other than the substrate stiffness, the topology of the substrates, that is the geometric patterns on the substrate, has been experimentally proven to regulate cellular responses. This method consists of imprinting or creating islands of ECM proteins on a substrate of controlled size and shape, known as micropatterns. MSCs cultured on micro-islands of large and small area differentiate towards osteogenic and adipogenic lineage respectively by modulating RhoA activity (McBeath *et al.*, 2004). Whereas, when hPSCs are cultured on substrates with various micropatterns, biochemical induction and mechanical signals from cell shape and cytoskeletal contractile forces, direct the differentiation of hPSCs towards endothelial cells (Kusuma *et al.*, 2017), cardiomyocytes via WNT/ β-catenin pathway (Myers *et al.*, 2013; Ma *et al.*, 2015) or neurons via BMP/SMAD signalling (Xue *et al.*, 2018). We can, therefore, better understand stem cell behaviour *in vitro* using bioengineered substrates. More studies which have reported the effect of different stiffness and biomaterials on mature and stem cells are highlighted in **TABLE 2.1.**

Table 2.1: Stiffness-induced biological response in various cell types				
Biomaterial	**Stiffness**	**Cell type**	**Cell response**	**Reference**
Natural Polymer				
Collagen (3D culture)	Not determined	MSCs	Differentiation towards tenogenic lineage	Kuo and Tuan, 2008
Collagen (2D culture)	Not determined	MSCs	Differentiate into tenogenic, osteogenic and chondrogenic lineages	Nöth *et al.*, 2007; Chen *et al.*, 2008; Park *et al.*, 2022
Alginate (3D culture)	10-30 kPa	mMSCs	Differentiation towards osteogenic lineage due to traction-mediated reorganisation of adhesion ligands	Huebsch *et al.*, 2010
	180 Pa (soft) 20 kPa (stiff)	Neural stem cells	Effective proliferation and expression of neuronal markers on soft stiffness	Banerjee *et al.*, 2009
Alginate-Gelatin-laminin (3D culture)	5 kPa	hiPSCs	Differentiation towards neurogenic lineage	Distler *et al.*, 2021
Matrigel (3D culture)	34 Pa - 480 Pa (3D Matrigel)	Primary extravillous trophoblast	Cells showed migration towards stiff region and differentiated into endometrium and placental cells	Abbas *et al.*, 2019
Vitronectin	As coating on tissue culture plate	hESCs	Cell attachment, growth and proliferation	Braam *et al.*, 2008
Synthetic Substrates				
Variable moduli interpenetrating polymer network (vmIPNs)	10 Pa	Adult neural stem cells	No cell spreading, self-renewal and differentiation observed	Saha *et al.*, 2008
	>/= 100 Pa		Cell proliferation	
	500 Pa		Differentiation towards neurons	
	1-10 kPa		Differentiation towards glial cells	

Polyacrylamide (PA) gel substrate				
PA gel substrate coupled with type I collagen	0.1-1 kPa (soft) 8-17 kPa (intermediate) 25-40 kPa (stiff)	hMSCs	Differentiated into neural lineage Differentiated into myogenic lineage Differentiated into osteogenic lineage	Engler *et al.*, 2006
PA gel substrate coupled with type I collagen	1 Pa (softest) 10 Pa (soft) 130 Pa (relatively stiff)	hMSCs	hMSCs on relatively stiff substrates have great spread area, less mature FAs and differentiated into various lineages compared to cell on other substrates	Cameron *et al.*, 2011
PA gel coated with type I collagen **PA gel coated with fibronectin**	80kPa 25 kPa	hMSCs	Differentiated into osteogenic lineage Differentiated into myogenic lineage	Rowlands *et al.*, 2008
PA gel functionalized with GAG peptides	0.7 kPa (soft) 10 kPa (stiff)	hESCs and hiPSCs	Better attachment, self-renewal and maintains pluripotency Cells adopted neural morphology and after addition of neuronal maintaining media developed into mature neurons	Musah *et al.*, 2012; Musah *et al.*, 2014
PA gel substrate coupled with Matrigel	3 kPa (soft) 165 kPa (stiff)	hESCs and hiPSCs	On soft substrate which showed stiffness similar to liver tissue, the cells differentiated into endoderm lineage whereas not on stiff substrate	Chen *et al.*, 2020
Poly-dimethyl-siloxane (PDMS) gel substrate				
PDMS coated with polydopamine	Not determined	Bone-marrow stromal cells	Promoted differentiation into osteogenic lineage in presence of osteogenic differentiation media	Chuah *et al.*, 2015
PDMS coupled with type I collagen	1.9 MPa – 2.7 MPa (stiff)	mESCs	Cells expressed pro-osteogenic gene markers	Evans *et al.*, 2009

PDMS coated with type I collagen	3 kPa (soft) 37 kPa (stiff)	Cardiac fibroblasts, 3T3 fibroblast, hMSCs	Cells of all three-cell line showed increased cell spreading on stiff substrate whereas on soft substrate these cells manifest small spread area Stiff substrate promoted myofibroblast activation of cardiac fibroblast	Yeh *et al.*, 2017
Electrospun Nanofibrous substrate				
PCL fibrous substrate **PET fibrous substrate** **PEKK fibrous substrate** **PCU fibrous substrate** **(All the substrates are either treated with plasma or conjugated with collagen)**	19 kPa (softest) 39 kPa (soft) 74 kPa (stiff) 193 kPa (stiffest)	hiPSCs	hiPSCs exhibit round 3D colony morphology on softest/soft substrate, on stiff/stiffest substrate cells spread and have flattened morphology. Long-term culture on soft(est) substrate led to ectodermal differentiation, no change in cells on other substrates. After adding defined growth factors, stiff substrate promoted motor neurons and soft substrate enhanced posterior foregut specification	Maldonado *et al.*, 2015; 2016; 2017
Semi-Synthetic Substrates				
Gelatin methyl acrylate (GelMa)				
5% GelMa, 10% Gelma	Not determined	Rat MSCs	Cells differentiated into osteoblast when supplemented with osteogenic media	Celikkin *et al.*, 2018
Mineralised GelMa with functionalized PEGDA	Not determined	hiPSCs	Cells underwent osteogenic differentiation devoid of biochemical signals compared with non-mineralized GelMA	Kang *et al.*, 2014
Hyaluronic acid (HA)				
HA conjugated with RGD peptide	3 kPa (soft) 30 kPa (stiff)	hMSCs	Soft substrate promotes adipogenesis Stiff substrate promotes osteogenesis	Guvendiren and Burdick, 2012

2.6 Role of YAP as TF in Stem Cell Self-Renewal and Differentiation

Yes-associated protein (YAP) and its homologue TAZ were first identified as primary effectors of the Hippo pathway, and then as a potent oncogene. However, growing body of literature suggests a complex and multiple functions of YAP/TAZ. Studies have shown that other than Hippo pathway, YAP/TAZ is regulated by GPCR (G-protein coupled receptors) (Yu *et al.*, 2012), Notch (Totaro *et al.*, 2017; Totaro *et al.*, 2018), TGF-β (Szeto *et al.*, 2016) and Wnt (Azzolin *et al.*, 2014; Jiang *et al.*, 2020) signalling pathways. As reviewed extensively by many (Morgan *et al.*, 2013; Totaro *et al.*, 2018; Ma *et al.*, 2019), YAP/TAZ orchestrates various signalling molecules and integrates mechanical signals into multiple proliferation and differentiation controlling pathways **(FIGURE 2.3).**

During the early stages of preimplantation development, YAP is expressed in the blastomeres of the morula and blastocyst. YAP expression has been shown to be highest in the ICM, which gives rise to the embryonic tissues, compared to the TE, which gives rise to the placenta. At 8-cell stage, YAP is nuclear in all the blastomeres, and from 16-cell stage till mid blastocyst stage, YAP is nuclear only in the outer cells which will form trophectoderm (Nishioka *et al.*, 2009). In late blastocyst stage, epiblast cells express nuclear YAP (Hashimoto and Sasaki, 2019). Inhibition of YAP activity in preimplantation embryos has been shown to increase differentiation of TE cells at the expense of ICM cells, suggesting that YAP plays a key role in maintaining the balance between these two cell lineages (Lorthongpanich *et al.*, 2013; Mizutani *et al.*, 2022). YAP activity is associated with suppression of genes involved in anterior primitive streak formation, and loss of YAP has shown to generate anterior primitive streak cells that effectively differentiate towards meso-endoderm and endoderm lineage. Interestingly YAP activation or suppression has no effect on the genes associated with posterior primitive streak (Hsu *et al.*, 2018). In an interesting study, Yoon and colleagues introduced retroviral vector expressing YAP into the embryonic neural stem cell niche *in utero* of the mouse embryonic brain (E13.5). YAP infected cells enhanced neural stem cell self-renewal and proliferation activity *in vivo* as observed by Sox2 expression, neural stem cell marker (Han *et al.*, 2015). YAP function in stem cell fate determination varies depending on the developmental stage and the plasticity of the stem cell population. Stem cells in early developmental stages require YAP for self-renewal and pluripotency maintenance,

whereas mature or lineage-committed stem cells require YAP for lineage specification or tissue regeneration.

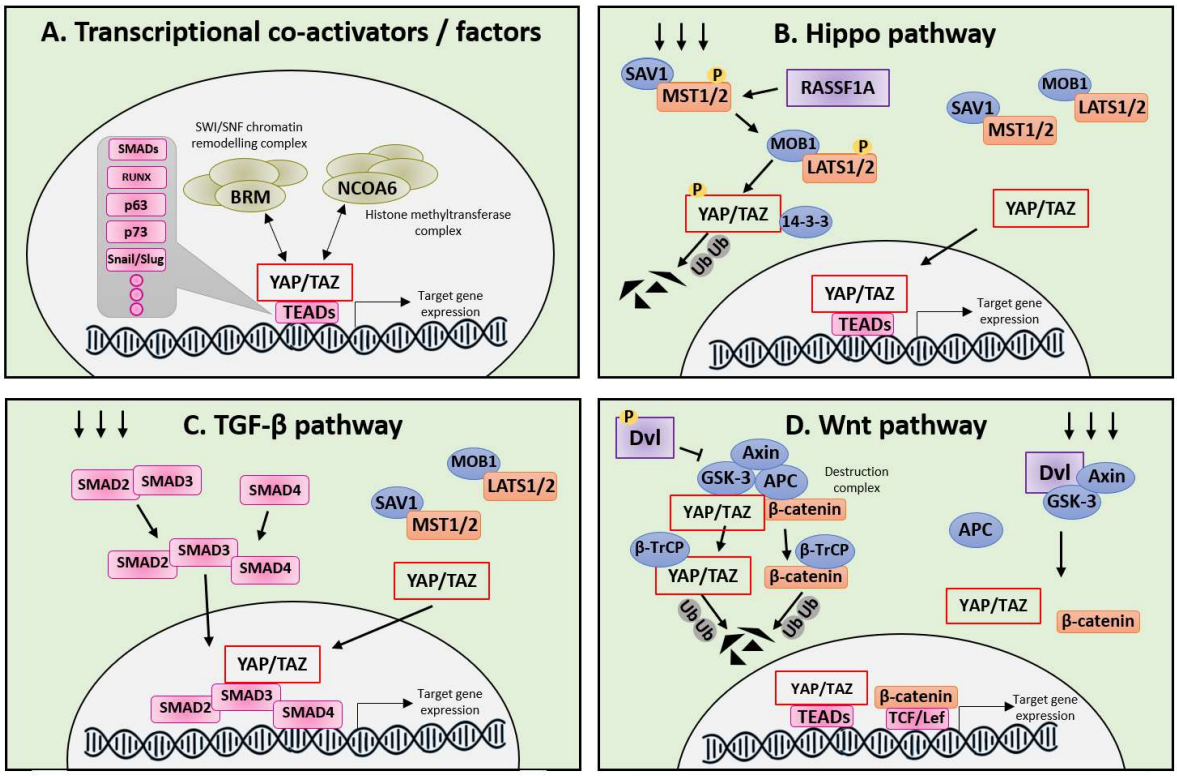


FIGURE 2.3: Nuclear and cytoplasmic YAP/TAZ regulation. (A) Transcriptional co-activators and factors. Nuclear YAP/TAZ bind to several transcriptional factors and recruits nuclear receptor coactivator 6 (NCOA6) and switch/sucrose nonfermentable (SWI/SNF) complex, which included Brahma (BRM) as one of the subunits. This assembly mediates TF target-specific gene expression. (B) Hippo pathway. Upstream signalling phosphorylates MST1/2, SAV1, LATS1/2 and MOB1/2 which leads to phosphorylation of YAP/TAZ and finally cytoplasmic retention by 14-3-3 protein and ubiquitination. The unphosphorylated state of core hippo proteins leave YAP/TAZ to translocate into the nucleus and bind to several TFs and respective DNA binding-proteins. (C) TGFβ signalling pathway. Upon activation of cell surface receptor, SMAD2/3 forms a complex with R-SMAD (SMAD4) and translocates into the nucleus, where nuclear YAP binds to SMAD2/3/4 complex and activates SMAD-mediated transcription. (D) Wnt signalling. In the absence of stimulus Wnt destruction complex which consists of Axin, GSK-3 (glycogen synthase kinase 3), APC (adenomatous polyposis coli) and cytoplasmic YAP binds to beta-catenin where the YAP and β-catenin undergo ubiquitin ligase β-TrCP mediated degradation. Wnt stimulation inhibits the destruction complex to degrade β-catenin and promote nuclear localization of β-catenin. Nuclear YAP forms a complex with beta-catenin resulting in targeted gene expression.

In mouse ESCs, YAP is highly expressed under normal culturing conditions (Ramalho-Santos *et al.* 2002), and downregulated at the onset of differentiation. Additionally, under differentiation conditions, ectopic YAP expression has shown to block mESCs differentiation, thus maintaining the pluripotency and stem cell phenotype, whereas silencing of YAP resulted in differentiation towards endoderm and mesoderm

lineages (Lian *et al.*, 2010). Subsequently, leukemia inhibitory factor (LIF) (Tamm *et al.*, 2011) and inter-α-inhibitor (IαI), serum component (Pijuan-Galitó *et al.*, 2014), were identified to activate YAP and thereby, induce Oct3/4 and Nanog expression in mESCs. Interestingly, YAP has been dubbed as a 'switch' between the pluripotency and differentiation in mESCs. It has been reported that upon mESCs differentiation, Gata1, a differentiation factor, promotes the expression of RASSF1A (Ras-association domain family 1A), a tumor suppressor and one of the upstream components of the Hippo pathway, which forms a complex with Mst1/2. This activates the Hippo pathway, leads to YAP phosphorylation, and reduces the interaction of YAP-TEAD with the Oct4 promoter (Papaspyropoulos *et al.*, 2018).

In hESCs, self-renewal and pluripotency are regulated by bFGF (basic fibroblast factor) signalling (Xu *et al.*, 2005) instead of LIF signalling as in mESCs. It is, therefore, possible that the findings from mESCs reports cannot necessarily be compared with hESCs. Nevertheless, studies have shown that like mESCs, hESCs also exhibit elevated YAP activity, which is crucial for their self-renewal, pluripotency and stem cell phenotype (Ohgushi *et al.*, 2015; Hsiao *et al.*, 2016; Estarás *et al.*, 2017). Ohgushi *et al.* (2015) demonstrated that cultured hESCs survive, self-renew and proliferate when YAP expression is maintained through AKAP-Lbc (A-kinase anchor protein-lymphocyte blast crisis)/Rho-GTPase/F-actin molecular cascade. Hsiao *et al.* (2016) shed light on density-dependent neuroepithelial differentiation of hESCs by knockdown of YAP. This provides evidence that at higher cell densities, YAP is phosphorylated and retained into the cytoplasm, as a result, expression of YAP-mediated genes, including regulators of pluripotency is impeded, and neuroepithelial differentiation is induced (Hsiao *et al.*, 2016). Estaras *et al.* (2017) uncovered an unexpected role of YAP as a selective inhibitor of hESCs differentiation into mesoderm. They show that YAP binds to *WNT3* enhancer and prevents its activation by Activin; whereas in the absence of YAP, Activin upregulates *WNT3* expression and facilitates cardiac mesoderm differentiation.

YAP's role in human pluripotent stem cell self-renewal and pluripotency is perhaps best demonstrated through the reprogramming of adult somatic cells into iPSCs. A novel method showed that overexpression of only two reprogramming factors OCT4 and SOX2; instead of the four-reprogramming factor (Takahashi and Yamanaka 2006); along with ectopically expressed YAP, induced reprogramming of human amniotic

epithelial cells into iPSCs (Zhao *et al.* 2017). A study published in 2012, Qin *et al.* showed that knockdown of LATS2, an important component of the Hippo pathway that facilitates YAP phosphorylation and retention in the cytosol, permitted more efficient reprogramming of human somatic cells. Furthermore, another study by the same group showed that YAP overexpression in hESCs and hiPSCs promotes a naive state, which represent a pre-implantation stage of development that is difficult to mimic and sustain in vitro (Qin *et al.*, 2016).

In *MSCs*, YAP has been reported to play a crucial role in self-renewal and differentiation. Interactions between YAP and another TF Snail/Slug has shown to regulate self-renewal and differentiation of bone marrow derived MSCs (Tang *et al.*, 2016; Tang and Weiss, 2017). Moreover, increased YAP expression, either by a pharmacological activator or genetic manipulation, has shown to induce osteogenic differentiation of hMSCs and repress adipogenic differentiation despite culturing MSCs in adipogenic differentiation inducing media. Conversely, YAP inhibition enhanced adipogenic differentiation and supressed differentiation towards osteoblasts (Lorthongpanich *et al.*, 2019). Similarly, it has been demonstrated that YAP is crucial for regulating differentiation of hMSCs into neural crest stem-like cells (NCSCs). Knockdown of YAP expression by transfection significantly downregulated the expression of NCSC-related genes, including NESTIN, FOXD3, SLUG and SOX9 (Zhang *et al.*, 2018). YAP also plays a pivotal role in maintaining the neural stem cells (NSCs) like characteristics, i.e., formation of neurospheres, and enhanced self-renewal and proliferation capacity of NSCs *in vivo* and *in vitro* through its association with TEAD (Han *et al.*, 2015), and induces differentiation into functional glial cells through its interaction with SMAD1/5/8-BMP signalling (Huang *et al.*, 2016). In addition to the above-mentioned studies, YAP has been described in other studies as being crucial to self-renewal and differentiation of tissue-specific stem cells and other cells (reviewed in Mo *et al.*, 2014; Heng *et al.*, 2020).

As stated above, YAP activity is influenced by the integrations of multiple signalling pathways and thus interacts with various transcriptional factors. This results in different levels of YAP activation and target gene expression. To summarise the above section, YAP expression regulates pluripotency markers OCT4 and NANOG; thus, maintaining the pluripotency and self-renewal in mouse and human PSCs, whereas, YAP downregulation, either by knockdown or inhibition by pharmacological molecule, so far

has shown to initiate differentiation in both mouse and human PSCs. However, in MSCs, YAP causes differentiation towards adipogenic or neurogenic lineages, and its downregulation leads differentiation towards osteogenic lineage. Therefore, from the above reports we can say that the YAP activity is modulated by the presence of lineage-specific transcription factors that interact with YAP in a cell type-specific manner.

2.7 YAP as a Mechanotransducer

Numerous studies in stem cells and progenitors of mature cell types have shown that YAP expression can be modulated by engineered biomaterials (Brusatin *et al.*, 2018). YAP activity is controlled by cell shape, substrate stiffness, substrate topology and by shear fluid flow as overviewed in **FIGURE 2.4**. A study by Duport *et al.* (2011) was the first to identify YAP as nuclear transducers of mechanical signals generated due to the ECM rigidity and cell shape. Their work showed that stiff substrate, large adhesive areas and high contractile forces activate YAP and promote nuclear localization, which facilitates proliferation of primary mammary epithelial cells (MECs) and differentiation of MSCs towards osteogenic lineage. Conversely, YAP is inactive and retained in cytoplasm on soft substrate, small adhesive area and under low contractile forces, causing apoptosis of MECs and differentiation of MSCs towards adipogenic lineage. Since then, several studies have shown that subcellular localization of YAP is regulated by substrate stiffness and topology (Halder *et al.*, 2012; Musah *et al.*, 2014; Yamazaki *et al.*, 2021) which controls actin cytoskeleton remodelling (Wada *et al.*, 2011; Zhao *et al.*, 2012; Piccolo *et al.*, 2014); or by cell stretching (Aragona *et al.*, 2013).

The mechanosensing through YAP involves detection of mechanical forces at ECM-integrin and cell-cell adhesion sites, which activates FA downstream proteins such as vinculin, talins, and FAKs, and interconnect F-actin and RhoA with Hippo core proteins (Dupont *et al.*, 2011; Aragona *et al.*, 2013; Nardone *et al.*, 2017). YAP mechanotransduction requires specific organisation of actin cytoskeleton, specifically F-actin. Inhibiting F-actin has shown to abolish YAP activity (Wada *et al.*, 2011; Dupont *et al.*, 2011; Zhao *et al.*, 2012; Piccolo *et al.*, 2014); as revealed by the effects of F-actin capping proteins (Sansores-Garcia *et al.*, 2011; Fernández *et al.*, 2011; Aragona *et al.*, 2013) and F-actin regulatory proteins (Mana-Capelli *et al.*, 2014; Heng *et al.*, 2021). Additionally, Rho signalling is also essential for regulating YAP (Dupont *et al.*, 2011; Wada *et al.* 2011, Zhao *et al.*, 2012; Aragona *et al.*, 2013; Ohgushi *et al.*, 2015; Nardone *et al.*,

2017). YAP activation on stiff substrates can also be by myosin light chain kinases, non-muscle myosin type II (NMII) and ROCK inhibitors (Dupont *et al.*, 2011; Guo *et al.*, 2018). Blocking NMII and Rho activities by specific inhibitors, significantly downregulates the YAP expression (Zhang *et al.*, 2018).

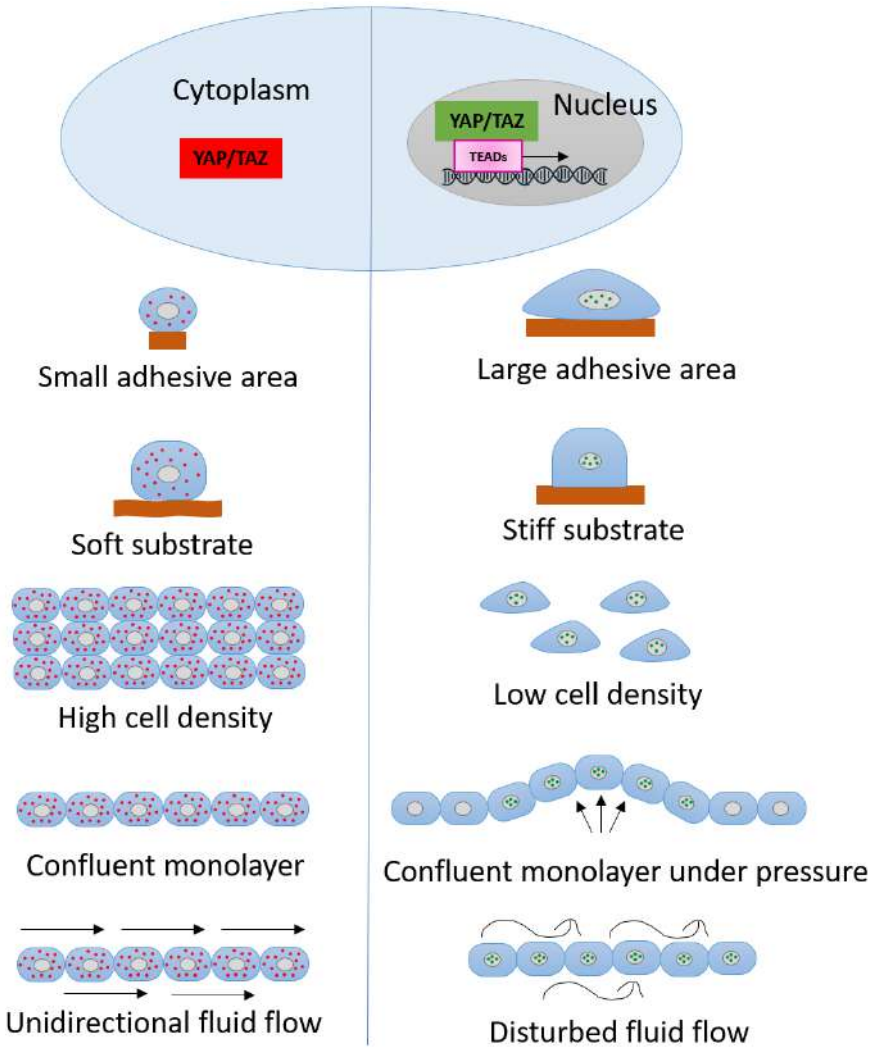


FIGURE 2.4: Schematic representation of different mechanical stimuli such as substrate stiffness, geometry, area, shear fluid flow and different physical conditions influencing YAP/TAZ regulation. The left panel illustrates conditions when YAP/TAZ is inactive (red) and retained in the cytoplasm, whereas the right panel depicts the conditions which promotes nuclear localization of YAP/TAZ (green). (Adapted from Panciera *et al.*, 2017).

In MSCs, many studies have reported that bioengineered substrate with high elastic modulus increases YAP activity and nuclear localization, thereby inducing differentiation into osteogenic lineage (Dupont *et al.*, 2011; Yang *et al.*, 2014; Driscoll *et al.*, 2015; Yuan *et al.*, 2016). In contrast, on soft substrate having low elastic modulus, YAP is phosphorylated and retained in the cytoplasm, resulting in MSCs differentiation into

adipogenic lineage (Dupont *et al.*, 2011; Yang *et al.*, 2014; Oliver de la Cruz *et al.*, 2019). Studies using hPSCs cultured on engineered biomaterials in presence of soluble signalling molecules have shown that stiff substrates promote YAP nuclear localization and self-renewal (Beyer *et al.*, 2013; Musah *et al.*, 2014; Ohgushi *et al.*, 2015). Conversely, soft substrate has shown to inhibit YAP expression which promote hPSCs differentiation towards neurogenic lineage (Musah *et al.*, 2012, 2014; Sun *et al.*, 2014; Ohgushi *et al.*, 2015; Hsiao *et al.*, 2016). The **TABLE 2.2** summarises the biological responses initiated by YAP under mechanical signalling in different cell. Activation of YAP due to various mechanical stimuli, in all the mature cell type leads to proliferation, in hESCs causes self-renewal and in MSCs induces osteogenic differentiation. Whereas, cytoplasmic localization of YAP due to various mechanical stresses either causes differentiation or apoptosis in mature and stem cells respectively. Therefore, the factors such as extracellular matrix composition, stiffness, the dimensionality of the microenvironment, the generation of contractile force and mechanical stress leads to a context-dependent function of YAP.

TABLE 2.2: Biomaterials and microfabrication-induced biological effects of YAP regulation

Cell type	Biomaterial/ mechanical stimuli	YAP 'ON'	YAP 'OFF'	Reference
hESCs	2D hydrogels	Self-renewal	Neurogenic differentiation	Musah *et al.*, 2014; Sun *et al.*, 2014
MSCs	2D hydrogels Stretching devices Photodegradable hydrogels Micro- and nanopillars Microfluidic devices Single cell micro-pipetting	Osteogenic differentiation	Adipogenic differentiation	Dupont *et al.*, 2011; Zhong *et al.*, 2013a, b; Tang *et al.*, 2013;
Endothelial cells	Microfluidic devices Single cell micro-pipetting	Proliferation	Apoptosis	Dupont *et al.*, 2011; Bertero *et al.*, 2015; 2016
Vascular smooth muscles cells	2D hydrogels	Proliferation	Apoptosis	Bertero *et al.*, 2015

Epithelial cells	2D hydrogels Stretching devices Micro- and nanopillars Patterning of cellular sheets Single cell micro-pipetting	Proliferation	Contact inhibition of proliferation or anoikis	Aragona *et al.*, 2013; Torato *et al.*, 2017;
Cardiac progenitors	3D hydrogel	Proliferation and cardiomyocytes differentiation	NA	Mosqueira *et al.*, 2014
Fibroblasts	2D hydrogels	Fibrosis	NA	Bertero *et al.*, 2015; Liu *et al.*, 2015
Hepatic stellate cells	2D hydrogels	Activation to myofibroblasts	NA	Caliari *et al.*, 2016; Mannaerts *et al.*, 2015
Somatic stem cells	2D hydrogels	Proliferation and self-renewal	NA	Gjorevski *et al.*, 2016; Panciera *et al.*, 2016; Hu *et al.*, 2017
Basal keratinocytes	2D hydrogels Single cell micro-pipetting	NA	Differentiation	Totaro *et al.*, 2017

Taken together, the diverse functions of YAP in different stem cells, in presence or absence of mechanical stress, arise from the intricate interplay between various signalling pathways, lineage-specific transcriptional factors, cellular context and the developmental stage of the stem cell population. Understanding these factors is crucial for deciphering the precise mechanisms underlying YAP role in stem cell biology and for harnessing its potential for regenerative medicine applications.

Chapter Three

Lacunae/Rationale

Since the past three decades, intense research has unravelled many biochemical pathways involved in signal transduction which control cell behaviour. We are also beginning to understand the cascade of protein-protein interactions and various gene expressions that regulate cell behaviour. It is now established that chemical signals alone do not regulate cell behaviour, especially from research on cancer cells has shown that physical cell-cell contact also affects gene expression (Whitfield *et al.*, 2002; Kamińska *et al.*, 2015; Sato *et al.*, 2021). Since cancer cells and stem cells share several similarities, hence we can assume that cell-cell physical contact would dictate gene expression in stem cells too (Shackleton, 2010; Riggs *et al.*, 2013). Mechanical signals and tissue architecture; which includes stiffness and topology of the ECM, interactions between ECM-cell, cell-cell adhesion, organization of the cytoskeletal network and tensile forces which maintains the integrity of the cell and tissue; also plays a crucial role in cell proliferation, differentiation, adhesion, migration, stem cell fate and apoptosis (McBeath *et al.*, 2004; Gupton and Waterman-Storer, 2006; Lock *et al.*, 2008; Wang and Pelling, 2012; Ankam *et al.*, 2013; Dado-Rosenfeld *et al.*, 2015; Vitillo *et al.*, 2016; Abagnale *et al.*, 2017; Sun *et al.*, 2018). Additionally, mechanical signals are now known to play a crucial role in embryogenesis and development of various tissues and organs. This has led to an increased interest in understanding the influence of mechanical signals on the differentiation of stem cells, specifically pluripotent stem cells (PSCs), including induced pluripotent stem cells (iPSCs) and human embryonic stem cells (hESCs).

The ability of the hPSCs to give rise to all the cell types present within an organism makes them an ideal tool in regenerative medicine and tissue engineering. However, because of this remarkable plasticity a major challenge associated with the use of ESCs and iPSCs is the difficulty to control the efficiency of their differentiation into specific cell types. To overcome this difficulty, many research groups have devised artificial microenvironment resembling the *in vivo* microenvironment to control stem cell differentiation. Stiffness of the substrate on which cells are cultured is major contributor of the mechanical signalling. A benchmark study by Engler and colleagues reported that naïve MSCs can be reprogrammed to neurogenic, myogenic and osteogenic lineages when cultured on substrates mimicking the stiffness of biological tissue, with the addition of soluble induction factors (Engler *et al.*, 2006). Similar study from Cooper-White lab showed that substrate stiffness coupled with appropriate ECM protein ligands in presence of differentiation inducing medium can direct the osteogenic and myogenic

differentiation of MSCs (Rowlands *et al.*, 2008). Numerous studies have demonstrated that substrate stiffness controls the differentiation of MSCs, yet paradoxically, all the studies have used known inducers soluble molecules to induce lineage-specific induction (Li *et al.*, 2010; Shih *et al.*, 2011; Park *et al.*, 2011; Lanniel *et al.*, 2011; Vincent *et al.*, 2013; Sun *et al.*, 2018; Yamazaki *et al.*, 2021).

There is limited literature highlighting the interplay between the substrate stiffness and human PSCs. Few studies which have used PSCs as culture model have used biochemical signalling along with mechanical stimulus. For instance, in hESCs, substrate stiffness in combination with soluble molecules help maintain self-renewal on stiff substrate and caused neurogenic differentiation on soft substrate (Maldonado *et al.*, 2015). Additionally, hPSCs on soft substrate differentiate into neuroectoderm in presence of differentiation inducing medium (Hindley *et al.*, 2016). However, no study has shown the interaction between hPSCs and substrate stiffness in absence of differentiation inducing medium. Understanding the interaction between hPSCs and stiffness is important because hPSCs are being used in many stem-cell based therapy especially in the light of new technologies such as 3D bioprinting and tissue engineered biomaterials. Also, by controlling the stiffness of the substrates we may have better control over the stem cell fate in bioartificial systems.

Furthermore, seminal work from the Piccolo lab identified Hippo signalling pathway effector YAP as the master regulator of mechanotransduction in MSCs (Dupont *et al.*, 2011). While the role of YAP as transcriptional coactivator in regulating organ growth during development and disease is well characterized, its role as mechanosensor is only beginning to be understood. Numerous studies have shown that YAP modulates the differentiated and undifferentiated state of MSCs on substrates of different stiffness through its interaction with various downstream signalling pathways and transcription factors, but in combination with inducing factors (Dupont *et al.*, 2011; Azzolin *et al.*, 2014; Driscoll *et al.*, 2015; Caliari *et al.*, 2016; Smith *et al.*, 2017; Totaro *et al.*, 2017; Oliver-De La Cruz *et al.*, 2019; Yamazaki *et al.*, 2021; Li *et al.*, 2022). Similarly, in regards to hPSCs, it was found that on stiff substrate YAP promotes pluripotent state of the hPSCs, whereas on soft substrate YAP is inactive which induces hPSCs differentiation towards neurogenic lineage, again, in presence of soluble differentiation factors (Musah *et al.*, 2012, 2014; Sun *et al.*, 2014; Maldonado *et al.*, 2015, 2016, 2017).

Thus, from the literature, we can say that there are several unknowns that need to be investigated with respect to YAP activity in human pluripotent stem cells, such as - expression levels and localization of YAP in undifferentiated and differentiated cells, activity of YAP in germ lineages other than ectoderm, expression of YAP in embryoid bodies in suspension culture which has been shown to promote ectoderm differentiation, how does hPSCs differentiate on stiff matrix and how is the activity of YAP affected during the process, does inhibition of YAP activity affect pluripotency or differentiation capacity of stem cells, how antagonistic signalling pathways affect YAP activity and the subsequent effect on fate of stem cells.

Our primary aim was to investigate the interaction between hESCs and substrate stiffness during their differentiation; and to understand if this interaction has any effect on the localization of YAP. Our study focused on the YAP expression during the differentiation of hESCs into endoderm lineage in response to substrate stiffness. We modulated the levels of YAP in differentiated cells cultured on substrates of varying stiffness and investigated whether the altered levels of YAP have any effect on the cell fate.

Chapter Four

Aim and Objectives

AIM of our project:

To study the activity of YAP and TAZ during differentiation of human pluripotent stem cells into endoderm and mesoderm lineages in response to different substrate stiffness.

To achieve this aim, we divided the study into the following three **OBJECTIVES**:

1. Investigate the expression of phosphorylated and non-phosphorylated YAP/TAZ in undifferentiated human pluripotent stem cells on substrates of different stiffness
2. Expression of phosphorylated and non-phosphorylated YAPZ/TAZ in hPSCs during endoderm differentiation on the substrates of different stiffness.
3. Effect of pharmacological inhibitor/activator of YAP/TAZ on hPSCs during endoderm lineage differentiation on substrates of different stiffness.

Chapter Five

Results

5.1 Characterization of Human Embryonic Stem Cells (hESCs) on TCP

The human embryonic stem cells line, KIND1 cells, was cultured on traditional tissue culture treated plastic plates (TCP) in xeno-free and feeder-free conditions with Essential 8™ medium with supplements. The plates were coated with 1X Vitronectin prior to the seeding and the cells were maintained in culture for five days **(FIGURE 5.1)**. [Note: TCP is considered as the stiffest substrate and the hESCs cultured on TCP is considered as control throughout the thesis].

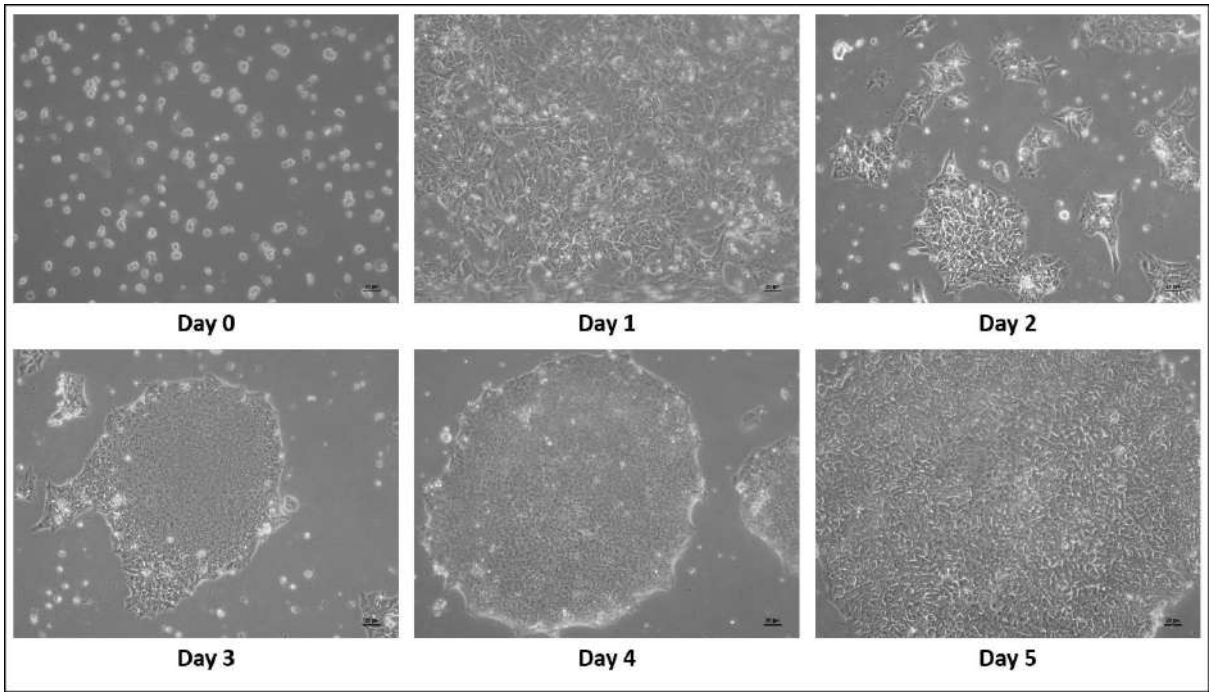
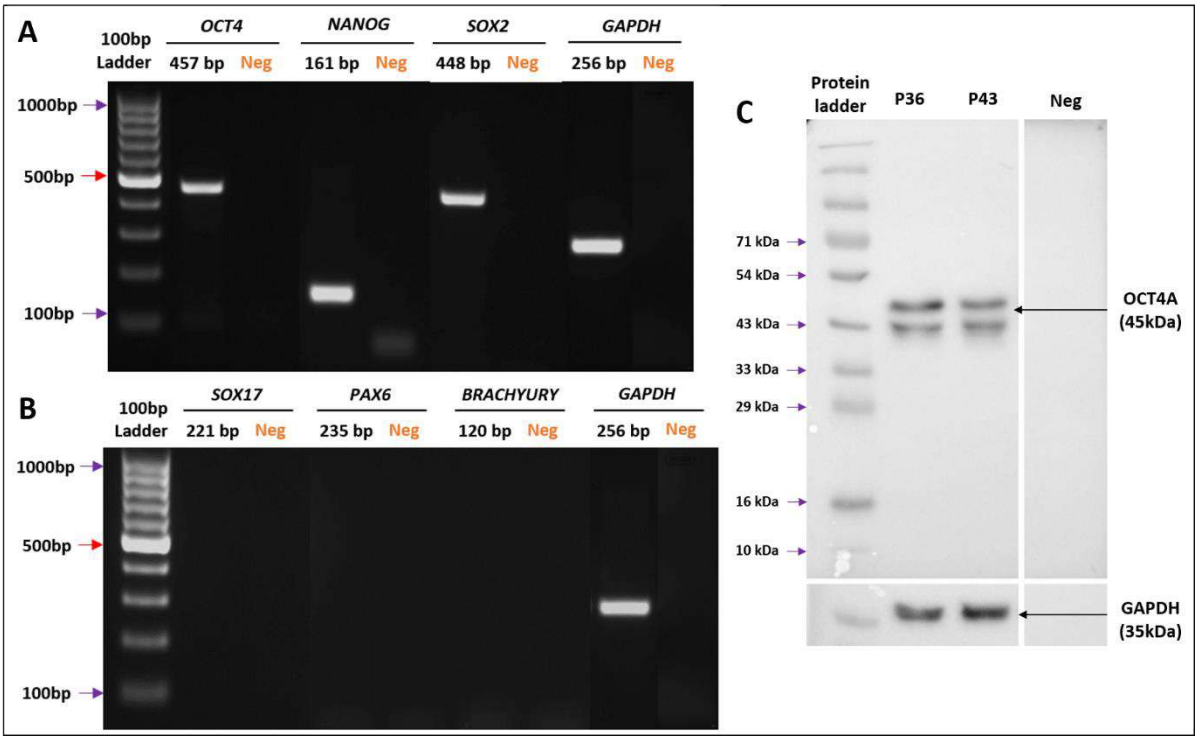


FIGURE 5.1: Phase-contrast images of the undifferentiated hESCs cultured on TCP at various days post passaging. KIND1 cells cultured on vitronectin coated culture dishes showed compact colonies a characteristic of undifferentiated pluripotent stem. The cells showed epithelial morphology with high nucleus to cytoplasm ratio. Scale bar, 20µm.

To check the pluripotency of the KIND1 cells, transcription levels of core pluripotency markers *OCT4, NANOG* and *SOX2* was checked by endpoint/reverse transcription polymerase chain reaction (RT-PCR) **(FIGURE 5.2A)**. We also checked the KIND1 cells for the expression of lineage specific markers: *SOX17* (endoderm), *PAX6* (ectoderm) and *BRACHYURY* (mesoderm) **(FIGURE 5.2B)**. The expression of the pluripotency markers and absence of the lineage-specific markers indicated an undifferentiated state of the KIND1 cells on TCP. Additionally, we also saw the levels of OCT4 by immunoblotting **(FIGURE 5.2C)**. Nuclear localization of OCT4 was seen in

KIND1 by immunofluorescence **(FIGURE 5.2D)**. These results further confirmed that the KIND1 cells cultured on TCP were undifferentiated.



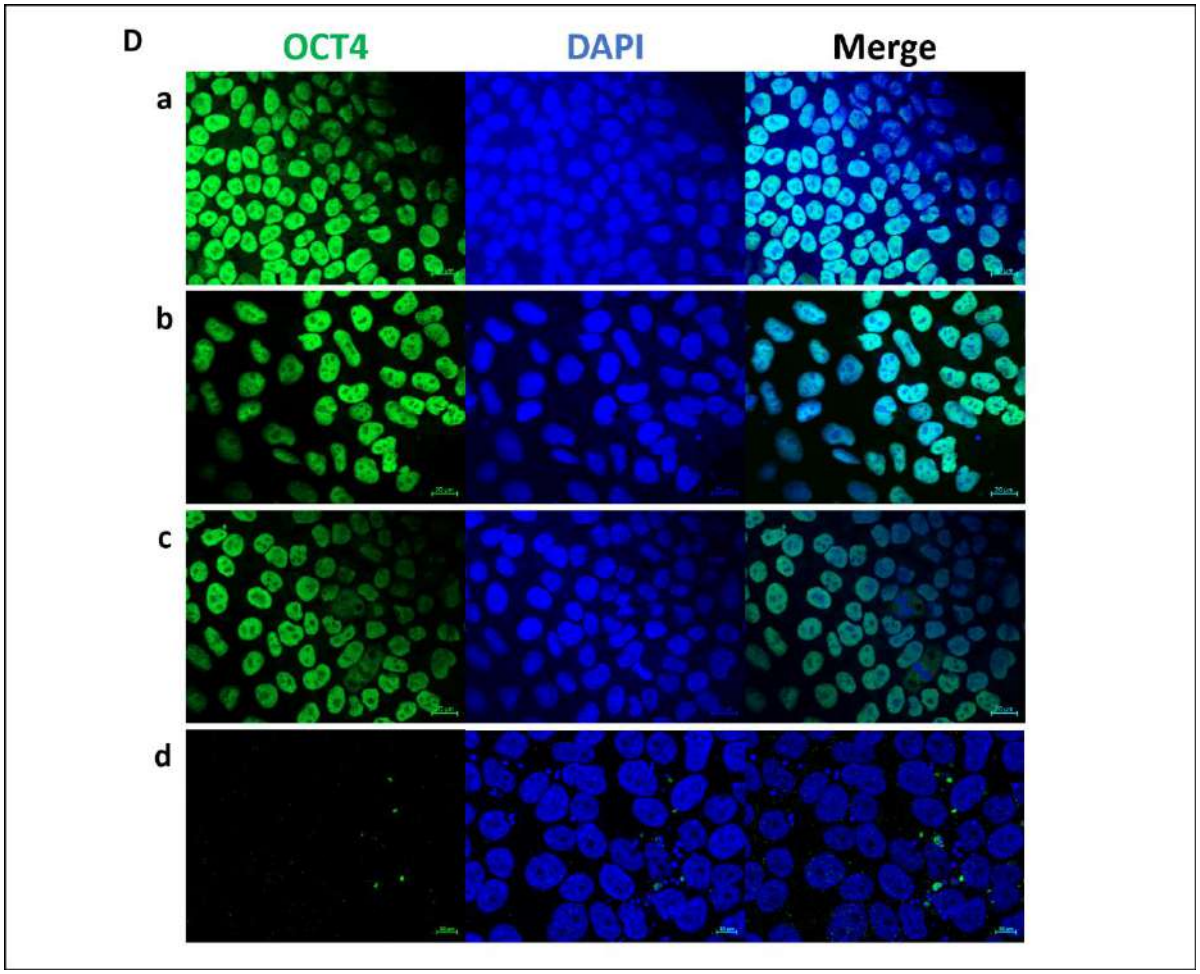


FIGURE 5.2: Characterization of undifferentiated KIND1 cells by RT-PCR, Immunoblotting and Immunofluorescence: The RT-PCR products were run on 2% agarose gel by electrophoresis, with ethidium bromide for imaging. **(A)** Pluripotency markers *OCT4*, *NANOG* and *SOX2,* and **(B)** lineage specific markers *SOX17* (ectoderm), *PAX6* (ectoderm) and *BRACHYURY* (mesoderm) was examined. *GAPDH* was used as internal standard control and a no temple control (NTC/Neg) was used as a negative control to check for any contamination. The bands of each PCR product were assessed using 100bp DNA ladder. **(C)** Immunoblotting of OCT4 was seen through various passages (P34 and P43) and GAPDH was used as a loading control. The negative control, where the protein samples were treated with only secondary antibody, was used for the possibility of any non-specific bands due to secondary antibody. **(D)** Immunofluorescence showed the expression of OCT4 (green) in the nucleus (a-c). A negative control, where the cells were treated with only secondary antibody, was used for the possibility of any non-specific bands due to secondary antibody (d). The nucleus was counterstained with DAPI (blue). Scale bar, 20µm.

5.2 Assessing Suitable Substrate for KIND1 Culture

To study substrate-induced changes in hESCs, we used various substrates such as GelMA, polyacrylamide (PA) hydrogels and ultra-low attachment dishes (ULAD). The GelMA and PA hydrogels were synthesised by following already established protocols and coupled with 1X Vitronectin **(Chapter 9, Section B)**.

5.2.1 Culturing KIND1 cells on GelMA

The KIND1 cells cultured on 10% GelMA substrate did not adhere to the hydrogel and aggregated to form clumps. Complete cell death was observed on day 2 of the culture **(FIGURE 5.3)**. The GelMA substrate was found incompatible as a biomaterial for KIND1 cells.

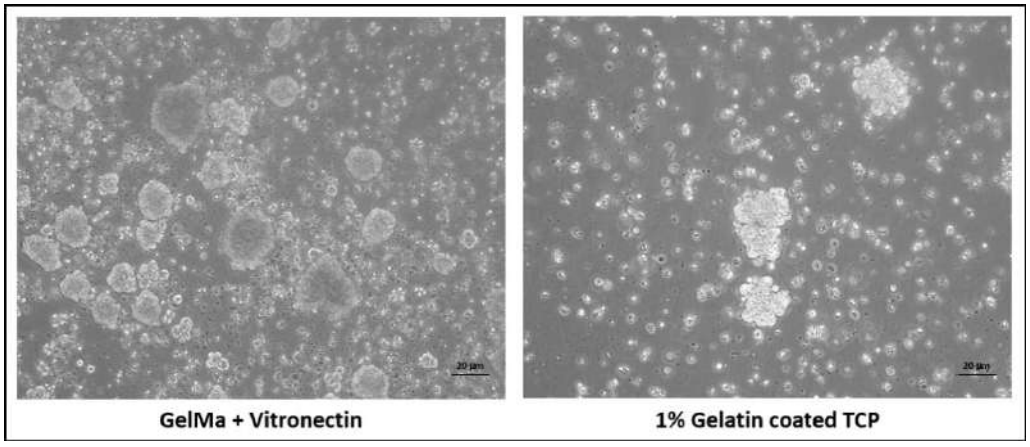
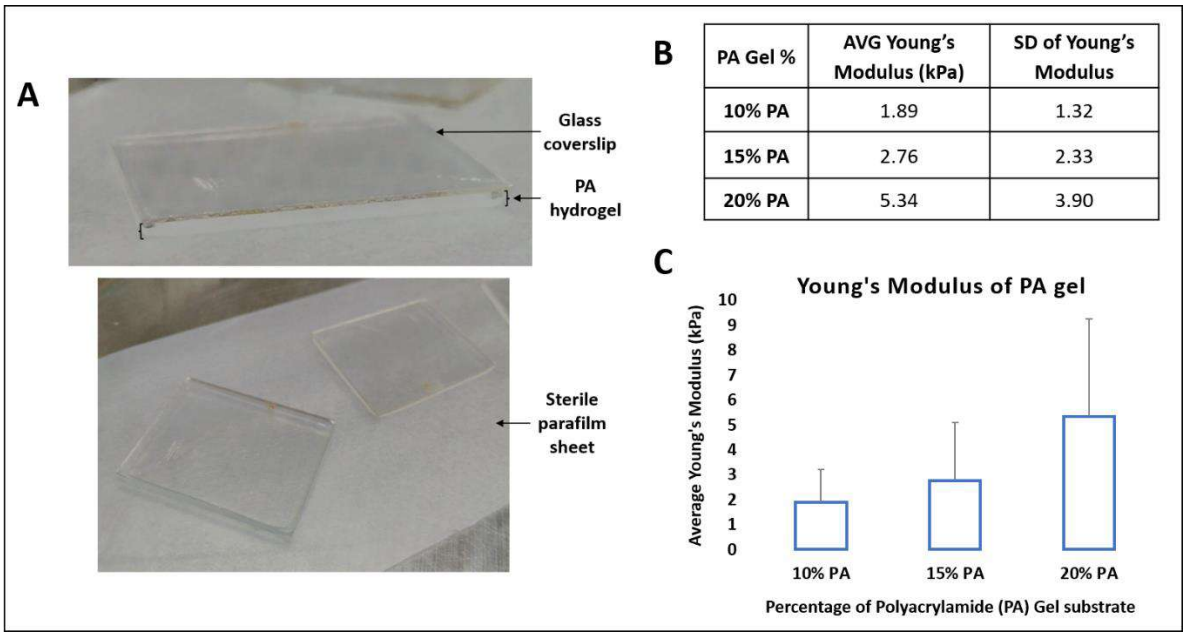


FIGURE 5.3: Phase contrast images of KIND1 cells cultured on 10% GelMA hydrogel substrate coupled with vitronectin and Gelatin coated TCP. The hESCs cultured on GelMA hydrogel aggregated to form cell clumps. The supernatant containing cell clumps from GelMA hydrogels when seeded on 1% Gelatin coated TCP, dead and floating cells were observed. Scale bar, 20µm.

5.2.2 Characterization of the Polyacrylamide (PA) Hydrogels

Since MSCs have been shown to undergo stiffness-dependent differentiation on PA hydrogels (Engler *et al.*, 2004, 2006; Dupont *et al.*, 2011), we synthesized PA hydrogels of various percentages: 10%, 15% and 20% using 30% acrylamide solution **(FIGURE 5.4A)**. The Young's modulus/stiffness of the hydrogels was determined by atomic force microscopy (AFM). The stiffness of the 10% PA hydrogel was calculated to be 1.9kPa, 15% PA hydrogel had stiffness of 2.7kPa and 20% PA hydrogel had stiffness of 5.3kPa **(FIGURE 5.4B, C)**.



PA Gel %	AVG Young's Modulus (kPa)	SD of Young's Modulus
10% PA	1.89	1.32
15% PA	2.76	2.33
20% PA	5.34	3.90

FIGURE 5.4: (A) Image of synthesized PA gel onto a sterile 22mm glass coverslip. **(B)** Young's modulus for the 10%, 15% and 20% PA hydrogel was determined by AFM and an average of 50 reading of each percentage PA substrate with their standard deviation (SD) was calculated. **(C)** Graph depicts the Young's modulus of PA gels of 10%, 15% and 20% hydrogel was plotted, the error bars represent the SD.

5.2.2.1 Culture and Characterization of hESCs on PA Hydrogel

KIND1 cells cultured on TCP were passaged onto PA hydrogels in complete Essential 8™ under normal culture conditions. The PA hydrogels supported cell morphology and colony characteristics better than the GelMA hydrogels. Few colonies were observed scattered around the edges of the hydrogel, while majority of the cells aggregated to form clumps **(FIGURE 5.5A)**. When compared to the TCP, few cells adhered to PA hydrogels. Therefore, to check for biocompatibility of the PA substrates, we cultured human colorectal adenocarcinoma cell line, HT29 cells, on TCP and 20% PA

hydrogel. We observed that compared to the KIND1 cells, HT29 cells showed better adherence and proliferation on 20% PA hydrogel **(FIGURE 5.5B)**.

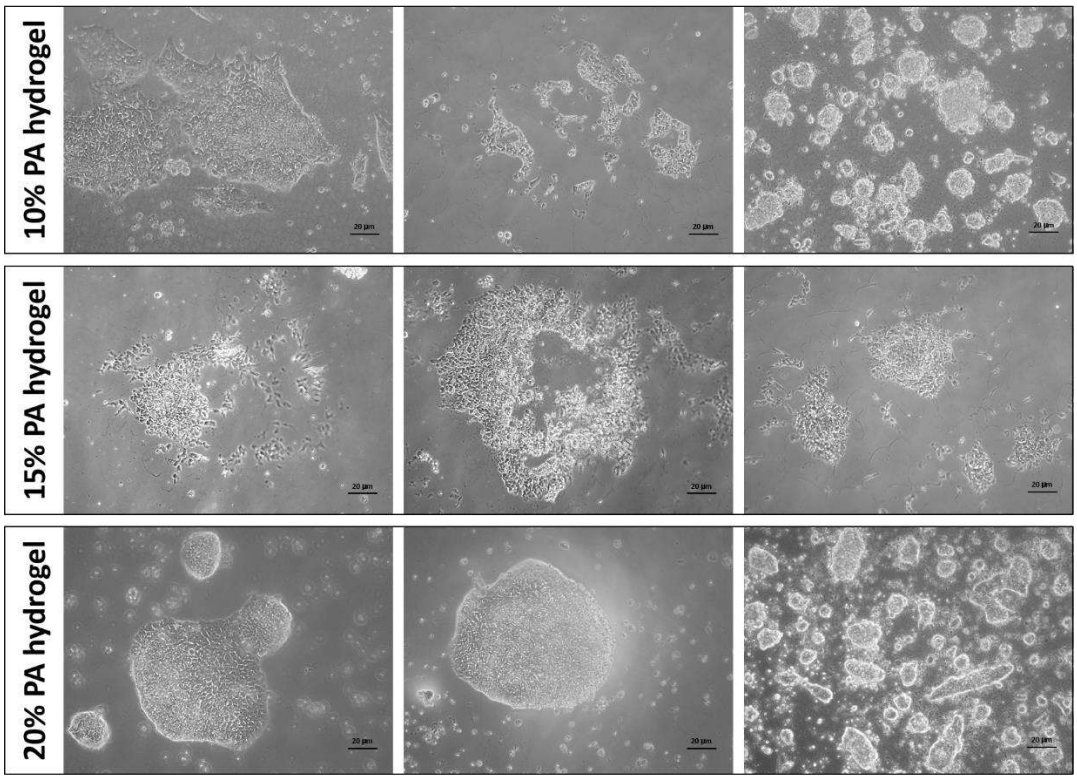


FIGURE 5.5 A: Phase contrast images of KIND1 cells cultured on 10%, 15% and 20% PA hydrogel substrate coupled with vitronectin. Scale bar, 20µm.

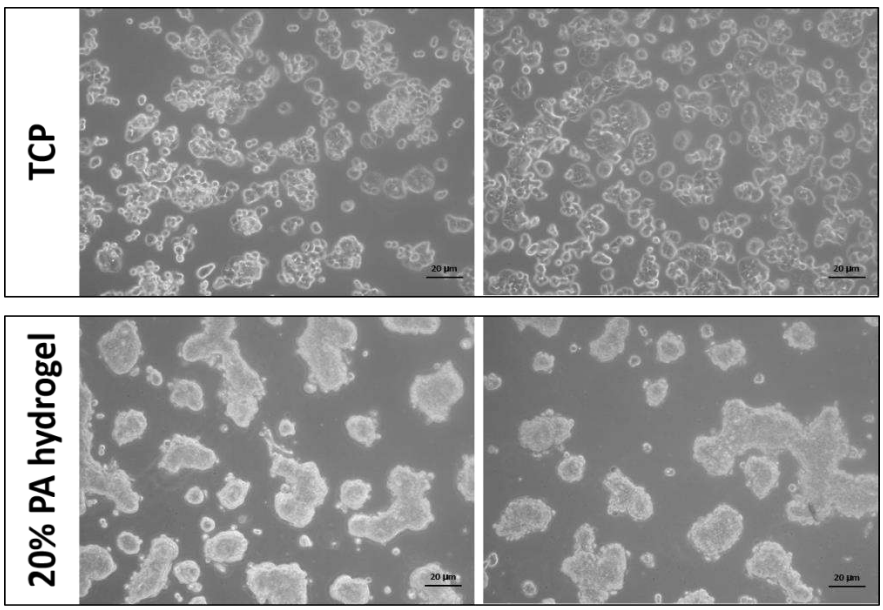


FIGURE 5.5 B: Phase contrast images of HT29 cells cultured on 20% PA hydrogel substrate coupled with vitronectin. Scale bar, 20µm.

The KIND1 cells cultured on 10% and 20% PA hydrogel were characterised for the expression of pluripotency markers *OCT4, NANOG* and *SOX2;* and lineage-specific markers *SOX17, PAX6* and *BRACHYURY* **(FIGURE 5.6A, B)**. Expression of pluripotency markers and no expression of lineage specific markers on PA hydrogels indicated that KIND1 cultured on soft hydrogels are pluripotent and have not undergone differentiation.

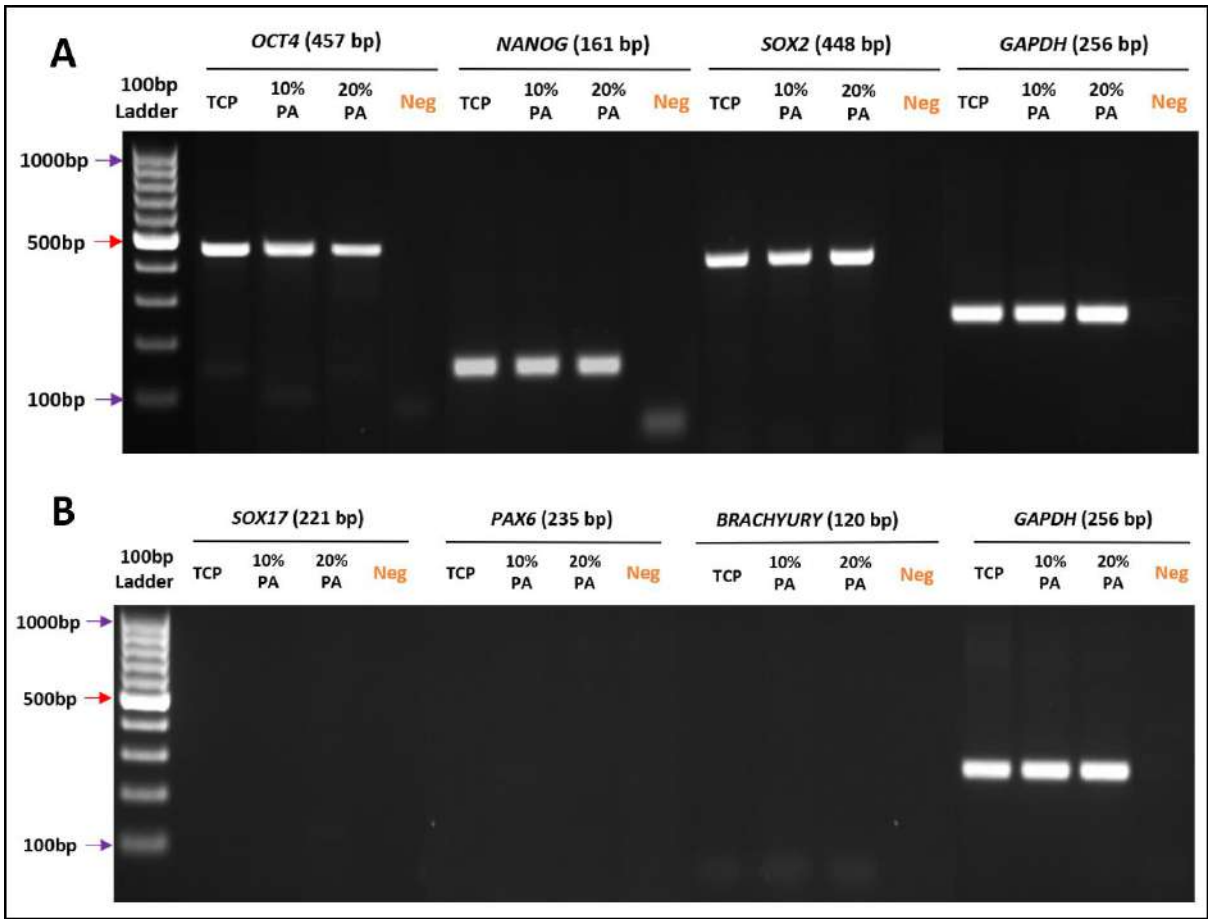


FIGURE 5.6: Characterization of KIND1 cells cultured on PA hydrogel by RT-PCR: The PCR products were run on 2% agarose gel by electrophoresis, with ethidium bromide for imaging. **(A)** Pluripotency markers *OCT*, *NANOG* and *SOX2,* and **(B)** lineage specific markers *SOX17* (ectoderm), *PAX6* (ectoderm) and *BRACHYURY* (mesoderm) was examined. *GAPDH* was used as internal standard control and a no temple control (NTC/Neg) was used as a negative control to check for any contamination. The bands of each PCR product were assessed using 100bp DNA ladder.

5.2.2.1 YAP expression in hESCs on PA Hydrogel

Next, we checked for the expression of *YAP*, a mechanotransducer reported to be affected by the substrate stiffness. *YAP* was equivalent in KIND1 cultured on 10% and 20% PA hydrogel as compared to TCP **(FIGURE 5.7)**.

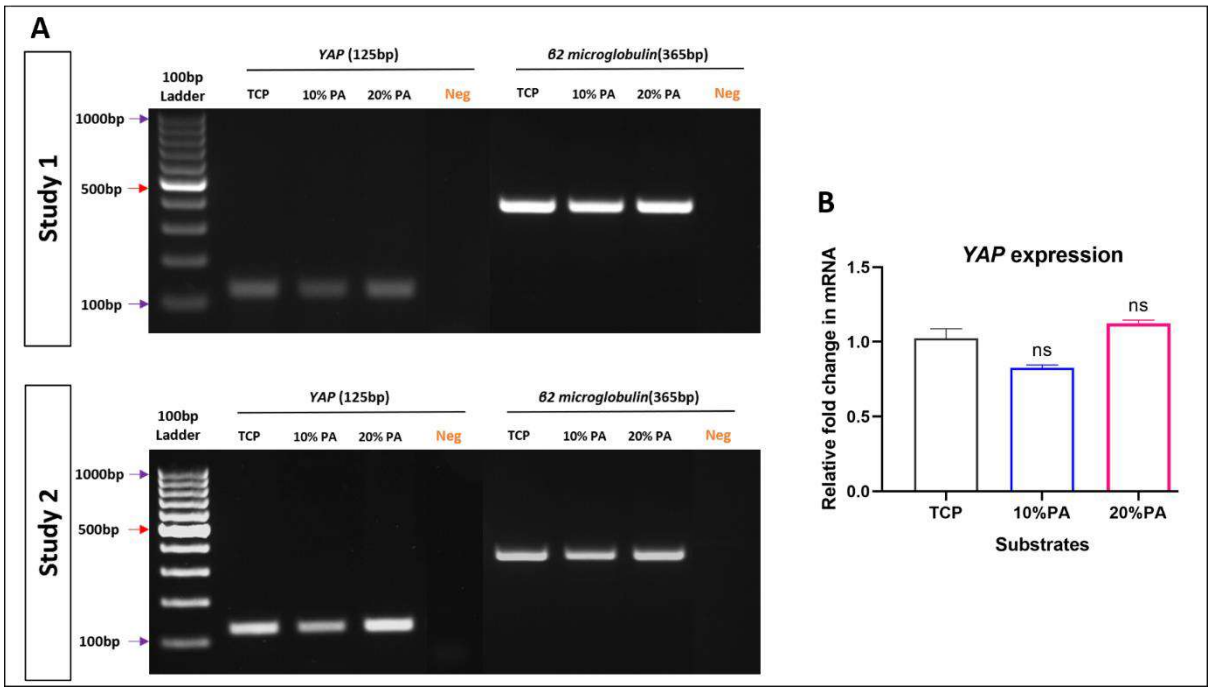


FIGURE 5.7: *YAP* expression in KIND1 cells cultured in PA hydrogel by RT-PCR: (A) The PCR products were run on 2% agarose gel by electrophoresis, with ethidium bromide for imaging. The data from two studies show that both the substrate TCP and PA showed *YAP* expression. *β2-Microglobulin* was used as internal standard control and a no temple control (NTC/Neg) was used as a negative control to check for any contamination. The bands of each PCR product were assessed using 100bp DNA ladder. **(B)** Semi-quantitative analysis by One-way ANOVA and Dunnett's multiple comparison test showed no significant difference of the *YAP* expression in TCP, 10% PA and 20% PA substrates. Data represents mean ± SD, n = two independent biological experiments, ns – not significant

Further studies with PA hydrogels were not possible due to multiple reasons: (i) most of the cells formed cell clumps, mainly in the centre of the PA gels, which loosely adhered to the substrates before completely detaching, (ii) few colonies were observed on each hydrogel and only at the edges of the substrates, no colonies were observed in the middle of the gels, (iii) the yield from cells on PA substrates was low even after pooling the cells from multiple gels, which made it impossible to passage the cells or extract sufficient protein for molecular biology assays, (iv) the cells were observed to undergo apoptosis beyond day 7 in culture.

5.2.3 Culture and Characterization of hESCs on Ultra Low Attachment Dishes (ULAD)

We hypothesized that 3D culture would provide us with a better model mimicking the *in vivo* microenvironment. 3D culture systems allow hESCs to grow in a more physiologically relevant microenvironment, resembling the conditions found *in vivo*. Moreover, in 3D cultures, hESCs can form complex spatial interactions between cells, promoting cellular communication and signalling that are crucial for their growth and differentiation. Therefore, to investigate whether near suspension culture can maintain pluripotency or initiate differentiation of hESCs, we cultured KIND1 cells in round bottom ultra-low attachment dishes. ULAD provided us with a no-substrate culture system while supporting cell survival. The KIND1 cells were maintained for seven days and three consecutive passages in Essential 8™ medium. Due to the absence of the adherent surface, the cells aggregated together and formed tight clumps, resembling embryoid bodies. By day 7, many small cell aggregates were observed at the bottom of the plate **(FIGURE 5.8)**. The cells were harvested for RNA and protein on the seventh day of each passage, while the remaining cells were reseeded into a fresh ULAD.

We checked the expression of pluripotency marker OCT4 by real-time PCR, immunoblot and immunofluorescence. The real-time PCR data showed slight variations in the OCT4 expression but no significant difference in the OCT4 expression was observed in all three passages with respect to (w.r.t) TCP **(FIGURE 5.9A, B)**. Additionally, immunoblotting also showed equal levels of OCT4 throughout the three passages w.r.t TCP **(FIGURE 5.9C, D)**. Nuclear localization of OCT4 was seen in KIND1 by immunofluorescence **(FIGURE 5.9F)**. To examine the differentiation status of the KIND1 cells cultured in ULAD over three passages, we performed real-time PCR with selective lineage-specific markers: SOX17 (endodermal marker), SOX1 (ectodermal marker), and BRACHYURY (mesodermal marker). The cells did not show the expression of any differentiation marker up till passage 2, the passage 3 samples showed insignificant expression of all the three lineage-specific markers as seen by their Ct values. The melt peaks showed us the nonspecific binding when compared to the standardized Tm of each gene **(FIGURE 5.9E)**.

As the cells were cultured in pluripotency-sustaining media (Essential 8TM) in the absence of specific differentiation-inducing molecules, it is possible that the expression of OCT4 did not reduce. Additionally, in the 3D culture, the aggregated cells might have

their own stiffness, which could contribute towards the maintenance of hESCs pluripotency in ULAD. Since YAP has been reported to maintain pluripotency of mESCs and hESCs by regulating OCT4 gene expression, we investigated the expression of YAP in KIND1 aggregates. We observed a similar trend with our system. The expression of the YAP transcript was observed in all the passage samples **(FIGURE 5.10)**. Therefore, we can hypothesize that YAP might be involved in maintaining the pluripotency of the hESCs in the 3D culture system, however, this requires further investigation. Therefore, in our ULAD study, we found that culturing the hESCs in ultra-low attachment conditions in the presence of pluripotency-maintaining factors did not cause them to differentiate, but rather preserved their pluripotency.

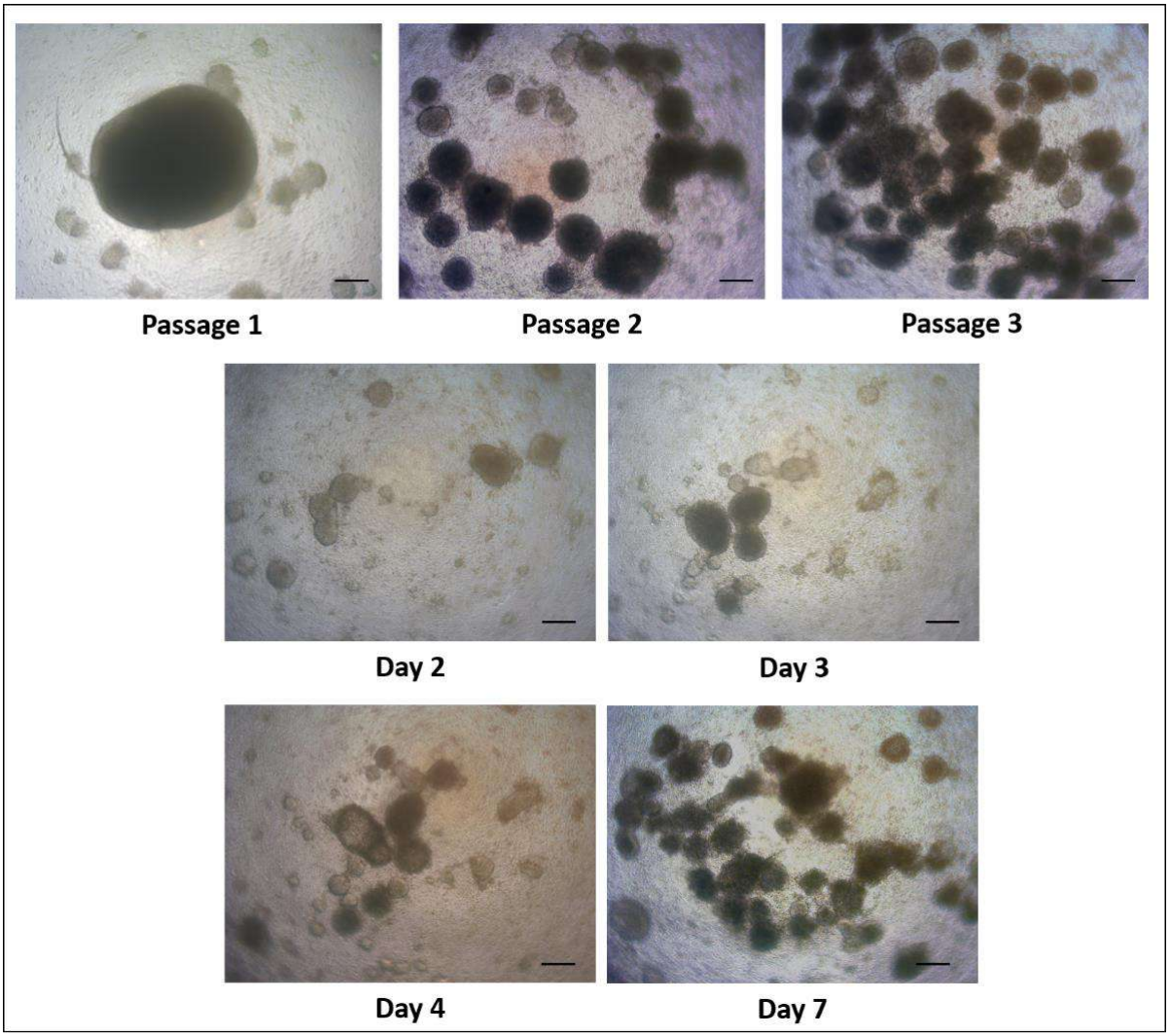


FIGURE 5.8: Phase contrast images of KIND1 cells cultured in ULAD. The upper panel shows cell aggregates formed at passage 1, passage 2 and passage 3. The lower two panels show the KIND1 cells at the bottom of the ULAD over the period of seven days. By the seventh day, tight clumps of KIND1 cells were observed. Scale bar, 5mm.

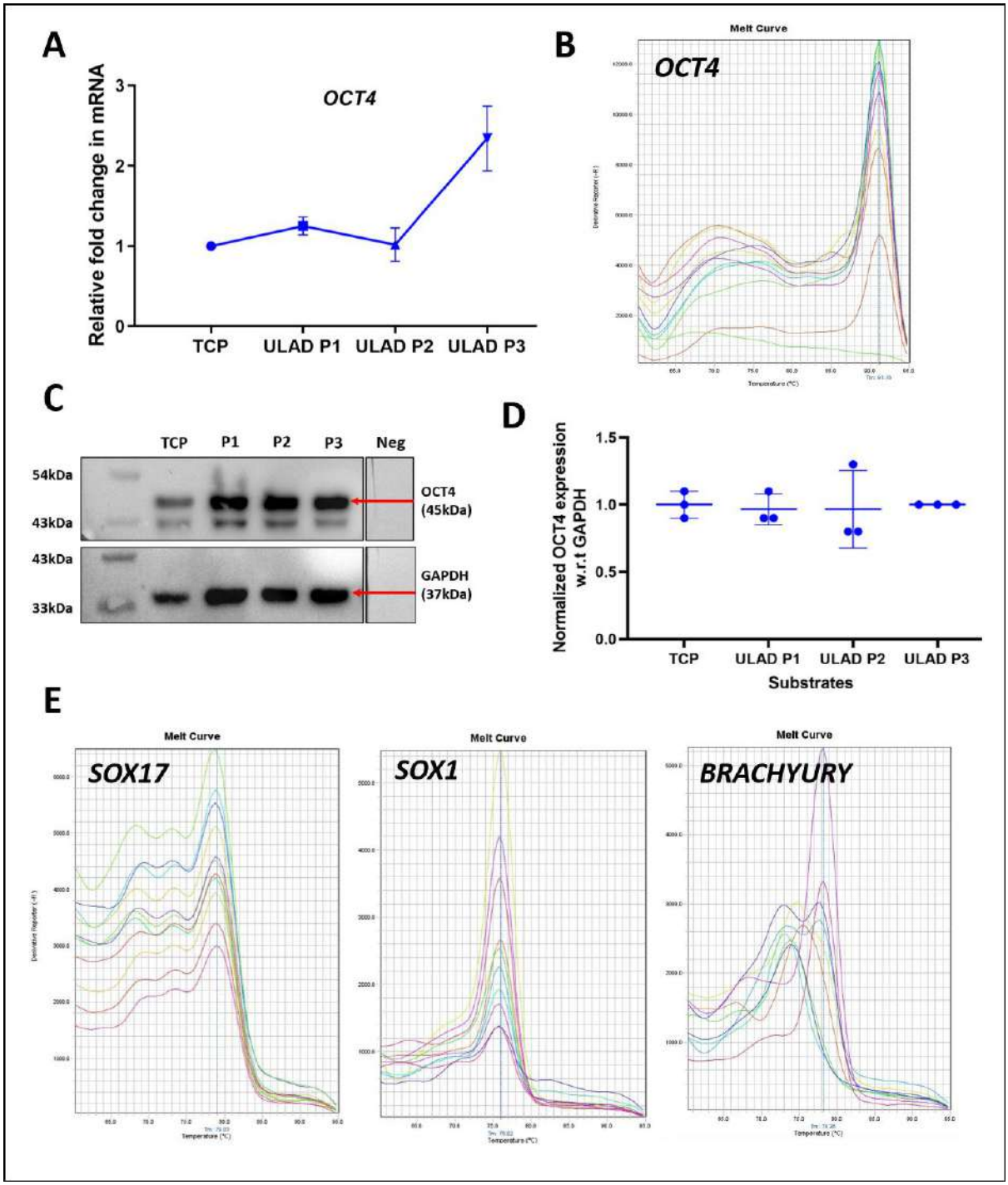


FIGURE 5.9: Characterization of KIND1 cells in ULAD using real time PCR, immunoblotting and immunofluorescence. (A) mRNA levels of pluripotency marker *OCT4* shown relative to the endogenous control *18S rRNA* and the expression is plotted relative to the levels in undifferentiated KIND1 cultured on TCP. **(B)** Melt curve of the PCR amplicons from various samples are shown in different colours. **(C)** Immunoblotting of OCT4 was seen through all the three passages in ULAD and in TCP passages. GAPDH was used as a loading control. **(D)** Protein level was quantified and plotted as bar graphs that represent individual values of OCT4 protein normalized to respective GAPDH. ULAD protein level plotted relative to TCP. **(E)** Melt curve of the PCR amplicons from various samples are shown in different colours. Statistical analysis was carried out by One-way ANOVA and Dunnett's multiple comparison. Data represents mean ± SD, n = technical triplicates of three independent biological experiments.

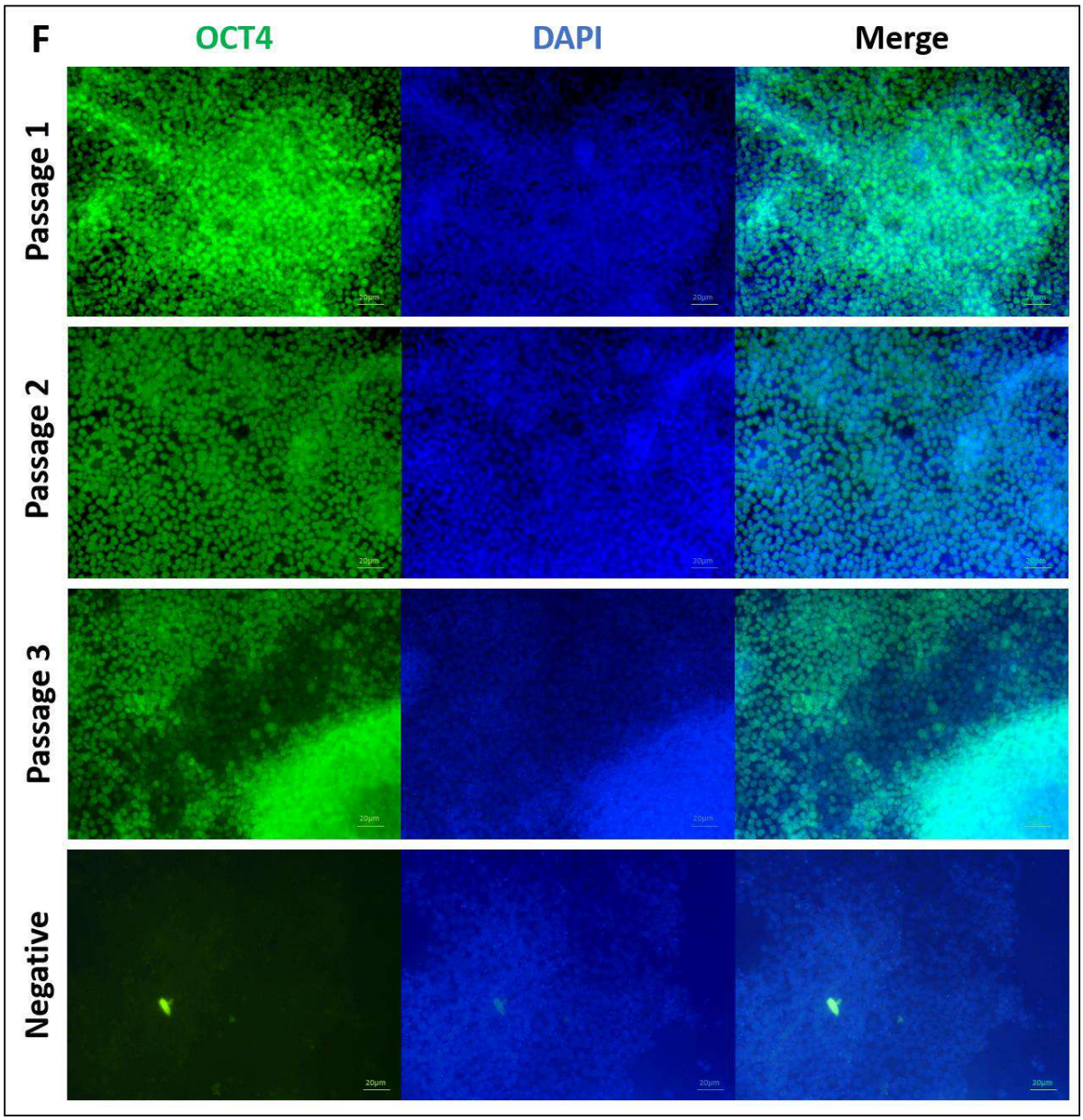


FIGURE 5.9: Characterization of KIND1 cells in ULAD using real time PCR, immunoblotting and immunofluorescence. (F) Immunofluorescence showed the expression of OCT4 (green) in the nucleus. A negative control, where the cells were treated with only secondary antibody, was used for the possibility of any non-specific bands due to secondary antibody. The nucleus was counterstained with DAPI (blue). Scale bar, 20µm.

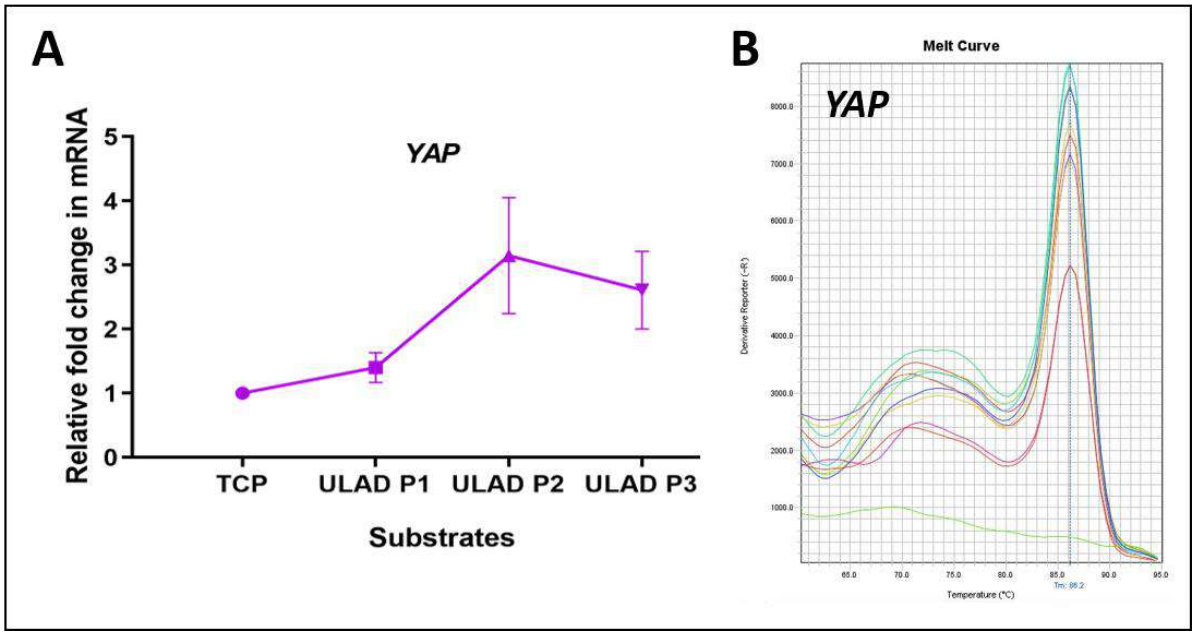


FIGURE 5.10: *YAP* expression in KIND1 cells in ULAD by real time PCR. (A) mRNA levels of *YAP* shown relative to the endogenous control *18S rRNA* and the expression is plotted relative to the levels in undifferentiated KIND1 cultured on TCP. **(B)** Melt curve of the PCR amplicons from various samples are shown in different colours. Tm for *YAP* = 86.2^{0}C. Statistical analysis was carried out by One-way ANOVA and Dunnett's multiple comparison. Data represents mean ± SD, n = technical triplicates of three independent biological experiments.

5.3 Culture and Characterization of hESCs on CytoSoft® (CS) Substrates in Pluripotency Maintaining Media (PMM)

To overcome cytotoxicity and batch to batch variations of the synthesized PA hydrogels, we selected commercially available CytoSoft® plates for our project. The CS substrates are biocompatible siloxane substrates of defined elastic moduli covering broad physiological range i.e., 0.2kPa, 2kPa, 5kPa, 8kPa, 16kPa, 32kPa and 64kPa. To identify whether the biophysical cues alone can induce differentiation, KIND1 cells were cultured on TCP and CS substrate plates in pluripotency maintaining medium i.e., complete Essential 8™ medium, for three consecutive passages **(FIGURE 5.11)**. The cells were collected for RNA and protein at day 5 of each passage. As expected, we observed compact colonies with characteristic epithelial morphology on TCP. Surprisingly, KIND1 cells cultured on CS substrates exhibited similar morphology and the cells were arranged in compact colonies **(FIGURE 5.12)**. We did not observe single cell culture or cell flattening, a sign of differentiation, in any of the passages.

Next, we checked for the levels of pluripotency markers OCT4 and NANOG, and cell cycle progression marker Cyclin D1 by immunoblotting. We observed that OCT4 and NANOG levels were maintained in all the three consecutive passages when compared to the OCT4 and NANOG levels in the samples of TCP. Densitometric analysis of the immunoblot by ImageJ revealed that OCT4 level was highest in the TCP and it gradually increased from 0.2kPa to 64kPa. Similarly, NANOG level was downregulated in all the CS substrates compare to TCP expect for in 8kPa and 64kPa which showed an equivalent peak to TCP **(FIGURE 5.13A, B)**. Cyclin D1 level indicated that our substrate culture system supports hESCs proliferation and survival **(FIGURE 5.13C)**. To confirm whether lineage differentiation had occurred, we studied the mRNA expression of representative markers of all three lineages - *SOX17*, *BRACHYURY* and *PAX6* by real time PCR. For endoderm and mesoderm positive control we used KIND1 cells differentiated on TCP by addition of Activin A **(TABLE A3, Annexure III)** and for ectoderm we used commercially available total adult human brain RNA as positive control. The gene expression analysis by $2^{-\Delta\Delta CT}$ method showed no expression of the endodermal marker *SOX17* and mesodermal marker *BRACHYURY* in TCP and CS substrates when compared to the positive control. *PAX6* showed positive expression in all the samples, but the high Ct values indicated late expression and the melt curve of *PAX6* showed nonspecific binding

at lower Tm than the standardized Tm value **(FIGURE 5.13D)**. Therefore, taken together our results showed that although subtle changes in expression of pluripotency markers was observed, hESCs remained pluripotent on soft substrates in presence of pluripotency maintaining factors after three consecutive passages.

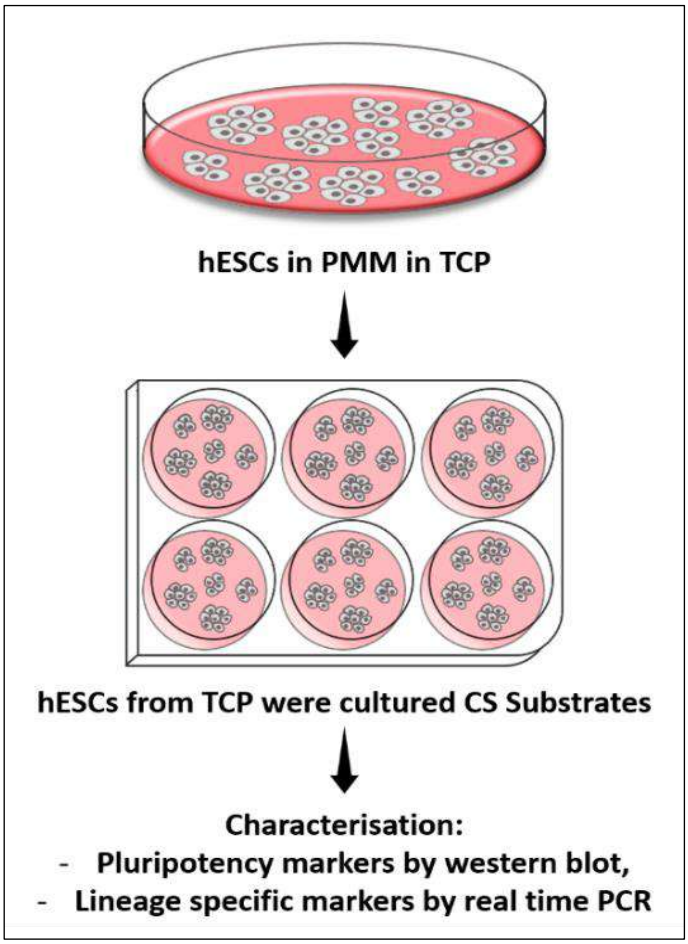


FIGURE 5.11: Schematic representation of the culturing protocol on CS substrates in pluripotency maintaining medium (PMM). KIND1 cells were cultured and maintained in complete Essential 8™ medium till the cells attained 90% confluency. A confluent 60mm TCP was passaged and seeded equally, into the six wells of one CS elastic modulus plate. The cells were maintained in complete Essential 8™ medium and were harvested for RNA and protein on day 5.

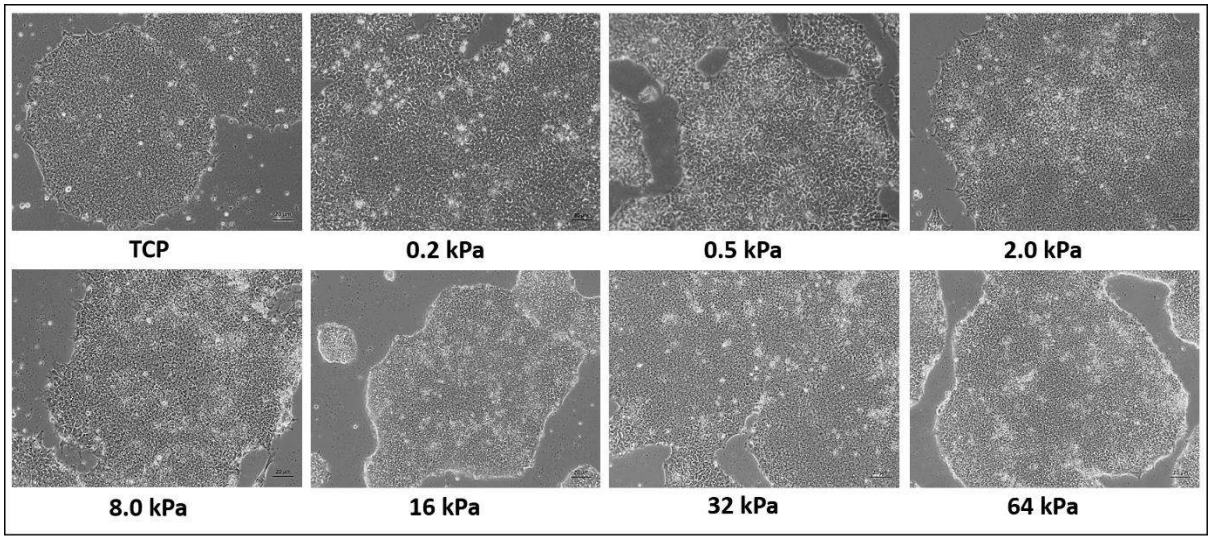


FIGURE 5.12: Phase contrast images of KIND1 cells cultured in TCP and CytoSoft® substrate of various modulus coupled with vitronectin and maintained in pluripotency maintaining medium. Scale bar, 20µm.

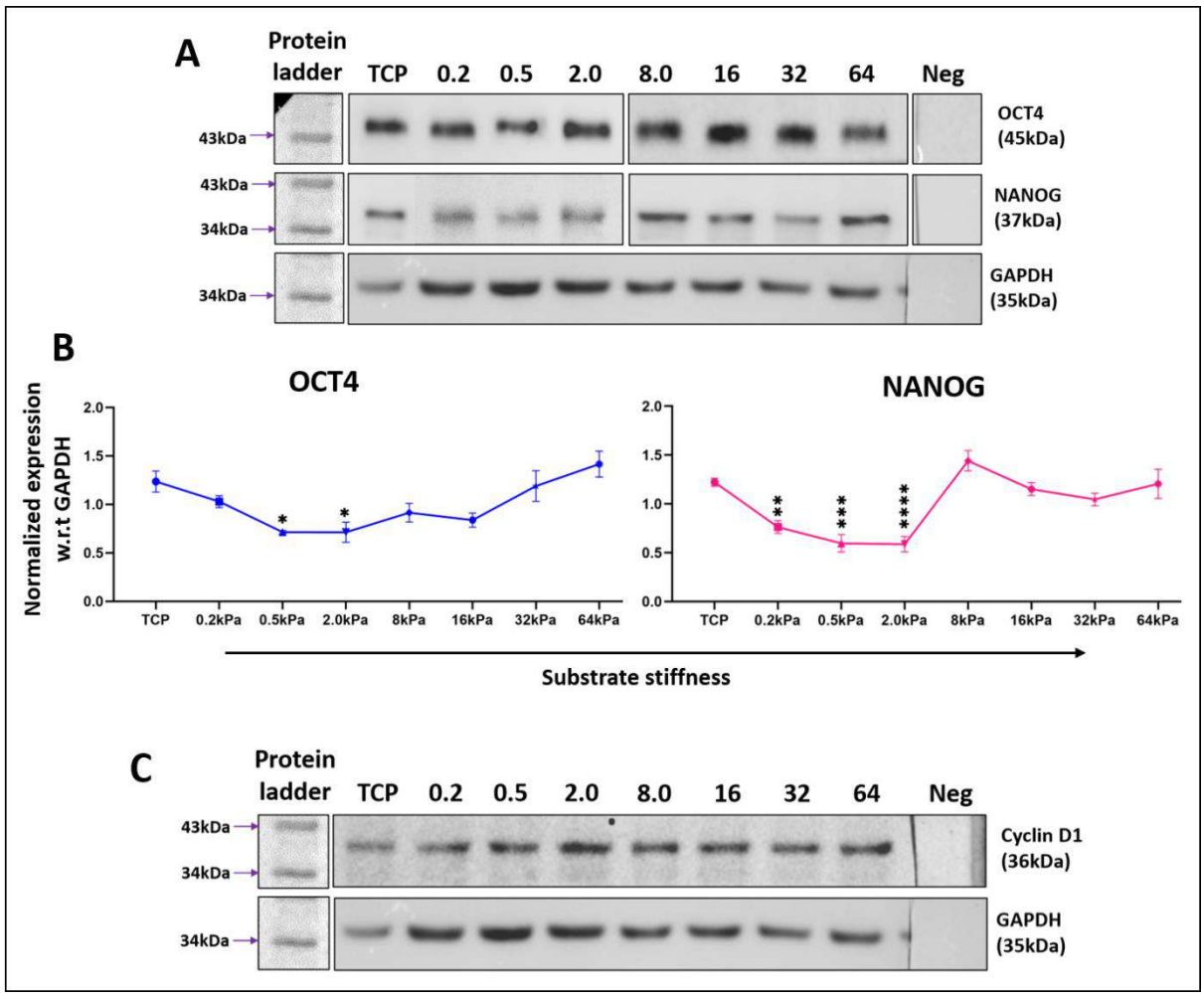


FIGURE 5.13: Characterization of KIND1 cells in TCP and CS in PMM by immunoblotting and real time PCR. (A) Immunoblotting of pluripotency markers OCT4 and NANOG was seen in TCP and all the stiffness of the CS substrates. GAPDH was used as a loading control. **(B)** Protein levels were

quantified and plotted as bar graphs that represent individual values of OCT4 and NANOG proteins normalized to respective GAPDH. The protein levels in CS were plotted relative to the protein levels in TCP. **(C)** Immunoblotting of cell cycle progression protein Cyclin D1 was seen in all the samples. Statistical analysis was carried out by One-way ANOVA and Tukey's multiple comparison. Data represents mean ± S.E.M, n = technical triplicates of three independent biological experiments. Asterisks (*) denote p values and represents statistical significance difference in the expression between the specific stiffness and TCP. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0.0005$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.

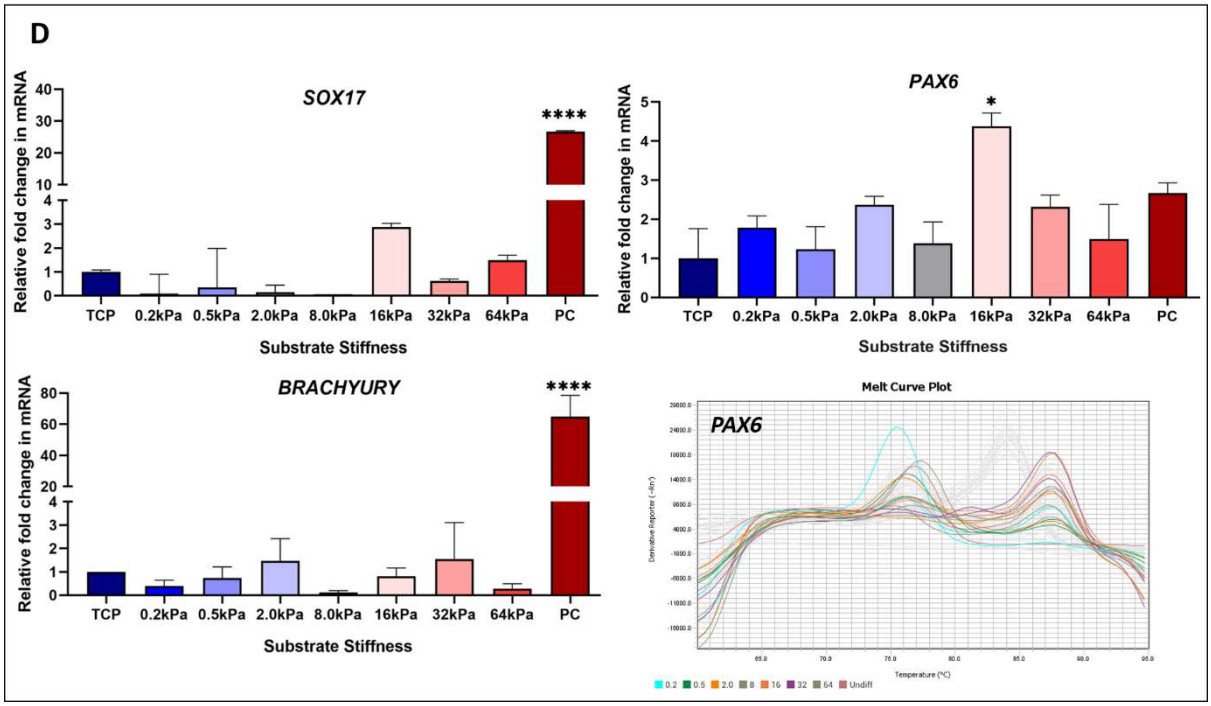


FIGURE 5.13: Characterization of KIND1 cells in TCP and CS in PMM by immunoblotting and real time PCR. (D) mRNA levels of lineage specific markers *SOX17*, *BRACHYURY* and *PAX6* shown relative to the endogenous control *18S rRNA* and the expression is plotted relative to the levels in KIND1 cultured on TCP. Melt curve of the *PAX6* PCR amplicons from various samples are shown in different colours. Statistical analysis was carried out by One-way ANOVA and Dunnett's/Tukey's multiple comparison tests. PC = positive control, endoderm differentiated day 4 cells for *SOX17* and *BRACHYURY*, and commercially procured total adult human brain RNA for *PAX6*. Data represents mean ± SD. n = technical triplicates of three independent biological experiments. Asterisks (*) denote p values and represents statistical significance difference in the expression between the specific stiffness and TCP. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0.0005$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.

5.4 Expression of YAP in hESCs cultured in CS substrates in PMM

We were curious about the expression of the mechanotransducer YAP in the hESCs cultured on the soft CS substrates because our results indicated that hESCs retained their pluripotency on soft CS substrates but according to literature YAP is known to maintain pluripotency in hESCs on TCP and cause differentiation on the soft substrate, where YAP

is inactive. Consequently, we wondered whether YAP might also be involved in maintaining the pluripotency state of hESCs on soft substrates. Immunoblot of YAP and pYAP showed equivalent levels of YAP and pYAP in all the samples **(FIGURE 5.14A)**. By immunofluorescence we saw the expression of YAP in the nucleus and the cytoplasm, this observation corelates with our western blot data **(FIGURE 5.14B)**. To determine whether Hippo pathway proteins are involved in YAP/pYAP regulation, we checked for the expression of three core Hippo proteins MST1, SAV1 and MOB1. The expression of the unphosphorylated state of these proteins explains the expression of unphosphorylated YAP into the nucleus. As we know from literature, YAP binds to its DNA binding partner TEAD1-4 in the nucleus and thereby regulates the expression of OCT4 and NANOG. Therefore, summarizing the above results, we can say that hESCs cultured on the soft substrates expresses both the nuclear and cytoplasmic YAP, and the nuclear YAP might be involved in maintaining the pluripotency of the hESCs.

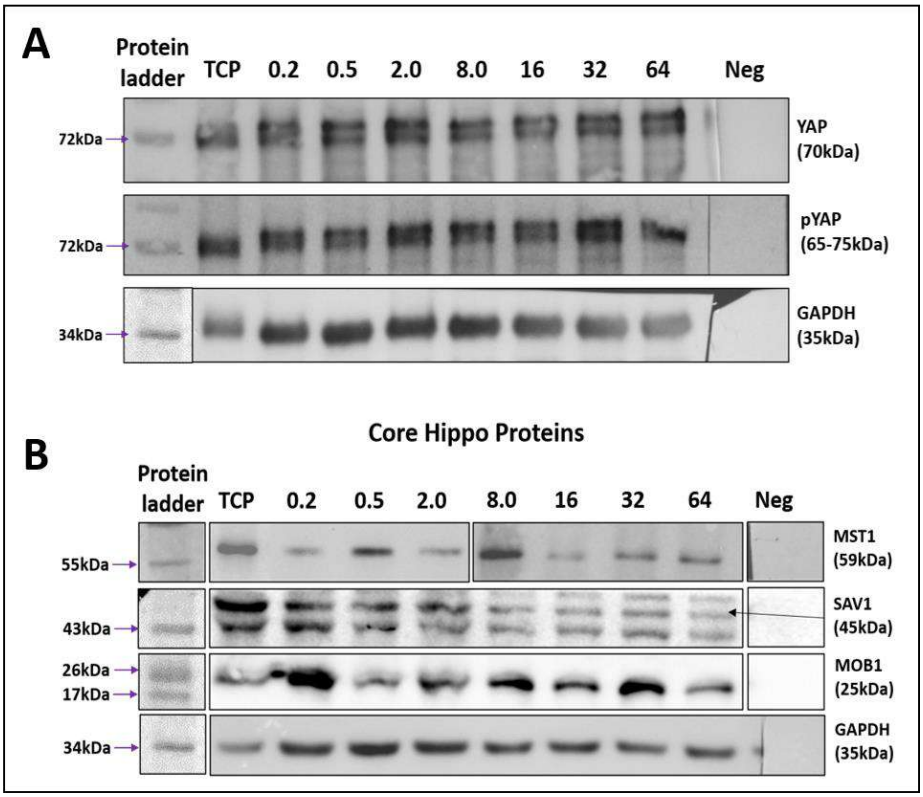


FIGURE 5.14: Expression of Hippo pathway proteins in KIND1 cells in TCP and CS in PMM by immunoblotting and immunofluorescence. Protein levels of **(A)** YAP and pYAP, and **(B)** core Hippo proteins MST1, SAV1 and MOB1 was seen in TCP and all the stiffness of the CS substrates. GAPDH was used as a loading control.

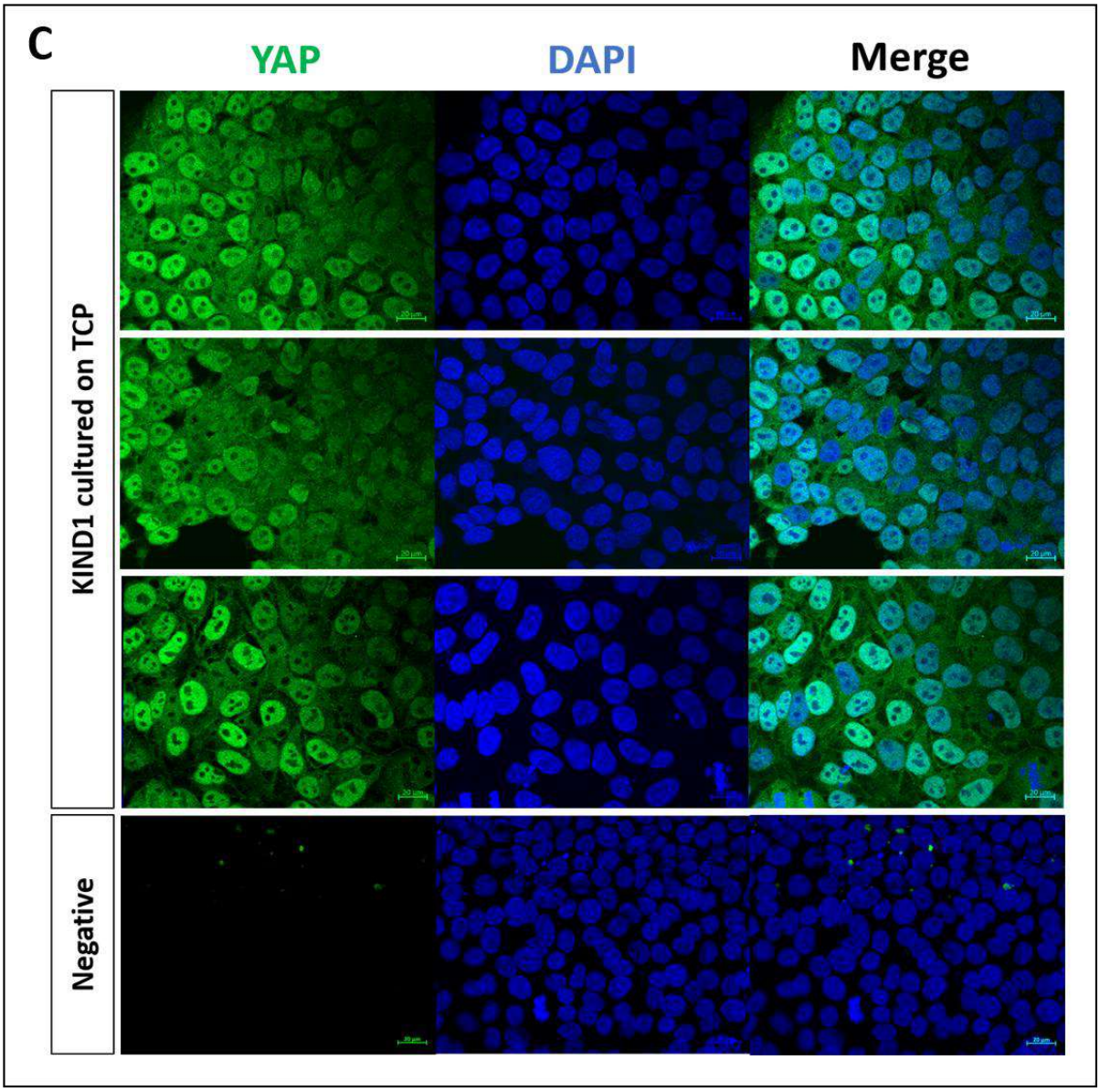


FIGURE 5.14: Expression of Hippo pathway proteins in KIND1 cells in TCP and CS in PMM by immunoblotting and immunofluorescence. (C) Immunofluorescence showed the expression of YAP (green) in the nucleus and the cytoplasm. A negative control, where the cells were treated with only secondary antibody, was used for the possibility of any non-specific bands due to secondary antibody. The nucleus was counterstained with DAPI (blue). Scale bar, 20µm.

5.5 Culture and Characterization of hESCs on CS® Substrates in Differentiation Inducing Media (DIM)

The above results roused our curiosity, if change in substrate stiffness alone does not induce differentiation as reported in MSCs and hESCs, then whether changing the medium to minimum differentiation inducing medium could lead to any specific lineage? To answer this question, we cultured KIND1 cells on TCP and CS substrate plates and maintained them in minimum differentiation inducing medium i.e., Advanced DMEM with 2% FBS, for two consecutive passages **(FIGURE 5.15)**. We did not add any differentiation specific molecule and intentionally kept the serum concentration to 2%, as we did not want the serum components to dictate the course of differentiation, but enough serum to keep the cells healthy. The cells were collected for RNA and protein at day 5 of each passage. Instead of compact colonies observed previously, KIND1 colonies in differentiation inducing medium appeared flattened and spread out, having lost their compactness and the epithelial morphology. Similar observations were noticed in KIND1 cultured on CS substrates **(FIGURE 5.16)**.

Next, we checked for the level of OCT4 by immunoblotting. OCT4 protein level was reduced in TCP as seen by the band intensity, however, surprisingly the cells grown on softer substrates showed relatively higher OCT4 level **(FIGURE 5.17A)**. The Ct values and the Tm indicated that the lineage specific markers are late expressing and the expression is less intense compared to the positive control. Also, a comparative analysis between positive control and other samples showed reduced expression of *SOX17*, *BRACHYURY*, and *PAX6* all the other substrates **(FIGURE 5.17B)**. Thus, we can say that the differentiation has been initiated in hESCs cultured on TCP and CS. Our results also hint that the hESCs cultured on soft substrates in the presence of low levels of morphogens undergoes non-specific differentiation.

Therefore, we can hypothesize that in presence of pluripotency maintaining medium, hESCs maintained the pluripotent state, and in differentiation inducing medium, the hESCs undergo differentiation irrespective of substrate stiffness. Furthermore, KIND1 does not seem to be as sensitive to the stiffness of the substrate as MSCs or other hESCs reported in literature.

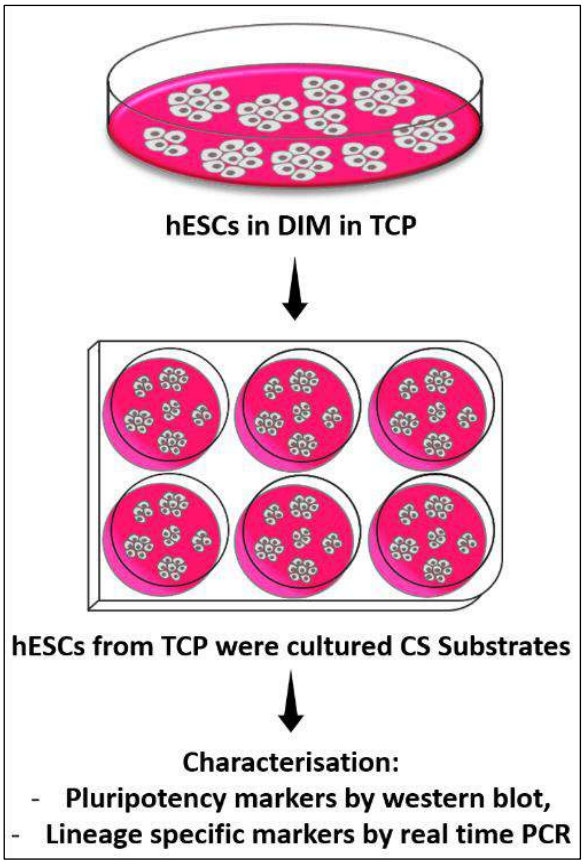


FIGURE 5.15: Schematic representation of the culturing protocol on CS substrates in differentiation inducing medium (DIM). KIND1 cells were cultured and maintained in Advanced DMEM medium with 2% FBS till the cells attained 90% confluency. A confluent 60mm TCP was passaged and seeded equally, in drop-wise manner, in the six wells of one CS elastic modulus plate. The cells were maintained in DIM and were harvested for RNA and protein on day 5.

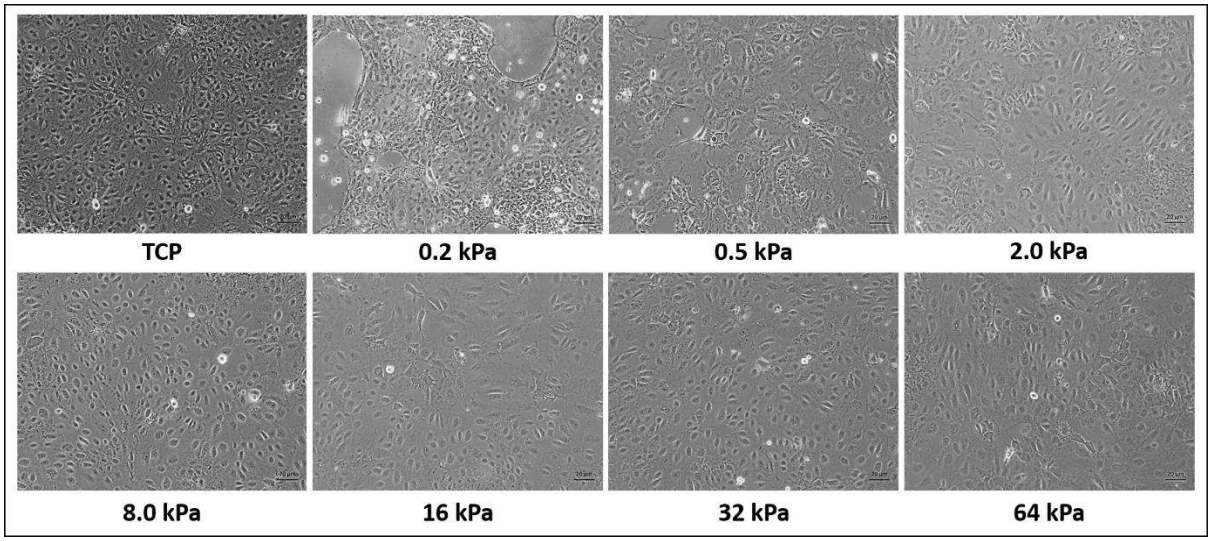


FIGURE 5.16: Phase contrast images of KIND1 cells cultured in TCP and CytoSoft® substrate of various modulus coupled with vitronectin and maintained in differentiation inducing medium. Scale bar, 20μm.

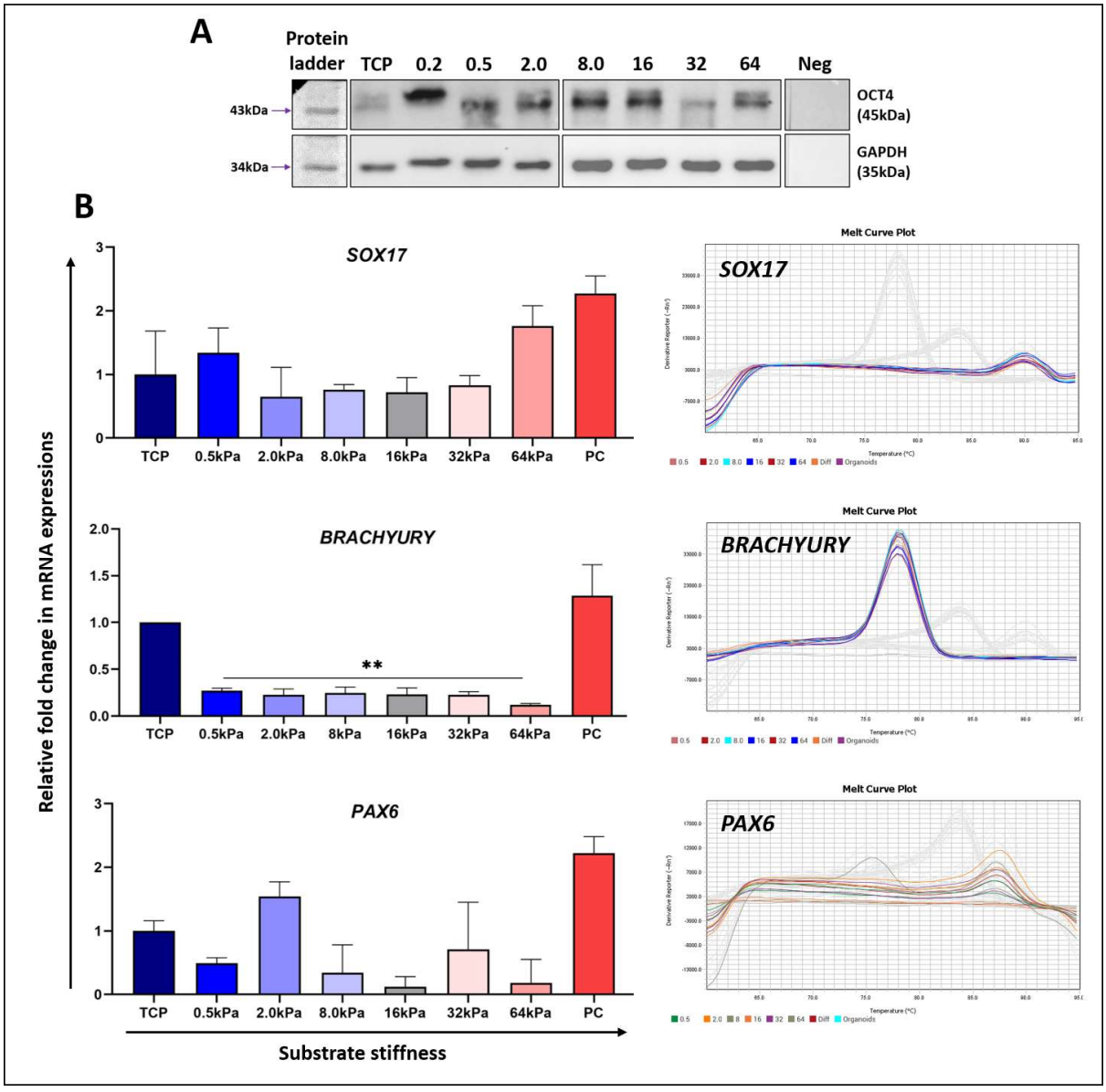


FIGURE 5.17: Characterization of KIND1 cells in TCP and CS in DIM by immunoblotting and real time PCR. (A) Immunoblotting of pluripotency markers OCT4 was seen in TCP and all the stiffness of the CS substrates. GAPDH was used as a loading control. **(B)** mRNA levels of lineage specific markers *SOX17*, *BRACHYURY* and *PAX6* shown relative to the endogenous control *18S rRNA* and the expression is plotted relative to the levels in KIND1 cultured on TCP. Melt curve of the respective PCR amplicons from various samples are shown in different colours. Statistical analysis was carried out by One-way ANOVA and Tukey's multiple comparison tests. PC = positive control, endoderm differentiated day 4 cells for *SOX17* and *BRACHYURY*, and commercially procured Brain cDNA for *PAX6*. Data represents mean ± SD. n = technical triplicates of two independent biological experiments. Asterisks (*) denote p values and represents statistical significance difference in the expression between the specific stiffness and TCP. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0.0005$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.

5.6 Expression of YAP in hESCs cultured in CS substrates in DIM

Since the differentiation had initiated in hESCs cultured on soft substrate, we investigated the expression of YAP and pYAP. According to the literature, YAP expression is downregulated when ESCs differentiates, therefore we were curious about the expression of YAP in these differentiating cells. Immunoblot of YAP and pYAP showed equivalent levels of YAP and pYAP in all the samples **(FIGURE 5.18A)**. To determine whether Hippo pathway proteins are involved in YAP/pYAP regulation, we checked for the levels of three core Hippo proteins MST1, SAV1 and MOB1 **(FIGURE 5.18B)**. The expression of the unphosphorylated state of these proteins explains the expression of unphosphorylated YAP into the nucleus. We next checked the expression of *YAP* by real time PCR. The early expression of YAP was seen from the Ct values and a single discrete peak for *YAP* amplicon is indicative of a positive *YAP* expression in all the samples. Based on our observation from the band intensities of the immunoblots we can hypothesize that the expression of YAP and pYAP in DIM seemed to be downregulated when compared to the YAP and pYAP in PMM. In both the case, the biochemical composition of the growth media determined the expression of YAP and not the substrate stiffness.

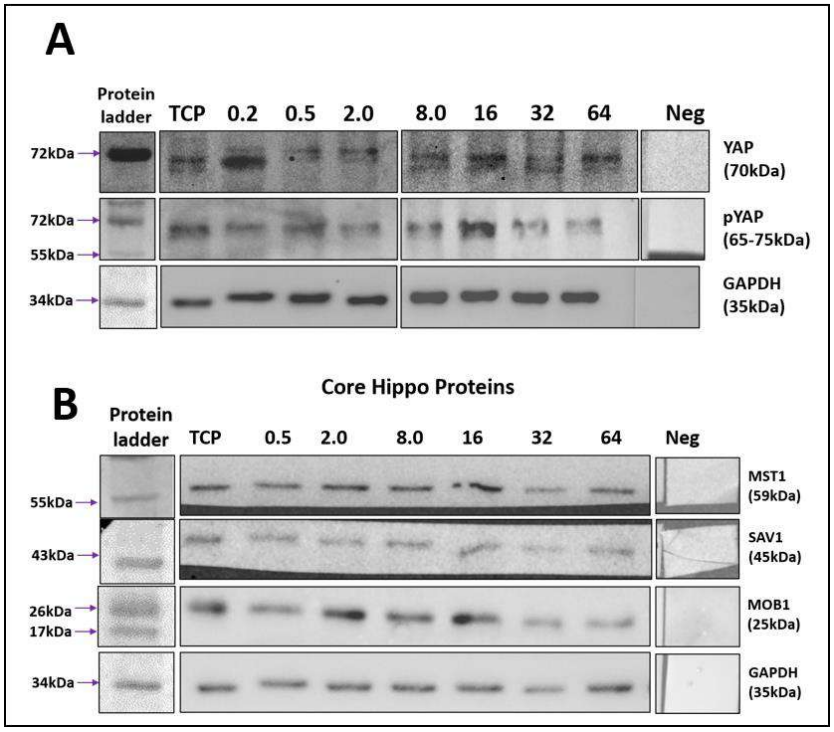


FIGURE 5.18: Expression of Hippo pathway proteins in KIND1 cells in TCP and CS in PMM by immunoblotting and real time PCR. Protein levels of **(A)** YAP, pYAP and **(B)** core Hippo proteins MST1, SAV1 and MOB1 was seen in TCP and all the stiffness of the CS substrates. GAPDH was used as a loading control.

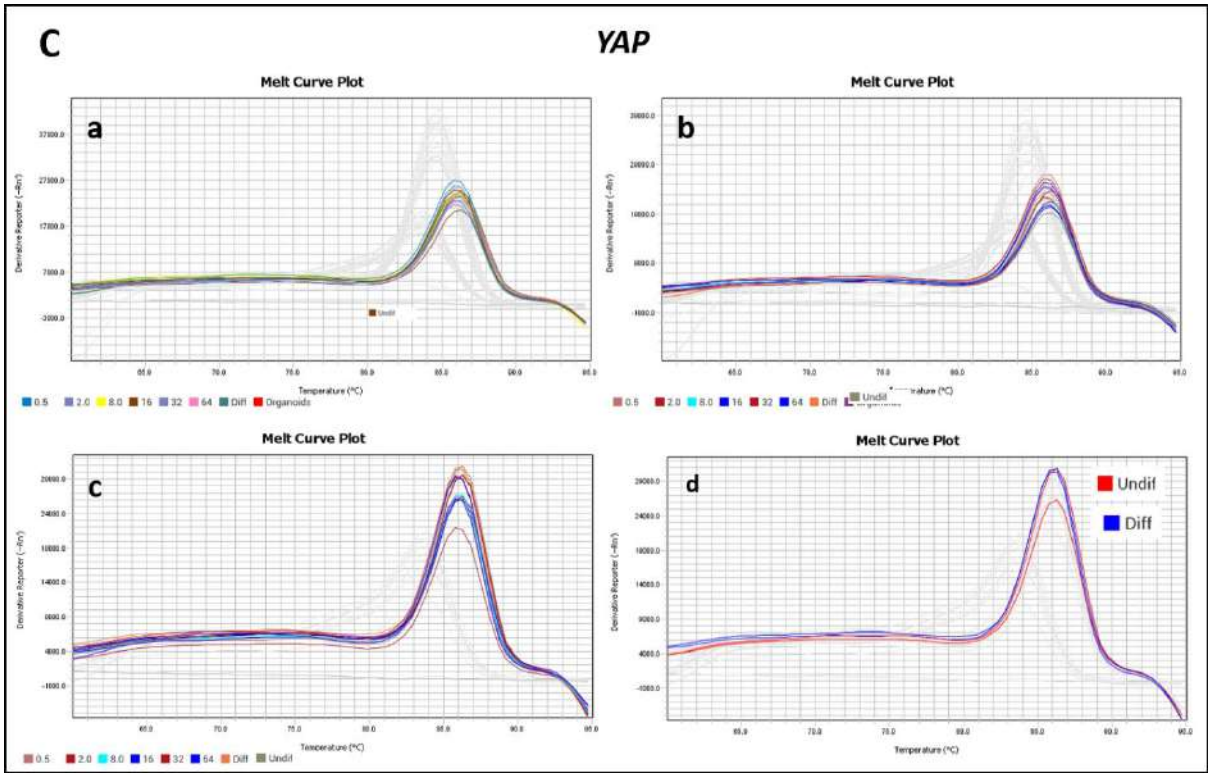


FIGURE 5.18: Expression of Hippo pathway proteins in KIND1 cells in TCP and CS in DIM by immunoblotting and real time PCR. (B) mRNA expression levels of YAP by real time PCR**. (a-c)** Melt curves show single discrete peak of the YAP amplicon through different passages in TCP and CS substrates. **(d)** Melt curve of YAP amplicon in undifferentiated KIND1 cells cultured in PMM on TCP and differentiated KIND1 cells cultured in DIM on TCP.

5.7 Culturing Human Mesenchymal Stem Cells (hMSCs) on CS® Substrates

From the above cell culture observations, we noted that hESCs cultured on soft CS® substrates does not undergo morphological changes when compared to the hESCs cultured on TCP. According to literature, studies using MSCs have reported that MSCs on soft substrate are round and less spread whereas MSCs on stiff substrate appeared flattened with large spread area. Therefore, we questioned the integrity and the width of the PDMS gel in the CS® substrates. To answer this question, we cultured human placental mesenchymal stem cells (hPMSCs) on TCP and CS® substrates and maintained them in culture for five days. On TCP, hPMSCs showed elongated fibroblast like morphology. hPMSCs cultured on 0.2kPa, 0.5kPa and 5kPa showed less spreading and cell count, however, hPMSCs cultured on 8kPa, 16kPa, 32kPa and 64kPa showed similar morphology to TCP **(FIGURE 5.19)**. Just from the visual confirmation we deduced that the CS® substrates may have induced changes in the cultured cells. Furthermore, now we demonstrated thathPMSCs are highly sensitive to the change in stiffness compared to hESCs, which resisted the change in stiffness.

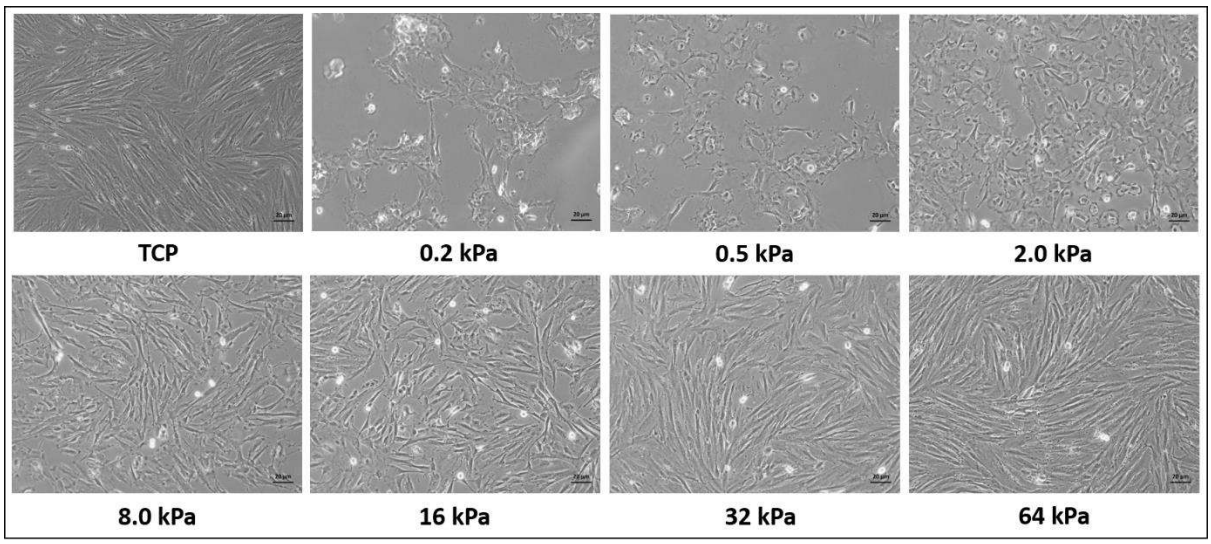


FIGURE 5.19: Phase contrast images of hPMSCs cultured in TCP and CytoSoft® substrate of various modulus coupled with collagen and maintained in IMEM medium. Scale bar, 20µm.

5.8 Directed Differentiation of hESCs on CS® Substrates towards Definitive Endoderm (DE)

KIND1 cells were differentiated towards definitive endoderm using a well-established protocol described (D'Amour *et al.* 2006; Pethe *et al.*, 2014; Dumasia *et al.*, 2021) with minor modifications as described in **TABLE A3 (Annexure 3)**. The differentiation towards definitive endoderm is a four-day procedure and it is the first step in the differentiation of hPSCs into pancreatic endoderm lineage. Once the hESCs cultured on TCP and CS substrates in Essential 8™ medium reached 90% confluency, the media was switched to DE-specific media containing high levels of Activin A, which pushed hESCs towards definitive endoderm fate. **FIGURE 5.20** schematically describes the modified differentiation procedure followed along with the key markers of each cell state. During first two days in the DE-specific medium, we observed high rate of cell death than the normal rate in Essential 8™ medium, and in the next two days proliferation of the cells was observed. Phase contrast images of the cells on day 4 of the differentiation protocol showed single, flattened, elongated, and outstretched cells (**FIGURE 5.21**). To confirm the transition of hESCs from the pluripotent state into the definitive endoderm state, real-time PCR, immunoblotting and immunofluorescence analysis were used to characterize the stage-specific markers indicative of a specific lineage.

Through the DE-stage in TCP and CS substrates, level of pluripotency marker OCT4 was detected by immunoblotting **(FIGURE 5.22A)**. OCT4 is known to present till day 4 of the endoderm differentiation and it becomes undetectable by day 8 of the differentiation with the addition of retinoic acid and FGF10 for further differentiation into primitive gut tube stage (Kroon *et al.*, 2008, Pethe *et al.*, 2014, Dumansia *et al.*, 2021). Although the levels of OCT4 in CS 8kPa stiffness substrate was significantly low, there was no significant change in the expression of other gene markers observed on this substrate. DE-stage specific markers *SOX17*, *FOXA2* and *CXCR4* were expressed in all the CS substrates, and a significant upregulation of these markers was observed in CS 0.2kPa and CS 64kPa stiffness substrates when compared to the TCP. DE day 4 cells also expressed mesoderm marker *BRACHYURY*. Therefore, addition of Activin A without the Wnt-signalling molecules induces mesoendodermal differentiation of ESCs and not just definitive endoderm fate in TCP and all the CS substrates **(FIGURE 5.22B)**. The chemokine

receptor CXCR4 increased upon DE induction with majority of day 4 cells staining positive for the cell surface receptor as seen by immunofluorescence assay **(FIGURE 5.22C)**.

Expression of mechanosensor YAP was also studied in the differentiated cells. According to the literature, YAP is downregulated in differentiated cells cultured on TCP, however we saw constant expression of YAP in all the differentiated cells on TCP and CS substrates **(FIGURE 5.23A)**. Protein analysis of YAP from the three independent biological experiments showed a downregulation of YAP in differentiated cells on CS substrates when compared with the differentiated cells on TCP. Similarly, pYAP level was also downregulated in CS substrates except in CS 0.2kPa stiffness substrate when compared to TCP **(FIGURE 5.23B)**.

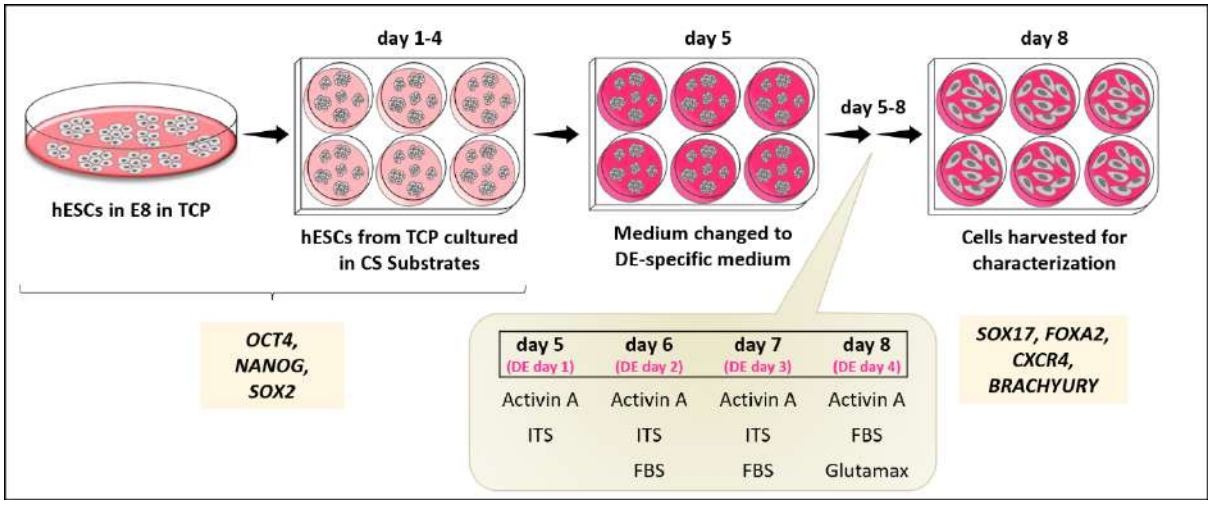


FIGURE 5.20: Schematic representation of the modified protocol employed for the differentiation of hESCs into the definitive endoderm. Day 1-4 represents cells cultured on TCP and CS substrates in Essential 8™ medium. The medium was switched to RPMI medium supplemented with Activin A to induce differentiation. Day 5 indicates day 1 in DE differentiation protocol and day 8 represents DE day 4 differentiation. The lineage specific markers are mentioned below each lineage stage.

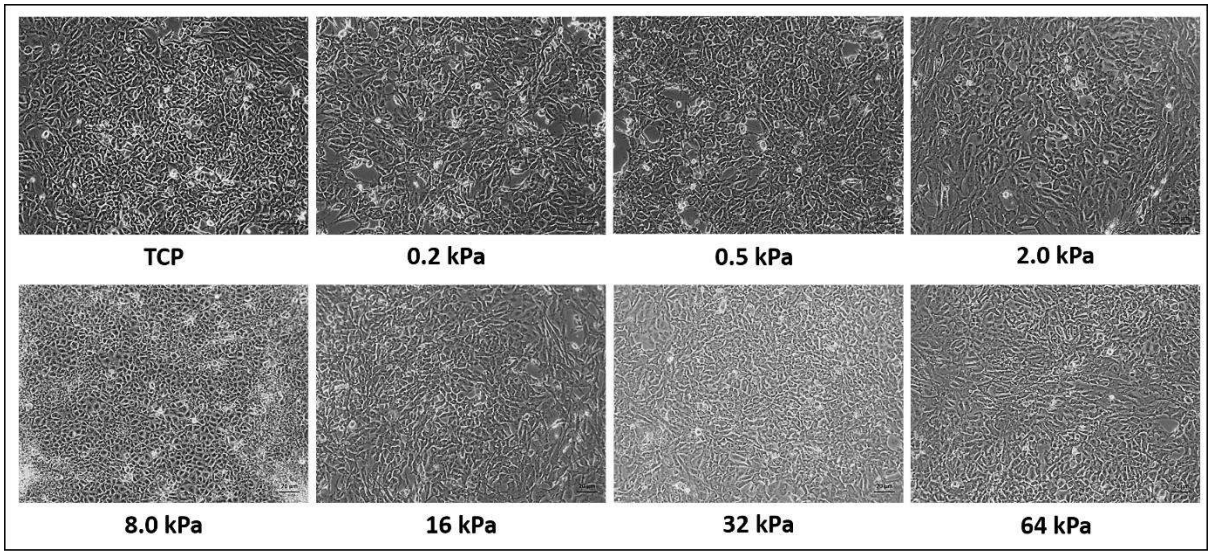


FIGURE 5.21: Phase contrast images of cells on the day 4 of the definitive endoderm differentiation stage. Scale bar, 20µm.

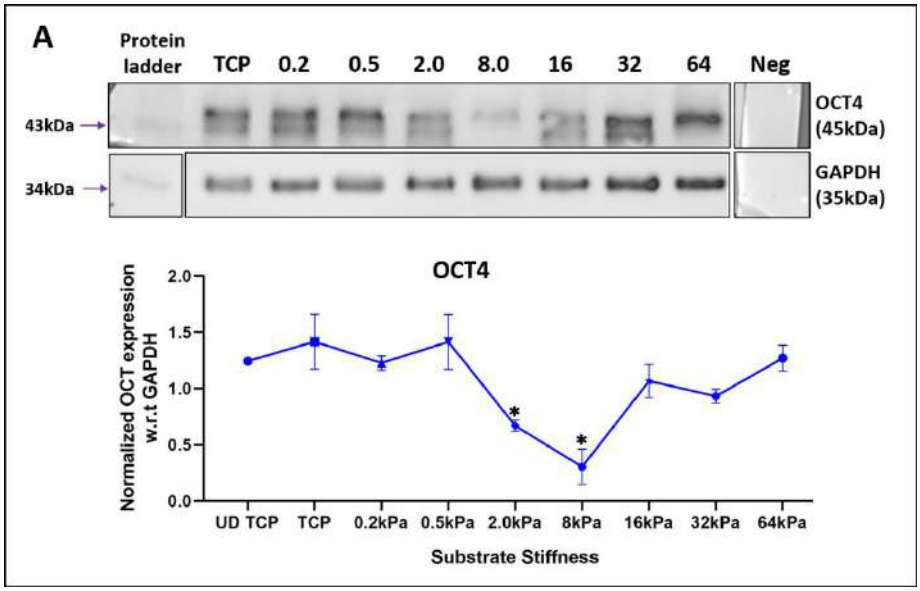


FIGURE 5.22: Characterization of DE differentiated cells in TCP and CS by immunoblotting, real time PCR and immunofluorescence. (A) Immunoblotting of pluripotency markers OCT4 was seen in TCP and all the stiffness of the CS substrates. GAPDH was used as a loading control. Protein levels were quantified and plotted as bar graphs that represent individual values of OCT4 normalized to respective GAPDH. The protein levels in CS were plotted relative to the protein levels in TCP. Statistical analysis was carried out by One-way ANOVA and Tukey's multiple comparison tests. Data represents mean ± SD. n = technical triplicates of three independent biological experiments. Asterisks (*) denote p values and represents statistical significance difference in the expression between the specific stiffness and TCP. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0.0005$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.

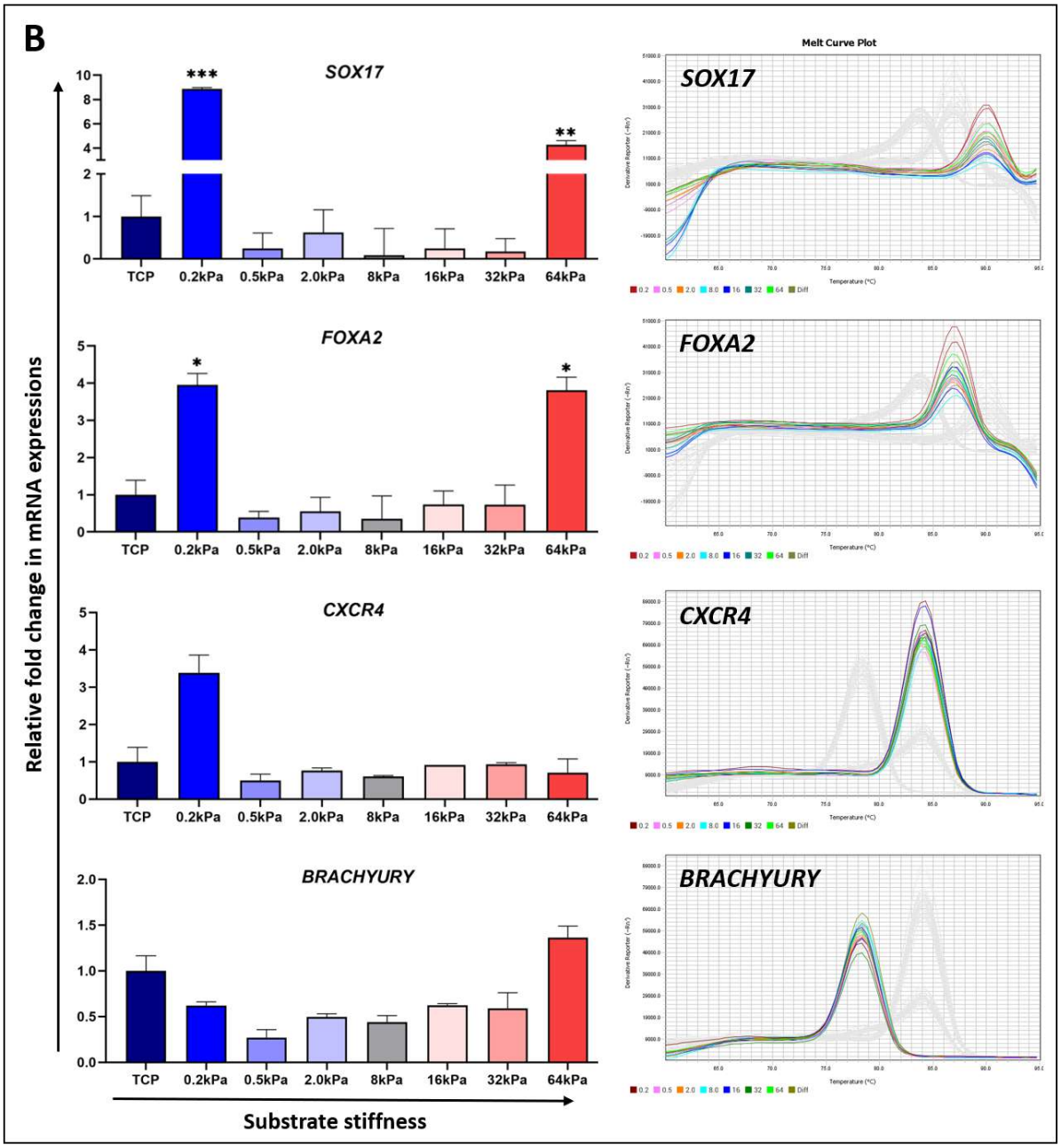


FIGURE 5.22: Characterization of DE differentiated cells in TCP and CS by immunoblotting, real time PCR and immunofluorescence. (B) Expression of DE lineage specific markers *SOX17*, *FOXA2, CXCR4*; mesoderm marker *BRACHYURY,* and ectoderm marker *PAX6* was studied using real time PCR. mRNA levels are shown relative to the endogenous control *18S rRNA* and the expression is plotted relative to the levels in the cells cultured on TCP. Melt curve of the respective PCR amplicons from various samples are shown in different colours. Statistical analysis was carried out by One-way ANOVA and Tukey's multiple comparison tests. Data represents mean ± SD. n = technical triplicates of three independent biological experiments. Asterisks (*) denote p values and represents statistical significance difference in the expression between the specific stiffness and TCP. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0.0005$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.

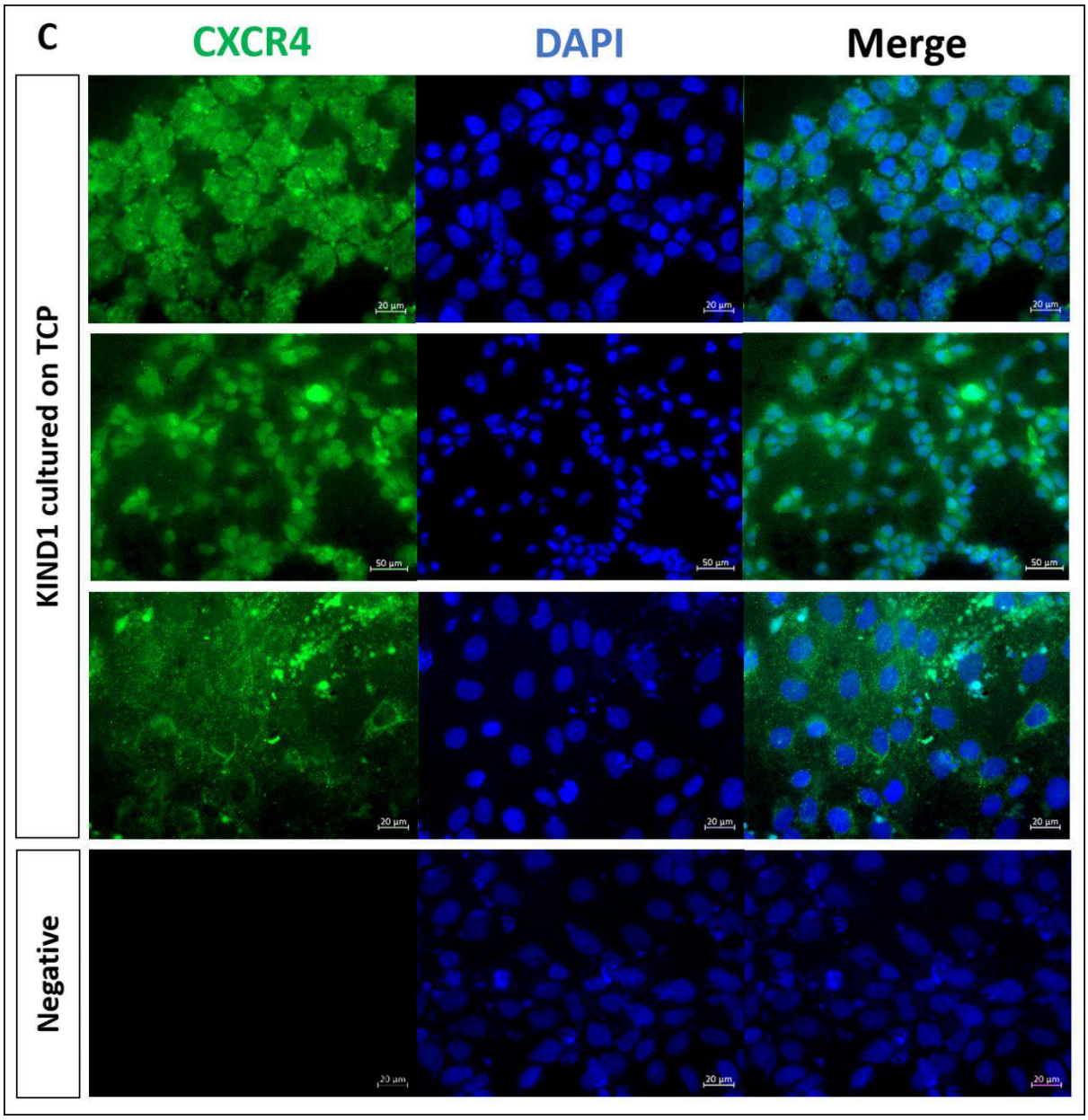


FIGURE 5.22: Characterization of DE differentiated cells in TCP and CS by immunoblotting, real time PCR and immunofluorescence. (C) Immunofluorescence showed the expression of CXCR4 (green) in the cytoplasm. A negative control, where the cells were treated with only secondary antibody, was used for the possibility of any non-specific bands due to secondary antibody. The nucleus was counterstained with DAPI (blue). Scale bar, 20µm and 50µm.

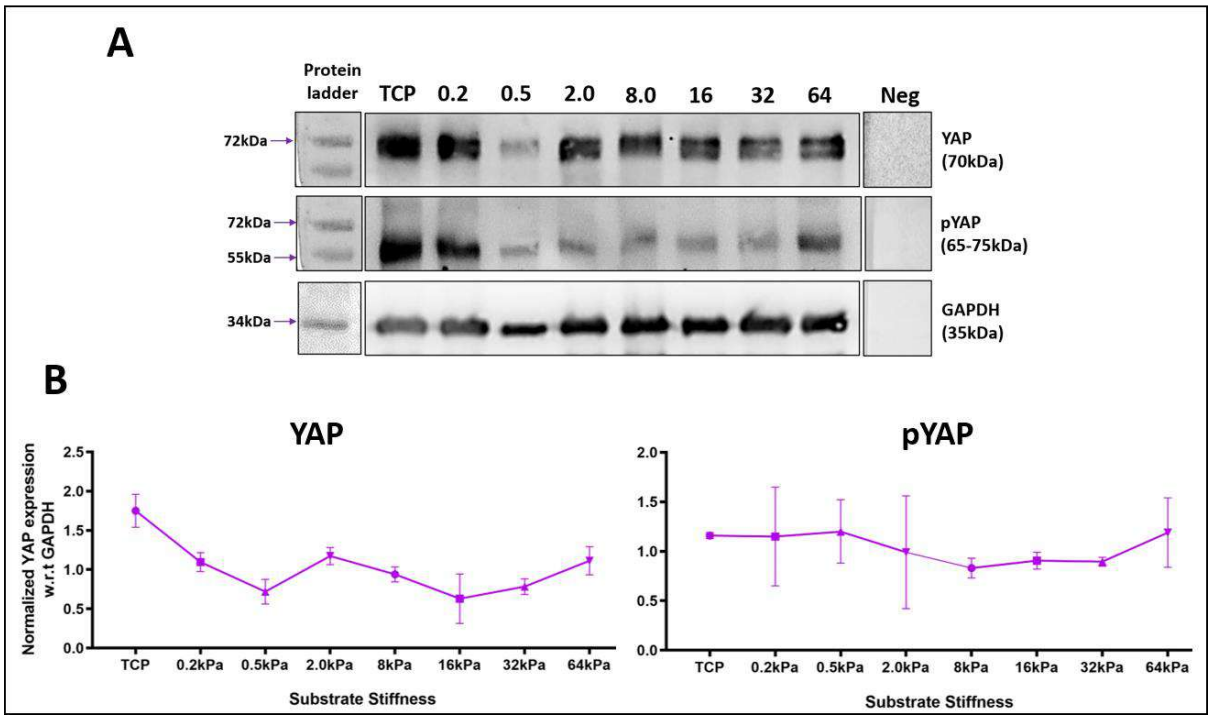


FIGURE 5.23: Protein levels of YAP and pYAP in DE cells in TCP and CS by immunoblotting. (A) YAP and pYAP protein levels in TCP and all the stiffness of the CS substrates was studied by western blot method, and GAPDH was used as a loading control. **(B)** Protein levels were quantified and plotted as graphs that represents individual values of YAP and pYAP proteins normalized to respective GAPDH and YAP respectively. The protein levels in CS substrates were plotted relative to the protein expressions in TCP. Statistical analysis was carried out by One-way ANOVA and Tukey's multiple comparison. Data represents mean ± SD, n = technical triplicates of three independent biological experiments.

5.9 YAP Inhibition by Verteporfin in Endoderm Differentiated cells on CS® Substrates

Based on the results we observed in DE differentiation study, we wished to study whether inhibiting the YAP expression in differentiating hESCs would affect the differentiation potential of hESCs. YAP inhibition has shown to enhance differentiation of hESCs (Rosado-Olivieri *et al.*, 2019; Quan *et al.*, 2021). Several studies in hESCs, MSCs, adult stem cells and even cancer cells have used Verteporfin (VP) to demonstrate the effect YAP inhibition has on the stem cell regulation and cancer progression (Rosado-Olivieri *et al.*, 2019; Dong *et al.*, 2020; Quan *et al.*, 2021). However, inhibition of YAP in differentiated hESCs cultured on substrates with varying stiffness has not yet been demonstrated. The exact mechanism of action of Verteporfin is unknown, however it has been reported that VP selectively binds to YAP causing some conformational changes in YAP structure, thereby eliminating YAP interaction with DNA binding proteins. Another

mechanism is that, VP increases the levels of 14-3-3σ protein in cytoplasm thereby blocking the YAP nuclear localization (Wang *et al.*, 2016) as illustrated in **FIGURE 5.24**.

For standardization of VP concentration for KIND1 cells, we used a large range of VP concentration: 10nM, 20nM, 30nM, 40nM, 50nM, 60nM, 70nM and 80nM. hESCs were cultured on TCP in complete E8™ medium and treated with the above-mentioned concentrations on day 3 after cell seeding. DMSO was used as vehicle control. We observed that hESCs treated with concentration above 30nM showed complete apoptosis of all the cultured hESCs. The cells from remaining three concentrations were harvested for protein, and YAP protein levels were observed. The VP concentration of 30nM VP showed significant downregulation in YAP level compared to its respective DMSO control **(FIGURE 5.25)**, hence this concentration was used for further studies.

Next, we went ahead to understand the effect of VP on endoderm differentiation. KIND1 cells were cultured on TCP, CS 0.2kPa stiffness substrate and CS 64kPa stiffness substrate. KIND1 cells were differentiated towards definitive endoderm by a well stablished protocol as described in **TABLE A3 (Appendix 3)** and **FIGURE 5.20**. The differentiated cells were treated with 30nM of VP on day 4 of the differentiation protocol and the cells were harvested for RNA and protein after 24 hours. To confirm the differentiation state, mRNA levels of lineage specific markers were analysed **(FIGURE 5.25)**. Expression of definitive endoderm markers: *SOX17, FOXA2*, and *CXCR4*, and mesoderm marker *BRACHYURY* confirmed that the hESCs have undergone mesoendodermal differentiation. In TCP, compared to their respective DMSO control, TCP-VP treated cells showed slight upregulation of *SOX17* and *CXCR4*, and downregulation of *BRACHYURY.* Among CS substrates, CS 0.2kPa stiffness substrate seem to support upregulation of all the lineage specific genes. This might indicate that hESCs favour softest substrate for mesoendodermal differentiation. However, VP does not seem to have significant effect on the hESCs on CS substrates when compared to TCP.

In the differentiation-VP treated culture system, there are three parameters, which has shown to effect YAP activity in individual studies: (1) substrate stiffness, (2) differentiation, and (3) inhibitor. The differentiation of the cells and the inhibitor have been reported to downregulate YAP expression in hESCs, whereas, soft stiffness of substrates have shown to upregulate YAP expression. We wished to see the effect of all the three parameters on YAP expression. Although YAP levels in TCP-differentiated-VP

treated cells was lower than TCP-differentiated-DMSO control, CS-differentiated-VP treated cells shower significant upregulation, especially on CS 64kPa stiffness substrate. pYAP level seen in all the culture systems, except for in TCP-differentiated-VP treated cells **(FIGURE 5.27A, B)**. From the known mechanisms, VP does not affect the other Hippo proteins, but on a soft substrate Hippo pathway is inactive. Immunoblotting showed a downregulation of the unphosphorylated form of the core Hippo proteins LATS, MST1, SAV and MOB in differentiated cells on CS 0.2kPa and CS 64kPa stiffness substrates **(FIGURE 5.27C)**. From the results of the inhibition study, we can conclude that the YAP inhibitor does not have an effect on the differentiation of hESCs, but the substrate stiffness and differentiation together seem to regulate YAP and Hippo pathway. This can be potentially explored further.

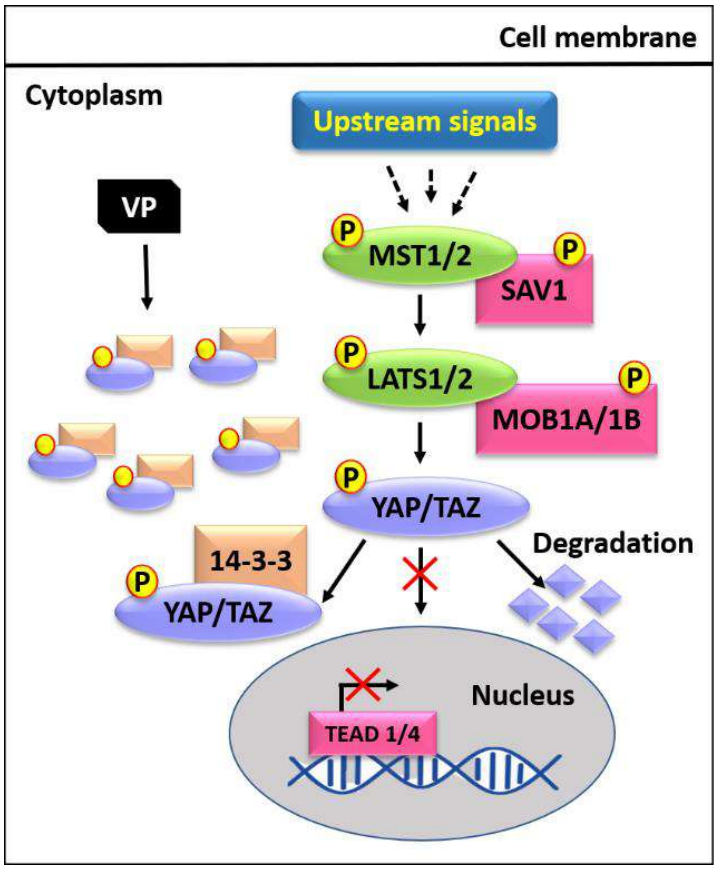


FIGURE 5.24: Schematic representation of the mechanism of action of Verteporfin in regulating YAP expression.

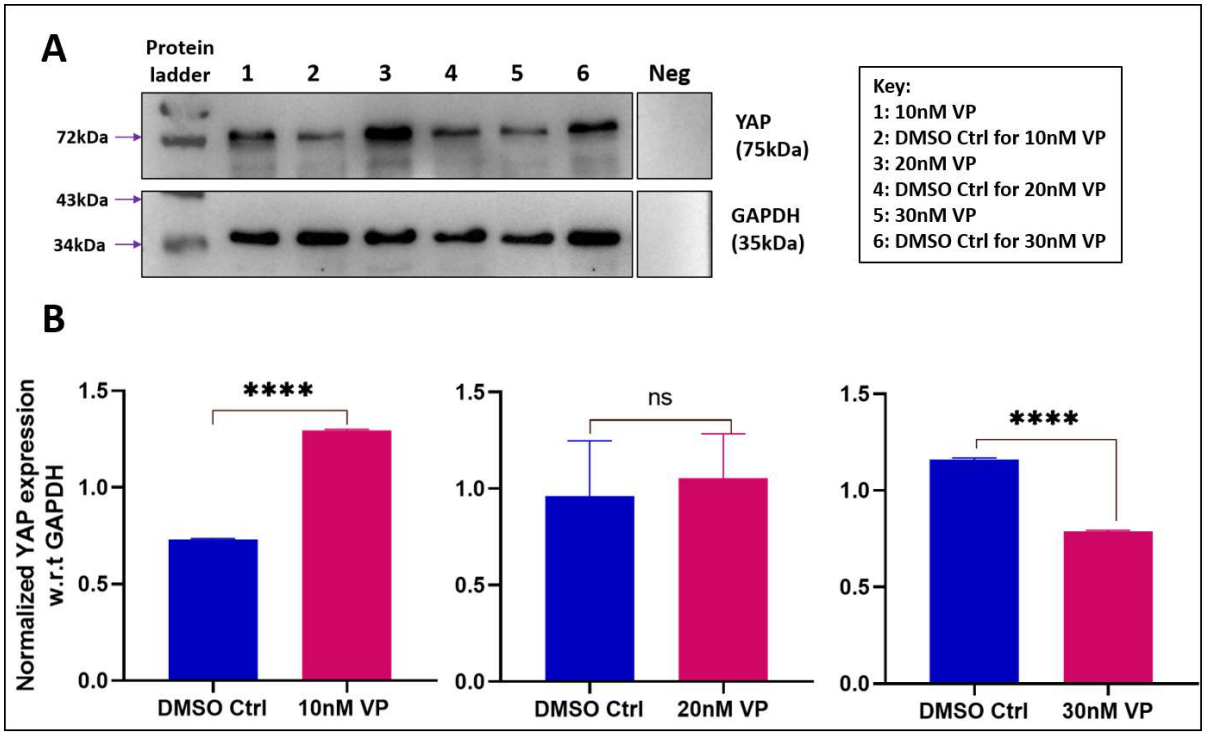


FIGURE 5.25: Standardization of Verteporfin concentration. Undifferentiated KIND1 cells cultured on TCP treated with various concentration of VP. DMSO was used as vehicle control for each concentration. **(A)** Immunoblotting showed YAP expression in the treated and control cells. GAPDH was used as a loading control. Protein levels was quantified and plotted as bar graphs that represent YAP proteins normalized to respective GAPDH. **(B)** The protein levels in treated cells were plotted relative to the protein levels in DMSO control cells. Statistical analysis was carried out by One-way ANOVA and Tukey's multiple comparison. Data represents mean $\pm$ SD. Asterisks (*) denote p values and represents statistical significance difference in the expression between the treated cells and the respective DMSO control. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0.0005$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.

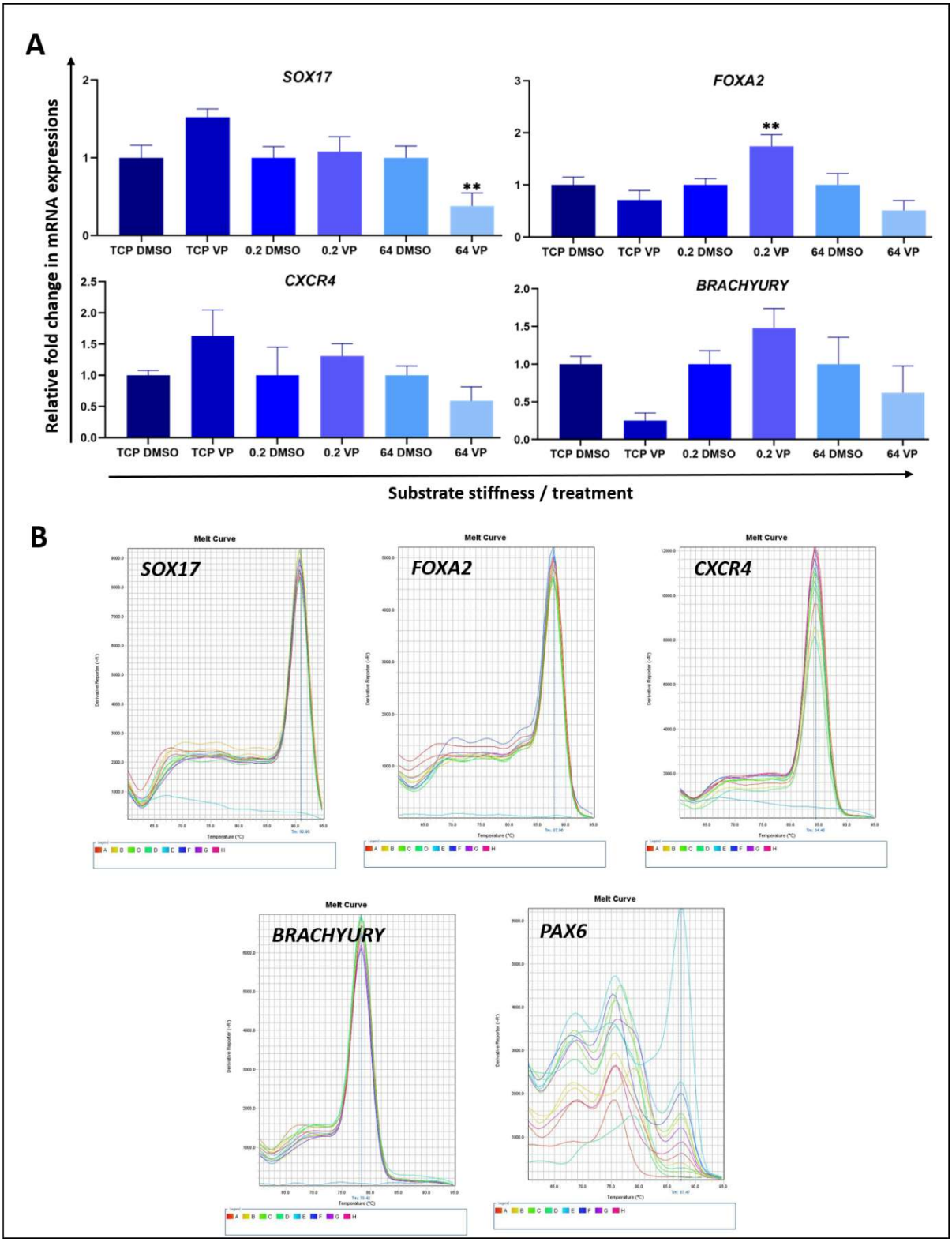


FIGURE 5.26: Gene expression analysis of DE differentiated- VP treated cells. Expression of DE lineage specific markers *SOX17*, *FOXA2, CXCR4*; mesoderm marker *BRACHYURY,* and ectoderm marker *PAX6* was studied using real time PCR. mRNA levels are shown relative to the endogenous control *18S rRNA* and the expression is plotted relative to the levels in the cells cultured on TCP and treated with DMSO (TCP DMSO control). Melt curve of the respective PCR amplicons from various samples are shown in different colours. Statistical analysis was carried out by One-way ANOVA and Tukey's multiple comparison tests. Data represents mean ± SD. n = technical triplicates of three independent biological experiments. Asterisks (*) denote p values and

represents statistical significance difference in the expression between the specific stiffness and TCP DMSO. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0.0005$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.

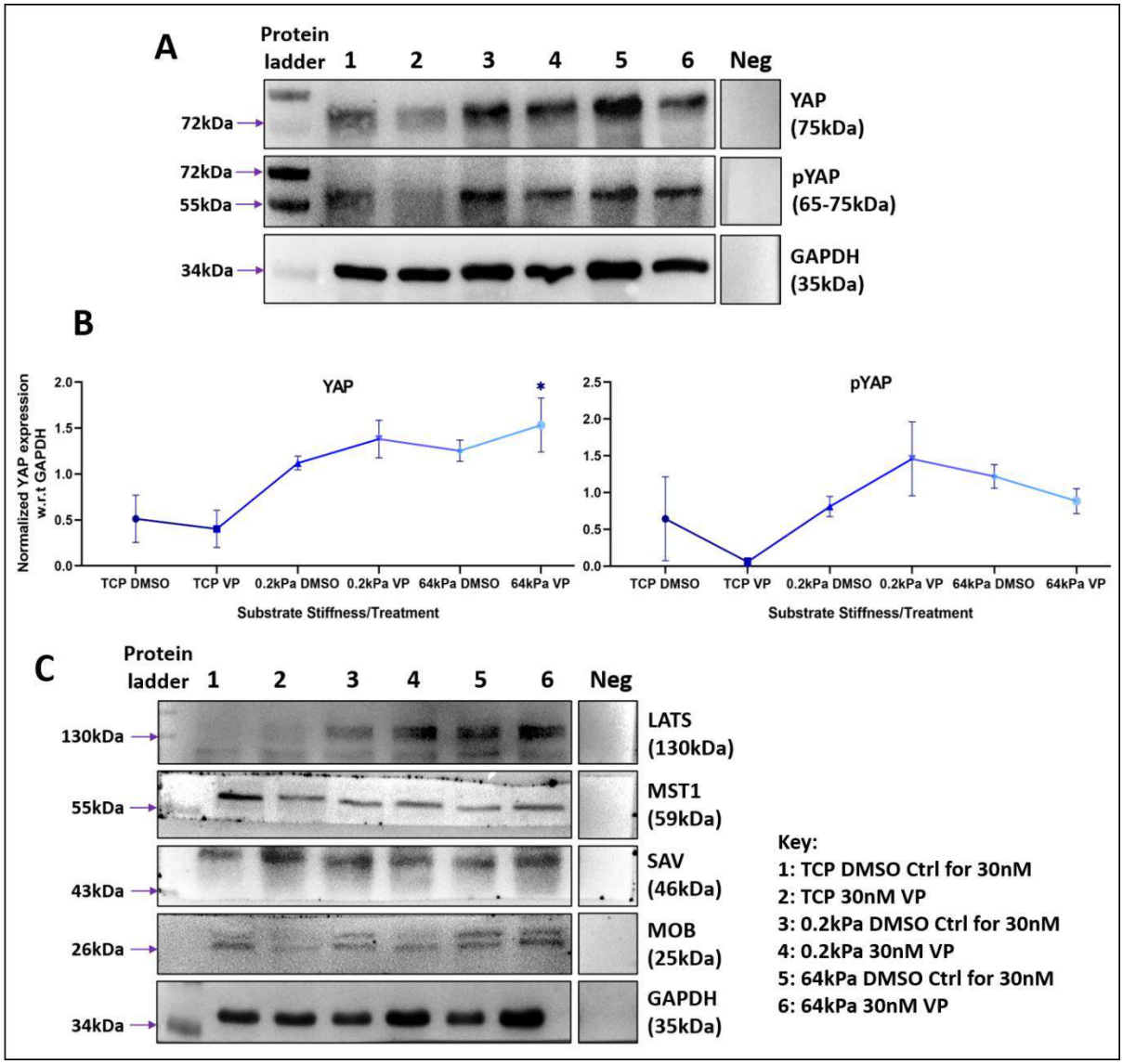


FIGURE 5.27: Expression of YAP and pYAP in DE differentiated-VP treated cells in TCP and CS by immunoblotting. Protein levels of **(A)** YAP, pYAP, and **(C)** core Hippo proteins LATS and MST1 with their co-effectors SAV and MOB were seen in TCP, CS 0.2kPa stiffness substrate and CS 64kPa stiffness substrate. GAPDH was used as a loading control. **(B)** Protein levels were quantified and plotted as graphs that represents individual values of YAP and pYAP proteins normalized to respective GAPDH and YAP respectively. The protein levels in CS were plotted relative to the protein levels in TCP and treated with DMSO (TCP DMSO control). Statistical analysis was carried out by One-way ANOVA and Tukey's multiple comparison. Data represents mean ± SD, n = technical triplicates of three independent biological experiments. Statistical analysis was carried out by One-way ANOVA and Tukey's multiple comparison tests. Data represents mean ± SD. n = technical triplicates of three independent biological experiments. Asterisks (*) denote p values and represents statistical significance difference in the expression between the specific stiffness and TCP DMSO. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0.0005$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.

5.10 YAP Stimulation by Lysophosphatidic acid in Endoderm differentiated cells on CS® Substrates

Intrigued by the inhibitor study, we hypothesized whether addition of YAP activator may show any change in the differentiation pattern across the substrate. YAP activator, lysophosphatidic acid (LPA) is a small lipid molecule which inhibits Hippo pathway by dephosphorylating LATS, thereby activating YAP expression in cells (Yu *et al.*, 2012) as illustrated in **FIGURE 5.28**. In 2016, Qin and colleagues found that activating YAP activity by supplementing lysophosphatidic acid (LPA) significantly induced the transition from the primed to the naïve state in multiple human ESC and iPSC lines, and the naïve state was prolonged in the culture medium supplemented with LPA. These results suggest an unexpected role of YAP in regulating the induction and maintenance of human naïve stem cells.

For standaridizing LPA concentration, we treated undifferentiated KIND1 cultured on TCP with 10µM, 15µM and 20µM concentration of LPA on day 3 after seeding. The YAP protein analysis showed that the 10µM of LPA significantly increases YAP levels in treated cell compared to respective DMSO control **(FIGURE 5.29)** and hence we used this concentration for differentiation studies. Next, we went ahead to understand the effect of LPA on endoderm differentiation in hESCs. KIND1 cells were cultured on TCP, CS 0.2kPa stiffness substrate and CS 64kPa stiffness substrate and were differentiated towards definitive endoderm by a well stablished protocol as described in **TABLE A3 (Appendix 3)** and **FIGURE 5.20.** The differentiated cells were treated with 10uM of LPA on day 4 of the differentiation protocol and the cells were harvested for RNA and protein after 24 hours. mRNA levels of lineage specific markers were analysed. LPA treated cells showed upregulation in the expression of *SOX17, FOXA2, CXCR4* and *BRACHYURY* confirming the mesoendodermal differentiation of KIND1 cells. While the expressions of *FOXA2, CXCR4* and *BRACHYURY* were significantly upregulated in CS 0.2kPa-VP treated cells, expressions of *SOX17* and *CXCR4* were significantly upregulated in CS 64kPa substrate **(FIGURE 5.30)**. From our observations, we can hypothesise that LPA treated cells on the softest substrate favour differentiation compared to their respective DMSO control.

In the differentiation-LPA treated culture system, there are three parameters, which has shown to effect YAP activity in individual studies: (1) substrate stiffness, (2) differentiation, and (3) activator. The soft stiffness of substrates and the activator have

been reported to increase YAP expression in hESCs, whereas, differentiation of the cells have shown to downregulate YAP expression. We wished to see the effect of all the three parameters on YAP expression. YAP level, when compared to the TCP-differentiated-DMSO cells, was equivalent or slightly upregulated in other systems, whereas, pYAP level was seen in CS-differentiated treated and non-treated cells. The protein analysis of YAP and pYAP levels showed an inverse relation as expected **(FIGURE 5.31A, B)**. LPA increases the concentration of unphosphorylated LATS which can be seen in **FIGURE 5.31C**. From our results we can hypothesis that LPA treatment and substrate stiffness might have an effect in regulating YAP expression in differentiated cells, however, the effect is not significantly noticed and needs to be studied further. Combined expression of lineage specific markers, YAP and pYAP in VP and LPA treated cells is illustrated in **FIGURE 5.32**.

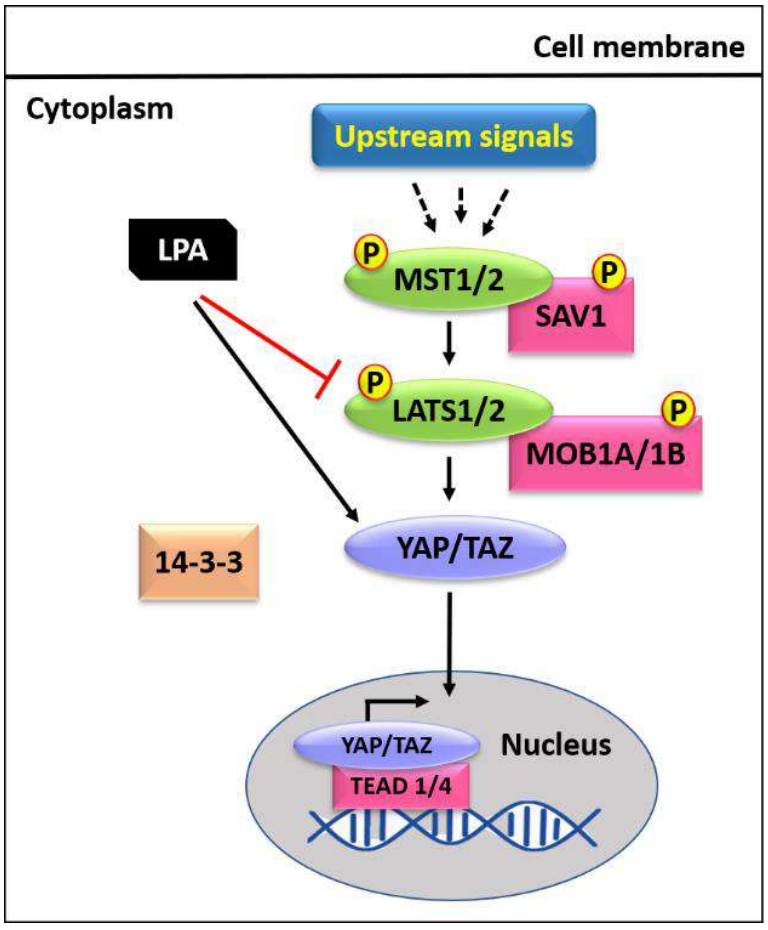


FIGURE 5.28: Schematic representation of the mechanism of action of Lysophosphatidic acid (LPA) in regulating YAP expression.

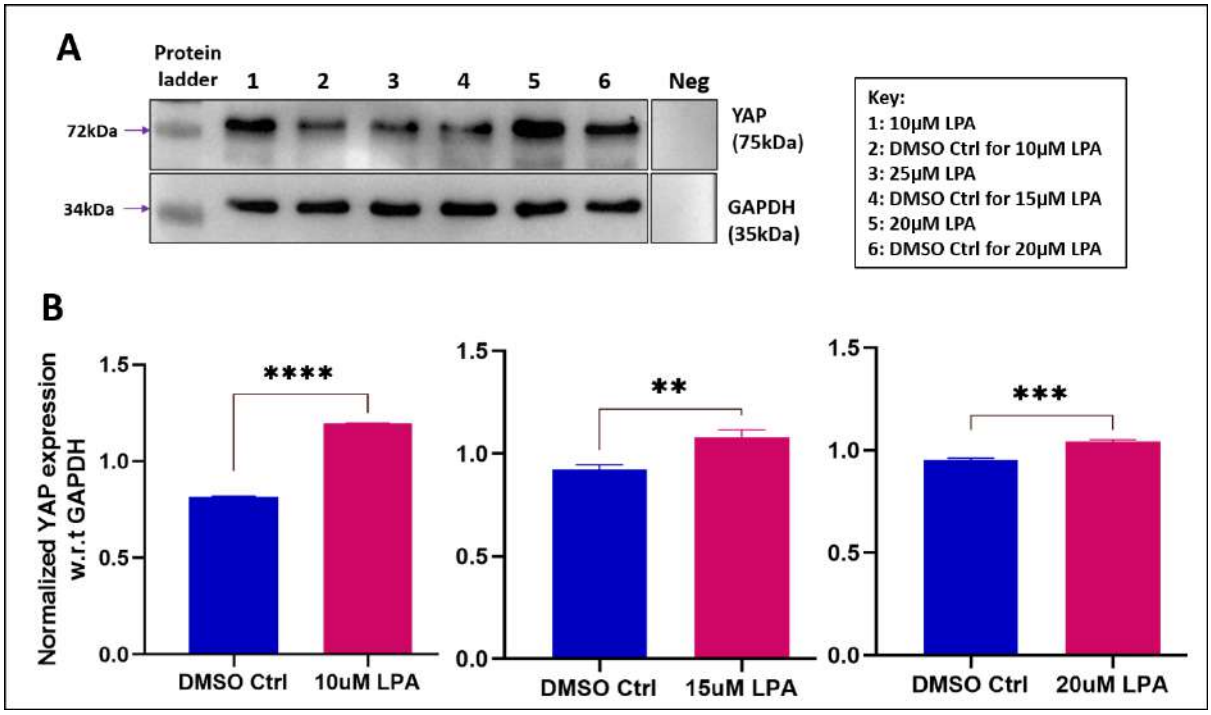


FIGURE 5.29: Standardization of Lysophosphatidic acid concentration. Undifferentiated KIND1 cells cultured on TCP treated with various concentration of VP. DMSO was used as vehicle control for each concentration. **(A)** Immunoblotting showed YAP levels in the treated and control cells. GAPDH was used as a loading control. **(B)** Protein expression was quantified and plotted as bar graphs that represent YAP proteins normalized to respective GAPDH. The proteins expression in treated cells was plotted relative to the protein expressions in DMSO control cells. Statistical analysis was carried out by One-way ANOVA and Tukey's multiple comparison. Data represents mean $\pm$ SD. Asterisks (*) denote p values and represents statistical significance difference in the expression between the treated cells and the respective DMSO. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0.0005$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.

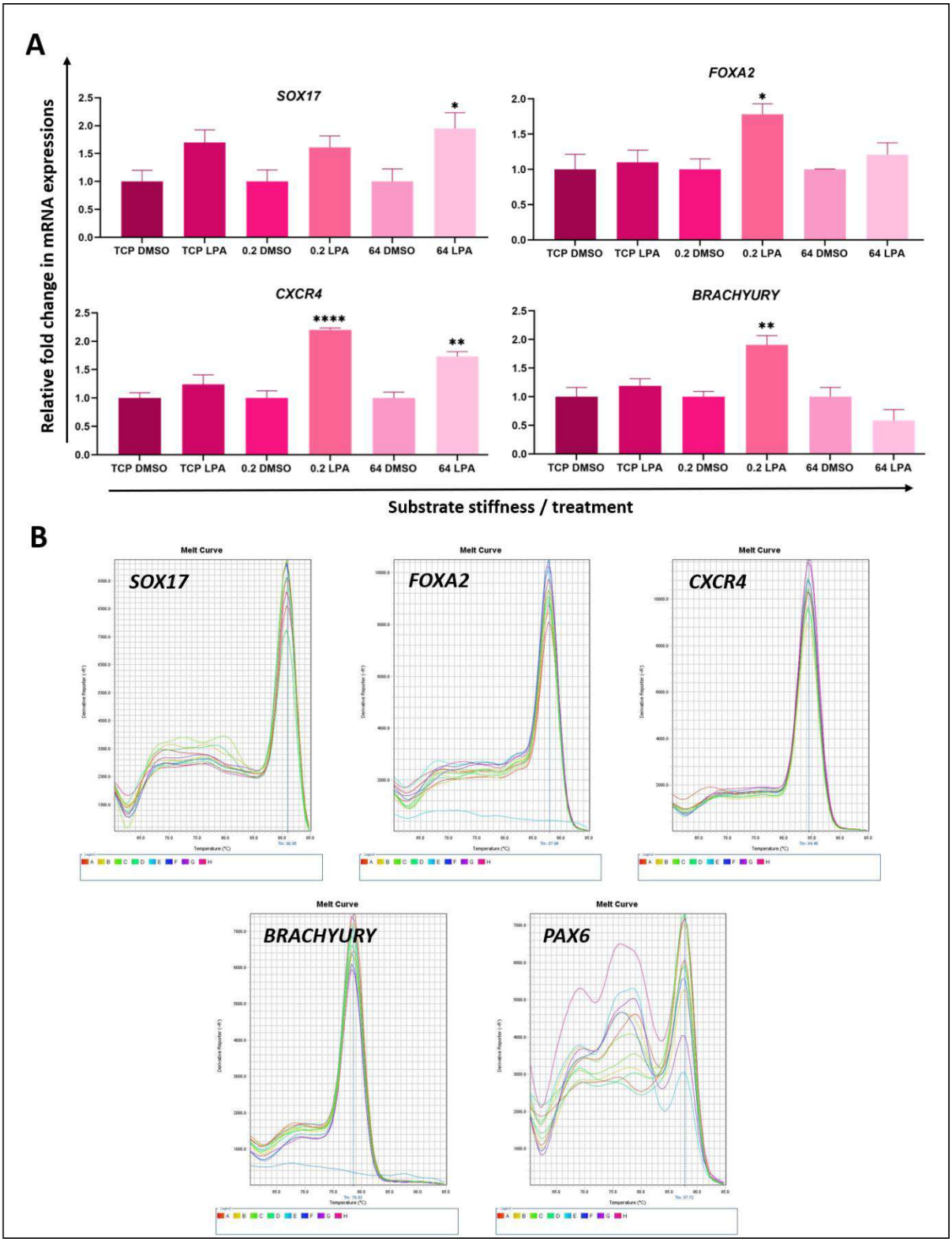


FIGURE 5.30: Gene expression analysis of DE differentiated-LPA treated cells. Expression of DE lineage specific markers *SOX17*, *FOXA2, CXCR4*; mesoderm marker *BRACHYURY,* and ectoderm marker *PAX6* was studied using real time PCR. mRNA levels are shown relative to the endogenous control *18S rRNA* and the expression is plotted relative to the levels in the cells cultured on TCP and treated with DMSO (TCP DMSO control). Melt curve of the respective PCR amplicons from various samples are shown in different colours. Statistical analysis was carried out by One-way ANOVA and Tukey's multiple comparison tests. Data represents mean ± SD. n = technical triplicates of three independent biological experiments. Asterisks (*) denote p values and

represents statistical significance difference in the expression between the specific stiffness and TCP DMSO. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0.0005$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.

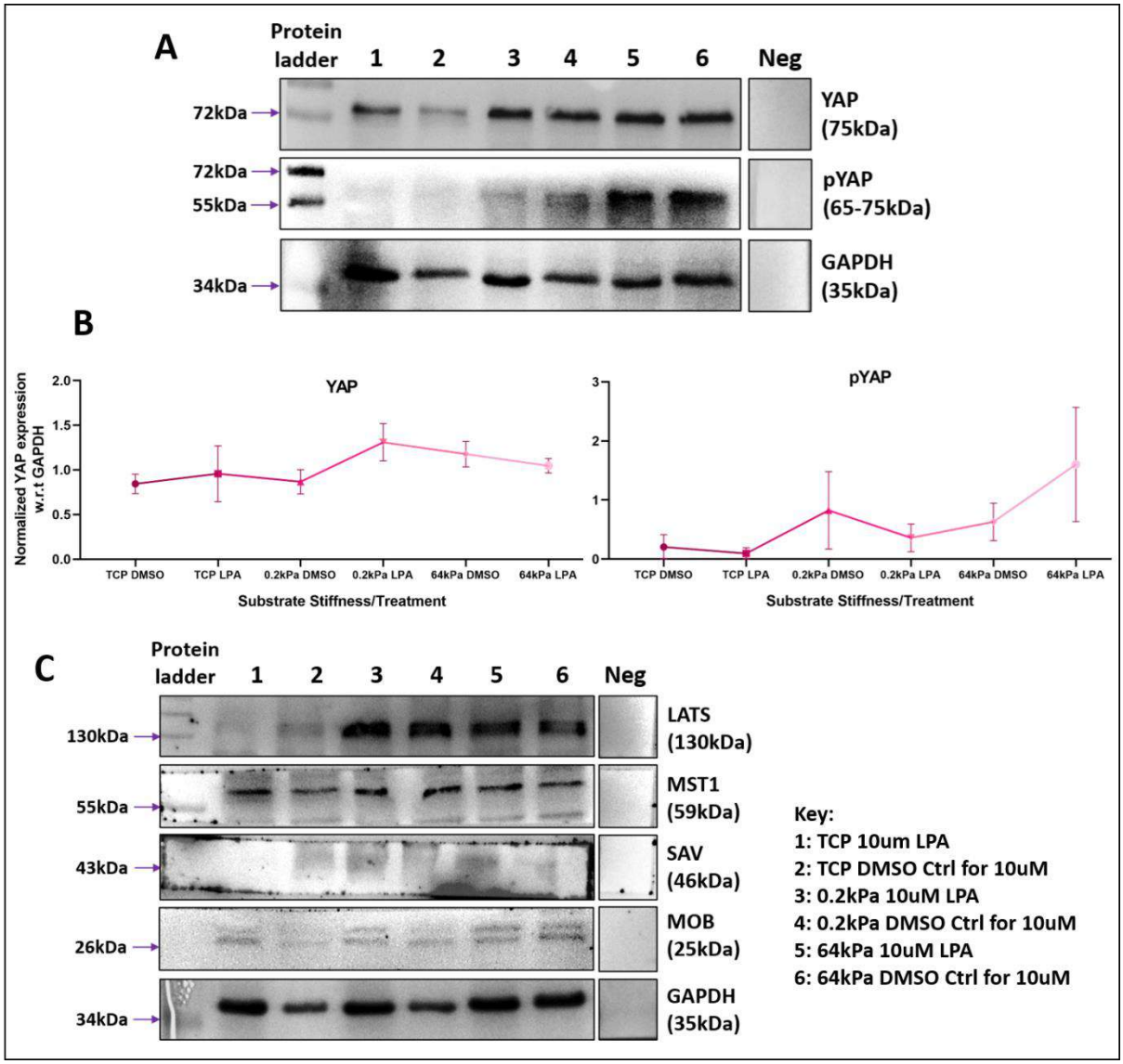


FIGURE 5.31: Expression of YAP and pYAP in DE differentiated-LPA treated cells in TCP and CS by immunoblotting. Protein levels of **(A)** YAP, pYAP and **(C)** core Hippo proteins LATS and MST1 with their co-effectors SAV and MOB was seen in TCP and two stiffnesses of CS substrates, 0.2kPa and 64kPa. GAPDH was used as a loading control. **(B)** Protein levels were quantified and plotted as graphs that represents individual values of YAP and pYAP proteins normalized to respective GAPDH and YAP respectively. The protein levels in CS were plotted relative to the protein expressions in TCP and treated with DMSO (TCP DMSO control). Statistical analysis was carried out by One-way ANOVA and Tukey's multiple comparison. Data represents mean ± SD, n = technical triplicates of three independent biological experiments. Statistical analysis was carried out by One-way ANOVA and Tukey's multiple comparison tests. Data represents mean ± SD. n = technical triplicates of three independent biological experiments. Asterisks (*) denote p values and represents statistical significance difference in the expression between the specific stiffness and TCP. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0.0005$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.

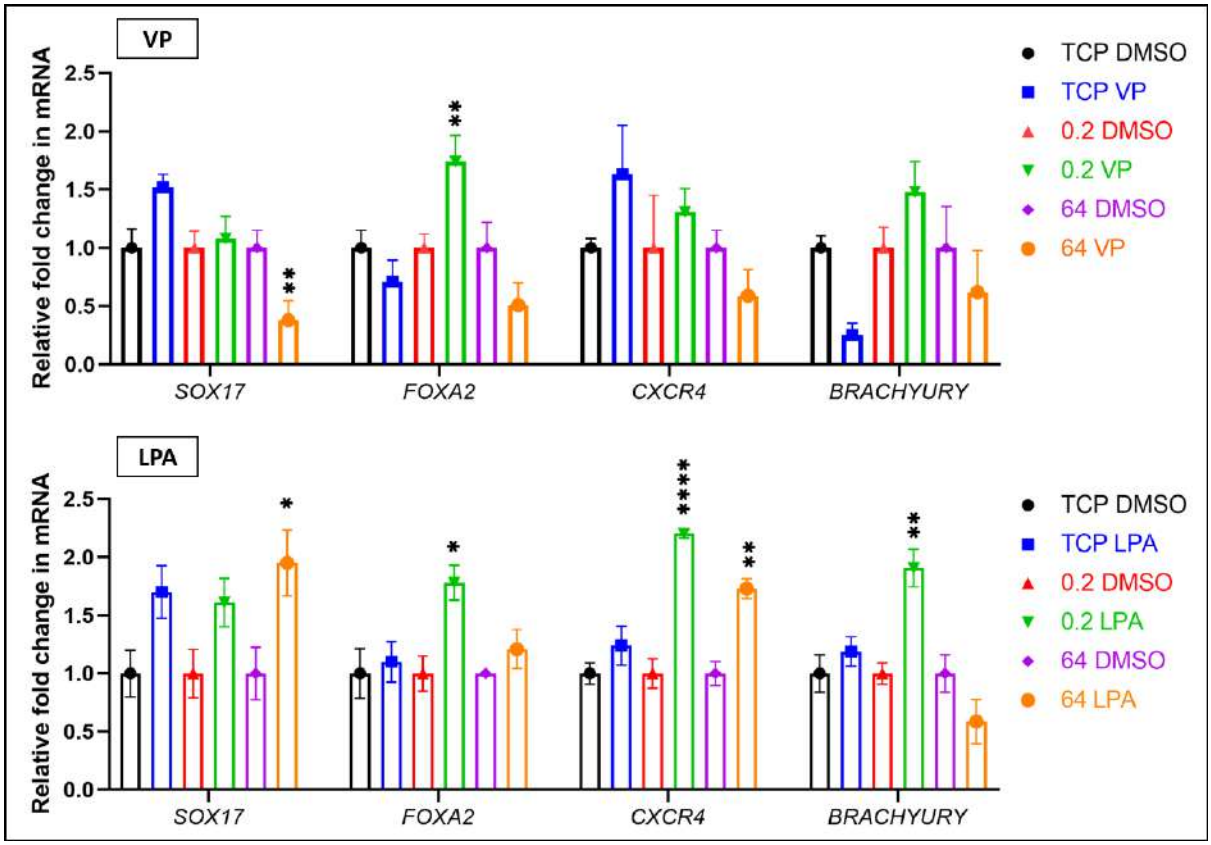


FIGURE 5.32: Combined data of differentiated cells treated with VP and LPA. The graphs show the expression of lineage specific genes in DE differentiated- activator (LPA) or inhibitor (VP) treated cells.

Chapter Six
Discussion

In this project, we aimed to investigate the corelation between the substrate stiffness, and the proliferation and differentiation potential of the human embryonic stem cells (hESCs), whilst focusing on the potential role played by the mechanosensor, YAP, during this interaction. The data, thus presented demonstrates that substrate stiffness alone does not induce differentiation in hESCs. We found that hESCs cultured on soft substrates maintained their stemness characteristics, and differentiated only when supplemented with differentiation inducing medium. Additionally, in both the culture condition, ubiquitous YAP expression was observed. Through directed differentiation of hESCs towards definitive endoderm (DE) stage on substrates of varying stiffness, we report that biochemical soluble molecules bring about the differentiational expression of DE-specific markers, *SOX17*, *CXCR4* and *FOXA2*, on all the stiffnesses alike, with high expression of active YAP. Through modulating YAP expression, we discovered that stimulating YAP enhanced the differentiation potential of the cells, however, inhibition of YAP did not show any significant effect on the differentiation potential. Overall, we shed light on the association between substrate stiffness, and YAP expression during the differentiation of hESCs towards definitive endoderm.

6.1 Pluripotent phenotype of hESCs on substrates of various stiffness

While plastic or glass plates, either uncoated or coated with feeder layer or ECM protein, is traditional way of culturing MSCs and ESCs (mouse and human), several studies have reported that stem cells respond to stiffness many folds less than the TCPs. While hMSCs differentiate towards neurogenic, myogenic or osteogenic lineage on substrate stiffness mimicking respective biological tissue stiffness (Engler *et al.* 2006; Driscoll *et al.* 2015; Hadden *et al.* 2017), mESCs either self-renew or undergo neurogenic differentiation on soft substrates and osteogenic differentiation on stiff substrate (Evans *et al.* 2009; Chowdhury *et al.*, 2010; Keung *et al.* 2012; Candiello *et al.*, 2013; Przybyla *et al.*, 2016). In our study we report that hESCs cultured on the soft substrates, self-renew and maintain their stemness as observed by their colony characteristics, protein expression of pluripotency markers OCT4 and NANOG, and concomitant absence of the lineage markers. Surprisingly, we observed similar results in hESCs cultured in ultra-low attachment dishes and maintained in Essential 8™ medium. However, the pluripotent state of the hESCs was due to the substrate stiffness or the pluripotency factors of the medium was unclear. Addition of minimum serum to the basal medium initiated

differentiation of hESCs, thus proving that soluble signalling biomolecules of the medium and not the substrate stiffness could be maintaining the hESCs in undifferentiated state and initiating differentiation.

Almost all the studies have used combination of differentiating factors and biologically similar substrate stiffness to induce differentiation in MSCs, mESCs and hPSCs (Li *et al.*, 2010; Shih *et al.*, 2011; Park *et al.*, 2011; Lanniel *et al.*, 2011; Vincent *et al.*, 2013; Hindley *et al.*, 2016; Sun *et al.*, 2018; Gerardo *et al.*, 2019; Yamazaki *et al.*, 2021), Although, very few report the mechanoresponsiveness of hESCs, one such finding showed that the hPSCs cultured on soft substrate developed round 3D colonies and remained pluripotent for seven days, however, prolonged culture on soft substrate induced spontaneous differentiation (Maldonado *et al.*, 2015). Contrary to this, we observed that hESCs cultured on 2D soft substrates displayed similar morphologies to hESCs cultured on TCP, and remained pluripotent for three consecutive passages. Moreover, round 3D cell aggregates obtained in ULAD did not undergo spontaneous differentiation. The substrates used by Maldonado and group are electrospun fibrous substrates having pores along the length of the fibre surface, therefore, the stiffness and surface topological variations might be controlling the cellular morphology and thereby, hESCs fate.

Interestingly, hPSCs have been cultured in suspension conditions for large-scale productions in the fields of cell-based therapy and drug discoveries. To achieve this, carrier beads and hESCs are suspended in pluripotency maintaining medium, like mTeSR™ or StemPro medium, with constant stirring to form cell aggregates. The hPSCs cultured in suspension conditions remain pluripotent as seen by the expression of OCT3/4, NANOG, SSEA-4, and TRA-1-60, and their potential to differentiate into the three germ layer cells (Amit *et al.*, 2010; Olmer *et al.*, 2010; Zweigerdt *et al.*, 2011; Abbasalizadeh *et al.*, 2012; Otsuji *et al.*, 2014). Therefore, our observation that hESCs cultured in ULAD under pluripotent and static conditions is in line with the fact that hPSCs can maintain their stemness without the support of the substrate.

Human ESCs cultured in conventional conditions are in a state called "primed pluripotency" that resembles the post-implantation epiblasts (Niakan *et al.*, 2012), unlike mouse ESCs which can remain in a naive ICM-like state after being induced by one growth factor LIF and two inhibitors MEK and GSK3 (Martello and Smith, 2014). Therefore, the

different cellular responses of hESCs and mESCs to substrate stiffness might be due to the naïve and primed states of the cells. It would be interesting to see the fate of naïve hESCs grown using a defined medium suitable for naïve pluripotency such as 5i/L/A (Khan *et al.*, 2021; Fischer *et al.*, 2022) on range of soft substrates. In human MSCs, multipotency is regulated by different set of factors and it has been reported that MSCs express higher levels of non-muscle myosin II (NMII), an important actin-binding protein crucial for cell adhesion, spreading and migration. As a result, changes in stiffness would have more significant effect on NMII regulation in MSC (Ma *et al.* 2010; Arora *et al.* 2015), and will increase their sensitivity to variations in stiffness than ESCs (Olivares-Navarrete *et al.*, 2017; Gerardo *et al.*, 2019). It has been observed that ROCK inhibitor (Y2632), NMII inhibitor, increases the expression of pluripotency regulators OCT3/4 and NANOG, and enhances revival of human pluripotent stem cells (Walker *et al.* 2010). Therefore, it is possible that the soft substrates are possibly preventing the activation of NMII when compared to the stiffer substrates and thereby allowing continuous expression of OCT4 and NANOG. The findings of these studies might explain why the hMSCs, and not hESCs, differentiates when cultured on substrates of different stiffness. In hESCs, the effect of soft substrate on the regulation of NMII deserves a separate investigation.

6.2 Ubiquitous YAP expression on all stiffnesses in Pluripotency Sustaining Media and Differentiation Inducing Media

YAP is a transcriptional coactivator, mainly regulated by Hippo pathway, and plays a crucial role in the regulating self-renewal, and differentiation of stem cells. However, YAP's role in stem cell fate determination is context-dependent and can vary among different stem cell types. The diverse functions of YAP in different stem cells arise from the intricate interplay between various signalling pathways, lineage-specific factors, cellular microenvironment, epigenetic regulation, and the developmental stage of the stem cell population as discussed in the chapters above. Understanding these dynamics is crucial for deciphering the precise mechanisms underlying YAP's role in stem cell biology and for harnessing its potential for regenerative medicine applications. Numerous studies have noted that on stiff substrates, YAP is active and localized in the nucleus where it helps in maintaining self-renewal and proliferation of stem cells; whereas on soft substrate, YAP gets phosphorylated which leads to its cytoplasmic retention and eventually destruction (Dupont *et al.* 2011; Musah *et al.*, 2014; Yamazaki

et al., 2021). The substrate stiffness has been reported to control YAP localization either by F-actin in hESCs and MSCs (Wada *et al.*, 2011; Zhao *et al.*, 2012; Piccolo *et al.*, 2014) or by NMII and Peizo1 in MSCs (Pathak *et al.* 2014).

Here, we assessed the effect of changing stiffness on YAP expression, and how this correlates with pluripotency and differentiation. We observed equivalent levels of YAP and pYAP on TCP and soft substrates in pluripotency sustaining medium and differentiation inducing medium. Our results contradict the many reports in the literature which state that in MSCs, YAP is inactive on soft substrate. Nevertheless, an interesting study showed that hESCs remain proliferative and pluripotent even on softest substrate from 150 Pa to 1.2 kPa and our results concur with their findings. They found that on stiff substrate ($E >$ 1kPa) YAP localizes to the nucleus and on soft substrates ($E <$ 450 Pa) YAP shows a heterogenous distribution with maximum nuclear localization at the periphery of the colony and mixed localization in the colony interior, this pattern resembles localization of YAP in the inner cell mass (ICM) of the early embryo (Price *at el.* 2017).

In order to determine the involvement of Hippo pathway, we examined the levels of upstream regulators of YAP. We observed expression of unphosphorylated MST1, SAV, LATS and MOB1, indicating that Hippo pathways is inactive in hESCs on TCP and soft substrates, however, determining the levels of phosphorylated form of these proteins could provide us with better understanding of Hippo pathway on these substrates. Studies revealing YAP activity on stiff substrates and inactivity on soft substrates indicate that actin cytoskeleton and Rho strongly regulate YAP activity, but do not mention Hippo core proteins as regulator of YAP expression (Dupont *et al.*, 2011; Zhao *et al.*, 2012). Indeed, the crosstalk between the Rho and Hippo signalling pathways via Src signalling pathway in regulating YAP activity has been reported in lung cancer (Hsu *et al.*, 2020), but such discovery has yet to be made in hESCs. There is also a possibility that the response of YAP is independent of the MST and LATS signalling pathway. Our results are first to explore the substrate induced role of core hippo proteins in pluripotency and differentiation of human embryonic stem cells.

Nonetheless, from the PMM and DIM results in TCP, CS substrates and ULAD, we can postulate that the biochemical composition of the growth media, and not the substrate

stiffness, is the main deciding factor in determining the fate of hESCs and in regulating the expression of YAP.

6.3 Directed Differentiation towards Definitive Endoderm from hESCs

Next, we explored the effect of directed differentiation of hESCs cultured on varying stiffness. Numerous stages of embryogenesis and foetal development are either affected by or generate mechanical forces (Ingber, 2003). More specifically, during gastrulation, blastula epiblast cells ingress (Keller at el., 2003; Beloussov and Grabovsky, 2003; Gadue *et al.*, 2005), the cells undergo changes in motility and shape, which is attributed to reorganization of the cytoskeleton within the cell (Odell *et al.*, 1981; Farge, 2003; Ingber, 2003; Beloussov and Grabovsky, 2006). This change in the cytoskeleton organization is caused by the mechanical forces acting on the cellular surfaces. Therefore, the mechanical forces play an essential role during the lineage-specification of the gastrulation phase. *In vitro*, optimal differentiation has been reported to be achieved on a substrate with the same stiffness as the natural microenvironment, for example, optimal scaffold stiffness obtained by controlling the ratio of PA gels or ploy-lactic acid (PLLA)/poly-lactic co-glycolic acid (PLGA) can direct the specific stages of myoblast differentiation and their organization into myotubes (Engler *et al.*, 2004; Levy-Mishali *et al.*, 2009). A benchmark study by Engler and colleagues demonstrated that MSCs cultured on soft PA substrate differentiate towards neurogenic lineage, those cultured on intermediate stiffness PA substrates favours myogenic lineage and MScs cultured on stiff PA substrates differentiates towards osteogenic lineage (Engler *et al.*, 2006). Most importantly, the MSCs used were first primed on the substrates and then differentiated using soluble factors. Another study demonstrated that soft PA substrate having stiffness similar to that of the liver enables maturation of hPSCs-derived hepatocytes better than stiff PA substrate (Mittal *et al.*, 2016). Jaramillo *et al.* (2015) revealed that mESCs cultured on soft stiffness substrates elicit strong upregulation of early endodermal related genes *Sox17*, *Afp* and *Hnf4* compared to stiff substrates.

Similarly, an intriguing study from Nam lab demonstrated the mechanoresponsiveness of hiPSCs at various stages of lineage commitment upon addition of specific growth factors. Immunofluorescent imaging showed that induction of hiPSCs towards ectoderm is enhanced on soft substrates as seen from the protein expression of PAX6, while the specification to neural progenitors and motor neurons

requires stiff substrates indicative from the expression of NESTIN and HB9 markers respectively. Unlike ectoderm differentiation, when hiPSCs were differentiated towards endodermal lineage, expression of mesoendodermal markers were higher on the stiff substrates compared to the soft substrate and TCP, while further differentiation towards posterior foregut and pancreatic endoderm were enhanced on soft substrates evident from the gene expression of *FOXA2* and *NKX2*.2. Conversely, BRACHYURY expression confirmed that mesodermal specification from the mesoendodermal cells require stiff substrate, but further specification towards chondrocytes require soft substrate. From these studies we can conclude that dynamic changes in the stiffness of substrates regulates the differentiation efficiency in hiPSCs (Maldonado *et al.*, 2017). Another study in hPSCs provided the evidence that soft substrate (Young's modulus ~3 kPa) promotes higher expression of endoderm-specific genes *SOX17* and *FOXA2* in comparison with stiff substrate (Young's modulus ~165 kPa) and TCP, by regulating SMAD2/3 via long non-coding RNA LINC00458 (Chen *et al.*, 2020).

DE formation is one of the most important outcomes of gastrulation, since it is the first germ layers to be form and it contributes to the formation of the other two germ layers (Lawson *et al.*, 1987;Kimmel *et al.*, 1990; Muhr and Ackerman, 2020). Thus, we wanted to determine the effect of substrate stiffness on the DE formation *in vitro*. Additionally, mechanical signals along promote formation of gastrulation (Keller *et al.*, 2003; von Dassow and Davidson, 2007; Muncie *et al.*, 2020). Therefore, based on literature, we hypothesized that substrate stiffness might play a vital role in initiating the differentiation towards a definitive endoderm (DE) lineage. Following a previously established protocol we differentiated hESCs, KIND1, towards definitive endoderm state using only one specific growth factor, ACTIVIN A (D'Amour *et al.* 2006; Pethe *et al.*, 2014; Dumasia and Pethe, 2021). Exogenous induction of high levels of ACTIVIN A has shown to induce mesoendoderm differentiation of hESCs by upregulating the expression of early endoderm markers: *SOX17* and *CXCR4* and mesodermal marker: *BRACHYURY* (Wang *et al.*, 2015).

We initiated the differentiation on TCP and CS substrates simultaneously, and after four days of continuous exposure to ACTIVIN A, we observed a consistent level of OCT4 protein in TCP and CS substrates. In undifferentiated hESCs, the pluripotency specific genes (*OCT4*, *NANOG* and *SOX2*) inhibit the differentiation towards DE by blocking the

expression of the mesoendodermal genes (Teo *et al.*, 2011), however, upon receiving DE signals the same pluripotency specific genes initiate mesoendoderm induction before their expression declines after day 4 (Pethe *et al.*, 2014; Dumasia *et al.*, 2021). Our observation of OCT4 levels at day 4 of DE stage are consistent with these findings. The expression of cell surface marker CXCR4 was detected at day 4 in DE-differentiated cells on TCP. CXCR4 is specific markers of definitive endoderm and expressed only during the early differentiation stage of hESCs (Liang *et al.*, 2020). Additionally, during *in vivo* endoderm induction, Cxcr4+ cells migrate through the primitive streak to form the definitive endoderm (McGrath *et al.*, 1999; Yasunaga *et al.*, 2005). CXCR4 positive cells also expressed *SOX17* and *FOXA2*, transcriptional regulators that are crucial for DE formation (Kanai-Azuma *et al.*, 2002; Kubo *et al.*, 2004; Burtscher and Lickert, 2009), and *BRACHYURY*, transcriptional regulator of mesoendoderm (Murry and Keller, 2008; Faial *et al.*, 2015). hESCs differentiated on the CS substrates also expressed DE specific markers: *SOX17*, *FOXA2* and *CXCR4*; and mesoendodermal marker: *BRACHYURY. SOX17* expression was highest on the CS 0.2kPa stiffness substrate by 10-fold increase and on the CS 64kPa stiffness substrate by 5-fold when compared to the expression on TCP. Similar pattern was observed for *FOXA2* expression. *CXCR4* expression was highest on CS 0.2kPa stiffness substrate only, whereas, *BRACHYURY* favoured stiff substrate. Our results concur with the findings of Maldonado *et al.* (2017) and Chen *et al.* (2020) which showed that early DE differentiation is enhanced on soft substrates while mesoendodermal differentiation favours stiff substrate.

Curiously, hESCs undergo differentiation towards ectoderm, endoderm and mesoderm lineages on TCP in presence of soluble differentiating factors (Pethe *et al.*, 2014). Therefore, lineage specifications are not likely to be greatly affected by mechanical stiffness. Further differentiation towards mature cell-type might give provide us with better understanding of the lineage-specific mechanomodulation of hESCs.

6.4 YAP expression in DE cells during Mechanomodulation

We studied the expression of mechanosensor YAP in these differentiated cells cultured on substrates of varying stiffness. According to literature, during differentiation of mESCs and hESCs on TCP, YAP is downregulated (Qin *et al.*, 2016; Heng *et al.*, 2020; Quan *et al.*, 2022; Zeevaert *et al.*, 2023). However, we saw constant levels of YAP in all the differentiated cells on TCP and CS substrates from the three independent biological

experiments when compared with the differentiated cells on TCP. Comparing these observations with the YAP and pYAP levels in PMM and DIM study, we can say that the YAP expression is not affected by the substrate stiffness, and the YAP levels are relative strong in DE differentiated cells than in undifferentiated cells and undirected differentiated cells. Therefore, our data contradicts the many published reports which states that: (1) YAP is inactive on the soft substrate, and (2) YAP is downregulated during differentiation of mouse and human ESCs.

ACTIVIN A, used for inducing differentiation towards DE stage, is a member of transforming growth factor family (TGF-β) superfamily of cytokines (Piek *et al.*, 1999). They are involved in numerous biological processes, including cell differentiation, proliferation, and tissue development. Using hESCs, Estarás *et al.* (2017) showed that YAP knockout cells treated with ACTIVIN A, enabled ACTIVIN-induced Wnt3 expression, and stabilized β-catenin, thereby synergizing SMAD signalling and activating mesodermal genes required to form the cardiac mesoderm. Another study showed that YAP$^{-/-}$ hESCs can be efficiently differentiated into anterior primitive streak by using ACTIVIN A (Hsu *et al.*, 2018). Few studies which have focused on the interaction between YAP and ACTIVIN A on TCP, has reported an inverse relation between the two. However, there is still a possibility that ACTIVIN A signalling could regulate the activity or localization of YAP within the cell. This interaction may involve modulating the Hippo pathway or influencing YAP's translocation into the nucleus. Alternatively, YAP and ACTIVIN A may cooperate in regulating gene expression by acting as transcriptional partners. ACTIVINs are known to activate various downstream effectors, including SMAD proteins, and YAP, as a transcriptional co-activator, can bind to specific transcription factors. Therefore, YAP and ACTIVIN A might work together to regulate the differentiation on soft substrates.

6.5 Verteporfin induced inhibition of YAP

Verteporfin (VP), a benzoporphyrin derivative, is a photosensitizer drug that was first used in photodynamic therapy (PDT) for the treatment of age-related macular degeneration (AMD) (Mellish and Brown, 2001; Parodi *et al.*, 2016). Recent studies have reported a significant role of VP in cancer therapy as well (Ma *et al.*, 2016; Wei *et al.*, 2017; Wei and Li., 2020 a; Wei and Li, 2020 b). Elevated levels of YAP have been observed in multiple tumours and as an oncoprotein, YAP has been linked-to increased tumour progression and metastasis in human cell lines and mouse models (Camargo *et al.*, 2007;

Orr *et al.*, 2011; Cordenonsi *et al.*, 2011; Kang *et al.*, 2011; Li *et al.*, 2012; Azzolin *et al.*, 2014; Noguchi *et al.*, 2014; Lau *et al.*, 2014; Zanconato *et al.*, 2015, 2016; Panciera *et al.*, 2017; Nguyen and Yi, 2019).

Liu-Chittenden *et al.* (2012) found that VP selectively binds to YAP and inhibits the YAP-TEAD complex in absence of ligand activation. There are more than fifty drugs that have been shown to inhibit YAP activity, however, VP is the only drug as it acts directly on the YAP (Juan and Hong, 2016). VP binds to YAP, bringing about conformational changes in YAP structure and disrupting YAP-TEAD interaction (Liu-Chittenden *et al.*, 2012) or VP sequester YAP in the cytoplasm by increasing the levels of 14-3-3σ, a YAP chaperon protein that retains YAP in cytoplasm, resulting in YAP degradation by proteasomal enzymes (Wang *et al.*, 2016). Consistent with this, YAP has been studied as potential VP target in many cancer cells and mouse models (Lin *et al.*, 2015; Pan *et al.*, 2016; Wei *et al.*, 2017; Li *et al.*, 2017; Kang *et al.*, 2018; Trautmann *et al.*, 2019; Li *et al.*, 2019). Majority of the VP-YAP studies are in cancer cells or cancer stem cells, there is limited literature on YAP-inhibition by VP in hPSCs and MSCs which focuses on inhibiting YAP to induce hPSCs differentiation towards cardiac lineage (Han *et al.*, 2020), trophoblast stem cells (Dong *et al.*, 2020) or chondrocytes (Yamashita *et al.*, 2021). Our study is the first to show the effect of VP in DE-differentiating cells on soft substrate.

In cancer cells, VP concentration between 2μM-15μM inhibited YAP expression and tumour progression (Pan *et al.*, 2016; Wang *et al.*, 2016; Kang *et al.*, 2018; Lui *et al.*, 2019), while 1nM-1μM concentration of VP downregulated YAP expression in hESCs and induced differentiation (Han *et al.*, 2020; Dong *et al.*, 2020; Yamashita *et al.*, 2021). VP concentration of 30nM reduced YAP expression in undifferentiated KIND1 cells compared to the DMSO control, however, in DE differentiated-VP treated cells, YAP was not significantly downregulated. Insufficient data is available on verteporfin effect in differentiated cells, but according to observations reported by Rosado-Olivieri *et al.* (2019), during directed differentiation of hPSCs towards β-cells, YAP was expressed throughout stages 3-6 of pancreatic differentiation, however, YAP expression declined gradually as the differentiation progressed. They used 350nM concentration of VP to downregulate YAP, and showed that YAP inhibition enhanced differentiation towards endocrine cells and β-cells. We found VP concentration higher than 30nM to be toxic in KIND1 cells cultured on TCP. During differentiation, YAP is indeed downregulated (Lian

et al., 2010; Zhang *et al.*, 2012; Sun *et al.*, 2020; Zeevaert *et al.*, 2023) and addition of YAP-inhibitor should enhance the differentiation, however, inconsistent YAP expression in our results may be due to an insufficient concentration of VP. Expression of unphosphorylated MST1, SAV, LATS, MOB on soft substrate might be regulating YAP expression, however, we also postulate that YAP expression could be regulated by an unknown mechanism other than Hippo pathway in differentiating cells on soft substrates.

6.6 Modulating YAP expression by Lysophosphatidic acid

Lysophosphatidic acid (LPA), a bioactive lipid, is a versatile soluble signalling molecule which can induce a wide-range of cellular responses, for example, cell proliferation and survival, cell migration, immune function, calcium mobilization, and act as a mediator for various pathological conditions (Ishii *et al.*, 2004; Yung *et al.*, 2014; Geraldo *et al.*, 2021). LPA signalling is essential in embryonic development, as deletion of autotaxin, an enzyme which produces extracellular LPA, or lipid phosphatase 3 (LPP3), an enzyme that catalyses LPA, has proven to be embryonic lethality in mice (Escalante-Alcalde *et al.*, 2003; van Meeteren *et al.*, 2006; Moolenaar *et al.*, 2013). Paradoxically, overexpression of autotaxin has also resulted in embryonic death due to severe vascular defects (Yukiura *et al.*, 2015).

In mouse and human ESCs, LPA binds to LPA-receptors or G-protein coupled receptors to activate a number downstream signalling pathways including extracellular signal regulated kinase (ERK), p38, c-jun N-terminal kinase (JNK), signal transducer and activator of transcription 3 (STAT3), and phospholipase C (PLC) to regulate ESC proliferation, self-renewal and pluripotency (Pitson *et al.*, 2009; Todorova *et al.*, 2009; Kime *et al.*, 2016). The interaction between the LPA and YAP was first established in 2012, when the authors reported that LPA and sphingosine-1-phosphate (S1P) can promote YAP dephosphorylation at Ser127 site, resulting in translocation of YAP into the nucleus (Yu *et al.*, 2012; Miller *et al.*, 2012). Serum-borne LPA can also regulate nuclear translocation of YAP by inhibiting LATS1/2 through Gα12/13-coupled receptors, while other core Hippo pathway proteins remain unaffected (Yu *et al.*, 2012; Cai and Xu, 2013). In human ESCs and iPSCs, LPA has been reported to activate YAP to induce naïve pluripotency (Qin *et al.*, 2016; Hsiao *et al.*, 2016). In human MSCs, LPA was found to promote differentiation towards osteogenic lineage by increasing YAP levels

(Lorthongpanich *et al.*, 2019). Our study is the first to show the effect of LPA in DE-differentiating cells on soft substrate.

On soft substrates, LPA treatment upregulated expression of endoderm differentiation markers. Although the reports of LPA in hPSCs differentiation is limited, one study demonstrated that constant treatment of hPSCs with LPA inhibits expansion of neural progenitor cells and differentiation of hPSCs towards neuronal lineage (Frisca *et al.*, 2013). Conversely, another study identified LPA as a major factor in expansion of hESCs-derived neural progenitor cells. Constant treatment of cells with LPA and in absence of neural or glial differentiating factors, resulted in the formation of neural rosette-like structures which was maintained over many passages (Medelnik *et al.*, 2018). However, in both the studies LPA activates Rho pathway and actin filaments to inhibit expansion and to induce morphological changes. Therefore, it is worth investigating Rho pathway and its downstream transcriptional factor SRF which is an important regulator of actin dynamics in LPA-treated DE differentiated cells. Furthermore, LPA has five distinct receptors and each receptors regulates different biological process, for instance, in hMSC-TERT cells, inhibition of LPA1 through LPA antagonist inhibits differentiation towards osteogenic lineage, whereas, inhibition of LPA4 with shRNA significantly increased osteogenesis of hMSC-TERT cells (Liu *et al.*, 2010). Therefore, we cannot rule out the possibility that LPA receptors might behave differently in DE differentiated cells on in response to soft substrate.

LPA concentration of 2-10μM has been reported to increase the YAP levels in hESCs on TCP (Qin *et al.*, 2016; Hsiao *et al.*, 2016), and we observed similar results in undifferentiated KIND1 cells, where 10μM of LPA showed significant upregulation of YAP protein levels compared to the DMSO control. The protein analysis of YAP and pYAP levels showed an inverse relation as expected. In the LPA-treated DE differentiated cells, we have combined three parameters which has shown to effect YAP activity in individual studies: (1) substrate stiffness, (2) differentiation, and (3) activator. The soft stiffness of substrates and the activator have been reported to increase YAP expression in hESCs, whereas, differentiation of the cells have shown to downregulate YAP expression. Therefore, from these results we can hypothesize that the combination of soft substrate and activator maintains the levels of YAP throughout the systems. This combinational

study needs special investigation to understand the interlink between stiffness-mediated YAP regulation during differentiation.

6.7 Summary

Taken together, our data suggests that after a prolonged culture, hESCs remain pluripotent when cultured in pluripotency maintaining medium on soft substrates and differentiate in minimum differentiation inducing medium but not in response to change in substrate stiffness. This implies that hESCs have different mechanism in sensing the substrate stiffness than hMSCs or mESCs; and for hESCs to differentiate, biochemical signals play a more crucial role than substrate stiffness. During directed differentiation of hESCs on varying stiffness, YAP expression was not effected by the differentiation or by substrate stiffness. There can be varying reasons attributing to these observations. It is possible that hESCs respond well to the signalling molecules and are resistant to the substrate stiffness. The CytoSift® substrate used in our study is PDMS hydrogel with limited stiffness range, whereas *in vivo* a wide range of stiffness of various tissue is determined by biologically active ECM proteins. Therefore, there is a possibility that hESCs did not reposnd to the selected stiffness used in our study.

Although YAP inhibition and overexpression did not show major changes in YAP expression in soft substrates, but we observed a pattern indicating that YAP inhibition slightly downregulated the differentiation potential whereas YAP stimulation by LPA increased the differentiation potential of hESCS into endoderm lineage. VP has been used in later stages of pancreatic differentiation on TCP (Rosado-Olivieri *et al.*, 2019), and widely used in studies focusing on cancer research, and our data provides an insight on the YAP inhibition in hESCs during early stages of endoderm differentiation. There has been no reports showcasing YAP stimulation during hESCs differentiation or on varying stiffness, and our data is the first presenting the complex interconnection between LPA and substrate stiffness during the early stages of endoderm differentiation.

Chapter Seven
Conclusion

Through this work, we present a different perspective on the relationship between the substrate stiffness, and Yes-associated protein (YAP) in maintaining the pluripotency and inducing the differentiation of hPSCs.

The undifferentiated hESCs line, KIND1 cells were successfully cultured on and maintained for a prolonged period on CytoSoft® substrates having stiffness of 0.2kPa, 0.5kPa, 2.0kPa, 8kPa, 16kPa, 32kPa and 64kPa. Our results showed that hESCs do not undergo substrate-induced differentiation on soft CS substrates, and for differentiation of hESCs soluble molecules plays a pivotal role. Expression of endoderm-specific markers showed that hESCs can be differentiated on soft substrates as efficiently as on traditional culture plates. The most significant finding was that YAP expression was substantial in undifferentiated and differentiated human embryonic stem cells on soft substrates as well as TCP, on the contrary, pYAP levels were downregulated on CS substrates compared to TCP. Our study on YAP modulation is one of the first to implement YAP inhibitor and activator for understanding the role of YAP during hESCs differentiation towards definitive endoderm on soft substrates. Addition of a YAP activator not only upregulated YAP levels in differentiating cells but also enhanced the differentiation potential of hESCs towards definitive endoderm. Surprisingly, the YAP inhibitor did not efficiently show any effect on YAP expression on CS substrates compared to TCP.

The present work suggests that substrate stiffness alone might not be most effective to drive lineage-specific and terminal differentiation in human embryonic stem cells, even though mechanical forces have been reported to play a crucial role in controlling stem cell self-renewal and differentiation. Furthermore, the mechanical cues generated from the substrate stiffness can be an important cofactor in the differentiation of hESCs caused by soluble biochemical factors, but they are not the only determining factor. With these new insights, we have shed light on the importance of biochemical and mechanical signals during the early stage of hESCs differentiation towards definitive endoderm in vitro. It is crucial to understand the interplay between substrate stiffness and YAP regulation in undifferentiated and differentiated hESCs to improve the protocols for regulating hPSCs differentiation for tissue engineering and stem cell therapies.

Chapter Eight

Implications and Future Prospects

Our study has contributed to the understanding of the biochemical and mechanical mechanisms in the human pluripotent stem cells during definitive endoderm differentiation. We show interactions between various parameters such as soluble growth factors, pharmacological modulators, mechanical stress and cues that influences hPSCs differentiation *in vitro*. The data presented in our study implies that hESCs might not be as mechanoresponsive as MSCs or mESCs, YAP is not affected by the differentiation of the hESCs as compared to the differentiation in MSCs, and mechanosensors other than YAP might sense stiffness in hESCs. Based on the findings of our study, and the above-mentioned implications, the current research provides a number of opportunities for further investigation and analysis. Some future prospects are listed below, (but are not restricted to these points):

- A comparative data using wide range of stiffness, i.e., from pascal to megapascal would provide insights in hESCs proliferation and differentiation due to mechano-responsiveness
- We used commercially available PDMS (CytoSoft®) substrates as a precaution against batch-to-batch variations, however, exploring, other biomaterials, such as Matrigel®, electrospun nanofibers or polyethylene glycol (PEG), could provide us with comparative data hESCs response to a variety of biomaterials.
- To study the expression of phosphorylated forms of other Hippo proteins
- Analyse the cytoplasmic and nuclear fractions of Hippo pathway components in undifferentiated hESCs and differentiated cells
- Investigating the various other signalling pathways, such as TGF-β, WNT, for the indication of their role in regulating YAP during differentaitaion from hESCs on varying stiffness
- Effect of YAP repression on Activin, SMADs, WNT3/β-catenin and epigenetic regulating proteins in undifferentiated and differentitaed hESCs on soft, intermediate and stiff substrates
- YAP binding to TEAD has been reported to induce *OCT4*, *NANOG* and *SOX2* expression, therefore exploring the YAP/TEAD interaction in hESCs cultured on various stiffness substrates in undifferentiated and differentiated state would help in validating YAP's role in hESCs
- Use of RNA-sequencing or Hi-C approach to examine the expression profile of known signalling pathway proteins, transcription factors, DNA binding proteins in

hESCs cultured on varying stiffness in pluripotency sustaining medium and during differentiation of hESCs

- Comparative study of the gene expressions between MSCs and hPSCs cultured on wide range of stiffness
- To study the later stages of endoderm differentiation on varying stiffness, and also explore the differentiation of hESCs towards mesoderm and ectoderm lineages on varying stiffness
- It would be interesting to conditionally knockout or directly target YAP by CRISPR or shRNA to delineate the role of Hippo pathways on hESCs pluripotency or during differentiation
- Study the biological effect of knockdown of YAP in hESCs cultured on varying stiffness with and without differentiation inducing soluble molecules
- Use of various concentrations of YAP inhibitor, Verteporfin and YAP activator, Lysophosphatidic acid on differentiating hESCs
- To study the moduations in YAP levels on varying stiffness during late endoderm differentiation
- Combinational study with two or more biophysical factors, such as stiffness and shear fluid flow, could provide with a better understanding on the effect of mechanical forces on the stem cell behvaiour
- Examining the YAP expression in hESCs cultured in 3D-culture system and mainatined in differentiation inducing medium
- Gene expression analysis in hESCs with knockdown or reduced YAP cultured in ULAD or hESCs-generated organoids

Chapter Nine

Procedures and Methodology

The list of all the reagents and materials used are mentioned in detail, along with the make and catalogue number, in **Annexure I**. The preparation of all the reagents mentioned below is explained in detail in **Annexure II**, unless stated otherwise.

A. Cell culture

i. **Ethical statement:** Human embryonic stem cells line KIND1 was provided by Dr. Deepa Bhartiya from the National Institute for Research in Reproductive and Child Health (NIRRCH), Mumbai, India. hESCs research was approved to use by Institutional Committee for Stem Cell Research (IC SCR), SVKM's NMIMS (deemed-to-be) University, Mumbai, India, Project proposal no.: NMIMS/IC SCR/022/2017 **(Annexure VII)**, and the Institutional Committee for Stem Cell Research (ICSCR) at Symbiosis Centre for Stem Cell Research (SCSCR), Symbiosis International (deemed) University, Project proposal no.: SSBS/ICSCR/2019/02 **(Annexure VIII)**.

ii. **Human Embryonic Stem Cell Maintenance:** KIND1 cells were regularly maintained in a serum-free complete Essential 8™ medium (with 1X supplement) and cultured on a feeder-free system of truncated-recombinant human ECM protein Vitronectin. Prior to culturing, tissue culture plates (TCP) were coated with 1X Vitronectin diluted in 2mL of sterile DPBS and incubated at RT/37^{0}C for 1 hour. Complete Essential 8™ medium containing 0.1% penicillin/streptomycin was pre-warmed as per the manufacturer's instructions before daily medium change or subculturing. The cells were incubated in a 5% CO_2 incubator under humified conditions at 37^{0}C (HERAcell VIOS 160i, Thermo Fischer Scientific, USA). For documentation, the unstained cells were photographed by an inverted phase-contrast light microscope (Carl Zeiss AG, Axio Vert.A1, Germany).

iii. **Human Embryonic Stem Cell Maintenance Sub-culturing:** KIND1 cells were passaged every 4th or 5th day (70% - 80% confluency). Before beginning the protocol, the cells were incubated with fresh medium for 30 minutes and later the medium was collected in a sterile 15mL tube labelled as spent medium. The cells were rinsed with 500µl of warm DPBS to remove residual medium and dead cells. The washed cells were incubated with 500µl of warm 0.5mM EDTA at 37^{0}C for 3-4 minutes, the EDTA was gently discarded and the cells were collected using the spent medium in a sterile

15ml tube. The cell suspension was centrifuged (Eppendorf, Hamburg, Germany) at 1000 rpm at RT for 5 minutes. The supernatant was discarded, while the pellet was resuspended in 1mL of the fresh complete Essential 8™ medium through gentle pipetting. Cells were plated onto vitronectin coated TCP or substrates containing 2mL of fresh complete Essential 8™ medium and incubated in a 5% CO_2 incubator under humified conditions at 37^0C (HERAcell VIOS 160i, Thermo Fischer Scientific, USA). The medium was not changed on the next day of the passage but was changed daily thereafter.

[NOTE: During resuspending the pellet, caution was taken to avoid generating single cell suspension and small cell aggregates were maintained.]

iv. **Human Embryonic Stem Cell Maintenance Cryopreservation:** The undifferentiated healthy cells were cryopreserved when the plate was 75% - 80% confluent. Before beginning the protocol, the cells were incubated with fresh medium for 30 minutes and later the medium was collected in a sterile 15mL tube labelled as spent medium. The cell pellet was obtained as per the above-mentioned protocol using 0.5mM EDTA, and cells were resuspended in a 500µL of complete Essential 8™ medium. The cell suspension was transferred to a labelled cryovial and to this, 500µL of a cold mix was added. The cold mix was prepared as follows - 400µL complete Essential 8™ medium and 100µL of sterile DMSO (a cryoprotectant) to make 10% DMSO solution. The 1mL of the cell suspension was transferred into a cryovial (Genaxy, India). During the resuspension step, care was taken to not obtain a single cell suspension. The cryovials were immediately stored at -80^0C refrigerator in Mr. Frosty for 20-24 hours before transferring to liquid nitrogen for long term storage.

v. **Human Embryonic Stem Cell Maintenance Revival:** The culture dishes were coated with 1X vitronectin as mentioned above. The desired cryovial was removed from the liquid nitrogen cannister and placed in a beaker containing warm water. The vial was the thawed by rubbing it between the palms and the cell suspension was transferred to a 15mL tube containing 9mL of pre-warmed complete Essential 8™ medium. The cell suspension was centrifuged at 1000 rpm at RT for 5 minutes. The supernatant was discarded and the cell pellet was gently resuspended in 1mL of the fresh complete Essential 8™ medium. The resuspended cells were seeded onto the

Vitronectin coated plates and incubated in a 5% CO_2 incubator under humified conditions at 37^0C. The medium was not changed the next day of the passage but was changed daily thereafter.

vi. **Undirected differentiation of hESCs:** The undifferentiated hESCs were cultured on Vitronectin coated TCP or substrates and maintained in differentiation inducing media (DIM) with minimum serum concentration and no lineage-specific soluble growth factors. Briefly, undifferentiated KIND1 cells with 80% confluency were detached using EDTA method as mentioned above. The pellet thus obtained was resuspended in Advanced Dulbecco's Modified Eagle Medium (Adv. DMEM) which was supplemented with 2% fetal bovine serum (FBS) and 1% antibiotic solution. The cells were incubated in a 5% CO_2 incubator under humified conditions at 37^0C (HERAcell VIOS 160i, Thermo Fischer Scientific, USA). The cells were maintained in DIM medium for 4 days and the medium was changed alternate day.

vii. **Endoderm differentiation from hESCs:** Directed differentiation of KIND1 cells towards definitive endoderm lineage was carried out by following a well-established protocol (Pethe et al., 2014; Dumasia et al 2021) with minor modifications. Briefly, undifferentiated KIND1 cells with 80% - 85% confluency were used for differentiation and harvested after day 4 of the differentiation protocol for RNA and protein. The differentiation was carried out as follows: day 4 undifferentiated cells were rinsed with DPBS followed by culturing in Rosewell Park Memorial Institute (RPMI)-1640 medium supplemented with 0.025X ITS, 100ng/mL ACTIVIN A and antibiotic solution for the first day and additional two days in RPMI-1640 medium containing 0.025X ITS, 100ng/mL ACTIVIN A with 0.2% FBS and 0.5% FBS on day 2 and day 3 respectively. On day 4, cells were cultured in RPMI-1640 medium containing 2% FBS and 1X GlutaMax.

The inhibitor and activator of YAP was used on the day 4 of the differentiation protocol. To inhibit the YAP expression, 30nM of Verteporfin was added to the culture medium and the studies are termed as 'VP'. Similarly, YAP expression was stimulated by adding 10uM of Lysophosphatidic acid in the culture medium and the study are labelled as 'LPA'. The 'control groups' are differentiated cells without

exogenous VP or LPA, but are exposed to the same quantity of the vehicle which was DMSO. Details of the protocol are summarized in **TABLE A3 (Annexure III)**.

viii. **Human Mesenchymal Stem Cell Maintenance:** Human placental mesenchymal stem cells (hPMSCs) were a kind gift from Dr. Vaijayanti P. Kale, Head and Professor, SCSCR, Symbiosis International University, Pune, India. The cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS and 1X GlutaMax. Prior to seeding, T75 flask was coated with collagen solution and incubated at RT/37^{0}C for 1 hour. The cells were incubated in a 5% CO_2 incubator under humified conditions at 37^{0}C (HERAcell VIOS 160i, Thermo Fischer Scientific, USA).

ix. **Human Mesenchymal Stem Cell Maintenance Sub-culturing:** The flasks showing 70% - 80% confluency were passaged. The spent medium was collected in a labelled tube, followed by one rinse with 9mLof warm DPBS to remove residual medium, FBS and dead cells. The washed cells were incubated with 3mL of TryplE at 37^{0}C for 5 minutes, and the cells were collected using the spent medium. The cell suspension was centrifuged (Eppendorf, Hamburg, Germany) at 1000 rpm at RT for 5 minutes. The supernatant was discarded, while the pellet was resuspended in 1mL of the fresh complete IMDM. For seeding in another T75 flask, 1mL of the cell suspension was plated onto collagen coated T75 flask, and for seeding on CS substrates, 6mL medium was added to the 1mL cell suspension to make up the volume up to 7mL, and 1mL of the cell suspension was seeded in each 6-well CS plates. The cells were incubated in a 5% CO_2 incubator under humified conditions at 37^{0}C (HERAcell VIOS 160i, Thermo Fischer Scientific, USA). The medium was changed every alternate day.
[NOTE: During each seeding, the cell suspension was gently mixed to get homogenous cell suspension.]

x. **Human Mesenchymal Stem Cell Maintenance Cryopreservation:** The cell pellet was obtained as per the above-mentioned protocol using TryplE, and cells were resuspended in a 600µL of IMDM. The cell suspension was transferred to a labelled cryovial and to this, 400µL of a cold mix was added. The cold mix was prepared as follows - 300µL FBS and 100µL of sterile DMSO (a cryoprotectant). The 1mL of the

cell suspension was transferred into a cryovial (Genaxy, India). The cryovials were immediately stored at -80^{0}C refrigerator in Mr. Frosty for 20-24 hours before transferring to liquid nitrogen for long term storage.

xi. **HT29 cells Revival and Maintenance:** The desired cryovial was removed from the liquid nitrogen cannister and placed in a beaker containing warm water. The vial was the thawed by rubbing it between the palms and the cell suspension was transferred to a 15mL tube containing 10mL of pre-warmed complete medium, i.e., Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and 1% penicillin-streptomycin solution. The cell suspension was centrifuged at 1000 rpm at RT for 5 minutes. The supernatant was discarded and the cell pellet was gently resuspended in 1mL of the fresh complete DMEM. The resuspended cells were seeded into T75 flask containing 5mL of the complete medium and incubated in a 5% CO_2 incubator under humified conditions at 37^{0}C (HERAcell VIOS 160i, Thermo Fischer Scientific, USA). The medium was changed every alternate day.

xii. **HT29 cells Sub-culturing:** The flasks showing 70% - 80% confluency were passaged. The spent medium was collected in a labelled tube, followed by one rinse with 5mL of the warm DPBS to remove residual medium, FBS and dead cells. The washed cells were incubated with 2mL of Trypsin at 37^{0}C for 5 minutes, and the cells were collected using the spent medium. The cell suspension was centrifuged (Eppendorf, Hamburg, Germany) at 1000 rpm at RT for 5 minutes. The supernatant was discarded, while the pellet was resuspended in 1mL of the fresh complete DMEM. For seeding on PA hydrogel substrates, 50ul of the cell suspension was seeded onto one 20% PA hydrogel and the remaining cell suspension was seeded into another into another T75 flask. The cells were incubated in a 5% CO_2 incubator under humified conditions at 37^{0}C (HERAcell VIOS 160i, Thermo Fischer Scientific, USA). The medium was changed every alternate day.

B. Synthesis of Various Substrates

i. **Preparing GelMa hydrogel:** GelMa was a kind gift from Dr. Prakriti Tayalia, Associate Professor, Indian Institute of Technology Bombay (IIT-B). 10% GelMa solution was prepared to which 0.25% of Irgacure 2959 was added, and the mixture was filtered using sterile 0.44-micron syringe filter. The filtered mixture was poured onto 35mm TCP and Irgacure 2959 in the mixture was activated by exposing the complete GelMa solution to using UV lamp (dual wavelength 254/366 nm, CAMAG®) set at 366nm for 5-7 minutes. The solidified GelMa hydrogel substrate was coated with 1X Vitronectin solution as per above-mentioned protocol. Undifferentiated KIND1 cells with 80% - 85% confluency cultured on TCP were sub-cultured onto the GelMa substrate and maintained in complete Essential 8™ medium. The cells were incubated in a 5% CO_2 incubator under humified conditions at 37^0C (HERAcell VIOS 160i, Thermo Fischer Scientific, USA).

ii. **Synthesis of Polyacrylamide (PA) hydrogel substrates:** PA gels are inert therefore they do not attach to the glass or plastic surfaces, and are non-adherent. To get a stable culture system the PA hydrogels were attached to a pre-treated glass coverslips by processes known as silanisation and derivatization. The glass coverslips used for preparing PA gels were autoclaved and just before use flame sterilized using 100% ethanol solution. In silanisation, the glass coverslips were cleaned with 1M HCl for 5 minutes, followed by three quick washes with distilled water to remove the traces of HCl. The cleaned coverslips were flooded with 2% of 3-aminopropyl trimethoxy silane for 5 minutes and rinsed thrice with autoclaved distilled water. The silane coated coverslips were incubated in oven set at 60^0C for 1 minute for drying (the coverslips can be air dried overnight at RT).

The next step was derivatization of glutaraldehyde, which is an effective cross-linking reagent. 5% glutaraldehyde solution was flooded onto the silane coated side of the coverslips for 5 minutes and then washed thrice with autoclaved distilled water. 10%, 15% and 20% PA gel mixtures were prepared using 30% acrylamide solution, 10% ammonium persulphate, tetramethylethylenediamine (TEMED) and autoclaved distilled water. 500µL of the gel mixture was placed on a sterile parafilm and the coverslips were placed onto the drop with silane coated side facing down.

After 15-20 minutes, the coverslips were flipped up gently using forceps and washed four-five times in autoclaved distilled water. The hydrogels were sterilized by UV radiation.

To make PA hydrogels suitable for cell adherence, the PA hydrogels were coated with a protein cross-linker sulfosuccinimidyl 6-(4′-azido-2′-nitrophenylamino) hexanoate or Sulfo-SANPAH (SS). Freshly prepared 10mM concentration of SS was flooded onto the hydrogels surface and exposed to UV lamp (dual wavelength 254/366 nm, CAMAG®) at 366nm for 7 minutes to active the cross-linking. The excess SS was removed and the hydrogels was washed five-six times with DPBS for 2 minutes. To couple the ECM protein vitronectin onto the PA hydrogels, 1X Vitronectin was allowed to covalently bound to the PA hydrogel overnight at 37^0C. The next day the PA hydrogels were placed in a 35mm TCP or 6-well plate and washed twice with DPBS. Prior to cell seeding, the PA hydrogels were incubated in fresh complete Essential 8™ medium for KIND1 cells and complete DMEM medium for HT29 cells at 37^0C for 1 hour. The protocol is illustrated in **FIGURE 8.1.** Some of the synthesized PA hydrogel of each percentage was checked for swelling by incubating them in DPBS for 48-72 hours. The stiffness of each PA hydrogel substrates coupled with Vitronectin was determined by Atomic Force Microscopy (AFM) at IIT-B. The AFM results were analyzed by Asylum Research software (Oxford Instruments).

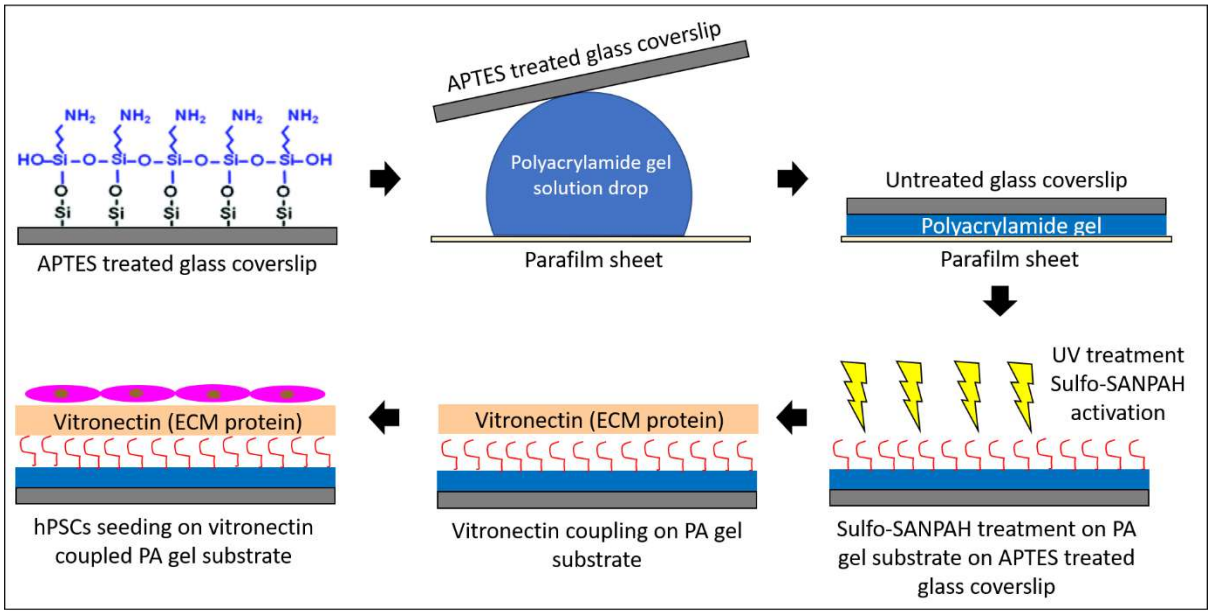


FIGURE 8.1: Diagrammatic representation of the protocol for the synthesis of polyacrylamide hydrogels and placement on glass coverslip for culturing human embryonic stem cells.

iii. **CytoSoft® substrates:** Due to cytotoxicity and to avoid batch-to-batch variations, commercially available CytoSoft® substrates plates were used for further. These 6-well plates have polydimethylsiloxane (PDMS) layer at the bottom of each well having elastic modulus of 0.2kPa, 0.5kPa, 2kPa, 8kPa, 16kPa, 32kPa, and 64kPa. The CytoSoft® (CS) substrates were activated as per manufacturer's instructions. Briefly, 3mL of 1X Vitronectin was dispensed into each well to thoroughly coat the surface of the hydrogel and incubated at RT/37^{0}C for 1 hour. After incubation, the solution was aspirated and the coated surfaces were immediately washed twice with DPBS. After last wash, 2mL of the culture medium was added to each well and the plates were incubated at RT/37^{0}C till cell-seeding. **[NOTE: Care was taken to not let the CytoSoft® surfaces become dry once the surfaces had been wetted].**

Undifferentiated KIND1 cells cultured on one 60mm TCP and having 70% - 80% confluency, were seeded into one 6-well CS plate of a particular stiffness, therefore seven 60mm TCP were seeded onto seven 6-well CS plates of all the stiffnesses. The cells were maintained in the suitable medium (either Essential 8™, DIM, IMDM, or DE differentiation medium), and were collected for subculturing or RNA or protein by EDTA method.

iv. **Ultra-Low Attachment Dishes:** The 96-well ultra-low attachment dishes (ULAD) were used for a suspension-like, no stiffness culture conditions. The ULAD plates were not coated with any ECM protein or given any pre-treatment. Undifferentiated KIND1 cells having 70% - 80% confluency were detached using EDTA and the cell pellet obtained after centrifugation was resuspended in complete Essential 8™ medium. 200µL of the cell suspension was seeded into each well of ULAD and the plate was incubated in a 5% CO_2 incubator under humified conditions at 37^{0}C. The medium was changed daily till seven days. For subculturing of the cell aggregates formed in ULAD, the medium along with the cell aggregates were collected in a sterile 15mL tube and centrifuged at 1000rpm at RT for 5 minutes. The supernatant was aspirated and the pellet was resuspended in fresh complete Essential 8™ medium. 200µL of the cell suspension was seeded into each well of fresh ULAD and the plate was incubated. For RNA and protein, the pellet was obtained by following the above-mentioned steps and dissolved in TRI-reagent, and 1X complete cell lysis buffer respectively.

C. Molecular assays

i. **RNA extraction, quantification and cDNA synthesis:** Total RNA was isolated using TRI reagent as per the manufacturer's instructions. The cells cultured on TCP and other substrates were collected as a pellet by EDTA method, and to the pellet 1mL of the TRI reagent was added. After complete homogenization of the cells by vortexing, the RNA was extracted by adding 100µL chloroform. The samples were vigorously shaken for 30-40 seconds and allowed to stand for 2 minutes at RT for phase separation. The three phases were separated by centrifugation at 12,000 g for 15 minutes at 4^0C, and the top aqueous layer was carefully transferred to a fresh vial. The RNA in the top aqueous layer was precipitated by adding 100% chilled isopropanol. The samples were centrifuged at 12,000 g for 10 minutes at 4^0C and incubated at -20^0C overnight for better yield. Next day, the samples were again centrifuged with above conditions and the pellet obtained was washed twice with 500µL of chilled 75% ethanol and once with 500µL of chilled 100% ethanol by centrifugating at 7,500 g for 7 minutes at 4^0C after every addition. The pellets were air-dried and reconstituted in 30µL - 40µL of DEPC treated distilled water. Purified RNA was quantified using Ultraviolet-Visible spectrophotometer (Perkin Elmer, USA) or the microplate spectrophotometer (BioTek Instruments, USA) and samples with A_{260}/A_{280} ratio > 1.8-2.0 were used for cDNA synthesis.

Complementary DNA (cDNA) was prepared from 1µg of total RNA as the starting template using Prime Script 1st Strand cDNA synthesis kit in the Thermal Cycler (Applied Biosystems, USA) as per the instructions given by manufacturer. The temperature profile of the reaction was 5 minutes at 65^0C, 10 minutes at 30^0C, 60 minutes at 42^0C and 15 minutes at 70^0C. All the samples were stored at -20^0C for long term storage.

ii. **Reverse transcription polymerase chain reaction (RT-PCR)/ Endpoint-PCR:** All RT-PCR assays were carried out using the Thermal Cycler (Applied Biosystems) in combination with the 2X EmeraldAmp GT PCR Master Mix. Target-specific primers listed in **TABLE A4 (Annexure IV)** were designed using NCBI Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and efficiency was checked using the cDNA serial dilutions (10-fold) and a standard curve method. Only primers

showing an efficiency between 90-110% were used for gene expression studies. For each RNA transcript, the DNA primers derived from separate exons to allow verification that the PCR product represented the cDNA and not the genomic DNA. The following thermal cycler conditions were used for 10µL or 20µL total volume reactions: initial denaturation at 94^0C for 3 minutes followed by 35 cycles of denaturation at 94^0C for 30 seconds, annealing at 55-61^0C for 30 seconds and elongation at 72^0C for 30 seconds. At the end of the amplification phase, PCR products were separated on 2% agarose containing 0.5µg/mL ethidium bromide. Product sizes were approximated by inclusion of a 100 bp DNA ladder. Images were captured on a molecular imager (Bio-Rad Laboratories). All measurements were normalized with the housekeeping *GAPDH*.

iii. **Quantitative reverse transcription PCR (qRT-PCR) or Real time PCR:** qRT-PCR was performed using StepOnePlus Real-Time PCR (Applied Biosystems, USA) or QuantStudio™ 3 Real-Time PCR (Applied Biosystems, USA) in combination with powerup SYBR Green Master Mix. Every qRT-PCR reaction was performed in three technical replicates of each of the three independent biological studies (n=3). The reaction mixture consisted of 1X Power Up SYBR master mix, 1µg of cDNA and 0.25µM of forward and reverse primer for a final volume of 20µL made up with DEPC treated distilled water (nuclease-free water). The PCR program involved following steps: initial denaturation at 95^0C for 2 minutes, followed by continuous 40 cycles of denaturation at 95^0C for 15 secs, gradient annealing from 50-61^0C for 15 secs and elongation at 72^0C for 1 minute. At the end of the amplification phase, a dissociation step known as melt curve analysis, was performed to identify a single melting temperature (Tm) for each primer set and to identify amplicon homogeneity. Target-specific primers provided in **TABLE A5 (Annexure V)** were designed using NCBI Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and only the primers showing an efficiency between 90-110% were used for gene expression studies. For each sample, the relative quantitation was determined using the comparative C_T ($2^{-\Delta\Delta CT}$) method. The normalized threshold cycle (ΔC_T) was calculated by subtracting C_T values of each gene with the corresponding C_T value of the sample of *18S rRNA* housekeeping gene. The expression of targets in the TCP

samples were used as the control for calculating relative fold change. The correct PCR-product was confirmed from the melt curve data.

iv. **Protein extraction and quantification:** The cells of TCP and other substrates were collected as a pellet by EDTA method, and the pellet was resuspended in 100µL of 1X complete cell lysis buffer. The homogenization of cells was carried out by intermediate vortexing for 30 minutes on ice. The cell lysate was clarified by centrifugation at 13,000 g for 30 minutes at 4^0C and the protein concentrations were measured by Folin-Ciocalteu's phenol reagent.

Total protein was determined using a standard solution of bovine serum albumin (BSA, 2mg/mL) along with 0.5% $CuSO_4$ (reagent A), 1% $C_4H_4O_6KNa$ (reagent B), 2% Na_2CO_3 in 0.1N NaOH (reagent C). Analytical reagents were mixed to obtain reagent D (1mL reagent A + 1mL reagent B + 48mL reagent C). A series of BSA dilutions ranging between 2µg/mL to 40µg/mL were prepared for a standard curve. Standard preparations and unknown samples (volume 5µL) were allowed to react with 5mL of reagent D at RT for 5 minutes in the dark. A lysis blank tube was also maintained which contained 5µL of the complete cell lysis buffer. 250µL of undiluted Folin-Ciocalteu's phenol reagent was added to the mixtures followed by incubation at room temperature for 30 minutes in the dark. Absorbance was measured at 700nm and 750nm using UV-Visible spectrophotometer (Perkin Elmer, USA) or the microplate spectrophotometer (BioTek Instruments, USA).

[NOTE: The mixtures were vortexed thoroughly at each step of addition as well as prior to the reads for obtaining appropriate values.]

v. **Immunoblotting/western blot assay:** 20µg of the protein extract was cooked with 5X Laemmli buffer (gel loading dye) at 95^0C for 10 minutes. The cooked samples were loaded in 10% SDS-PA gels using the Mini-Protean Tetra Cell System (Bio-Rad Laboratories, USA). Once the electrophoresis was complete, the gels were electroblotted onto polyvinylidene difluoride (PVDF) membranes for 90-100 minutes at constant voltage of 100V. The membranes were blocked with 3% bovine serum albumin (BSA, prepared in 1X TBST buffer) and probed overnight at 4^0C with primary antibody dilutions prepared in 1% BSA. The next day, the blots were washed thrice for 10 minutes with TBST to remove unbound primary antibody and probed

with HRP-conjugated anti-rabbit secondary antibody for detection. The details of all the antibodies are in **TABLE A6 (Annexure VI)**. The bands of the targeted proteins were detected using Clarity Western ECL kit or 20X LumiGlo, and imaged on a molecular gel documentation system (Molecular Imager ChemiDoc XRS+ System, Bio-Rad Laboratories or G:BOX Chemi XRQ, Syngene). Densitometric analysis of the targeted proteins was done using Java-based ImageJ (http://rsbweb.nih.gov/ij/) software from experiments performed in triplicates and GAPDH was used for normalizing protein levels across samples.

The PVDF blots were regularly stripped and re-probed multiple times for detecting proteins with lower expected signals before detection of more abundant proteins. The stripping protocol was based on the principle of reducing agent, β-mercaptoethanol. Approximately 5mL of the complete stripping buffer was added to the blot and incubated at 60^0C for 10 minutes. The stripping solution was discarded and the blots were washed 5-6 times, or until the smell of β-mercaptoethanol vanished, for 10 minutes at RT on a rocker. Before pre-probing, the blots were incubated with blocking buffer.

vi. **Immunofluorescence staining and image acquisition:** For the immunofluorescence assay, cells were cultured on the coverslips coated with vitronectin. For undifferentiated and differentiated states day 4 cells were taken for immunofluorescence, whereas for ULAD the day 7 cell aggregates were transferred onto vitronectin coated coverslips and incubated at 37^0C in 5% CO_2 incubator for one hour. After aspirating the medium and washing the cells with DPBS, the cells were fixed using freshly prepared 4% paraformaldehyde in 1X PBS for 10 minutes at RT followed by four washes with 1X PBS for 10 minutes each at RT. For nuclear proteins, cells were permeabilized with a permeabilization buffer for 25 minutes at RT, followed by quick washes of 1-2 minutes with wash buffer. Fixed cells were incubated in blocking buffer for 2 hours at RT followed by incubation with primary antibody overnight at 4^0C. The following day, the cells were given three washes of 10 minutes each with wash buffer to remove any residue of primary antibody. For detection, the cells were labelled with Alexa-488 conjugated secondary antibody for 2 hours at RT. Primary and secondary antibodies were prepared in 0.5% BSA **(TABLE A6, Annexure VI)**. Post incubation with antibody, the cells were washed with washing buffer and

counterstained with 300nM DAPI for 5 minutes. Images were acquired using inverted fluorescence microscopes (Carl Zeiss Microscope Apotome HXP 120, Germany, or Carl Zeiss Axio Vert.A1, Germany) at 20X, 40X and 63X magnification. Image analysis or enhancements were performed using Java-based ImageJ (http://rsbweb.nih.gov/ij/) software.

[NOTE: Since permeabilization of the cells disrupts the membranes, permeabilization step was not performed for cell membrane proteins. The fixed cells were directly treated with blocking buffer.]

vii. **Statistical analysis:** Data in the thesis are presented as the mean value ± standard error of the mean (S.E.M.), or mean value ± standard deviation (S.D.). Significance was calculated using One-way ANOVA and Tukey's multiple comparison tests unless stated otherwise. Asterisks indicate statistical significance as explained in each figure legend. All the graphs and statistical analyses were generated using GraphPad Prism version 8 (GraphPad Software, USA; www.graphpad.com).

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Annexures

List of Reagents and Materials used in Cell Culture and Molecular Biology experiments

(All the reagents listed below are reconstituted and stored as per the manufacturer's instructions, unless stated otherwise. The antibodies are listed in **Annexure VI**)

Reagent/Materials	Make	Catalogue #
2% 3-aminopropyl trimethoxy silane	Sigma-Aldrich	281778
ACTIVIN A, 50mg	R&D Systems	338-AC/CF
Adult human brain RNA sample, 50µg	Takara Bio	636530
Advanced Dulbecco's Modified Eagle Medium	Thermo Fischer Scientific	12634010
Clarity Western ECL Substrate	Bio-Rad	170-5060
Color-coded pre-stained Protein Marker, broad-range	Cell Signaling Technology	74124
CytoSoft® plates	Advanced Biomatrix	5190
DAPI	Thermo Fisher Scientific	D1306
Diethyl pyrocarbonate	Sigma-Aldrich	D5758
Dithiothreitol	Thermo Fisher Scientific	R0861
Dulbecco's Modified Eagle Medium	Thermo Fischer Scientific	11995065
Dulbecco's Phosphate Buffered Saline	Life Technologies	14040133
EmeraldAmp GT PCR Master Mix	Takara Bio	RR310Q
Essential 8™ Medium Kit	Thermo Fisher Scientific	A1517001
Ethylenediaminetetraacetic acid tetrasodium salt dihydrate	Sigma-Aldrich	E6511
Foetal bovine serum	Life Technologies	10270106
Folin & Ciocalteu's phenol reagent	Sigma-Aldrich	F9252
GelMa	Gift from Dr. Prakriti Tayalia, IIT, Bombay	
GeneRuler 100 bp DNA Ladder	Thermo Fisher Scientific	SM0243
GlutaMAX-I, 100X	Life Technologies	35050-061

Hippo Signalling Pathway Kit	Cell Signaling Technology	8579
Insulin Transferrin Selenium, liquid media supplement, 100X	Sigma-Aldrich	I3146
Irgacure 2959	Sigma-Aldrich	410896
Iscove's Modified Dulbecco's Medium	Thermo Fisher Scientific	12440053
LumiGlow 20X	Cell Signaling Technology	7003
Lysophosphatidic acid	Sigma-Aldrich	L7260
Penicillin-Streptomycin, liquid	Life Technologies	15140122
Phenol:Chloroform:Isoamyl Alcohol 25:24:1 pH 8.0	Sigma-Aldrich	P2069
Phenylmethylsulfonyl fluoride	MP Biomedical	195381
PhosStop	Roche	03115836001
Polyvinylidene difluoride membrane, Hybond P0.45	Amersham	10600023
PowerUp SYBR Green Master Mix	Applied BioSystems	A25741
PrimeScript 1st strand cDNA Synthesis Kit	Takara Bio	6110A
ProLong Gold antifade	Molecular Probes	P36930
Protease inhibitor cocktail	Sigma Aldrich	P8340
PureCol, collagen solution, 3mg/ml	Advanced BioMatrix	5005
Rosewell Park Memorial Institute 1640 medium	Thermo Fisher Scientific	11875101 and 31800022
Sulfosuccinimidyl 6-(4′-azido-2′-nitrophenylamino) hexanoate	Thermo Fisher Scientific	A35395
TRI Reagent	Sigma-Aldrich	T9424
TryplE	Thermo Fisher Scientific	12604013
Trypsin-EDTA (0.25%), phenol red	Thermo Fisher Scientific	25200056
Ultra-low attachment dishes	Corning	CLS7007

Verteporfin	Sigma-Aldrich	SML0534
Vitronectin, truncated recombinant human	Life Technologies	A14700

Stock Preparation of Buffers/Reagents used in Cell Culture and Molecular Biology experiments

(All the preparations and procedures for the cell culture and substrate synthesis were carried out under sterile conditions, using sterile plasticware/glassware)

Cell Culture

1. *Complete Essential 8™ medium (For KIND1 cells)*

   48.5mL of the Essential 8™ medium was aliquoted in a sterile 50mL tube to which 1mL of the 50X supplement and 0.5% of penicillin-streptomycin solution was added. The medium was stored at 4^0C and warmed before use.

2. *Advanced DMEM medium (For KIND1 cells)*

   48.5mL of the Advanced DMEM medium was aliquoted in a sterile 50mL tube to which 2% of fetal bovine serum (FBS) and 0.5% of penicillin-streptomycin solution was added. The medium was stored at 4^0C and warmed before use.

3. *Complete DMEM medium (For HT29 cells)*

   44.5mL of the DMEM was aliquoted in a sterile 50mL tube to which 10% FBS and 0.5% of penicillin-streptomycin solution was added. The medium was stored at 4^0C and warmed before use.

4. *Complete IMDM medium (For hPMSCs)*

   44mL of the IMDM medium was aliquoted in a tube to which 10% FBS, 1X GlutaMax and 0.5% of antibiotic solution was added. The medium was stored at 4^0C

5. *1X Vitronectin*

   20µL of 100X vitronectin was mixed in 2mL of sterile DBPS for one 60mm TCP and was incubated at 37^0C for 1 hour. Before using, the excess solution was discarded.

   NOTE: 1X Vitronectin was always prepared fresh right before use.

6. *PureCol®*

   100ug/ml of the solution was prepared from the 3mg/mL of the PureCol by dilution in DPBS for one T75 flask and was incubated at 37^0C for 1 hour. Before using the excess solution was discarded.

   NOTE: Collagen dilution was always prepared fresh right before use.

7. *ACTIVIN A*

   50µg of ACTIVIN A was reconstituted in 100µl of 4mM HCl in 1X DPBS with 0.1% bovine serum albumin (BSA) to get a stock solution of 500µg/mL and was stored at -20^0C. 100ng/mL of the working stock was used for differentiation protocol.

8. *Verteporfin*

   5mg of the Verteporfin was dissolved in 2.4mL of warm and sterile DMSO. The vial was covered with aluminium foil to protect from light and stored at -20^{0}C. 1:100 dilution of the working stock was prepared in DMSO just before using.

9. *Lysophosphatidic acid*

   1mg of the lysophosphatidic acid was dissolved in 7.142mL of the 1X DPBS containing 0.1% BSA to get a stock solution of (0.3mM (0.14mg/mL). 1:10 dilution was prepared to get a working stock solution of 0.03mM. The vial was covered with aluminium foil to protect from light and stored at -20^{0}C.

10. *0.5mM EDTA (cell culture grade)*

    104.05 mg of EDTA added to 50mL of 1X DPBS and filter sterilised using 0.22µm syringe filter and stored at 4°C.

GelMa Substrate

1. 10% (w/w) GelMa solution

   1gm of dry GelMa was dissolved in 10mL of warm distilled water just before use.

2. 0.25% Irgacure 2959

   1% stock of Irgacure 2959 was prepared in warm distilled water from which 0.25% was added to the 10% GelMa solution, just before use. The mixture was filtered using 0.44micron syringe filter, and sterilized by UV radiation.

Polyacrylamide Gel Synthesis

1. *3-Aminopropyl trimethoxy silane (for Silanisation)*

   0.05mL of APTMS was mixed in 10mL of acetone to get 0.5% of working solution

2. *0.5% Glutaraldehyde (for Derivatization)*

   0.05mL of the glutaraldehyde was mixed in 10mL of 1X PBS to get 0.5% of working stock solution. The solution is prepared fresh before using.

3. *10mM SulfoSANPAH*

   1mg pellet of SulfoSANPAH was dissolved in 40µL of DMSO. 10µL of the reconstituted SulfoSANPAH was aliquoted in vials and stored at -20^{0}C. Just before use, 240µL of autoclaved distilled water was added to each vial.

RNA Extraction

1. *RNA blaster*

   100mM NaOH, 0.3% SDS, 10mM EDTA and 1mM DTT was added in autoclaved distilled water to prepare a blaster stock of 1L to be use in molecular biology experiments. The buffer was stored at RT.

2. *0.1% DEPC*

   500µL of Stock DEPC solution was added in 500mL of MilliQ distilled water. The solution was shaken vigorously and incubated at RT/37°C overnight under dark conditions followed by autoclaving the next day. The DEPC water was aliquoted and filter sterilised using 0.45µm syringe filter and stored at RT.

Agarose Gel Electrophoresis (AGE)

1. *50X Tris Acetate EDTA (TAE) buffer*

   40mM Tris and 1mM EDTA was added to MilliQ distilled water and 20mM Glacial Acetic Acid to prepare a stock of 500mL of 50X TAE buffer which was autoclaved and stored at RT.

2. *2% Agarose gel*

   2g of Agarose was weighed in 100mL of 1X TAE and cooked for 2 to 3 mins in a microwave. The gel was cooled under tap water followed by addition of 4µL of 10mg/mL EtBr solution. The gel was casted in the casting tray and used for electrophoresis.

3. *Ethidium Bromide (EtBr) preparation*

   10mg of EtBr was weighed and dissolved in distilled water under dark conditions. The stock was wrapped in aluminium foil and stored at 4°C.

4. *Coomassie Brilliant Blue (CBB) Satin*

   50% Methanol, 10% Glacial Acetic Acid and 0.1% CBB stain was dissolved in distilled water to make up the volume to 50mL. The stain was stored at RT.

5. *Destaining Solution*

   40% Methanol, 10% Glacial Acetic Acid was dissolved in distilled water to make up the volume to 50mL. The stain was stored at RT.

Protein Extraction and Quantification

1. *PhosSTOP*

   10X stock solution was prepared by dissolving 1 tablet in 1mL of ultra-pure distilled water. The stock solution was stored at -20^{0}C.

2. *200mM PMSF*

   34.84mg of PMSF was added to 1mL of 100% isopropanol, vortexed to dissolve all the crystals and stored at – 20°C. A final working stock of 2mM was used in 1X cell lysis buffer just prior to use.

3. *1X Cell Lysis Buffer*

   50mM Tris, 150mM NaCl, 1mM EDTA, 1mM NaF, 0.1% SDS, 1% Triton X-100 were dissolved in 30mL of the distilled water and later the volume was made up to 50mL. pH was adjusted to 7.5 and stored at 4°C.

   NOTE: PhosSTOP, Protease Inhibitor Cocktail and 2mM PMSF was added just before use to make complete cell lysis buffer

4. *2mg/mL BSA (for standard curve)*

   2mg of the BSA was dissolved in 1mL of the distilled water and stored at 4^{0}C.

5. *Reagent A*

   0.5% of copper sulphate ($CuSO_4$) was added in to 30mL of the distilled water

6. *Reagent B*

   1% potassium sodium tartrate was dissolved in 10mL of the distilled water and stored at room temperature

7. *Reagent C*

   0.1N sodium hydroxide (NaOH) and 2% sodium carbonate (Na_2CO_3) was dissolved in 150mL of the distilled water. Later volume was made up to 200mL and the solution was kept in dark conditions.

   NOTE: The solution was prepared fresh before use

8. *Reagent D*

   Prepared fresh by mixing reagents A, B, and C at 1:1:48 ratio respectively. 4mL of reagent A + 4mL of Reagent B + 192mL of reagent C was mixed to give 200mL of reagent D.

Immunofluorescence

1. *Grease free cleaning and lysine coating of slides for immunofluorescence*
   - 20g of potassium dichromate was dissolved in minimum volumes of water (10mL-20mL) followed by addition of 300mL of conc. H_2SO_4 slowly from the slides, the solution turns reddish brown
   - The glass slides were kept in this solution for overnight
   - Next day, the slides were washed several times running tap water until the yellowish tinge was removed
   - The slides were air dried and coated with 10µL of Poly-L-Lysine solution on both the slides of slides. The slides were air dried and stored at RT
2. *10X Phosphate Buffered Saline (PBS) solution*

   1.37M NaCl, 27mM KCl, 100mM Na_2HPO_4, 18mM KH_2PO_4 was added to 100mL of MilliQ distilled water and pH adjusted to 7.4 using 1N NaOH. The solution was filter sterilised using 0.45µm syringe filter to remove residual particles and stored at RT.
3. *4% Paraformaldehyde (PFA) solution*

   0.4g of PFA was added to the 10mL of 1X PBS and this solution were kept in 60°C water-bath for 30 minutes to dissolve the particulates of PFA with intermittent vortexing. The solution was always prepared fresh prior to usage.
4. *Washing Buffer*

   0.02% Tween 20 was dissolved in 20mL of 1X PBS solution and stored at room temperature.
5. *Permeabilization Buffer*

   0.3% Triton X-100 was dissolved in 10mL of 1X PBS solution and stored at room temperature.
6. *Blocking Buffer*

   2% BSA was dissolved in 1mL of warm 1X PBS, for antibody addition, 0.5% BSA was dissolved in appropriate volumes of 1X PBS. The buffer was always prepared fresh prior to usage.

Western Blot

1. *5X Laemmli Buffer (Gel Loading dye)*

   250 mM Tris, 10% SDS and 50% Glycerol dissolved in 8mL distilled water; pH was adjusted to 6.8 with concentrated HCl. 0.05% Bromophenol blue was added to the solution and the volume was made to 10mL with distilled water. The buffer was stored at 4°C.

2. *20X DTT solution*

   308.5mg of Dithiothreitol was dissolved in 1mL of MilliQ distilled water and stored at 4°C. 1X DTT was added to 5X gel loading dye to make tracking dye of 4X to be used in SDS-PAGE.

3. *Resolving gel buffer*

   1.5M Tris was dissolved in 80 mL distilled water and pH adjusted to 8.8 with concentrated HCl. The volume was made up to 100 mL with distilled water and stored at 4°C.

4. *Stacking gel buffer*

   0.5 M Tris was dissolved in 80 mL distilled water and pH adjusted to 6.8 with concentrated HCl. The volume made up to 100 mL with distilled water and stored at 4°C.

5. *30% Acrylamide solution*

   29.2g Acrylamide and 0.8g of Bis-acrylamide were dissolved in 50 mL distilled water. Once dissolved the volume was made up to 100 mL with distilled water in dark. The solution was stored in amber coloured bottle at 4°C.

6. *10% SDS PAGE*

10% Resolving PAGE		
Components	**Final Concentration**	**Volume for 10mL**
30% Acrylamide Solution	10%	3.33mL
Resolving gel buffer	25%	2.5mL
10% SDS	0.10%	100µL
10% APS	0.10%	100µL
TEMED	0.01%	10µL
Distilled water	make up the volume to 10mL	

4% Stacking PAGE		
Components	**Final Concentration**	**Volume for 5mL**
30% Acrylamide Solution	4%	0.667mL
Stacking gel buffer	25%	1.25mL
10% SDS	0.10%	50µL
10% APS	0.10%	50µL
TEMED	0.01%	5µL
Distilled water	make up the volume to 5mL	

7. *1X Running Buffer*

   24.8 mM Tris, 192 mM Glycine and 0.1% SDS were dissolved in 1L of distilled water. The solution was stored at RT.

8. *1X Transfer Buffer*

   24.8 mM Tris and 192 mM Glycine were dissolved in 800 mL of distilled water. 20% Methanol was added to make up the volume to 1L. The solution was stored at RT. But prior to use, the buffer was stored at -20°C for over 2h.

9. *1X Tris Buffer Saline - Tween 20 (TBST)*

   10 mM Tris, 150 mM NaCl and 0.05% Tween 20 were dissolved in 800 mL of distilled water and pH adjusted to 7.6 with concentrated HCL. Once the pH was adjusted volume made up to 1000 mL with distilled water. The solution was stored at RT.

10. *Blocking Buffer for western Blotting*

    5% Non-Fat dry milk (NFDM) or 3% Bovine Serum Albumin (BSA) powder dissolved in 10 mL of 1X TBST. For antibody addition, 1% NFDM or 1% BSA was dissolved in appropriate volume of 1X TBST. The buffer was always prepared fresh prior to usage.

11. *Poncheau S Stain*

    0.1% (w/v) Poncheau stain was dissolved in 5% (v/v) glacial acetic acid and the rest of the volume made up to 50mL with distilled water. The solution was stored at RT.

12. *Sodium Azide solution*

    A stock of 5% sodium azide was prepared in distilled water and filtered it to store at 4°C. A working stock of 0.05% was used in the antibody dilution to prevent the bacterial and fungal contamination.

13. Stripping Buffer (Mild low pH acidic stripping buffer)

200mM Glycine, 0.1% SDS and 1% Tween-20 was dissolved in 100mL of distilled water after adjusting the pH to 2.2 distilled water and stored at RT.

14. Stripping Buffer (Harsh stripping buffer)

62.5mM Tris and 2% SDS was dissolved in 80mL of the distilled water/ pH was adjusted to 7.4 using 1M HCl and volume was made up to 100mL. the buffer was stored at RT. 100mM β-mercaptoethanol was added to 10mL of the stripping buffer just before use.

TABLE A3: Details of the Differentiation Protocol used for Directed Differentiation towards Definitive Endoderm

State/Days	Basal medium	Cytokines	Preparation/Stock conc.
UD Day 1 - 4	Essential 8	1X Supplement	1mL of the 50X supplement in 50mL of Essential 8 medium
DE Day 1	RPMI-1640	100ng/mL ACTIVIN A	50µg reconstituted in DPBS consisting of 4mM HCl and 0.1% BSA to get 500µg/mL stock
		0.025X ITS	from 100X stock
		0.5% Antibiotic	-
DE Day 2	RPMI-1640	100ng/mL ACTIVIN A	As above
		0.025X ITS	As above
		0.2% FBS	from 100X FBS
		0.5% Antibiotic	-
DE Day 3	RPMI-1640	100ng/mL ACTIVIN A	As above
		0.025X ITS	As above
		0.5% FBS	from 100X FBS
		0.5% Antibiotic	-
DE Day 4	RPMI-1640	100ng/mL ACTIVIN A	As above
		2.0% FBS	from 100X FBS
		1X GlutaMax	from 100X GlutaMax
		0.5% Antibiotic	-

Details of inhibitor and activator:

Treatment day	Reagent	Conc. Used	Stock conc. prepared	Reconstitution medium
DE day 4	Verteporfin (YAP inhibitor)	30nM	2.7mM (2mg/mL)	Sterile DMSO
DE day 4	Lysophosphatidic acid (YAP activator)	10uM	0.3mM (0.14mg/mL)	0.1% (w/v) BSA

TABLE A4: List of primers used for Reverse-Transcription PCR (RT-PCR) or End-point PCR

Gene symbol	Primer sequence (5' to 3')	Size (bp)	Annealing temp (^{0}C)	Accession ID
OCT4	F – AGCCCTCATTTCACCAGGCC R – TGGGACTCCTCCGGGTTTTG	456	58	NM_002701.5
NANOG	F – AGTCCCAAAGGCAAACAACCCACTTC R – GCTGGAGGCTGAGGTATTTCTGTCTC	161	57	NM_024865.3
SOX2	F – CCCCCGGCGGCAATAGCA R – TCGGCGCCGGGGAGATACAT	448	57	NM_003106.3
SOX17	F – AAGGGCGAGTCCCGTATC R - TTGTAGTTGGGGTGGTCCTG	221	56	NM_022454.3
PAX6	F – AGAGCGAGCGGTGCATTTG R – CTCAGATTCCTATGCTGATTGGTG	235	59	NM_000280.4
BRA	F – TGCTTCCCTGAGACCCAGTT R - ATCACTTCTTTCCTTTGCATCAAG	120	60	NM_003181.3
YAP	F – TGACCCTCGTTTTGCCATGA R – GTTGCTGCTGGTTGGAGTTG	125	57	NM_001282101.1
β2MG	F – GAGATGTCTCGCTCCGTGG R – GCTTACATGTCTCGATCCCA	365	55	NM_004048.2
GAPDH	F – GTCAGTGGTGGACCTGACCT R - CACCACCCTGTTGCTGTAGC	256	60	NM_001289746.1

β2MG: β2-MICROGLOBULIN

BRA: BRACHYURY

TABLE A5: List of primers used for quantitative Reverse-Transcription PCR (qRT-PCR)

Gene symbol	Primer sequence (5' to 3')	Size (bp)	Annealing temp (^{0}C)	Tm (^{0}C)	Accession ID
OCT4	F – AGCCCTCATTTCACCAGGCC R – TGGGACTCCTCCGGGTTTTG	456	58	90.99	NM_002701.5
SOX17	F – AAGGGCGAGTCCCGTATC R - TTGTAGTTGGGGTGGTCCTG	221	56	90.2	NM_022454.3
FOXA2	F - GCTGGTCGTTTGTTGTGGC R - CGTGTTCATGCCGTTCATCC	182	56	87.21	NM_021784.4
CXCR4	F – GGCAGCAGGTAGCAAAGTGACGC R – AGAGGAGGTCGGCCACTGACA	334	61	83.99	NM_005189.2
SOX1	F – TGTAATCACTTTAACGAATGAGTGG R - AGTTTAATGAGAACCGAATTCAGC	134	60	75.52	NM_005986.2
PAX6	F – AGAGCGAGCGGTGCATTTG R – CTCAGATTCCTATGCTGATTGGTG	235	59	87.21	NM_000280.4
BRA	F – TGCTTCCCTGAGACCCAGTT R - ATCACTTCTTTCCTTTGCATCAAG	120	60	78.02	NM_003181.3
YAP	F – TGACCCTCGTTTTGCCATGA R – GTTGCTGCTGGTTGGAGTTG	125	57	86.73	NM_001282101.1
18S rRNA	F – GGAGAGGGAGCCTGAGAAAC R - CCTCCAATGGATCCTCGTTA	171	56	84.38	NR_003286.2

BRA: BRACHYURY

TABLE A6: List of primary and secondary antibodies used for Immunoblotting/Western Blot (WB) and Immunofluorescence (IF)

Antibody	Make/Catalogue number	Host species	Dilution (WB)	Dilution (IF)
OCT4	Cell Signalling Technology (CST) Catalogue # C30A3	Rabbit	1:1000	1:200
NANOG	AbCam Catalogue # ab109250	Rabbit	1:1000	-
YAP	Cell Signalling Technology Catalogue # 14074	Rabbit	1:1000	1:200
KO Validated YAP	ABClonal Catalogue # 1002	Rabbit	1:1000	-
pYAP (Ser127)	AbCam Catalogue # ab76252	Rabbit	1:1000	-
pYAP (Ser127)	Hippo Signalling Kit, CST Catalogue # 13008	Rabbit	1:1000	-
MST1	Hippo Signalling Kit, CST Catalogue # 3682	Rabbit	1:1000	-
SAV	Hippo Signalling Kit, CST Catalogue # 13301	Rabbit	1:1000	-
LATS1	Hippo Signalling Kit, CST Catalogue # 3477	Rabbit	1:1000	-
MOB1	Hippo Signalling Kit, CST Catalogue # 137301	Rabbit	1:1000	-
CYCLIN D1	Cell Signalling Technology Catalogue # 2978	Rabbit	1:1000	-
CXCR4	AbCam Catalogue # ab124824	Rabbit	-	1:400
GAPDH	Cell Signalling Technology Catalogue # 2118	Rabbit	1:1000	-
Anti-rabbit IgG, HRP-linked antibody	Cell Signalling Technology Catalogue # 7074	Goat	1:1000	-
Anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific Catalogue # A-11008	Goat	-	1:250

SVKM's INSTITUTIONAL COMMITTEE ON STEM CELL RESEARCH

(IC-SCR)

8th July, 2017

To

Principal Investigator

School of Science, NMIMS

Sub: Project proposal No: **NMIMS/IC SCR/ 002/2017**

The Committee has reviewed and approved the proposal entitled "Understanding the activity of mechanosensors - YAP and TAZ in differentiating human pluripotent stem cells "in its meeting held on July 7th, 2017, for a period of three years. The progress reports and final report of the proposal should be submitted to the Committee.

Signature of the Member-Secretary
(Dr. Prasad Pethe)

Signature of the Chairman
(Dr. Harikesh Buch)

Copy forwarded to protocol applicant:

Copy in File

SYMBIOSIS SCHOOL OF BIOLOGICAL SCIENCES
Symbiosis International (Deemed University)
(Established under section 3 of the UGC Act, 1956)
Re-accredited by NAAC with 'A' grade (3.58/4) | Awarded Category - I by UGC
Founder: Prof. Dr. S. B. Mujumdar, M. Sc., Ph. D. (Awarded Padma Bhushan and Padma Shri by President of India)

Approval of the stem-cell related research project

30/05/2019

To
Dr. Prasad Pethe
Symbiosis Centre for Stem Cell Research
Symbiosis International (Deemed University)
Pune

Dear Dr. Pethe

This is with reference to the meeting of the Institutional Committee for Stem Cell Research (ISCSR) for reviewing following project proposal related to stem cell research submitted by you.

Project no. SSBS/ICSCR/2019/02

Title: Understanding the activity of mechanosensors - YAP and TAZ in differentiating human pluripotent stem cells

I am happy to inform that the committee has approved this project.

Best Wishes

Dr. Surendra Ghaskadbi
Chairperson

॥वसुधैव कुटुम्बकम्॥
SSBS

Dr. Ram Kulkarni
Member Secretary

Lavale, Pune 412115, Maharashtra, India. | Tel : 020-39116496 | Fax: 020-39116440 | Email: info@ssbs.edu.in|
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Conferences and Publications

CONFERENCES

- **International Society for Stem Cell Research 2021 (ISSCR 2021). 21st – 26th June 2021:** Presented poster entitled "Role of Hippo pathway in regulating pluripotency of human embryonic stem cells on soft substrate"

- **Summer Biomechanics, Bioengineering and Biotransport conference 2021 (SB3C2021). 14th – 18th June 2021:** Presented poster entitled "Role of substrate stiffness on pluripotency and differentiation of human embryonic stem cells"

- **Advances in Materials Science and Applied Biology (AMSAB). 8th – 10th January 2019:** Presented poster entitled "Substrate stiffness regulates YAP/TAZ activity in human pluripotent stem cells."

PUBLICATIONS

- Virdi, J.K. and Pethe, P., 2022. Soft substrate maintains stemness and pluripotent stem cell-like phenotype of human embryonic stem cells under defined culture conditions. Cytotechnology, 74(4), pp.479-489.
  DOI: https://doi.org/10.1007/s10616-022-00537-z
  Cytotechnology Journal Impact Factor (2022): 2.3

- Virdi, J.K. and Pethe, P., 2021. Biomaterials regulate mechanosensors YAP/TAZ in stem cell growth and differentiation. Tissue Engineering and Regenerative Medicine, 18(2), pp.199-215.
  DOI: https://doi.org/10.1007/s13770-020-00301-4
  Tissue Engineering and Regenerative Medicine Journal Impact Factor (2022): 3.6

- Virdi, J.K. and Pethe, P., 2021. Dynamic Interactions Between Stem Cells and Biomaterials. In Engineering Materials for Stem Cell Regeneration (pp. 381-398). Springer, Singapore.
  DOI: https://doi.org/10.1007/978-981-16-4420-7_15

Synopsis

SYNOPSIS OF THE THESIS TO BE SUBMITTED TO THE UNIVERSITY OF NMIMS (DEEMED-TO-BE UNIVERSITY) FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (BIOLOGICAL SCIENCES)

NAME OF THE CANDIDATE:	**Jasmeet Kaur Virdi**
DOCTORAL COMITTEE (TAC):	**1. Dr. Dhanjit Das** Scientist (E), Stem Cell Biology Department, National Institute for Research in Reproductive and Child Health (NIRRCH), Parel, Mumbai **2. Dr. Prakriti Tayalia** Associate Professor, Bioscience and Bioengineering Department, Indian Institute of Technology (IIT), Powai, Mumbai
NAME AND DESIGNATION OF MENTOR:	**Dr. Prasad Pethe** Assistant Professor, Symbiosis Centre for Stem Cell Research (SCSCR), Symbiosis International University (SIU), Lavale, Pune
TITLE OF THE THESIS:	Understanding the activity of mechanosensors - YAP and TAZ in differentiating human pluripotent stem cells
PLACE OF RESEARCH:	1. Sunandan Divatia School of Science (SDSOS), SVKM's NMIMS (deemed-to-be) University, Vile Parle, Mumbai, India. Pin – 400056. 2. Symbiosis Centre for Stem Cell Research (SCSCR), Symbiosis International University (SIU), Lavale, Pune
REGISTRATION NUMBER:	75109160002
DATE OF REGISTRATION:	26th September 2017
DATE OF SUBMISSION OF SYNOPSIS:	28th January 2023
SIGNATURE OF CANDIDATE:	
SIGNATURE OF MENTOR:	

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Chapter 1: Introduction

Biophysical information from the cellular microenvironment such as extracellular matrix rigidity or mechanical forces, triggers intracellular signalling which results in various cellular functions such as proliferation, differentiation, migration and apoptosis through a process termed as mechanotransduction. The ability of cells to sense and respond to mechanical stimuli is essential for several developmental processes and postnatal homeostasis (Morin-Kensicki et al., 2006; Tian et al., 2007; Makita et al., 2008).

The stiffness of the substrate can regulate mesenchymal stem cells (MSCs) differentiation, with the stiffest substrate leading to osteogenic differentiation while soft substrate causing adipogenic differentiation (Engler et al., 2006; Dupont et al., 2011). In hESCs, substrate stiffness in combination with soluble molecules help maintain self-renewal on stiff substrate and caused neurogenic differentiation on soft substrate (Maldonado et al., 2015). Similar studies have reported that hPSCs on soft substrate differentiate into neuroectoderm (Hindley et al., 2016). However, no study has shown the interaction between hPSCs and substrate stiffness in absence of differentiation inducing medium. Understanding the interaction between hPSCs and stiffness is important because hPSCs are being used in many stem-cell based therapy especially in the light of new technologies such as 3D bio printing or tissue engineering biomaterials. Also, by controlling the stiffness of the substrates we may improve the regulation of the stem cell fate in bioartificial systems. Yes-associated protein (YAP) is a key mechanosensor that relays the mechanical signals into the nucleus. YAP is a transcriptional coactivator of the evolutionary conserved Hippo signalling pathway, which is an important regulator of organ size, cell proliferation and apoptosis (Wu et al. 2003; Huang et al. 2005; Dong et al. 2007).

Our aim was to study the effect of varying substrate stiffness on hPSCs differentiation into endoderm lineage.

Chapter 2: Review of Literature

Pluripotent stem cells (PSCs) are cells that have the capacity to self-renew and to develop into the three primary germ cells of the early embryo but not extra embryonic tissues such as the placenta. Embryonic stem cells (ESCs) are pluripotent cells, isolated from the inner cell mass of pre-implantation blastocyst, which can grow indefinitely and differentiate into any specialized cell of the body (Evans and Kauffman, 1981). Induced pluripotent stem cells (iPSCs) too are pluripotent stem cells but are generated by reprogramming of the adult stem cells using factors such as Oct3/4, Sox2, c-Myc and Klf4. They have properties similar to embryonic stem cells; therefore, can self-renew and differentiate into all cell types of the body (Takahashi & Yamanaka, 2006).

2.1 Mechanobiology

The microenvironment in which the stem cells reside consists of chemical, biological and mechanical signals which have a great influence on their survival, proliferation and differentiation. Along with signalling molecules, biophysical cues generated from extracellular matrix (ECM) stiffness, cell morphology or substrate topology plays an important role in regulating the signalling pathways in cells (Pelham & Wang, 1997; Li *et al.,* 2011). The cells sense these biochemical and mechanical stimuli generated from the extracellular environment by means of mechanical sensors present around the cell surface and transduced into the nucleus by mechanotransducers. This process is known as mechanotransduction. Integrins, cadherins and stretch-activated ion channels such as transient receptor potential channels (TRP channels) have been implicated as mechano-receptors which sense the signal from the extracellular environment. They relay the signals to the signal transducing molecules such as actin binding proteins, focal adhesion kinases, β-catenin, talin, Src, members of MAPK family and Rho family GTPases present in the cytoplasm. The signal is then transduced into the nucleus leading to target-specific gene expression (Holle *et al.,* 2013; Liu & Lee, 2014).

The effects of the substrate stiffness on the cellular behaviour have been studied extensively. Pelham and Wang (1997) showed that the kidney epithelial and fibroblast cells cultured on polyacrylamide gel respond to the difference in the substrate flexibility by altering their adhesion structures and motile behaviour. Furthermore, Engler *et al.* (2004a, 2006)

reported that MSCs cultured on substrates of varying stiffness commit to phenotype corresponding to their biological tissue. Soft stiffness that mimics brain induces cells to commit towards neurogenic lineage, stiffer stiffness that mimic muscle induces cells to commit towards myogenic lineage, and comparatively rigid stiffness that mimic bone commit towards osteogenic lineage. Adding to this, Evans *et al.* (2009) demonstrated that the cell spreading, growth rate, gene expression and differentiation of ESCs is influenced by the change in the substrate stiffness. Many research groups have studied the importance of substrate stiffness in cell behaviour. Transcriptional co-activators: Yes-associated proteins (YAP) has emerged as mechanosensor which respond to the substrate stiffness and cell morphology (Dupont *et al.,* 2011; Aragona *et al*., 2013; Brusatin *et al.,* 2018; Lee *et al*., 2019).

2.2 YAP – Yes associated protein

YAP plays an important role in regulating organ size, normal tissue regeneration and also as potential therapeutic targets in cancer. They are the primary effectors of the Hippo pathway and are known to interact with TGFβ signalling pathway, WNT pathway, biophysical pathways and several others (Morgan *et al.,* 2013). The two kinases: MST and LATS, of the Hippo pathway regulate the localization of YAP. Phosphorylation of these two kinases by the upstream signals, causes phosphorylation of YAP, causing pYAP to be sequestered into the cytoplasm by 14-3-3 protein, and thereby degraded by proteosomes (Low *et al.,* 2014). In absence of the upstream signals, MST and LATS are unphosphorylated, causing YAP to translocate into the nucleus and bind to the DNA-binding transcription factors, such as TEADs, RUNX2, p-related p73 and the ErbB4 cytoplasmic domain, to activate the expression of certain genes (Lei *et al.,* 2008; Hong *et al.,* 2005).

In mouse ESCs, YAP is highly expressed under normal culturing conditions (Ramalho-Santos *et al.,* 2002). Previous studies demonstrated that YAP promotes stem cell self-renewal and pluripotency, and that loss of YAP leads to the loss of pluripotency in human and mouse ESCs (mESCs) (Lian *et al.,* 2010; Estaras *et al.,* 2017; Papaspyropoulos *et al.,* 2018). In addition, it was reported that YAP prevents hESCs differentiation and YAP overexpression suppresses mESCs differentiation (Lian *et al.,* 2010). Conversely, another study reported that YAP is dispensable for self-renewal, depletion of YAP inhibits differentiation, and overexpression of YAP stimulates differentiation in mESCs (Chung *et al.,* 2016). It has also been shown that YAP

depletion does not affect any of the normal stem cell characteristics in human induced pluripotent stem cells (hiPSCs) (Lorthongpanich *et al.,* 2020). These results suggest that the function of YAP is context specific and its role during human pluripotent stem cell differentiation needs has not been uncovered.

2.3 YAP as Mechanotransducer in stem cells

Dupont *et al.* (2011) identified YAP and TAZ as nuclear transducers of mechanical signals exerted by the ECM rigidity and cell shape. They reported that stiff substrate, large adhesive areas and in cells with high contractile forces activates YAP and promotes proliferation of primary mammary epithelial cells (MECs) and promotes MSCs differentiation towards osteogenic lineage. Conversely, YAP is inactive and in cytoplasm on soft substrate, small adhesive area and in cells with low contractile forces, causing apoptosis of MECs and differentiation towards adipogenic lineage of MSCs. Piccolo's laboratory reported that the subcellular localization and activity of YAP is regulated by actin cytoskeleton remodelling, cell substrate rigidity and topography, and cell stretching. The stiff substrate and high filamentous actin (F actin) levels have been shown to result in their nuclear translocation (Aragona *et al.,* 2013). Thus, confirming that substrate stiffness regulates the YAP activity in MSCs and hPSCs.

However, effect of substrate stiffness on human pluripotent stem cells (hPSCs) is not clearly understood. hESCs cultured on varying substrate stiffness, in presence of soluble pluripotency factors, show YAP nuclear localization and differentiation towards post mitotic neurons (Musah *et al.,* 2014). While one study states that hPSCs maintained the stemness on stiff substrates and undergo neurogenic differentiation of soft substrates (Maldonado *et al.,* 2015). Another study reports that neural induction is initially enhanced on soft substrate, but differentiation into neural progenitors and motor neurons occurs on stiff substrate. Additionally, mesoendodermal differentiation enhances on stiff substrate but further specification to posterior foregut requires soft substrate (Maldonado *et al.,* 2017).

Smith *et al.* (2017) highlighted that by changing the stiffness of the polydimethylsiloxane (PDMS) substrate the mesoderm differentiation kinetics of hiPSCs can be modulated and this mechanical change activates YAP during mesoderm induction. The main aim was to achieve endothelial commitment of the hiPSCs when cultured on substrates

of varying stiffness. These contrary results in hPSCs indicates that dynamic changes of substrate stiffness effects differentiations.

Chapter 3: Lacunae/Rationale, Aim and Objectives

3.1 Lacunae/Rationale

Growth factors and chemical cues are well known regulators of stem cells, but recent studies demonstrated the role of biophysical signals in regulating stem cell proliferation and differentiation through mechanotransduction pathways. Many research groups have reported the effect of artificial substrate on stem cells by varying its stiffness. Additionally, Hippo signalling pathway and its downstream effectors YAP have been shown to synchronize various signalling pathways and physical interactions between cells and its surrounding environment to bring about change in gene expression (Dupont *et al.*, 2011). YAP and TAZ are also known to play a crucial role in maintaining the "stemness" of the mouse pluripotent stem cells (Lian *et al.*, 2010), however, whether YAP regulates lineage specification in human pluripotent stem cell differentiation has not been demonstrated.

The studies on YAP and TAZ have focused on the fate of MSCs on different substrates and the role played by YAP during this process (Engler *et al.*, 2006; Dupont *et al.*, 2011; Smith *et al.*, 2017). Few studies that have used human pluripotent stem cells have uncovered role of YAP activity in maintaining the undifferentiated state and their inhibition led to neuronal differentiation. There are several unknowns that need to be studied with respect to YAP activity in human pluripotent stem cells such as: expression levels and localization of YAP in undifferentiated and differentiated cells, activity of YAP in germ lineages other than ectoderm, expression of YAP in embryoid bodies in suspension culture which has been shown to promote ectoderm differentiation, how does hPSCs differentiate on stiff matrix and how is the activity of YAP affected during the process, does inhibition of YAP activity affect pluripotency or differentiation capacity of stem cells, how antagonistic signalling pathways affect YAP activity and the subsequent effect on fate of stem cells.

Our primary aim was to investigate the interaction between hPSCs and substrate stiffness during their differentiation. And to understand if this interaction has any effect on the localization of YAP and TAZ. The study focuses on the YAP expression during the differentiation of hPSCs into endoderm lineage in response to substrate stiffness. We have modulated the levels of YAP by changing substrate stiffness and investigated if the altered

levels of YAP have any effect on the cell fate. These basic interactions will help us understand whether YAP is the integral part of hPSCs differentiation or not.

3.2 Aim of the project

To study the activity of YAP and TAZ during differentiation of human pluripotent stem cells (hPSCs) into endoderm lineages in response to different substrate stiffness.

3.3 Objectives

1. Investigate the expression of phosphorylated and non-phosphorylated YAP/TAZ in undifferentiated human pluripotent stem cells on substrates of different stiffness
2. Expression of phosphorylated and non-phosphorylated YAPZ/TAZ in hPSCs during endoderm differentiation on the substrates of different stiffness.
3. Effect of pharmacological inhibitor/activator of YAP/TAZ on hPSCs during endoderm lineage differentiation on substrates of different stiffness.

Chapter 4: Materials and Methods

4.1 Ethical statement

hESCs cell line KIND1 (Kumar *et al*., 2009) was procured from National Institute for Research in Reproductive Health (NIRRH), Mumbai, India; the use of these cell line was approved by the Institutional Committee for Stem Cell Research (ICSCR), NMIMS (deemed-to-be) University, Mumbai, India. Human placental mesenchymal stem cells (hPMSCs) were procured from Symbiosis Centre for Stem Cell Research (SCSCR). The cell line was used in NCCS & SCSCR and approved by the IC SCR committee of NCCS.

4.2 Substrates used in the study

For positive control, traditional polystyrene tissue culture treated plastic dishes (TCP) (Corning, USA) were used. For softer substrates, commercially available CytoSoft® plates (Advanced BioMatrix, USA) were used. These plates have polydimethylsiloxane (PDMS) layer and elastic modulus of 0.2kPa , 0.5kPa, 2kPa, 8kPa, 16kPa, 32kPa, to 64kPa. The Cytosoft® (CS) substrates were activated as per manufacturer's instructions. Briefly, each well was coated with3mL of 1X vitronectin (Thermo Fisher Scientific, Life technologies, CA, USA) for 1 hour at 37˚C, followed by three washes with 1x DPBS for 5 mins and cell seeding. The 96-well ultra-low attachment dishes (ULAD) (Corning, USA) was also used which gave us the near-suspension culture conditions. In TCP and CS cells were cultured for 4-5 days on each substrate before passaging and in ULAD cells were cultured for 7 days. KIND1 cells were maintained for three consecutive passages on all the substrate.

4.3 Maintenance of hESCs

hESCs, KIND1 cells, were cultured and maintained in xeno- and feeder-free system using 1X vitronectin. All the substrates i.e., TCP and CS were coated with 1X vitronectin. hESCs were maintained in pluripotency maintaining medium (PMM), Essential 8 basal medium (E8; Thermo Fisher Scientific, Gibco) supplemented with 1X Essential 8 supplement (Life Technologies) and incubated at 37˚C, 5% CO_2. The media was changed daily. When the cells attained 80% - 90% confluency, they were passaged using 0.5mM EDTA (Sigma Aldrich, MO, USA) at a passage ratio of 1:4. In ULAD, 200µl of the cell suspension was added to each well

and incubated at 37˚C, 5% CO_2. The cells were cryopreserved in cryoprotectant medium containing E8 and 10% DMSO (Sigma Aldrich) and stored in liquid nitrogen.

4.4 Differentiation of hESCs with minimum differentiation inducing media

To study whether substrate stiffness alone can induce differentiation of hESCs, we cultured hESCs in differentiation inducing media (DIM) with minimum serum concentration. KIND1 cells were maintained in Advanced Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fischer Scientific, USA) which was supplemented with 2% foetal bovine serum (FBS) (Thermo Fischer Scientific, USA) and 1% antibiotic, without any lineage specific growth factors for 4 days.

4.5 Directed differentiation towards definitive endoderm

Undifferentiated KIND1 cells showing 80% - 85% confluency were used for differentiation and RNA was extracted on day 4 of the differentiation protocol. Briefly, cells were rinsed with DPBS followed by culturing in RPMI-1640 (Sigma-Aldrich) media supplemented only with 0.025× ITS (Sigma-Aldrich) and 100 ng/ml ACTIVIN A (R&D Systems) on the first day and an additional three days with increasing concentration of foetal bovine serum from 0.2%, 0.5% and 2% on days 2, 3 and 4 respectively. KIND1 differentiated on TCP were used as control for all the studies.

4.6 Inhibitor and Activator

For modulation study, KIND1 cells were differentiated on TCP, and CS plates of stiffness 0.2kPa and 64kPa. 30nM of YAP inhibitor, Verteporfin (Sigma-Aldrich), and 10uM of YAP activator, Lysophosphatidic acid (Sigma-Aldrich) was added to the differentiated cells on day 4 of the differentiation protocol. Equal volume of DMSO was added to each substrate as vehicle control. The cells were collected for RNA and protein post 24 hours after treatment.

4.7 Maintenance of hMSCs

For hPMSCs, TCP and CS substrates were coated with 1X collagen. hPMSCs were maintained on TCP and CS substrates in Improved Minimum Essential Medium (IMEM) (Thermo Fischer Scientific, USA) medium supplemented with 10% FBS and antibiotics and maintained them in culture for four days.

4.8 RNA extraction and Quantitative Reverse Transcription RCR

Total RNA was isolated using TRI reagent (Sigma Aldrich, USA), and was quantified using BioTek™ Epoch™ Microplate Spectrometer (BioTek, USA). Complementary DNA (cDNA) was prepared from 1µg of total RNA using Prime Script 1st Strand cDNA synthesis kit (TakaraBio, Japan) as per the manufacturer's recommendations in Nexus Thermal Cycler (Eppendorf, Germany). The quantitative real time PCR was performed in QuantStudio 3 and Thermal cycler (Applied Biosystems, CA, USA). For each sample, the relative fold change was calculated by $2^{-\Delta\Delta Ct}$ method. Each PCR reaction was carried out in duplicates using samples from three biological replicates. Endoderm differentiated day 4 (ED D4) cDNA was used as positive control for endoderm and mesoderm; and commercially available adult human brain RNA sample (Takara Bio) was used as positive control for ectoderm. The following primers were designed using NCBI Primer Blast and used in the study: Pluripotency: *OCT4, NANOG* and *SOX2*; Lineage specific primers: Endoderm specific – *SOX17*, *FOXA2*, *CXCR4*; Mesoderm specific – *BRACHYURY*; Ectoderm specific – *PAX6*; Housekeeping: *18S rRNA*

4.9 Protein extraction and Western blot

Proteins were extracted by resuspending the cell pellet (obtained after EDTA treatment) in chilled complete cell lysis buffer containing 1X Protease inhibitor cocktail (Sigma Aldrich) and Roche PhosSTOP (Sigma Aldrich). The total protein concentration was by the Lowry assay using Folin–Ciocalteu's phenol reagent (Sigma Aldrich). 20ug-50ug of the total protein was loaded on 10% SDS-PAGE. For transfer, PVDF membrane (Biorad, USA) was used. The primary antibodies used were: anti-OCT4A, anti-Cyclin D1, Hippo signalling pathway kit (CST, USA); anti-YAP (Sigma Aldrich); anti-NANOG (Abcam, USA); and loading control anti-GAPDH (CST, USA) followed by detection with the anti-rabbit HRP linked secondary antibody (CST).

4.10 Immunofluorescence

KIND1 cells cultured on glass coverslips in pluripotency maintaining medium (control, TCP) and KIND1 cells cultured in ULAD were transferred to glass coverslips and allowed to expand for 24 hours, before fixing them with 4% paraformaldehyde. The primary antibody used for immunofluorescence are: anti-OCT4A and anti-YAP XP® (Cell Signalling Technology). The secondary antibody Anti-Rabbit IgG (H+L) Alexa Fluor 488 (Thermo Fisher) was used.

Chapter 5: Investigate the expression of phosphorylated and non-phosphorylated YAP/TAZ in undifferentiated human pluripotent stem cells on substrates of different stiffness

We have cultured hESCs on TCP, CS substrates and ULAD in pluripotency maintaining media. TCP was used as control, CS substrate provided a range of stiffness: 0.2kPa, 0.5kPa, 5kPa, 8kPa, 16kP, 32kPa and 64kPa, and ULAD gives us no-substrate system. On soft substrate, hESCs have been reported to under matrix-induced differentiation towards neurogenic lineage. We wished to see how hESCs respond under stress.

5.1 Characterization of hESCs cultured on TCP

KIND1 cells cultured in E8 media on TCP, were first characterized for pluripotency by end point PCR and confirmed by immunofluorescence. Expression of pluripotency markers *OCT4, NANOG* and *SOX2* were seen and no expression of lineage specific markers *SOX17*, *BRACHYURY* and *PAX6* was observed. Moreover, localization of OCT4 protein in the cell nuclei confirmed that cells were in the undifferentiated state.

5.2 Maintaining hESCs on CS substrates in Pluripotency maintaining media (PMM)

To identify whether the biophysical cues alone can induce differentiation in pluripotency supporting media, we cultured hESCs on tissue culture treated plastic (TCP) dishes and on CytoSoft® (CS) substrate plates in pluripotency maintaining for three consecutive passages. The cells were collected for RNA and protein at day 4 of each passage. In pluripotency maintaining medium hESCs on TCP were present in compact colonies with characteristic epithelial morphology. Surprisingly, hESCs cultured on CS substrates exhibited similar morphology and cells were arranged in compact colonies. We characterized the hESCs cultured on TCP and CS substrates for OCT4 and NANOG protein expression. Compared to their expression on TCP, OCT4 and NANOG levels were maintained in all the three consecutive passages. To confirm whether lineage differentiation had occurred, we checked the mRNA expression of lineage specific markers - *SOX17* (endoderm), *BRACHYURY* (mesoderm) and *PAX6* (ectoderm). No expression of lineage specific markers was observed by real time PCR. Our results showed that hESCs remained pluripotent on soft substrates in presence of pluripotency maintaining factors.

5.2.1 Correlation between YAP and substrate stiffness

In hESCs, YAP has shown to maintain pluripotency on TCP and overexpression of YAP has been correlated to naive pluripotency (Qin *et al*., 2016). So, we wondered whether YAP plays any role in maintaining the pluripotent state of hESCs on soft substrates as well. Protein samples isolated from TCP and CS substrates were probed with YAP and pYAP antibodies. The western blot data showed almost equivalent expression of YAP and pYAP on all the substrates. KIND cells were cultured on glass coverslips (stiff substrate) and probed with YAP antibody. We saw the expression of nuclear YAP in the nucleus and cytoplasm corelating with the western blot data. Summarizing the above results, we can say that contrary to the earlier reports, we did not see any change in pluripotency of hESCs despite culturing them on substrates with stiffness several thousand-fold lower than conventional plastic dishes.

5.2.2 Maintaining hESCs in ULAD in PMM

Since all our substrates retained stemness, we were intrigued to see whether culturing KIND1 in near suspension like conditions can affect stemness. We maintained KIND1 cells on ULAD for seven days in pluripotency maintaining media. We subsequently passaged hESCs till three passages. We observed almost equal expression of OCT4 protein in ULAD in all the three passages. OCT4 expression was confirmed by immunofluorescence staining. No expression of lineage specific markers seen., therefore, we can say that hESCs cultured without substrate retained their stemness. Significant upregulation of YAP was observed in ULAD compared to TCP. We can speculate that YAP might regulate pluripotency but this needs extensive evaluation. We did not carry this study further because our other systems are 2D and the cells on ULAD form tight clumps which are 3D. Therefore, comparing 2D and 3D results might give misleading results.

5.3 Maintaining hESCs on CS substrates in Differentiation inducing media (DIM)

The above results roused our curiosity, if change in substrate stiffness alone does not induce differentiation, then whether changing the medium to minimum differentiation inducing medium could lead to any specific lineage as reported previously with hMSCs. We intentionally kept the serum concentration to 2%, as we did not want the serum components to dictate the course of differentiation, but still added enough serum to keep cells healthy.

We switched the media to Advanced DMEM with 2% FBS and checked the expression of pluripotency markers, lineage specific markers, YAP and pYAP.

The hESCs colonies in presence of differentiation inducing medium lost their compact epithelial morphology and appeared flattened. As expected, the induction of differentiation was marked by downregulation of OCT4 expression in cells grown on TCP, however, surprisingly the cells grown on softer substrates showed relatively higher OCT4 expression. Next, we studied gene expression of lineage specific markers by quantitative reverse-transcription PCR to assess lineage specification. A comparative analysis between positive control and other samples showed reduced expression of *SOX17*, *BRACHYURY*, and *PAX6* in TCP and CS. These results hint that the hESCs on soft substrates in the presence of low levels of morphogens differentiates but not towards specific lineage.

5.3.1 Correlation between YAP and substrate stiffness

Next, we performed immunoblotting to check the dynamics of YAP and pYAP proteins in differentiation inducing media on soft substrates. The comparative study of YAP and pYAP showed low expression of YAP and relatively high expression of pYAP. Low expression of YAP has been reported to cause loss of stem cell pluripotency (Lian *et al*., 2010; Rosado-Olivieri *et al*., 2019) and pYAP gets sequestered in the cytoplasm leading to no YAP expression. Our results imply that on soft substrates and in presence of minimum differentiation inducing signals, hESCs start losing their pluripotency and undergo differentiation.

5.4 Pilot study of hPMSCs

Most of the substrate studies use MSCs, therefore, we wished to replicate the reported results with hPMSCs. On TCP, hPMSCs showed elongated fibroblast like morphology. hPMSCs cultured on 0.2kPa, 0.5kPa and 5kPa showed less spreading and cell count, however, hPMSCs cultured on 8kPa, 16kPa, 32kPa and 64kPa showed similar morphology to TCP. Just from the visual confirmation we can deduced that MSCs are sensitive to the change in stiffness compared to hESCs, which resisted the change in stiffness.

Chapter 6: Expression of phosphorylated and non-phosphorylated YAPZ/TAZ in hPSCs during endoderm differentiation on the substrates of different stiffness

To investigate the stiffness-induced changes during stem cells differentiation, we cultured hESCs substrates of different stiffness. KIND1 cells were differentiated towards definitive endoderm lineage on TCP and CS plates. After day 4 of the differentiation procedure, cells were harvested for RNA and protein. The hESCs on TCP and CS substrates showed transition from pluripotent cell morphology (compact colonies) to elongated, flattened morphology. Expression of pluripotency marker OCT4 at day 4 of endodermal differentiation was observed (Pethe *et al*., 2014; Dumasia *et al*., 2021), however upregulation of lineage specific gene markers *SOX17*, *FOXA2*, *CXCR4,* and *BRACHYURY* by reverse transcription PCR, indicated the differentiation of hESCs into mesendoderm lineage. No expression of ectoderm marker *PAX6* was observed.

Our next step was to assess the expression of YAP and pYAP in differentiating cells. The purpose of this study was to determine whether substrate stiffness as well as differentiation affects YAP expression. YAP is a critical component of Hippo signalling pathway which when unphosphorylated, relays mechanical signal into the nucleus thereby deciding cellular fate. pYAP expression normalised with relative YAP levels did not show significant change in the expression levels compared to the TCP. Whereas, YAP levels when normalised with the relative GAPDH levels showed slight downregulation compared to TCP. Our results suggests that substrate stiffness does not affect YAP levels, and the cells maintain total YAP expression during the differentiation.

Chapter 7: Effect of pharmacological inhibitor/activator of YAP/TAZ on hPSCs during endoderm lineage differentiation on substrates of different stiffness

In this chapter we discuss the results of modulation of YAP levels during differentiation on substrates of varying stiffness.

7.1 YAP inhibition by Verteporfin

Verteporfin (VP) has been used in to demonstrate the effect of YAP inhibition on stem cell regulation (Rosado-Olivieri *et al*., 2019; Dong *et al*., 2020; Quan *et al*., 2021). However, inhibition of YAP in hESCs cultured on varying stiffness has not been demonstrated. The exact mechanism of action of Verteporfin is unknown, however it has been reported that VP selectively binds to YAP causing some conformational changes in YAP structure, thereby eliminating YAP interaction with DNA binding proteins. Another mechanism is that, VP increases the levels of 14-3-3 protein in cytoplasm thereby blocking the YAP function (Wang *et al*., 2016). YAP inhibition has shown to enhance differentiation of hESCs (Rosado-Olivieri *et al*., 2019; Quan *et al*., 2021).

For our study we used a large range of VP concentration from 10nM, 20nM, 30nM, 40nM, 50nM, 60nM, 70nM and 80nM to standardize the VP concentration. hESCs were cultured on TCP in complete E8 medium and treated with the mentioned concentrations on day 4. DMSO as vehicle control was used. We observed that hESCs treated with concentration above 30nM showed abnormal cell death. The cells from remaining three concentrations were harvested for protein, and YAP expression was observed. 30nM VP concentration showed significant downregulation in YAP expression compared to the DMSO control, hence this concentration was used for further studies.

Next, we went ahead to understand the effect of YAP inhibition on endoderm differentiation. KIND1 cells were cultured on TCP, CS substrates with stiffnesses 0.2kPa and 64kPa. KIND1 was differentiation was per above mentioned protocol and the differentiated cells were treated with 30nM of VP on day 4 of the differentiation. Next day, the cells were harvested for RNA And protein. To confirm the differentiation state mRNA levels of lineage specific markers were analysed. Expression of definitive endoderm markers: *SOX17, FOXA2*, and *CXCR4*, and mesoderm marker: BRACHYURY confirmed that the hESCs have undergone

differentiation into definitive endoderm stage. *SOX17* and *CXCR4* showed similar pattern throughout the various stiffness, with gradual decrease from TCP to 64kPA. High expression of *FOXA2* and *BRACHYURY* in 0.2kPa VP treated cells indicate that cells might favour soft substrate for mesoendoderm differentiation. To confirm whether YAP inhibition promoted differentiation we checked for the YAP proteins levels. YAP levels were downregulated in TCP which corelates to the literature. Surprisingly, we observed an upregulation of YAP in 0.2kPa and 64kPa compared to the TCP DMSO control. From the inhibition study, we can conclude that the substrate stiffness and the inhibitor have an effect on the differentiation of hESCs other than the one reported in the literature and this can be potentially explored further.

7.2 YAP activation by Lysophosphatidic acid

Intrigued by the inhibitor study, we added YAP activator, LPA during definitive endoderm. LPA is a small lipid molecule which inhibits Hippo pathways by dephosphorylating LATS, thereby activating YAP expression in cells (Yu *et al.*, 2012). In 2016, Qin and colleagues found that activating YAP activity by supplementing lysophosphatidic acid (LPA) significantly induced the transition from the primed to the naïve state in multiple human ESC and iPSC lines, and the naïve state was prolonged in the culture medium supplemented with LPA. These results suggest an unexpected role of YAP in regulating the induction and maintenance of human naïve stem cells. For standaridizing LPA concentration, we treated undifferentiated cells cultured on TCP with 10µM, 15µM and 20µM concentration of LAP on day 4. The protein expression study showed that 10µM of LPA showed significant increase in YAP expression compared to respective DMSO control and hence we used this concentration for differentiation studies (Qin *et al.*, 2016).

Next, we went ahead to understand the effect of YAP inhibition on endoderm differentiation in hESCs. KIND1 cells were cultured on TCP, CS substrates with stiffnesses 0.2kPa and 64kPa. KIND1 was differentiation was per above mentioned protocol and the differentiated cells were treated with 10uM of LPA on day 4 of the differentiation. Next day, the cells were harvested for RNA And protein. To confirm the definitive endoderm differentiation and also the effect of stiffness on the differentiation, mRNA levels of lineage specific markers were analysed. LPA treated cells showed upregulation of *SOX17, FOXA2,* and *CXCR4* confirming the definitive endoderm stage of differentiated cells. Additionally, LPA treated cells seemed to favour differentiation compared to the respective DMSO control

more in 0.2kPa stiffness compared to TCP and 64kPa stiffness. YAP protein expression was high on CS substrates compared to TCP DMSO control, however, no significant change in YAP expression was observed.

Chapter 8: Discussion

In the project, we intended to determine the role of substrate stiffness and YAP in human pluripotent stem cells during their undifferentiated and differentiated state. Our data showed that for hESCs the biochemical signals can keep cell in undifferentiated or differentiate them, but changes in substrate stiffness does not affect the hESCs. Differentiation of hESCs on TCP and soft substrates was observed after the addition of differentiation inducing media, as seen by the expression of selective lineage markers.

We shed a light on the connection between the biochemical and biophysical signals during endoderm differentiation of hESCs cultured on substrate of varying stiffnesses, which is still an active area of research. Several research groups have demonstrated that stem cells specifically hMSCs and mESCs respond to the changes in substrate stiffness and differentiate into neurogenic, myogenic or osteogenic lineage on substrate stiffness mimicking respective biological tissue stiffness (Engler *et al*., 2006; Evans *et al*., 2009; Keung *et al*., 2012), and that YAP is an important transcriptional regulator in maintaining pluripotency and stiffness-induced stem cell behaviour (Dupont *et al*., 2011; Oliver-De La Cruz *et al*., 2019; Musah *et al*., 2014; Brusatin *et al*., 2018; Lee *et al*., 2019). Under normal culture condition, downregulation of YAP in mESCs has shown to cause differentiation into endoderm and mesoderm lineages (Lian *et al*., 2010). *In vitro,* ectopic expression of YAP inhibits ESC differentiation and cells maintain stem cell phenotype under differentiation conditions. Subsequently, LIF (Leukaemia inducing factor) (Tamm *et al*., 2011) and Inter-α-Inhibitor (IαI), a component of serum (Pijuan-Galitó *et al*., 2014), were identified to facilitate YAP expression and induce OCT3/4 expression. A novel method showed that overexpression of two reprogramming factors OCT4 and SOX2 (Takahashi and Yamanaka 2006) and YAP, key component of Hippo pathway, together induced reprogramming of human amniotic epithelial cells into induced pluripotency stem cells (Zhao *et al*. 2017). However, there are no reports on how YAP regulates with pluripotency in human embryonic stem cells.

Intrigued by these studies, we asked whether culturing the hESCs in pluripotency maintaining medium on TCP and CS substrates affects pluripotency and when induced to differentiate whether hESCs favour a specific lineage like hMSCs. We found that despite reducing the substrate stiffness to ten-fold, hESCs remain pluripotent in media with pluripotency factors and differentiation of hESCs occurred only in presence of soluble

signalling molecules. It should be noted that the mESCs are naïve cells while hESCs are primed cells (Ginis *et al*., 2004) and this might affect how cells respond to change in substrate stiffness. The YAP expression in hESCs maintained in pluripotency maintaining medium on TCP and CS substrates remained constant through the stiffness, indicating the YAP expression is not affected by stiffness and helps in maintaining pluripotency.

Next, we explored the effect of differentiation of hESCs cultured on varying stiffness. In certain cell types, such as myotubes (Engler *et al*., 2004; Levy-Mishali *et al*., 2009) and mesenchymal stem cells (Engler *et al*., 2006), optimal differentiation was achieved on a substrate with the same stiffness as the natural microenvironment. Numerous stages of embryogenesis and foetal development are either affected by or generate mechanical forces (Ingber, 2003). More specifically, during gastrulation, blastula epiblast cells ingress (Keller *et al*., 2003; Beloussov and Grabovsky, 2003; Gadue *et al*., 2005), the cells undergo changes in motility and shape, which is attributed to reorganization of the cytoskeleton within the cell (Odell *et al*., 1981; Farge, 2003; Ingber, 2003; Beloussov and Grabovsky, 2003). This change in the cytoskeleton organization is caused by the mechanical forces acting on the cellular surfaces. Therefore, the mechanical forces play an essential role during the lineage-specification of the gastrulation phase.

Our results show that hESCs efficiently differentiated into definitive endoderm lineage upon adding suitable signalling molecules on all the stiffness, TCP and soft substrates alike, as seen by the expression of *SOX17, FOXA2* and *BRACHYURY.* We also checked for the expression of YAP in these differentiating cells, and observed that differentiation has no apparent effect on the YAP expression.

We find that modulating the expression of YAP in differentiating hESCs gives an interesting perspective to the YAP regulation by substrate stiffness. On traditional stiffness (TCP) YAP inhibition has been shown to promote endothelial cell differentiation in hESCs (Quan *et al*., 2021) and also enhanced differentiation of functional stem cell-derived insulin producing beta cells (Rosado-Olivieri *et al*., 2019). When YAP was inhibited by pharmacological inhibitor, TCP showed concurrent result with the literature of decreasing YAP expression during differentiation. Surprisingly, soft substrates showed upregulation of YAP compared to TCP DMSO control. Overexpression of YAP, by supressing Hippo pathway has been shown to promote proliferation of stem cells. Additionally, YAP overexpression induces

transition from primed to the naïve state of hPSCs (Yu *et al*., 2012; Qin *et al*., 2016). Suprisingly, we observed that YAP overepxression favoured hESCs differentiation into endoderm lineage and that YAP expression remains unaffected during the differentiation.

Taken together, our data suggests after a prolonged culture, hESCs remain pluripotent when cultured in pluripotency maintaining medium on soft substrates and differentiate in minimum differentiation inducing medium. This implies that hESCs have different mechanism in sensing the substrate stiffness than hMSCs or mESCs; and for hESCs to differentiate, biochemical signals play a more crucial role than substrate stiffness. During directed differentiation of hESCs on varying stiffness, YAP expression was not effected by the differentiation or by substrate stiffness. This can be varying reasons attributing to these results. It is possible that hESCs respond well to the signalling molecules and are resistant to the substrate stiffness.

Although YAP inhibition and overexpression did not show major changes in YAP expression in soft substrates, but we observed a pattern indicating that YAP inhibition slightly downregulated the differentiation potential whereas YAP overexpression increased the differentiation potential of hESCS into endoderm lineage. Most of the studies using VP and LPA as modulators of YAP have shown YAP effect during the later stages of differentiation in TCP. Our study was focused on understanding the early stages of differentiation, therefore it would be interesting to study the moduations in YAP levels on varying stiffness during late endoderm differentiation. Directly targetting YAP by CRISPR or shRNA would provide further details into the relationship between stiffness and YAP during differentiation. Also, a combinational study with two or more biophysical factors will give us a better understnading on the effect of mechanical forces on the stem cell behvaiour.

Chapter 9: Summary and Conclusion

- ✓ hESCs remain pluripotent when cultured in pluripotency maintaining medium on all stiffness and differentiate in minimum differentiation inducing medium
- ✓ hESCs differentiation does not seem be affected by substrate stiffness
- ✓ Compared to the undifferentiated hESCs and differentiated cells cultured on TCP, no significant change in YAP and pYAP protein levels were seen during endoderm differentiation on soft stiffness
- ✓ YAP expression if affected by the substrate stiffness. On soft stiffness, YAP expression is almost similar in YAP inhibited and overexpressed cells during differentiation.

Chapter 10: Significance of the Study

Our study sheds light into the interplay between the substrate stiffness, hESCs differentiation and YAP regulations in undifferentiated and differentiated hESCs. hPSCs is being actively used in stem cell-based therapies, and regulating hPSCs differentiation will promote better outcome. Our study reveals that more research needs to be conducted in understanding the intrinsic connection between stiffness and hESCs differentiation.

Publications and Poster Presentations

Publications:

1. Manuscript entitled "Impartial effect of substrate stiffness on YAP expression and differentiation in human embryonic stem cells" (under preparation)
2. Virdi, J.K. and Pethe, P., 2022. Soft substrate maintains stemness and pluripotent stem cell-like phenotype of human embryonic stem cells under defined culture conditions. *Cytotechnology, 74(4)*, pp.479-489.
3. Virdi, J.K. and Pethe, P., 2021. Biomaterials regulate mechanosensors YAP/TAZ in stem cell growth and differentiation. *Tissue Engineering and Regenerative Medicine, 18(2),* pp.199-215.
4. Virdi, J.K. and Pethe, P., 2021. Dynamic Interactions Between Stem Cells and Biomaterials. *In Engineering Materials for Stem Cell Regeneration* (pp. 381-398). Springer, Singapore.

Poster presentation:

1. International Society for Stem Cell Research 2021 (ISSCR 2021). 21st – 26th June 2021: Presented poster entitled "Role of Hippo pathway in regulating pluripotency of human embryonic stem cells on soft substrate"
2. Summer Biomechanics, Bioengineering and Biotransport conference 2021 (SB3C2021). 14th – 18th June 2021: Presented poster entitled "Role of substrate stiffness on pluripotency and differentiation of human embryonic stem cells"
3. Advances in Materials Science and Applied Biology (AMSAB). 8th – 10th January 2019. Presented poster entitled "Substrate stiffness regulates YAP/TAZ activity in human pluripotent stem cells."

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ORIGINAL RESEARCH

Soft substrate maintains stemness and pluripotent stem cell-like phenotype of human embryonic stem cells under defined culture conditions

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Abstract Human embryonic stem cells (hESCs) are derived from the inner cell mass (ICM) of the pre-implantation blastocyst. Prior to embryo implantation, the ICM cells are surrounded by trophoblasts which have mechanical stiffness ranging from Pascal (Pa) to kilopascal (kPa). However, under in vitro conditions these cells are cultured on stiff tissue culture treated plastic plates (TCP) which have stiffness of approximately 1 gigapascal (GPa). This obvious dichotomy motivated us to investigate the fate of hESCs cultured on softer substrate, and to probe if the hESCs undergo differentiation or they retain pluripotency on soft substrates. We investigated the expression of pluripotency markers, and lineage-specific markers; we particularly looked at the expression of transcriptional coactivator YAP (Yes-associated protein), an important mediator of extracellular matrix (ECM) mechanical cues and a known downstream transducer of Hippo pathway. Downregulation of YAP has been correlated to the loss of multipotency of human mesenchymal stem cells (hMSCs) and pluripotency in mouse ESCs (mESCs); but we report that hESCs maintain their stemness on soft substrate of varying stiffness. Our findings revealed that on soft substrate hESCs express pluripotency markers and does not undergo substrate-mediated differentiation. Interestingly we show that hESCs maintained basal level of YAP expression for cell survival and proliferation, but YAP expression does not correlate directly with pluripotency in hESCs. To summarize, our results show that hESCs retain their stemness on soft substrate despite downregulation of YAP.

Keywords Human embryonic stem cells · Substrate stiffness · Differentiation · Pluripotency · YAP · Mechanobiology

Introduction

Human embryonic stem cells (hESCs) are unique cell type with indefinite self-renewal property and capability to differentiate into specialized cell types and these unique characteristics make them suited for use in regenerative medicine. When grown in vitro, hESCs maintain a balance between the pluripotent and differentiation states, which is mainly attributed to the microenvironment. Prior to implantation, the

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ICM microenvironment is a complex environment made up primarily of adjacent cells, the extracellular matrix (ECM), and soluble biomolecules but devoid of stiff cell support provided by the endometrial cells. hESCs like other cells receive multitude of biochemical and mechanical signals from its microenvironment (Pelham and Wang 1997; Ireland and Simmons 2015; Vining and Mooney 2017). The ability of a biochemical signals to direct stem cell behaviour has been well established, however in the past decade numerous findings has highlighted the significant role of mechanical signals in stem cell differentiation and maturation (Engler et al. 2006; Dupont et al. 2011; Oliver-De La Cruz et al. 2019; Heng et al. 2020).

Yes-associated protein (YAP) is a key mechanosensor that relays the mechanical signals into the nucleus. YAP is a transcriptional coactivator of the evolutionary conserved Hippo signalling pathway, which is an important regulator of organ size, cell proliferation, and apoptosis (Wu et al. 2003; Huang et al. 2005; Dong et al. 2007). Upstream signals from growth factors, mechanical stimulus, and cell–cell contact can activate the Hippo pathway which causes phosphorylation of mammalian Ste20-like kinases 1/2 (MST 1/2) facilitated by Salvador (SAV1), large tumor suppressor kinase 1/2 (LATS1/2), and monopolar spindle-one-binder proteins (MOB1) complex (Wu et al. 2003; Chan et al. 2005). LATS1/2-MOB1 complex, in turn, phosphorylates and inactivates YAP and its homologous protein transcriptional coactivator with PDZ binding motif (TAZ also known as WWTR1) (Oka et al. 2008). Phosphorylated YAP (pYAP) is anchored in the cytoplasm by 14-3-3 and degraded by proteosomes leading to reduced cell proliferation and enhanced cell differentiation (Huang et al. 2005; Udan et al. 2003; Rosado-Olivieri et al. 2019). Conversely, non-phosphorylated YAP/TAZ heterodimer enter the nucleus, where it binds to DNA-binding transcriptional factors, such as the transcriptional enhanced associate domain 1-4 (TEAD 1-4) protein family, which regulates the expression of genes required to initiate cell cycle, cell proliferation and inhibition of apoptosis (McClatchey and Yap 2012; Kim et al. 2018).

YAP plays a key role in maintaining a delicate balance between self-renewal, proliferation, and differentiation in mouse ESCs (mESCs; Lian et al. 2010; Tamm et al. 2011) as well as hESCs (Varelas et al. 2008; Ohgushi et al. 2015; Hsiao et al. 2016). Numerous studies have shown that the substrate stiffness, a measure of Young's modulus (E) and matrix elasticity, strongly influences the differentiation of human mesenchymal stem cells (hMSCs; Engler et al. 2006; Dupont et al. 2011; Wen et al. 2014; Driscoll et al. 2015; Hadden et al. 2017) and hESCs (Musah et al. 2014; Sun et al. 2014). In vivo the human blastocyst attaches to endometrial cells which have stiffness of approximately $E \sim 1000$ Pa (Abbas et al. 2019), however, hESCs are routinely grown on stiff polystyrene tissue culture treated plastic dishes (stiffness $E \sim 1$ GPa). It is unclear whether substrate stiffness or the activation of Hippo pathway affects the pluripotency of the hESCs. To investigate whether change in substrate alone can induce differentiation of hESCs we examined the cellular response of hESCs when grown on soft substrate with pluripotency maintaining and minimum differentiation inducing media. Our results show that hESCs maintained their stemness even on the soft substrate in absence of any differentiation inducing signals, but differentiated only when exposed to differentiation inducing media. Thus, our results demonstrate that change in substrate stiffness alone does not affect pluripotency of hESCs.

Materials and methods

Ethical statement

KIND1, a hESCs cell line, was procured from National Institute for Research in Reproductive Health (NIRRH), Mumbai, India (Kumar et al. 2009; Pethe et al., 2015; Dumasia et al., 2021) and was approved to use by Institutional Committee for Stem Cell Research (IC SCR), NMIMS (deemed-to-be) University, Mumbai, India.

Cell culture and maintenance

hESCs were cultured on 1 × vitronectin (Thermo Fischer Scientific, USA) coated plastic culture dishes and maintained in complete Essential 8 (E8) media with supplement (Thermo Fischer Scientific, USA). For differentiation, hESCs cells were maintained in Advanced Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fischer Scientific, USA) supplemented with 2% foetal bovine serum (FBS; Thermo Fischer Scientific, USA) and

1% antibiotic, without any lineage specific growth factors for 4 days. Briefly, for 60 mm culture plate, 20 µl of 100 × vitronectin was diluted in 2 ml of DPBS for coating the plate/substrate surface, followed by incubation at 37 °C for 1 h, after which the DPBS was discarded and 2 ml of complete pluripotency or differentiation media was added to each plate. When the cells were about 70–80% confluent, 0.5 mM EDTA (Sigma Aldrich, USA) was used to detached the cells. After EDTA was removed, cells were centrifuged at 1000 rpm for 5 min at room temperature. The pellet was resuspended in pluripotency or differentiation media and seeded in 1:4 seeding ratio (i.e., cells from 1 × 60 mm plate was seeded into 4 × 60 mm plates). The cells were cultured for 4–5 days on each substrate and maintained for three consecutive passages (approximately 12–15 days) before harvesting for protein/RNA. Differentiation of KIND1 into endoderm lineage was performed out using a well-established protocol (Pethe et al., 2015; Dumasia et al., 2021). Briefly, KIND1 cells were cultured in RPMI 1640 media containing 100 ng/ml Activin A (R&D Systems, USA) and 100 × ITS for 24 h, with subsequent addition of 0.2% of FBS on day 2, 0.5% FBS on day 3 and 2% FBS on day 4. On day 4 cells were harvested to extract RNA.

Substrates used in the study

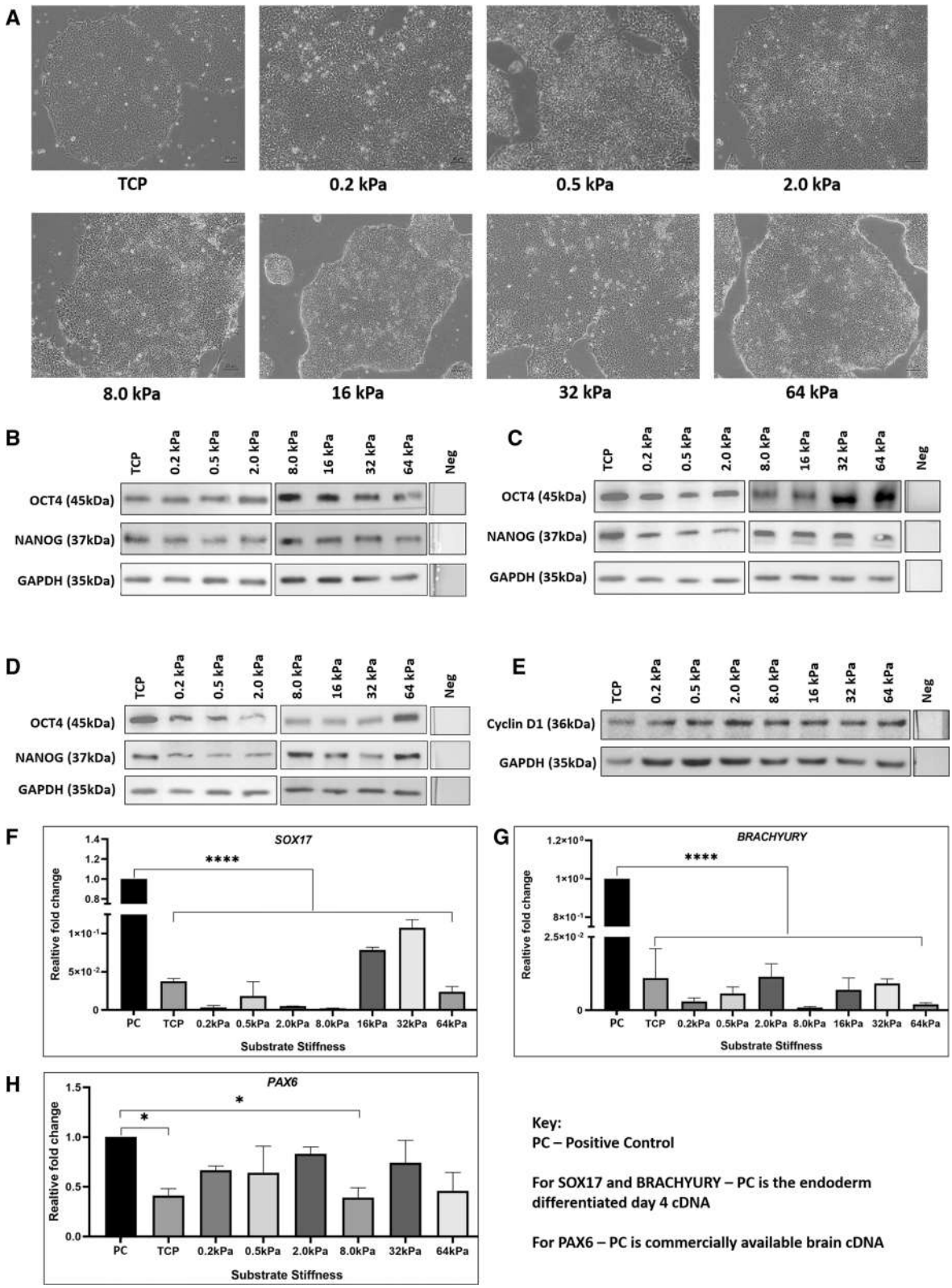
For positive control, traditional polystyrene tissue culture treated plastic dishes (TCP; Corning, USA) were used. For softer substrates, commercially available CytoSoft® plates (Advanced BioMatrix, USA) were used. These plates have polydimethylsiloxane (PDMS) layer and elastic modulus of 0.2 kPa, 0.5 kPa, 2 kPa, 8 kPa, 16 kPa, 32 kPa, to 64 kPa. The Cytosoft® substrates were activated as per manufacturer's instructions and from here on will be referred to as CS plates throughout the manuscript. Briefly, each well was given three washes with 1 × DPBS for 5 min and coated with 1 × vitronectin followed by cell seeding as per above mentioned protocol. The ultra-low attachment dishes (ULADs) (Corning, USA) were also used which gave us the near-suspension culture conditions. The cells were cultured for 4–5 days on each substrate before passaging and were maintained for three consecutive passages on the respective substrate.

Protein extraction and western blot

Proteins were extracted by resuspending the cell pellet (obtained after EDTA treatment) in chilled complete cell lysis buffer containing 1 × protease inhibitor cocktail (Sigma Aldrich, USA) and Roche PhosSTOP (Sigma Aldrich, USA). The cell lysate was incubated on ice for 30 min with intermediate vortexing, followed by centrifugation at 13,000 × g for 20 min. The supernatant was collected and the total protein concentration was by the Lowry assay using Folin–Ciocalteu's phenol reagent (Sigma Aldrich). For protein separation, 10% SDS PAGE was prepared and 20–50 µg of total protein was loaded per well. The proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Biorad, USA), followed by incubation with blocking buffer [5% bovine serum albumin (BSA) + 0.5% Tween 20 in 1 × TBS] 2 h at room temperature (RT). The proteins were probed overnight at 4 °C with monoclonal rabbit antibodies: anti-OCT4A, anti-Cyclin D1, and anti-pYAP (Cell Signalling Technologies, USA); anti-YAP (Sigma Aldrich, USA); anti-NANOG (Abcam, USA); and loading control anti-GAPDH (Cell Signalling Technologies, USA) followed by detection with the anti-rabbit HRP linked secondary antibody (Cell Signalling Technologies, USA). The bands were developed by LumiGlo (Cell Signalling Technologies, USA), imaged using GeneSys Gel Doc (Syngene, USA) and analysed by ImageJ software (https://imagej.nih.gov/ij/download.html).

RNA isolation and quantitative reverse transcription PCR

Total RNA was isolated using TRIreagent (Sigma Aldrich, USA), and was quantified using BioTek™ Epoch™ Microplate Spectrometer (BioTek, USA). Complementary DNA(cDNA) was prepared from 1 µg of total RNA using Prime Script 1st Strand cDNA synthesis kit (TakaraBio, Japan) as per the manufacturer's recommendations in Nexus Thermal Cycler (Eppendorf, Germany). The quantitative real time PCR was performed in QuantStudio 3 (Applied Biosystems, USA), and the reaction mixture contained Power Up SYBR Green master mix (Applied



◂**Fig. 1** Characterization of hESCs in pluripotency maintaining medium: **A** bright-field images of hESCs cultured with Essential 8 media on tissue culture treated plastic dish (TCP) with stiffness of ~ 1 GPa and Cytosoft® (CS) substrates of varying stiffness (0.2 kPa to 64 kPa). Scale 20 µm for all the images. Western blot images show expression of pluripotency markers OCT4 and NANOG in **B** passage 1, **C** passage 2, and **D** passage 3, and **E** proliferative marker Cyclin D1 along with housekeeping gene GAPDH. mRNA levels of lineage specific markers **F** *SOX17*, **G** *BRACHYURY*, and **H** *PAX6* was analysed using quantitative reverse-transcription PCR in hESCs cultured on TCP and CS substrates. Transcript levels were normalized to house-keeping *18S rRNA*, and the expression was plotted relative to levels in positive controls denoted as PC [Day 4 endoderm differentiated cells (ED D4) for *SOX17* and *BRACHYURY*, and commercially available human brain mRNA for *PAX6*]. Quantitative reverse-transcription PCR was performed in biological triplicates and two technical repeats. Error bars indicate standard deviation. Statistical significance (p) was computed using one-way ANOVA variance analysis and Bonferroni's multiple comparison test between each sample and is denoted as *, where *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$

Biosystems, USA). Target specific primers were designed using Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/prime r-blast/) and the primers with efficiency between 90 and 110% were used for gene expression studies. The forward primer (F) and reverse primer (R) used for the study are provided in Supplementary Table S1. For each sample, the relative fold change was calculated by $2^{-\Delta\Delta Ct}$ method. Each PCR reaction was carried out in duplicates using samples from three biological replicates. Endoderm differentiated day 4 (ED D4) cDNA was used as positive control for endoderm and mesoderm; and commercially available adult human brain RNA sample (Clonetech Laboratories, Inc., CA, USA) was used as positive control for ectoderm.

Immunofluorescence and image acquittance

KIND1 cells were cultured on treated and vitronectin coated glass coverslips up till 50–60% confluency was obtained. The cells were fixed with freshly prepared 4% paraformaldehyde in 1×PBS for 10 min at room temperature, followed by two rinses with 1×PBS. Fixed cells were placed in permeabilization buffer (30 µl of Triton X in 10 ml of 1×PBS) for 25–30 min at room temperature, followed by two quick washes with wash buffer (10 µl Tween 20 in 50 ml 1×PBS). The permeabilized cells were placed in blocking buffer (2% bovine serum albumin) for 2 h at room temperature, followed by incubation with primary antibody overnight at 4 °C in a moist chamber. After three washes with washing buffer, cells were incubated with Alexa-488 conjugated (Thermo Fisher Scientific, USA) secondary antibody for 2 h at room temperature. Primary and secondary antibody solutions were prepared in DPBS containing 0.5% bovine serum albumin, and antibodies source are given in Supplementary Table S2. Post-secondary antibody incubation, cells were counterstained with 300 nM DAPI (Invitrogen, USA) for 5 min. Images were acquired on an inverted fluorescence microscope (Carl Zeiss Microscope Apotome 2.0, Germany) at×40 magnifications. Image analyses or enhancement was performed using Java-based ImageJ software (http://rsbweb.nih.gov/ij/).

Statistical analysis

All the graphs and statistical analysis was generated using GraphPad Prism 8 software (https://www.graphpad.com/) and were plotted as ± standard deviation from three biological replicates. Statistical significance (*p*, indicated by *) was calculated using One-way ANOVA variance followed by Bonferroni's multiple comparison test (where *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$). The confidence intervals were kept at 95%.

Results

Assessment and characterization of hESCs on TCP and CS substrates in pluripotency maintaining medium:

To identify whether the biophysical cues alone can induce differentiation in pluripotency supporting media, we cultured hESCs on tissue culture treated plastic (TCP) dishes and on CS substrate plates in pluripotency maintaining medium. hESCs on TCP were present in compact colonies with characteristic epithelial morphology. Surprisingly, hESCs cultured on CS substrates exhibited similar morphology and cells were arranged in compact fashion to generate colonies (Fig. 1A). Intrigued, we characterized the hESCs cultured on TCP and CS substrates for protein expression of OCT4 and NANOG. Compared to their expression on TCP, OCT4 and NANOG

levels were downregulated in the consecutive passages (Fig. 1B–D). Although some change in OCT4, and NANOG was seen but the densitometric analysis showed these changes to be statistically insignificant (Supplementary Fig. S1). Statistically there was no difference observed in expression of pluripotency markers after three consecutive passages on soft substrates. To confirm whether lineage differentiation had occurred, we checked the mRNA expression of representative key transcription factors—*SOX17* (endoderm), *BRACHYURY* (mesoderm) and *PAX6* (ectoderm). The quantitative reverse-transcription PCR results showed no expression of *SOX17*, and *BRACHYURY* compared to positive controls (Fig. 1F, G); *PAX6* expression was however observed in CS substrates with respect to positive control (statistically not significant). *PAX6* expression needs to be further investigated. We were intrigued to know whether similar pattern will be observed if hESCs were cultured in ultra-low attachment conditions. hESCs cultured on ULAD expressed OCT4 after three consecutive passages as analysed by protein and mRNA expression, whereas no expression of lineage specific markers was observed (Supplementary Fig. S2). This confirmed that pluripotency of hESCs is governed only by pluripotency sustaining media and changing substrate stiffness does not result in the loss of pluripotency.

Expression of Hippo proteins in hESCs on TCP and CS substrates in pluripotency maintaining medium

In hESCs, YAP has shown to maintain pluripotency on TCP and overexpression of YAP has been correlated to naive pluripotency (Qin et al. 2016). So, we wondered whether YAP plays any role in maintaining the pluripotent state of hESCs on soft substrates as well. Protein samples isolated from TCP and CS substrates were probed with YAP and pYAP antibodies. The western blot data showed almost equivalent expression of YAP and pYAP on all the substrates. Since the expression of pYAP in CS substrates with respect to TCP is not changing we can imply that the hESCs remain pluripotent and does not undergo substrate-induced differentiation (Fig. 2). Our western blot data on OCT4 and YAP in KIND1 cells cultured on glass coverslips (stiff substrate) was confirmed by the immunofluorescence data. We saw the expression

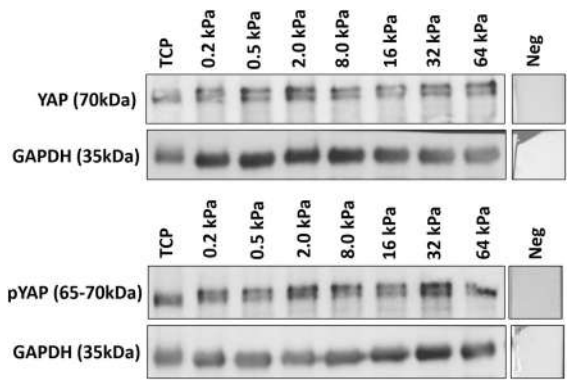


Fig. 2 Expression of Hippo pathway proteins in hESCs grown in pluripotency maintaining medium: western blot images show protein expression of Hippo signalling pathway core kinases YAP, pYAP and housekeeping control GAPDH on tissue culture treated plastic dish (TCP) with stiffness of ~ 1 GPa and Cytosoft® (CS) substrates of varying stiffness (0.2 kPa to 64 kPa)

of nuclear OCT4, whereas YAP was seen both in the nucleus and cytoplasm (Supplementary Fig. S3).

Summarizing the above results, we can say that contrary to the earlier reports, we did not see any change in pluripotency of hESCs despite culturing them substrates that have several thousand-fold lower stiffness than conventional plastic dishes.

Characterization of hESCs on TCP and CS substrates in differentiation inducing medium

The above results roused our curiosity, if change in substrate stiffness alone does not induce differentiation, then whether changing the medium to minimum differentiation inducing medium could lead to any specific lineage as reported with hMSCs (Engler et al. 2006). We intentionally kept the serum concentration to 2%, as we did not want the serum components to dictate the course of differentiation, but still have enough serum to keep cells healthy. We switched the media to Advanced DMEM with 2% FBS and checked the expression of pluripotency markers, lineage specific markers, YAP and pYAP.

The hESCs colonies in presence of differentiation inducing medium lost their compact epithelial morphology and appeared flattened (Fig. 3A). As expected, the induction of differentiation was marked by downregulation of OCT4 expression in cells grown on TCP, however, surprisingly the cells

grown on softer substrates showed relatively higher OCT4 expression (Fig. 3B). Next, we studied gene expression of lineage specific markers by quantitative reverse-transcription PCR to assess lineage specification. A comparative analysis between positive control and other samples showed reduced expression of *SOX17*, *BRACHYURY, and PAX6* in TCP and CS (Fig. 3C–E). These results hint that the hESCs on soft substrates in the presence of low levels of morphogens differentiates but not towards specific lineage. However, since there are multiple markers for every lineage, there is a possibility that mixture of other lineage cells could also be present.

Expression of YAP in hESCs on TCP and CS substrates in differentiation inducing medium

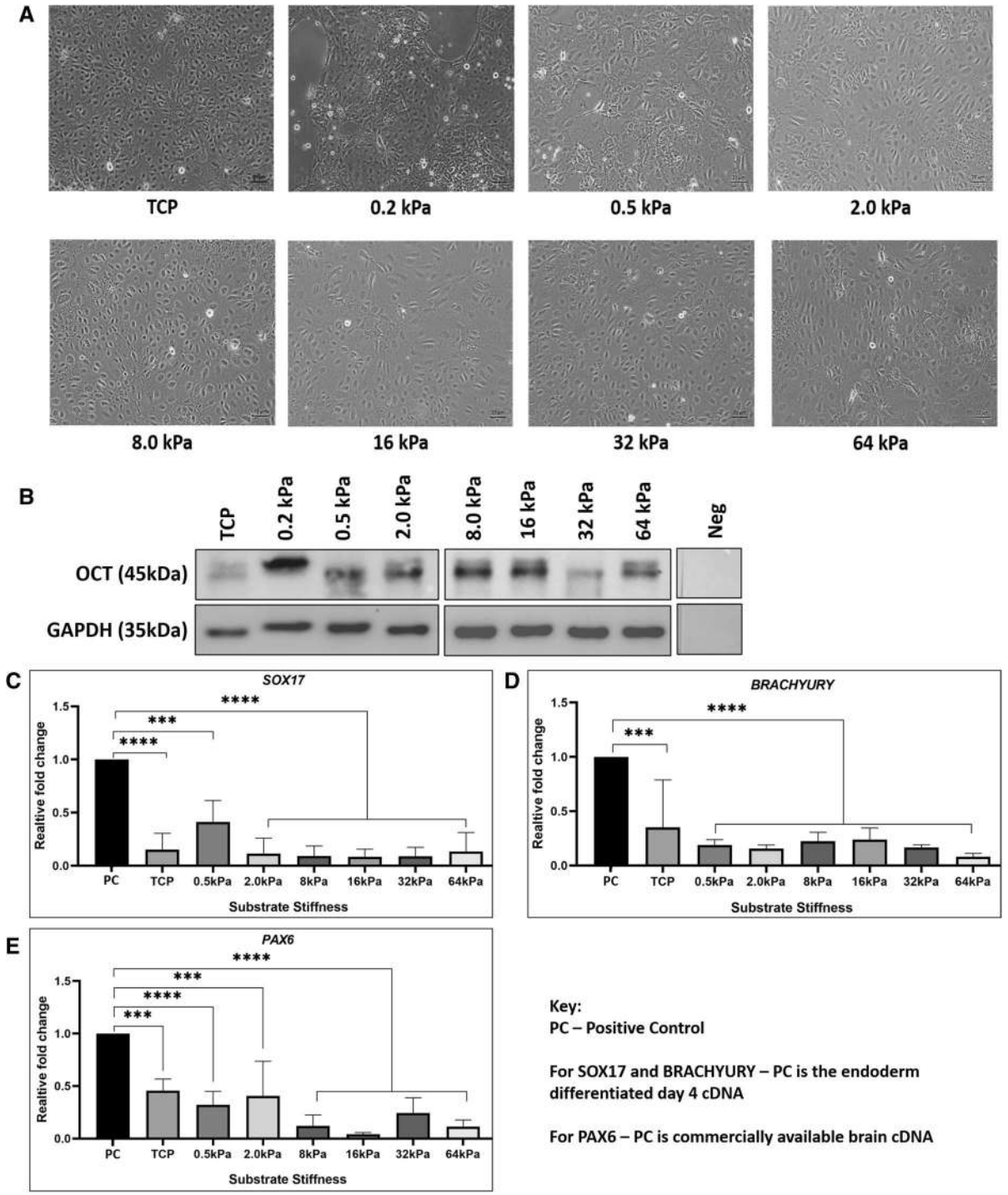
Next, we performed immunoblotting to check the dynamics of YAP and pYAP proteins in differentiation inducing media on soft substrates. The comparative study of YAP and pYAP shows a low expression of YAP and relatively high expression of pYAP. Low expression of YAP has been reported in loss of stem cell pluripotency (Lian et al. 2010; Rosado-Olivieri et al. 2019) and pYAP is sequestered in the cytoplasm leading to no YAP expression (Fig. 4). These results imply that on soft substrates and in presence of minimum differentiation inducing signals, hESCs start losing their pluripotency and undergo differentiation.

Discussion

In the present study, we show that hESCs maintained their stemness and did not undergo differentiation when cultured on soft substrates over three consecutive passages, however when differentiation inducing media was added, selective lineage markers were expressed. Previous studies have shown that stem cells, specifically hMSCs and mESCs, respond to changes in substrate stiffness and differentiate into neurogenic, myogenic or osteogenic lineage on substrate stiffness mimicking respective biological tissue stiffness (Engler et al. 2006; Evans et al. 2009; Keung et al. 2012), and that YAP is an important transcriptional regulator in maintaining pluripotency and stiffness-induced stem cell behaviour (Dupont et al. 2011; Oliver-De La Cruz et al. 2019; Musah et al. 2014; Brusatin et al. 2018; Lee et al. 2019).

For instance, one study reported that downregulation of YAP in mESCs led to differentiation into endoderm and mesoderm lineages (Lian et al. 2010). In vitro, ectopic expression of YAP inhibits ESC differentiation and cells maintain stem cell phenotype under differentiation conditions. Subsequently, LIF (leukaemia inducing factor) (Tamm et al. 2011) and Inter-α-Inhibitor (IαI), a component of serum (Pijuan-Galitó et al. 2014), were identified to facilitate YAP expression and induce OCT3/4 expression. A novel method showed that overexpression of two reprogramming factors OCT4 and SOX2 (Takahashi and Yamanaka 2006) and YAP, key component of Hippo pathway, together induces reprogramming of human amniotic epithelial cells into induced pluripotency stem cells (Zhao et al. 2017). However, there are no reports on how YAP regulates with pluripotency in hESCs.

Intrigued by these studies, we asked whether culturing the hESCs in pluripotency maintaining medium on TCP and CS substrates affects pluripotency and when induced to differentiate whether hESCs favour a specific lineage like hMSCS. The previous studies have reported that mESCs and hMSCs differentiate towards specific lineage when cultured on substrates mimicking biological tissue stiffness (Engler et al 2006; Dupont et al. 2011; Lian et al. 2010; Evans et al. 2009) however our results are contrary to these published reports. It should be noted that the mESCs are naïve cells while hESCS are primed cells (Ginis et al. 2004) and this might affect how cells respond to change in substrate stiffness. It would be interesting to see the fate of naïve hESCs grown using 2i media on range of soft substrates. Non-Muscle Myosin II (NMII) plays an important role in cell spreading and migration, it has been observed that ROCK inhibitor (Y2632), NMII inhibitor, increases the expression of pluripotency regulators OCT3/4 and NANOG, and enhances revival of human pluripotent stem cells (Walker et al. 2010). Soft substrates possibly prevent the activation of NMII as compared to stiffer substrates and thereby allow for continued expression of OCT4 and NANOG. In human MSCs multipotency is regulated by different set of factors and it has been reported that MSCs expressed higher levels of NMII therefore the change in stiffness would affect NMII regulation to a greater extent in MSC (Ma et al. 2010; Arora et al. 2015). This might explain why the hMSCs differentiate when cultured on substrates of



different stiffness. The regulation of NMII via soft substrates in hESCs deserves a separate investigation.

Our observations indicate that hESCs respond differently to substrate stiffness than hMSCs or mESCs, and that in vitro substrate stiffness might not play important role in hESCs differentiation. This is interesting since both hMSCs and hESCs are culture adapted to grow on stiff plastic dishes. Moreover,

◂**Fig. 3** Characterization and expression study of hESCs in differentiation inducing medium: **A** bright-field images of hESCs cultured with Advanced DMEM + 2% FBS media, on tissue culture treated plastic dish (TCP) with stiffness of ~ 1 GPa and Cytosoft® substrates of varying stiffness (0.2–64 kPa). Scale 20 µm for all the images. **B** Western blot images show pluripotency markers OCT4 and housekeeping protein GAPDH. mRNA levels of lineage specific markers **C** *SOX17*, **D** *BRACHYURY* and **E** *PAX6* was analysed using quantitative reverse-transcription PCR in hESCs cultured on TCP and CS substrates. Transcript levels were normalized to house-keeping *18S rRNA*, and the expression was plotted comparatively to levels in positive controls denoted as PC [Day 4 endoderm differentiated cells (ED D4) for *SOX17* and *BRACHYURY*, and commercially available human brain mRNA for *PAX6*]. Quantitative reverse-transcription PCR was performed in biological triplicates and two technical repeats. Error bars indicate standard deviation. Statistical significance (p) was computed using one-way ANOVA variance analysis and Bonferroni's multiple comparison test between each sample and is denoted as *, where *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$

mESCs that are cultured on stiff plastic dishes are routinely used to generate chimera (Czechanski et al. 2014), since the mESCs contribute to chimera it implies that stiffness substrates do not affect mESC pluripotency. Our findings also show that change in substrate stiffness does not alter the pluripotency of hESCs.

Importantly, we assessed the effect of changing stiffness on YAP expression, and how this correlates with pluripotency or differentiation. Many studies have noted that on stiff substrates YAP is active and localized to the nucleus where it helps in maintaining self-renewal and proliferation of stem cells; whereas on soft substrate YAP get phosphorylated which leads to cytoplasmic retention and eventual destruction (Dupont et al. 2011). YAP localization is also controlled by NMII and Peizo1 in MSCs (Pathak et al. 2014). Our results showed that there was not much change in Hippo pathway proteins in substrates of different stiffness, but we see change in YAP expression upon differentiation. From our observations, we speculate that in differentiating cells, YAP levels could be regulated by Peizo1 or NMII instead of Hippo pathway.

An interesting study showed that hESCs remain proliferative and pluripotent even on softest substrate from 150 to 1.2 kPa and our results concur with their findings. They found that on stiff substrate ($E > 1$ kPa) YAP localizes to the nucleus and on soft substrates ($E < 450$ Pa) YAP shows a heterogenous distribution with maximum nuclear localization at the periphery of the colony and mixed localization in the colony interior, this pattern resembles localization of YAP in the inner cell mass (ICM) of the early embryo (Price et al. 2017). The localization study by immunofluorescence might provide better understanding of YAP regulation.

We have looked at the overall YAP expression, studying the expression of phosphorylated forms of other Hippo proteins and also immunostaining will provide a better understanding on the function and localization of YAP in hESCs. Using conditional knockout/over expression strategies for YAP and other hippo components, we can delineate the role of Hippo pathways on hESCs pluripotency. It would also be interesting to study the functionality of differentiated hESCs on softer substrates, since differentiated cells most likely will encounter stiffness lower than that of plastic dishes. Nonetheless, we can say that hESCs maintain a basal level of YAP activity sufficient for survival and proliferation on soft microenvironment. Our study has focused on how upstream mechanical signals generated due to substrate stiffness, and YAP expression with hESCs cellular state. We have initiated the process of unravelling how mechanical and biochemical cues contribute together to regulate YAP expression in hESCs.

Conclusion

Taken together, our data suggests hESCs remain pluripotent when cultured in pluripotency maintaining medium on soft substrates and differentiate in minimum differentiation inducing medium. This implies that hESCs have different mechanism in sensing the substrate stiffness than hMSCs or mESCs; and for hESCs to differentiate, biochemical signals play a more crucial role than substrate stiffness. Interestingly, YAP levels change only during differentiation. Further intensified studies are necessary to unravel the underlying contribution of the mechanical and biochemical cues together to regulate YAP expression in hESCs.

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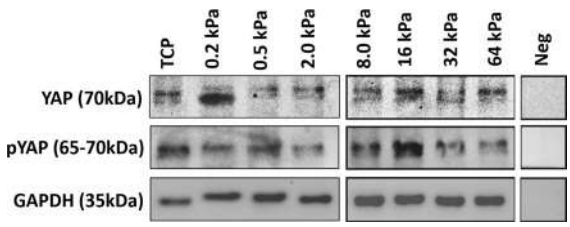

Fig. 4 Expression of Hippo signalling pathway proteins in hESCs in differentiation inducing medium: western blot images show protein expression of Hippo signalling pathway core kinases YAP, pYAP and housekeeping control GAPDH on tissue culture treated plastic dish (TCP) with stiffness of ~ 1 GPa and Cytosoft® (CS) substrates of varying stiffness (0.2 kPa to 64 kPa)

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Author contributions PP proposed the concept of the study. JKV performed the experiments and wrote the manuscript. All authors contributed in design of the project, acquisition, data analysis and data interpretation. All authors were involved in revising the manuscript for important intellectual content and approval of the final version of the manuscript.

Data availability The data that support the findings of this study are available from the corresponding author upon a reasonable request.

Declarations

Conflict of interest The authors declare that there was no conflict of interests.

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REVIEW ARTICLE

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Abstract Tissue-resident stem cells are surrounded by a microenvironment known as 'stem cell niche' which is specific for each stem cell type. This niche comprises of cell-intrinsic and -extrinsic factors like biochemical and biophysical signals, which regulate stem cell characteristics and differentiation. Biochemical signals have been thoroughly studied however, the effect of biophysical signals on stem cell regulation is yet to be completely understood. Biomaterials have aided in addressing this issue since they can provide a defined and tuneable microenvironment resembling in vivo conditions. We review various biomaterials used in many studies which have shown a connection between biomaterial-generated mechanical signals and alteration in stem cell behaviour. Researchers probed to understand the mechanism of mechanotransduction and reported that the signals from the extracellular matrix regulate a transcription factor yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ), which is a downstream-regulator of the Hippo pathway and it transduces the mechanical signals inside the nucleus. We highlight the role of the YAP/TAZ as mechanotransducers in stem cell self-renewal and differentiation in response to substrate stiffness, also the possibility of mechanobiology as the emerging field of regenerative medicines and three-dimensional tissue printing.

Keywords Mechanobiology · Human pluripotent stem cells · YAP/TAZ · Biomaterials

1 Introduction

Stem cells are unique cells that can self-renew and differentiate into specialized cells. Stem cell research has opened a new field of regenerative medicine, it has modernized the fields of drug discovery and our understanding of the physiological processes associated with disease or injury [1, 2]. Even though adult human stem cells have been used in clinical settings, their use is impeded because of their limited expansion capabilities, differentiation potential, and availability [3–5]. Human pluripotent stem cells (hPSCs) on the other hand exhibit unlimited expansion potential and unique property to differentiate into three germ layer cells, which makes them an ideal cell source for basic and clinical research [6, 7]. The microenvironment surrounding the stem cells, also known as 'niche', maintains a balance between self-renewal and differentiation and is specific for each type of stem cell population. It is mainly composed of tissue-specific extracellular matrix (ECM) proteins, other cells, and soluble factors such as Wingless-Type MMTV integration site family member (WNT), bone morphogenetic proteins (BMPs), ACTIVIN/NODAL, fibroblast growth factor (FGF) among others, all of which regulate cellular functions [8–10].

Signalling pathways originating from soluble factors are well known in regulating stem cell proliferation and differentiation. A number of studies have demonstrated that

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the physical properties of ECM and the mechanical forces generated from the surrounding niche also play an integral part in defining the stem cell behaviour, inducing specific differentiation pathways. The mechanical forces such as stress, tension, pressure, and stretch can be generated internally or externally in response to fluid flow, substrate stiffness, gas partial pressures or from adjacent cells [11, 12]. The mechanism by which the mechanical signals originating from the ECM are transduced into biological signals is collectively known as mechanotransduction and has been of great interest to the researchers. The knowledge gained so far has been invaluable in understanding metastatic cancers, neuronal regeneration, wound healing, liver regeneration, bone repair, and several other cellular phenomena. Mechanotransduction involves sensing of mechanical signals by cell surface receptors and translating it into biochemical signals by initiating a signalling cascade and eventually regulate the activity of specific genes [13]. Among all the mechanical forces, the role of ECM has been extensively researched; as the ECM plays important role in determining cellular functions [12, 14].

There has been a lot of excitement around organoids, generated from pluripotent and adult stem cells. Advances in the field of organoid research have shown the significance of cell–matrix interactions on stem cell self-renewal and differentiation [15]. For organoid formation, stem cells are embedded in complex ECM matrix which creates small, three-dimensional (3D) and self-organized tissue-like cell clusters. Organoids resembling complex tissues of intestine [16], retina [17], thyroid [18], brain [19], inner ear [20], kidney [21], liver [22], lungs [23], gastrointestinal tract [24] and blood vessels [25] have been generated. Mechanobiology behind organoid formation can shed some light upon the self-organization behaviour of stem cells in organogenesis.

Fundamental understanding of mechanical forces on stem cell behaviour can provide insights for developing an artificial niche, to support stem cells for regenerative medicine as well as it will add to our knowledge of developmental biology. In this review, we have tried to give the reader comprehensive information about the studies that have demonstrated the effect of mechanical signals mediated through hydrogels and artificial substrates on stem cell behaviour. We focus particularly on transcriptional activators yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ), their regulation by mechanical signals and their critical role in stem cell proliferation and differentiation.

2 Extracellular matrix regulated stem cell differentiation

The extracellular matrix is an essential component of the stem cell microenvironment and is indispensable for proliferation and differentiation. Apart from providing physical support to the cells, ECM regulates cell proliferation, growth, differentiation, cell shape, and migration. It is a complex assembly of proteins (mainly composed of collagen, fibronectin, elastin, laminin, and vitronectin), proteoglycans and small integrin-binding glycoproteins secreted by the cells; however, the precise composition of the matrix is different between tissues [26]. For instance, the extracellular matrix of bone is composed of highly insoluble collagen type I fibres, with traces of collagen III, V, XI, and XIII, with many intra- and inter-molecular crosslinks. Based on the composition and structure of the collagen fibres, the estimated stiffness; represented as Young's modulus (E) expressed in Pascals (Pa); for bone is approximately 100 kPa–1GPa [27]. Whereas, the brain extracellular matrix primarily consists of lecticans, a proteoglycans family, crosslinked by tenascin (a glycoprotein), and hyaluronic acid (a glycosaminoglycan). ECM of the brain has a low content of fibrous proteins such as collagen, fibronectin, and vitronectin, making it soft compared to bone ECM with E of approximately 1 kPa [28–30]. Several experimental studies have shown that changing the biochemical composition of ECM, changes the mechanical properties (stiffness and resulting stress) which in turn regulates cell morphology, growth, differentiation, migration, and gene expression [14, 31, 32].

The substrate stiffness has been shown to change cellular morphology that leads to the reorganization of the intracellular cytoskeletal network [14, 31]. Human mesenchymal stem cells (hMSCs) develop broader and flattened morphology on the stiff substrate ($E \sim$ 25–40 kPa) and show high expression of osteoblast-specific genes such as SRY-box transcriptional factor 9 (*SOX9*) and core-binding factor subunit alpha-1 (*CBFA1*) [33, 34], whereas, on the softer substrate ($E \sim$ 0.1–1 kPa) hMSCs have shown upregulation of neuronal-specific protein markers such as Nestin, Tubulin beta 3 (TUBB3), Neuro Filament Light (NFL) chain, and Neural Cell Adhesion Molecule (NCAM) [33]. Mouse primary spinal cord neural cells grow and extend to neurite on the soft substrate but not on the stiff substrates [35]. On the soft substrate, cells show reduced spreading, reduced organization of actin into stress fibres and morphologically they appear round [36, 37]. Moreover, muscle stem cells maintain stemness and self-renew when cultured on soft synthetic hydrogel which mimics the elasticity of muscle ($E \sim$ 12 kPa) [38]. Such diverse responses of stem cells to substrate stiffness

represent a fundamental role of matrix in regulating cell behaviour. This shows that actin filaments regulate cell morphology in response to matrix stiffness. These biosystems help us in understanding the basic principles of tissue and organ functions.

3 Tools to engineer stem cell niche ex vivo and study ECM-cell interactions

In vitro studies are the ideal way to investigate the ECM-cell interactions; however, the complexity of the niche and variations in ECM composition makes it challenging and difficult. Traditional *in vitro* culturing methods involves the use of glass and plastic culture dishes for cell culture with $E \sim 1$GPa, which is very stiff compared to *in vivo* conditions [39]. Researchers have been using various biocompatible materials to engineer stem cell niches *in vitro* which mimic the stiffness of biological tissues. Natural polymers such as collagen, agarose, collagen, chitin [40], alginate, and hyaluronic acid or its conjugated hydrogel [32] are used to synthesize scaffolds due to their similarity with native ECM. However, given their limited mechanical properties focus has now shifted to synthetic hydrogels such as polyacrylamide (PA) gels [37], poly-dimethylsiloxane (PDMS) [41], polyethylene glycol hydrogel [38], polyvinyl alcohol [42] to name a few. These synthetic hydrogels provide a wide array of stiffness range similar to physiological tissue stiffness but synthetic polymers provide limited cellular interactions due to lack of functional group present. To overcome the disadvantage of natural and synthetic polymers, semi-synthetic hydrogels such as gelatin methyl acrylate (GelMa) [43] was synthesized which combines the biocompatibility of natural hydrogel polymer and mechanical properties of synthetic biomaterials. Other than the hydrogels, artificial micropatterned and nanopatterned substrates [44], flexible micropillars [45], and electrospun nanofiber [46] have also been used as scaffolds to understand the effect of different substrate and their stiffness on cell migration, growth, and differentiation. In the following section, we have briefly discussed few natural and synthetic substrates and their effect on stem cells. Table 1 gives an overview of the various biomaterials discussed below and their influence on cell behaviour.

3.1 Natural polymers

Conventional protocols for growing pluripotent stem cells use mouse embryonic fibroblast cells (MEFs) as a natural substrate. MEFs is known as 'feeder cells' are maintained in metabolically active but non-proliferating state, thus allowing them to express soluble, membrane-bound growth factors and extracellular matrix proteins, which controls cell growth and acts as a substrate [63, 64]. In 1998, the first derivation of human embryonic stem cells (hESC) line described the importance of inactivated MEFs feeder layer for maintaining their undifferentiated state [6]. However, owing to the risk of xeno-contamination and variability, a feeder-free culture system was introduced for the maintenance and proliferation of hESCs. The feeder-free culture system uses ECM protein namely laminin [65], collagen [66], fibronectin [67], vitronectin [48, 68], Matrigel [69–71].

Collagen is the abundant ECM protein, which is widely used as a natural substrate by many researchers. Since the majority of the native ECM is composed of collagen, collagen-based biomaterials are highly biocompatible and biodegradable. Collagen alone has very low mechanical property since the mechanical properties depend on the composition of the scaffold, collagen along with co-polymer has shown to support self-renewal and differentiation of hPSCs [66] and regeneration of various tissues [72–75] in several individual studies. Fibronectin is the second most abundant ECM protein after collagen and is often used for increasing cell adhesion [67]. Several studies have shown that fibronectin supports long-term self-renewal of hPSCs [76, 77]. Fibronectin along with another polymer, for example, silk fibroin, have been used to develop a hybrid fibre, which mimics the mechanical properties of desired tissue and sustains cell growth and proliferation [78].

Matrigel is a commercially available basement membrane-like protein matrix made up of laminin, collagen IV, heparan sulphate proteoglycan and a number of growth factors in a non-defined proportion and has been reported in several studies to support the growth of undifferentiated hESCs [79, 80]. By changing the composition of the biopolymer and protein growth factors, the elastic modulus of Matrigel varies from 34 Pa–480 Pa [81, 82]. Given such low elastic moduli, Matrigel has been an ideal substrate to study cell migration in 3D culture and for the generation of many 3D organoid culture models, for example, the organoid culture of endometrium and placenta has been well established [47]. Since Matrigel is not well-defined, it may not be suitable for large-scale manufacturing for therapeutic purposes. It is generated from mouse sarcoma cells and may contain carcinogenic or xenogenic factors; thus, it is not suitable for human clinical trials [83]. Alternatively, other ECM components have been coupled with synthetic polymer matrices with specific compositions. For example, hyaluronic acid (HA), glycosaminoglycans are critical in modulating neural and hematopoietic stem cell behaviour [84, 85]. Also, HA in conjugation with tyramine can form a tuneable 3D microenvironment that modulates chondrogenesis and may impact the spatial organization of cells [32].

Table 1 Examples of various biomaterials and their influence on mammalian cell behaviour

Biomaterial	Stiffness	Cell type cultured	Cell response	References
Natural polymer				
Matrigel (3D culture)	34 Pa–480 Pa (of 3D Matrigel)	Primary extravillous trophoblast	Cells showed migration towards stiff region and differentiated into endometrium and placental cells	[47]
Vitronectin	As coating on tissue culture plate	hESCs	Cell attachment, growth and proliferation	[48]
Synthetic substrates				
Polyacrylamide (PA) gel substrate				
PA gel substrate coupled with type I collagen	10 Pa (soft) 90 Pa (stiff)	Kidney epithelial cells and 3T3 fibroblasts	Less spreading of cells on soft substrate compared to stiff substrate Cells migrate from soft substrate towards stiff substrate	[14, 31]
PA gel substrate coupled with type I collagen	0.1–1.0 kPa (soft) 8–17 kPa (intermediate stiff) 25–40 kPa (stiff)	hMSCs	Differentiated into neural lineage Differentiated into myogenic lineage Differentiated into osteogenic lineage	[33]
PA gel functionalized with GAG peptides	0.7 kPa (soft) 10 kPa (stiff)	hESCs and hiPSCs	Better attachment, self-renewal and maintains pluripotency Cells adopted neural morphology and after addition of neuronal maintaining media developed into mature neurons	[49] [50]
PA gel substrate coupled with Matrigel	3 kPa (soft) 165 kPa (stiff)	hESCs and hiPSCs	On soft substrate which showed stiffness similar to liver tissue, the cells differentiated into endoderm lineage whereas not on stiff substrate	[51]
Poly (dimethyl siloxane) (PDMS) gel substrate				
PDMS coated with polydopamine	Not determined	Bone-marrow stromal cells	Promoted differentiation into osteogenic lineage in presence of osteogenic differentiation media	[52]
PDMS coupled with type I collagen	1.9 MPa–2.7 MPa (stiff)	mESCs	Cells expressed pro-osteogenic gene markers	[53]
PDMS printed with Fibronectin or Laminin	5 kPa (soft)	PC12 (rat adrenal gland derived cell line), C2C12 (mouse muscle derived cell line)	PC12 cells differentiated into neurons on soft substrate, whereas C2C12 formed myotubes when cultured stiff substrate	[54]
PDMS coated with type I collagen	3 kPa (soft) 37 kPa (stiff)	Cardiac fibroblasts, 3T3 fibroblast, hMSCs	Cells of all three-cell line showed increased cell spreading on stiff substrate whereas on soft substrate these cells manifest small spread area Stiff substrate promoted myofibroblast activation of cardiac fibroblast	[55]
Electrospun nanofibrous substrate				
PCL fibrous substrate PET fibrous substrate PEKK fibrous substrate PCU fibrous substrate (all plasma treated or collagen-conjugated)	19 kPa (softest) 39 kPa (soft) 74 kPa (stiff) 193 kPa (stiffest)	hiPSCs	hiPSCs exhibit round 3D colony morphology on softest/soft substrate, on stiff/stiffest substrate cells spread and have flattened morphology Long-term culture on soft(est) substrate led to ectodermal differentiation, no change in cells on other substrates After adding defined growth factors, stiff substrate promoted motor neurons and soft substrate enhanced posterior foregut specification	[46] [56] [57]
Semi-synthetic substrates				
Gelatin methyl acrylate (GelMa)				

Table 1 continued

Biomaterial	Stiffness	Cell type cultured	Cell response	References
5% GelMA 10% GelMA 20% GelMA 30% GelMA	3.08 kPa (softest) 34.9 kPa (stiffer) ~75 kPa (data not provided, estimated from the graph) 184.52 kPa (stiffest)	PC12 (rat adrenal gland derived cell line)	Cells show highest adhesion rate on 5% GelMa compared to 20% and 30% Cells on 10% GelMA have optimum spreading rate and longest neurite length	[58]
5% GelMa, 10% Gelma	Not determined	Rat MSCs	Cells differentiated into osteoblast when supplemented with osteogenic media	[59]
3D GELMA-PEGDA	30 Pa–150 Pa (soft)	MDA-MB-231	Soft substrate support spindle-like morphology	[60]
3D PEGDA-GELMA	1 kPa–8 kPa (stiff)	(breast cancer cell line)	Stiff substrate showed tumor-like spheroidal morphology, cells from these spheroids showed epithelial to mesenchymal transition and drug resistance	[61]
Mineralised GelMa with functionalized PEGDA	Not determined	hiPSCs	Cells underwent osteogenic differentiation devoid of biochemical signals compared with non-mineralized GelMA	[62]

3.2 Synthetic polymers

Synthetic substrates are promising alternative to natural substrates. Some advantages of synthetic polymers over natural polymers are their tuneable mechanical properties, ease of synthesis, available and low cost.

3.2.1 Polyacrylamide gel

Polyacrylamide gel consists of a linear monomer of acrylamide and a crosslinker bisacrylamide which links the linear acrylamide monomers. PA-gels have been used as a substrate for a diverse range of cell cultures for a long time because of its ideal physical and mechanical properties. By changing the ratio of acrylamide and bisacrylamide, PA-gel substrates can be generated having elastic modulus ranging from 0.1 kPa to 100 kPa, which is equivalent to the physiological elastic moduli of soft tissues namely adipose tissue, brain tissues, endothelial tissue or lung tissue [86–88]. Polyacrylamide is inert in nature, therefore, does not support cell growth and proliferation. For it to be used as a cell culture substrate, PA-gels are first treated with UV-activated cross-linker which couples' extracellular matrix proteins to the substrate. A number of studies have reported that chemical and physical properties of the various PA substrates have a profound effect on cell locomotion, growth, and differentiation. Early studies with kidney epithelial cells and 3T3 fibroblasts on PA substrates reported that cells on flexible substrates with E between 10 Pa (soft)–90 Pa (stiff) displayed less spreading by limiting the amount of phosphotyrosine at adhesion sites, and more cell death [14]. A similar study with 3T3 fibroblast showed that cells migrate from the soft side towards the stiff side of the PA substrate [31]. Furthermore, PA substrate of varying stiffness has been shown to direct lineage specificity for human mesenchymal stem cells (hMSCs). hMSCs cultured on soft collagen-coated PA substrate ($E \sim 0.1$–1 kPa), the intermediate stiff substrate ($E \sim 8.0$–17 kPa) and stiff substrate ($E \sim 25$–40 kPa) differentiate into neural, myogenic and osteogenic lineages respectively [33]. Musah *et al*. [49] reported that stiff PA substrate ($E \sim 10$ kPa), when functionalized with glycosaminoglycans (GAG) peptides, offer better hESCs and human induced pluripotent stem cells (hiPSCs) attachment, self-renewal and promotes pluripotency evident from the expression of octamer binding transcriptional factor-4 (OCT4), stage-specific embryonic antigen-4 (SSEA4) and other pluripotency specific markers. In a different study, conducted by the same research group, they reported that PA substrate ($E \sim 0.7$ kPa) functionalized with GAG-binding peptide selectively differentiates hESCs. After several weeks of culture on these compliant substrates and in absence of neuronal inducing factors, hPSCs adopted neuronal morphology and expressed neuronal-specific tubulin beta 3 chain (TUJ1) protein. When the media was switched to neuronal maintaining media, cells showed expressions of microtubule-associated protein 2 (MAP2), a mature neuronal marker [50]. These results indicate that substrate alone can direct differentiation of hESCs, independent of soluble factors. A similar conclusion was drawn by Chen et al. [51], where they demonstrated that hPSCs cultured on soft PA-substrate with $E \sim 3$ kPa expresses high levels of the anterior primitive streak and definitive-endoderm-specific gene expression such as Eomesodermin (*EOMES), Brachyury (T),* Forkhead box protein A2 (*FOXA2)*, and SRY-box transcription factor 17 (*SOX17)*

compared to hPSCs on a stiff substrate with $E \sim 165$ kPa and tissue culture-treated plastic plates. Another study by Maldonado et al. [56] in hiPSCs cultured on electrospun nanofibrous scaffolds reported a similar observation, which we have discussed under subsection *Electrospun Nanofibrous substrate.*

3.2.2 Poly (dimethyl siloxane) (PDMS)

PDMS is a silicon-based flexible elastomer having a wide range of applications in medicine and cosmetics, soft lithography even as anti-foaming agents and surfactants [52]. PDMS has been extensively used to understand cell behaviour in the field of mechanobiology. These rubber-like elastomers have E up to 2 MPa [89], thus making them suitable for investigating cellular responses to changing substrate stiffness. Stiff PDMS substrate ($E \sim 1.9$ MPa-2.7 MPa) has been reported to support proliferation and cell spreading of mouse embryonic stem cells (mESCs). These cells showed significant upregulation of pro-osteogenic transcription factors runt-related transcription factor (*Runx2*) and secreted phosphoprotein 1 (*Spp1*), compared to mESCs cultured on soft PDMS [53]. PC12 (rat adrenal pheochromocytoma cell line) differentiate into neurons and develop longer neurites on soft PDMS substrate with elastic modulus similar to brain tissue ($E \sim 5$ kPa) compared to the cells on stiffer PDMS substrate. The same study also showed that C2C12 (mouse skeletal muscle cell line) when cultured and differentiated on stiff PDMS substrates formed longer myotubes compared to cells cultured on a soft substrate [54].

Many a time because of high surface hydrophobicity of PDMS cell attachment to the substrate is poor. For enhanced cell adhesion and proliferation bio-inspired polydopamine-coated PDMS was used instead of protein-coated PDMS. Polydopamine-coated PDMS promoted long term bone-marrow stromal cells culture while maintaining their multipotency [52]. In regard to cell spreading, 3T3 fibroblasts, cardiac fibroblasts and MSCs showed increased spreading on stiffer PDMS substrate coated with collagen compared to soft substrate, an observation similar to the PA substrate study. Interestingly, cells cultured on soft PDMS respond to secondary crosslinking-induced stiffness, representing similar spread area to the cells on stiff PDMS [55]. These reports indicate that PDMS hydrogels influences cell adhesion and spreading, potency and differentiation. It would be interesting to see whether PDMS modified to low stiffness help maintain the pluripotency of hPSCs or direct them towards differentiation.

3.2.3 Electrospun nanofibrous substrate

These substrates have been around for many years now and researchers have shown keen interest in using these substrates for as biological scaffolds in tissue engineering. They provide a porous mesh of nanoscale and microscale fibrous structures which highly resembles natural ECM [90]. A notable report by Maldonado et al. (2015 and 2016) [46, 56] studied the proliferation and differentiation of hiPSCs on various synthesized electrospun nanofiber substrates exhibiting different chemical and mechanical properties. These substrates were poly(ε-caprolactone) (PCL), polyethylene terephthalate (PET), polycarbonate-urethane (PCU), and poly(etherketoneketone) (PEKK). The measured Young's modulus of these substrates ranged from $\sim$ 19 kPa to $\sim$ 313 kPa. Short- and long-term culture of hiPSCs showed characteristic pluripotent colony morphology and expressed pluripotency-associated markers OCT4, homeobox protein Nanog (NANOG) and DNA (cytosine-5-)-methyltransferase3 beta (DNMT3B) on all the substrates. However, after 12 days in culture, cells on 19 kPa substrate highly expressed ectodermal markers PAX6 and NEUROD1, whereas these makers showed minimal expression on 193 kPa substrate and no expression on tissue culture-treated plastic dish.

Electrospun fibrous substrates induce substrate-dependent changes in colony morphology and gene expressions in hiPSCs. hiPSCs, when subjected to defined growth factors of specific lineages, tend to differentiate into motor neurons on a soft substrate during early differentiation, conversely, stiff substrates promoted motor neuron specification during late stages. In contrast, hiPSCs differentiated into the mesendodermal lineage on the stiff substrate, but soft substrate enhanced further their specification into the posterior foregut [57]. These observations are different from the studies that have used hydrogels as substrates, suggesting that differentiation is not restricted to single stiffness and dynamic changes in the mechanical microenvironment may help in enhancing the differentiation efficiency of hiPSCs.

3.3 Semi-synthetic polymers

Biohybrid or semi-synthetic polymers combine the best of both the natural and synthetic polymers. A number of scaffolds with at least one natural polymer such as collagen, fibrin, laminin in well-defined proportion with synthetic polymer namely polyethylene glycol (PEG), polyglycolic acid (PGA) or polycaprolactone (PCL) have been synthesized for 2D and 3D culture of many cell lines including stem cells [91]. Recently, GelMA has emerged as an attractive candidate for mimicking native ECM conditions *in vitro*.

3.3.1 Gelatin methyl acrylate (GelMa)

GelMa is a photo-reactive hydrogel made up of gelatin and methacrylic groups. First synthesized in 2000 by Van Den Bulcke *et al*. [92], GelMa is an inexpensive, natural polymer synthesized from hydrolysis and denaturation of collagen which makes it suitable biomaterial for *in vitro* studies. GelMa has proven to provide optimal conditions for cell culture due to the presence of arginine-glycine-aspartic acid (RGD) motifs for adhesions and matrix metalloproteinase (MMP) degradation motif for cellular enzymatic degradation. Depending upon the percentage of GelMa (w/v) or the concentration of the other polymers, such as alginate or hyaluronic acid, mixed with it, stiffness of GelMa can range from 1 kPa to 200 kPa [58, 93, 94]. Owing to such a large physiological stiffness range, GelMa hydrogels have been thoroughly investigated for its physical and biochemical properties as 2-D and 3-D scaffolds in tissue engineering [95]. Notable application of GelMa 3-D scaffolds for tissue engineering had been reported for bone [96], skin [97, 98], cardiac tissue [99], endochondral bone [100], skeletal muscles [101], and vascular networks [102].

To determine whether soft GelMa substrate supports neuronal development, PC12 cells were cultured on GelMa substrates having $E \sim 3$ kPa–184 kPa. The cells showed maximum adhesion on softest substrate of 3 kPa compared to other stiffness, however, the longest neurite length was observed on substrate with intermediate stiffness ($E \sim 34.9$ kPa) [58]. The authors, however, did not show the gene expression profile of the differentiated cells. It would be interesting to know whether these substrate support neurite maturation for a long duration and generate mature as well as functional neurons. In another study, rat bone-marrow derived MSCs were cultured on 5% (soft) and 10% (stiff) GelMa substrates and when they were supplemented with osteogenic media, MSCs differentiated into osteoblasts. The stiffness of these two GelMa substrates was not determined; hence which stiffness range of GelMa supports osteoblast differentiation still remains a question [59].

For 3D cell culture, a blend of GelMa and polyethylene (glycerol) diacrylate (PEGDA) was developed to study cell metastasis using cancer cells. By increasing the concentration of PEGDA, the elastic moduli of non-degradable 3D PEGDA-GELMA scaffold was between $E \sim 1$ kPa to 8 kPa whereas soft degradable 3D scaffolds were obtained by increasing GelMa concentration ($E \sim 30$ Pa to 150 Pa). Encapsulated MDA-MB-231 breast cancer cells in these substrates showed tumor-like spheroidal morphology in stiff PEGDA-GELMA substrate, while they showed spindle-like morphology in soft GELMA-PEGDA substrate. Invasion chick aorta arch assay showed that the softer GELMA-PEGDA substrate supports the invasion of both MDA-MB-231 and endothelial cells, along with sprouting of endothelial cells from chick aortic arches [60]. The gene expression profile of the spheroids derived cell from stiff PEGDA-GELMA showed epithelial-mediated transition (EMT) behaviour and drug resistance [61]. GelMa substrate mineralized with calcium and phosphate minerals induce osteogenic differentiation of hiPSCs in 2D and 3D, evident from gene expression study and immunofluorescent staining for osteogenic specific transcription factors RUNX2, OCN and SPP1. The hiPSCs were cultured devoid of growth factors and the differentiation was achieved through substrate-based biomechanical cues. However, non-mineralized GelMa or gelatin-coated dishes functionalized with PEGDA does not support the growth and differentiation of hiPSCs [62]. Though GelMa has shown great promise in tissue engineering, questions such as—whether GelMa directs differentiation into lineages other than osteogenic differentiation, is GelMa a better substrate than normal culture dishes or other substrates for differentiation, how stem cells differentiated on GelMa functionally differ from differentiated cells generated via the normal stiff culturing conditions, need to be addressed before GelMa could be routinely used for basic and possibly for translational purposes.

4 Mechanobiology: Intracellular sensory system

We have described the effect of various natural and synthetic biomaterials on cell proliferation and differentiation, this implies that cells have a mechanism to sense substrate stiffness and make modifications to generate specific proteins. In order to generate supportive biomaterial, it is essential to understand what changes occur to mechanosensing machinery inside the cells. Cell–matrix interactions are mediated by cell surface receptor integrins, focal adhesion kinases (FAK), and cytoskeletal filaments that connects the extracellular matrix to the intracellular environment. When cells come in contact with mechanical signals from the extracellular region, a multiprotein complex is formed near the cell surface receptor, known as focal adhesions (FA). FA comprises of talin, vinculin, paxillin, alpha-actinin, p-130^{cas}, FAK, SRC, and FAs act as a mediator between integrins and actin filaments (Fig. 1). Other than FA, Rho family small GTPases, its downstream effectors Rho-associated kinases (ROCK), and myosin light chain kinases also relay the mechanical signals to cytoskeletal filaments [103–107]. The cytoskeletal components mainly actin filaments, microtubules, and non-muscle myosin undergo conformational changes (stretching or relaxing, shortening or elongating) [108, 109] which affects gene-specific transcription due to cytoplasmic or nuclear localization of gene-specific transcriptional factors.

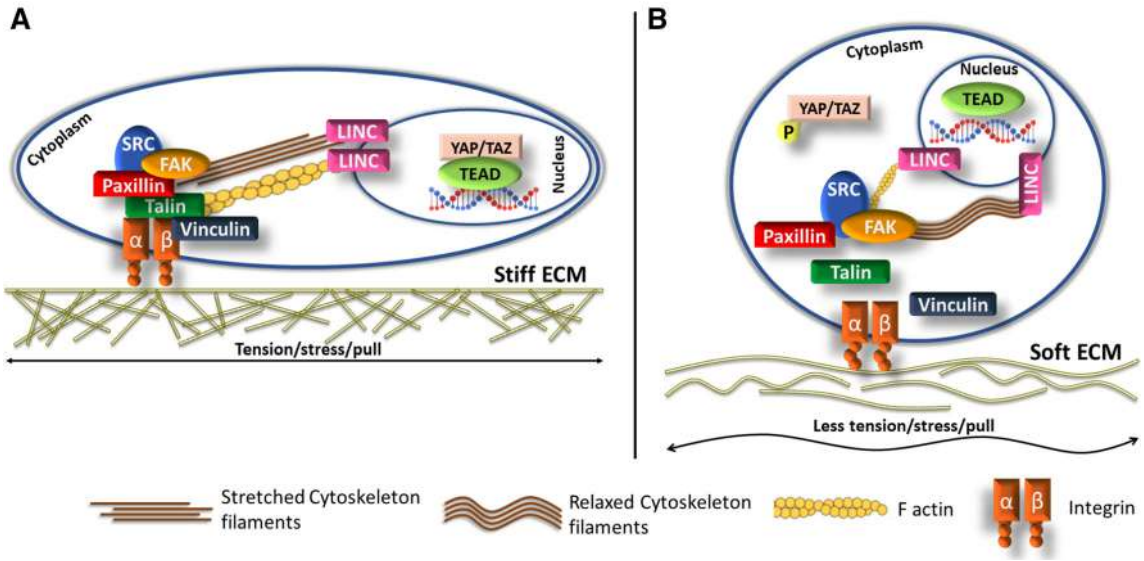

Fig. 1 The effect of substrate stiffness on YAP/TAZ. **A** Stiff substrate activates focal adhesion proteins—talin and vinculin, they bind to and activate integrin, resulting in cell adhesion and spreading. Formation of integrin-talin-vinculin phosphorylates FAK which forms a complex with Src and phosphorylates paxillin, contributing in mechanosignalling and cell spreading. This entire complex creates tension on actinomycin and cytoskeletal filaments which causes stretching of LINC; nuclear envelope protein; ensuring nuclear localization of YAP/TAZ and subsequent gene expression. **B** On the contrary cells on soft substrate experience less tension, forming an unstable integrin-talin-vinculin complex. Loosely formed FAK-SRC complex does not form tension-dependent stretching of actinomycin, cytoskeletal filaments, and LINC, causing cytoplasmic retention of YAP/TAZ

For example, the formation of stress fibres causes nuclear localization of transcriptional coactivators YAP and TAZ or cytoskeletal remodeling activates and translocates beta-catenin into the nucleus [110, 111], where these two proteins interact with their respective coactivators and activate specific gene expression.

Apart from these, other transcriptional factors which have been reported to respond to different mechanical signals are tight junction protein ZO-1 [112], tyrosine kinase c-Abl [113], myocardial-related transcriptional factor (MRTF) [114], Nuclear Factor-kappa-B (NFκB) [115], nuclear factor erythroid-2-related factor 2 (NRF2) [116], epigenetic regulator HDAC3 in both mouse and human cell lines [117]. An important point to be noted here is that, these transcriptional factors have been known to be regulated by specific signalling pathways and do not solely function as mechanotransducers. The shuttling of these transcriptional factors between cytoplasm and nucleus is gated by linker of nucleoskeleton and cytoskeleton (LINC) complex present on the nucleus envelope. The tensed actin cytoskeleton causes stretching of actin binding LINC complex component Nesprin1, resulting in stiffening of nuclear envelope and localization of transcriptional factors [118, 119]. The mechanical signal-regulated signalling pathways are complex; numerous studies have identified YAP/TAZ proteins as mechano-transmitters that respond to signals from substrate stiffness, substrate topology, surface area, cell density, cell polarity, and cell geometry. Given such a vast array of regulators, YAP/TAZ has garnered the attention of many research groups. Here, we review the data highlighting the functions of YAP/TAZ as transcriptional co-activators and as mechanosensors.

5 Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ)

Yes-associated protein (YAP) was first discovered in *Drosophila* and was termed Yorkie due to its association with Src family kinase Yes [120] and transcriptional co-activator of PTZ binding motif (TAZ) was first identified as 14-3-3 binding protein as a paralog to YAP [121]. These transcriptional co-activators share 46% sequence homology and display a similar domain organization, except for a proline right domain, one WW domain and SH3 binding region [122]. YAP and TAZ are primary effectors of the Hippo pathway however; these coactivators also interact with TGF β, WNT, BMP, ERK and FGF signalling pathways [123, 124]. Figure 2 shows the core pathway consists of a cascade of kinase activations in which the upstream signals phosphorylates mammalian Ste20-like kinases 1/2 (MST 1/2; *Drosophila* homologous Hippo [Hpo]) which phosphorylates and binds to SAV1 (*Drosophila* homologous Salvador [Sav]). This complex then phosphorylates and activates large tumor suppressor kinase 1/2 (LATS1/2;

Drosophila homologous Warts [Wts]) and monopolar spindle-one-binder proteins (MOB1; *Drosophila* homologous Mats) complex. LATS1/2 in turn activates YAP/TAZ by phosphorylating at specific serine residues, thereby causing it to localize in the cytoplasm. Besides LATS1/2 several other proteins—Angiomotin (AMOT), 14-3-3, SMADs, casein kinase 1 isoform epsilon and delta (CSNK1E/D), glycogen synthase kinase 3 beta (GSK3B), catenin beta (CTNNB), CAPZ and beta transducing repeat-containing protein (BTRC) also affect YAP/TAZ localization [121, 125–128] (Fig. 2A). Since YAP/TAZ does not have their own DNA binding motifs, unphosphorylated nuclear YAP/TAZ interacts with various DNA-binding transcription factors regulating targeted gene expression. The function of YAP/TAZ depends upon the upstream signal and its binding partner [129] (Fig. 2B). Their nuclear localization results in cell proliferation, differentiation, migration, epithelial-mediated transition (EMT), cancer development or suppression, and loss of contact inhibition [129–133].

YAP and TAZ share high protein sequence similarity (Fig. 3) and thereby have similar functions. However, there are few structural differences that suggest non-overlapping functions of YAP and TAZ [134, 135]. YAP regulates TAZ cytoplasmic retention, but the exact mechanism is still not clear, although interestingly, the same cannot be said for TAZ because an increase in TAZ expression does not show any effect on YAP levels [136]. Transgenic expression of only YAP in mouse liver showed a dramatic increase in liver mass [137]. YAP and TAZ exhibit some physiological differences as well, and knockout studies have shown that *YAP* knockout mice embryo having normal *TAZ* develop severe developmental defects and subsequent embryonic lethality by embryonic day 8.5 [138]. Conversely, *TAZ* knockout embryos with normal *YAP* exhibit partial embryo lethality, where only half of the embryos survive while the other half die [139–141]. From these observations, it can be said that TAZ is unable to compensate for the loss of YAP.

The differentiation of MSCs into adipocytes, osteoblasts or myocytes has been shown to be regulated by YAP/TAZ activation [142, 143], whereas, YAP/TAZ has been associated in maintaining the pluripotent state and self-renewal in mESCs [137]. Qin et al. (2016) [144] reported that YAP overexpressing hPSCs cultured in DMEM F12 supplemented with bFGF, N2B27, ERK, GSK inhibitors, Forskolin and human LIF did not undergo differentiation as opposed to primed hESCs. Even in differentiating media these hESCs overexpressing YAP strongly expressed pluripotency markers such as OCT4, NANOG, SSEA3, SSEA4, alkaline phosphatase (AP), TRA-1-60 and TRA-1-81. The YAP overexpressing cells when cultured for more than 70 passages showed increased growth rate and dome-like colony morphology. They further proved the role of YAP in maintaining the naïve state by generating clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9)-generated $YAP^{-/-}$cells. *YAP* knockout impairs hESCs ability to exhibit naïve-specific colony characteristics. Apart from this, YAP was shown to play a crucial role in anterior–posterior streak specification. $YAP1^{-/-}$ pluripotent stem cells specify into anterior–posterior streak progenitors in the presence of ACTIVIN which further differentiate into cardiac mesoderm and endoderm [145]. Other than stem cells, YAP/TAZ activation is well known in cancer cells where they function as an oncogene and play an important role in cancer initiation, progression, and metastasis [146]. Numerous knockouts, conditional knockout, deletion studies in various cancer models have revealed the potential role of YAP/TAZ in promoting tumorigeneses in skin, liver, breast and ovarian cancer. We would suggest the readers refer to other excellent reviews on the role of YAP/TAZ in cancer [147–150].

5.1 YAP and TAZ as mechanosensors in stem cells

Apart from acting as transcriptional activators, YAP/TAZ have emerged as key mechanotransducers, acting as nuclear relays in response to cell polarity, substrate stiffness, topology, surface area, and cell density [151–153]. YAP/TAZ activity as mechanosensors has been widely associated with the focal adhesion (FA) components namely integrins, talins, and FAKs. Indeed, on larger stiff substrates cells are well spread, in these conditions they exhibit high ROCK, non-muscle myosin II and F-actin levels, experience high contractile forces which activate YAP/TAZ. Conversely, cells on the soft or small substrates with reduced adhesive area display round morphology, low F-actin, low contractile forces causing cytoplasmic retention, and inactivation of YAP/TAZ [151, 154]. Additionally, the F-actin-capping and -severing proteins Cap Z, Cofilin and Gelsolin have shown to bind to YAP/TAZ thus, limiting YAP/TAZ nuclear localization in cells experiencing low mechanical stress [153]. hESCs on stiff PA-gel substrate ($E \sim 10$ kPa); showed high levels of nuclear YAP/TAZ, F-actin and OCT4 which is indicative of pluripotency [49]. Conversely, hESCs on soft PA-substrate ($E \sim 0.7$ kPa) showed more cytoplasmic YAP and differentiation into post-mitotic neurons [50]. These findings highlight that, substrate stiffness affects the formation of F-actin and further regulates YAP/TAZ activity. Interestingly, a recent study reported that by changing the stiffness of the PDMS substrate, the mesoderm differentiation kinetics of hiPSCs can be modulated and this mechanical change activates YAP during mesoderm induction [155]. This curious observation can be explored further by

differentiating hiPSCs into other lineages on PDMS substrate of varying stiffness and studying the YAP/TAZ dynamics.

The nuclear translocation of YAP in response to mechanical stimuli has also been associated with the FAK activity. Computational model studies suggest that substrate rigidity affects the FAK activation levels which

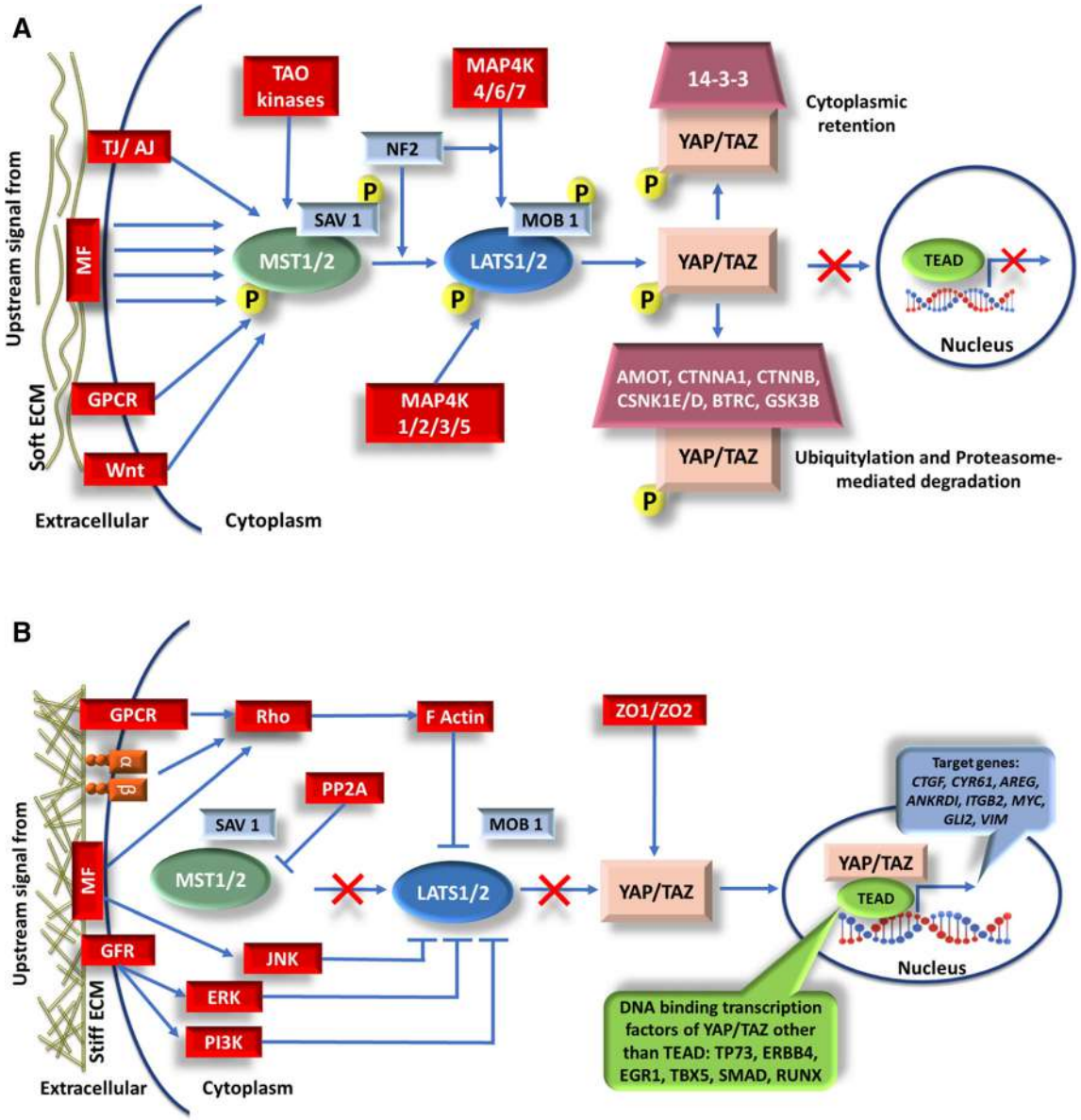

Fig. 2 The Mammalian Core Hippo Signaling pathway. **A** When Hippo pathway is ON, YAP and TAZ are cytoplasmic and do not bind to TEAD (DNA binding transcriptional factor). Upstream signals from various cell surface receptors such as GPCR, WNT, TAO Kinases, tight junction (TJ), adhesion junctions (AJ) or mechanical forces (soft substrate, high cell density, small surface area for adhesion etc.) initiates a cascade of phosphorylation reactions of core Hippo pathway proteins. These signals and scaffold protein SAV1 phosphorylate MST1/2. LATS1/2 is phosphorylated by MST1/2 and MAP4K, facilitated by MOB1 and NF2 (also known as Merlin), which subsequently phosphorylates YAP/TAZ at various serine residues (refer Fig. 3). After phosphorylation by LATS1/2-MOB1, YAP/TAZ either binds to 14-3-3 protein resulting in its cytoplasmic retention or binds to angiomotin (AMOT), casein kinase 1 isoform epsilon and delta (CSNK1E/D), β-transducing repeat-containing protein (BTRC), glycogen synthase kinase 3 Beta (GSK3B) or catenin beta (CTNNB) resulting in ubiquitylation and proteasomal-mediated degradation. YAP/TAZ cannot shuttle into the nucleus and bind to one of its DNA binding transcription factors such as TEAD when Hippo pathway is ON. **B** When Hippo pathway is OFF i.e. in absence of upstream signaling, MST1/2 and LATS1/2 are unphosphorylated and inactive. Unphosphorylated YAP/TAZ is active and is free to move into the nucleus, bind to transcriptional factors, such as TEAD, TP73, ERBB4, EGR1, TBX5, SMAD, or RUNX, and depending upon the binding partner promote specific gene expression

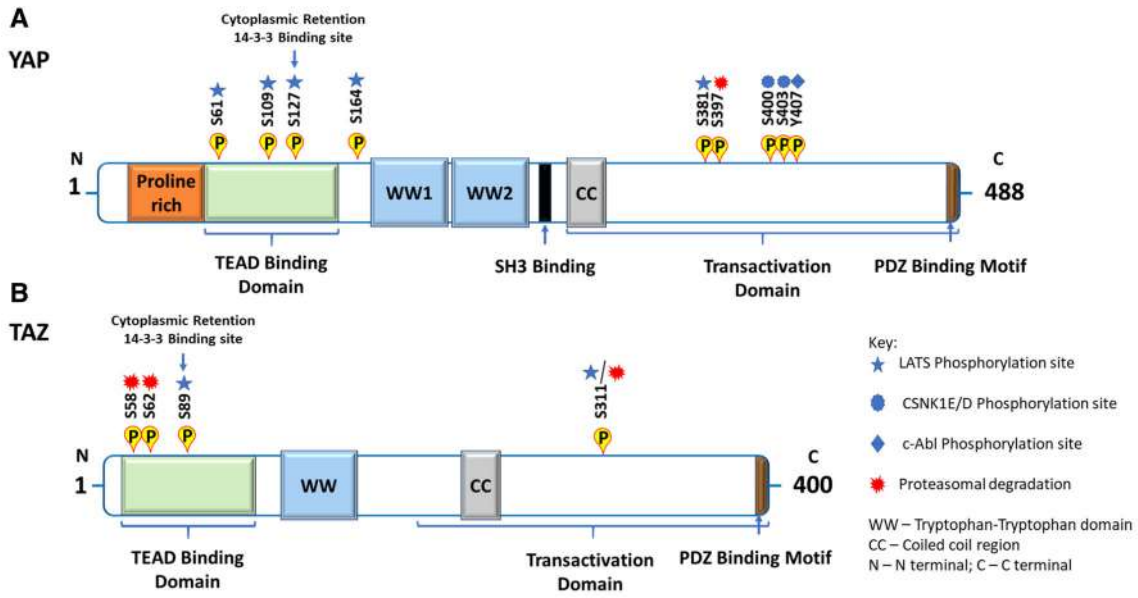

Fig. 3 Schematic representation of **A** YAP and **B** TAZ primary protein sequence: Phosphorylation sites on YAP and TAZ by LATS, CSNK1E/D, c-Abl and for proteasomal degradation are highlighted, including phosphorylation of YAP at S381 by LATS1/2 leads to SCF-βTRCP-mediated proteasomal degradation whereas phosphorylation of YAP at S58/62 causes GSK-3β mediated proteasomal degradation. Phosphorylation at Serine 127 (for YAP) and at Serine 89 (for TAZ) by LATS creates a binding site for 14-3-3 thereby retaining YAP/TAZ in the cytoplasm. YAP and TAZ both have a TEAD binding domain, WW domain, CC (coiled-coil region) domain, transactivation domain, and PDZ binding motif; with an additional proline-rich region, WW domain, and SH3 domain

mediate YAP nuclear localization, and subsequent activations of downstream proteins and eventually gene regulation [119]. Among all the mechanosensing molecules, Talin directly links Integrins to Actin, thus the ECM-Integrin-Talin-Actin clutch is of particular interest in understanding mechanotransduction by matrix rigidity. Substrate stiffness above 5 kPa triggers conformational changes in Talin, it unfolds, binds to and activates vinculin, leading to an increase in focal adhesions and nuclear localization of YAP/TAZ as illustrated in Fig. 1. This role of Talin was demonstrated by culturing *Talin*-depleted cells on PA-gel, these cells showed normal spreading however they neither develop focal adhesions nor caused nuclear localization of YAP [153].

A common observation across current literature shows that hPSCs attach poorly on the soft substrate due to lack of binding sites on substrates, which directly affects their survival. To overcome this, a semi-interpenetrating matrix of PA gel and Matrigel was synthesized ranging from 150 to 12000 Pa in stiffness. hESCs on all the substrates maintain the expression of pluripotency markers even in small colonies. In response to the substrate stiffness, hESCs mimicked colony characteristics of epiblast, inner cell mass, and proamnion. hESCs on soft substrates with $E < 450$ Pa exhibit decreased nuclear YAP compared to cells on stiff substrates having $E > 1000$ Pa. Immunofluorescence probing of single cells grown on soft substrate showed a heterogeneous distribution of YAP in peripheral cells and nuclear localization in the colony interior, indicating a significant variation in YAP distribution within a single colony. When the substrate was switched from soft PA to glass coverslips, an increase in nuclear YAP was observed. Further analysis showed that YAP expression drastically decreased on soft substrate ($E \sim 450$ Pa and 150 Pa) whereas no significant changes in OCT4 expression were seen. This implies that on soft substrate, YAP expression in pluripotent stem cells is sufficient for proliferation and survival but it does not seem to play any role in maintaining pluripotency [156]. Substrate stiffness has a significant effect on cell migration, human adipose-derived stromal/stem cells, human hepatic stellate cells (HHSteC) and MSCs cultured on PA-gel substrate of stiffness spanning the *in vivo* physiological range, migrate towards the stiff region of the rigidity gradient substrate, a process known as durotaxis. To understand the mechanism behind migration, cells were treated with FAK inhibitor and siRNA YAP. Treated cells showed less motility compared to untreated cells indicating that the directed movement of cells from soft region towards stiff region is controlled by FAK and YAP [157–159].

To summarize, YAP/TAZ regulation is largely controlled by the integrity of the cytoskeleton mainly actin-myosin contractility [151]. Actin-severing proteins and unfolded Talin affects the YAP/TAZ nuclear localization

[153, 154], while actin-capping proteins, impairment, and depletion of LINC or Nesprins, and finally stiffness of the substrates cause the cytoplasmic retention of YAP/TAZ in the cytoplasm [119, 154]. Elosegui-Artola et al. [107] have shown that by applying force to the nucleus, nuclear import of the YAP/TAZ and other proteins increases irrespective of their active or inactive state. Whether this passive transport initiates expressions of YAP/TAZ-regulated genes is yet to be shown.

6 Mechanobiology: future perspectives

Bone marrow transplantation or blood transfusions harbouring stem cells have been used for decades and have been reported to be very successful, however when isolated stem cells that are cultured on stiff substrates are transplanted, the results are variable. Sudden change in substrate stiffness may cause changes at gene-level leading to tumour formation or death of the injected cells. This might be the reason why many of the stem cell-based therapies have failed to show the desired effect. 3D bioprinting is a rapidly developing field, where cells are printed onto synthetic or natural biomaterials to generate tissues that closely resemble natural cell arrangement. Recently many newer biomaterials have been designed [160, 161], these biomaterials need to be stiff enough to support the growth of epithelial cells and pliable enough to support angiogenesis, to be clinically successful. Therefore, it is important that we understand the relationship between substrate stiffness and stem cells, so that cells can be cultured on substrate having similar physiological stiffness in order to achieve better results. We hope that with the knowledge about YAP/TAZ and the mechanism by which they regulate genes in response to varied substrate stiffness, would help us design functional 3D printed tissues. Most of the research work done to understand the effect of substrate stiffness on cell behaviour, has been carried out with MSCs, however not much data is available for primary tissues/cells or human pluripotent stem cells.

On a very fascinating note, researches have been focused on understanding the effect of microgravity on stem cell growth and differentiation, necessitated by the advances in space travel and challenges it poses to human physiology. Microgravity has a reversible effect on cell adhesion and microfilament rearrangements of MSCs as shown by the expression levels of vinculin, integrin, VCM1, and actin filaments [162]. Recently it has been demonstrated that the reduced gravity of the international space station (ISS) altered the functioning of neonatal and adult human cardiac progenitor cells [163]. These newer observations demonstrate that there is a lot to be uncovered about how living cells respond to external cues. Mechanobiology would be important discipline that should be studied if humans have to travel to distant planets or survive in low gravity environments of space stations.

7 Conclusion

Until recently, most of our understanding of molecular biology was from the cells cultured on very stiff tissue culture-treated plastic dishes. Cellular functions were defined by the signalling molecules used during culturing; however, the overarching function of mechanical signals suggests that mechanotransduction contributes to defining cellular activity. This raises profound questions such as- in the absence of signalling molecules are the external signals such as substrate stiffness or topology sufficient to direct the cellular fate of stem cells, the answers as and when are uncovered will have significant clinical impact.

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Compliance with ethical standards

Conflict of interest The authors have no financial conflict of interest.

Ethical statement There are no animal experiments carried out for this article.

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Dynamic Interactions Between Stem Cells and Biomaterials

15

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Abstract

The cellular microenvironment has been known to direct the cell behaviour through biochemical and mechanical signalling. Different biomaterials have been fabricated to study the impact of biophysical cues on proliferation and stem cell differentiation in vitro. Stem cells have immense promise in regenerative medicine. Therefore, there is a pressing need to understand the interdependency of biophysical signals and biochemical signals in regulating stem cell potency and differentiation. In this chapter, we explore the different types of biomaterials commonly used for studying mechanobiology in stem cells and highlight the primary mechanism and pathways behind extracellular matrix (ECM)-mediated cellular response. Furthermore, we discuss how the understanding of stem cell mechanobiology influences the fields of tissue engineering and regenerative medicine. We also touch upon the importance of mechanobiology in cancer. In short, we have tried to convey to our readers that although current expansion and differentiation methods use biochemical molecules alone, it is crucial to understand that biophysical cues from the stem cell microenvironment can also regulate the proliferation and differentiation of stem cells.

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15.1 Introduction

Human pluripotent stem cells (hPSCs), which include both human-induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs), have a unique ability to differentiate into cells of three germ layers and have unlimited expansion potential; hence, they can be used for tissue engineering. Multipotent stem cells, for example, mesenchymal stem cells and hematopoietic stem cells, are often used for various clinical researches, and there are several clinical trials conducted with these cells. However, most applications remain at the clinical trial stage due to the non-functionality of transplanted cells, cell death after transplantation, deposition of cells into the lungs, or teratoma formation (Lodi et al. 2011; Naji et al. 2019). This can be due to sudden changes in the microenvironment from in vitro to in vivo. Many researchers have been trying to study interactions between stem cells and their surrounding microenvironment to overcome this.

In vivo, stem cells reside in a specific microenvironment, also known as "niche." This niche maintains an equilibrium between stem cell self-renewal and differentiation and is unique to every stem cell type. The critical regulatory components within the niche include dynamic and complex interactions between cells, macromolecules of extracellular matrix (ECM), biochemical components such as signaling molecules and hormones, and biophysical components such as ECM stiffness, pressure, shear fluid flow, stress, and strain (Pelham and Wang 1997; Vining and Mooney 2017). While the role of biochemical factors is well established, recent scientific literature points to evidence which indicates that the mechanical and biophysical signals generated from the extracellular milieu affect stem cell proliferation and differentiation (Gerardo et al. 2019; Gungordu et al. 2019). All cells, including stem cells and cancer cells, respond to mechanical cues. In stem cells, biophysical signaling control stem cell differentiation and self-renewal; and, in cancer cells, these signals lead to tumor invasiveness and metastasis (Lee et al. 2019; Choudhury et al. 2019). All these recent developments have led to the emergence of a new discipline—mechanobiology, which combines physical forces with the biological phenomenon.

The emergence of biomaterials has facilitated to artificially recreate biophysical signals experienced by cells under in vivo conditions. These biomaterials can be employed as a carrier for the transplantation of stem cells or to recruit endogenous progenitor cells at the site to repair and reconstruct damaged tissues or organs. A common hurdle in the use of biomaterials in regenerative medicine is the immune response. After transplantation, the biomaterials are extensively infiltrated by immune cells. These cells facilitate in removing cellular debris caused by injury; however, they can evoke inflammatory responses, which might hinder tissue repair and cell differentiation (Mokarram and Bellamkonda 2014). The development of new strategies has made biomaterials more sophisticated with respect to

biocompatibility, biological cues, and the potential to reduce damage by an immune response and facilitate in vivo tissue development and direct repair.

In this chapter, we have explored the mechanical and functional interactions between stem cells and their microenvironment. We begin with a brief overview of the importance of ECM in mechanobiology, along with the fundamental molecular mechanisms and the emerging field of biomaterials for stem cell culture. We touch upon cancer mechanobiology and the implications of stem cell mechanobiology and regenerative medicine. We finally provide a perspective on the use of biomaterials to create a modified 3D microenvironment for stem cell culture, which will provide a model to uncover fundamental aspects of mechanobiology and hold tremendous potential in cell-based therapies.

15.2 Unique Tissue-Specific ECM Stiffness in Normal Physiology

The ECM is composed of fibrous proteins such as collagen, fibronectin, elastin, vitronectin, laminin; proteoglycans, and glycoproteins secreted by cells and matricellular-associated proteins such as CNN family, osteopontin, fibulin, periostin, and secreted protein acidic and rich in cysteine (SPARC); however, the ratios of these proteins vary between tissues (Yue 2014; Mouw et al. 2014). Therefore, each tissue has different stiffness, which is defined as elasticity or Young's modulus (E) and is measured in a unit called pascal (Pa). For instance, bone ECM is primarily made up of collagen, which makes it stiff, and the estimated stiffness is approximately within the range of 100 kilopascal (kPa)–1 gigapascal (GPa). On the other hand, brain ECM has low fibrous proteins and higher amounts of proteoglycans compared to bone with E of approximately 1 kPa (Fig. 15.1) (Ruoslahti 1996; Wells

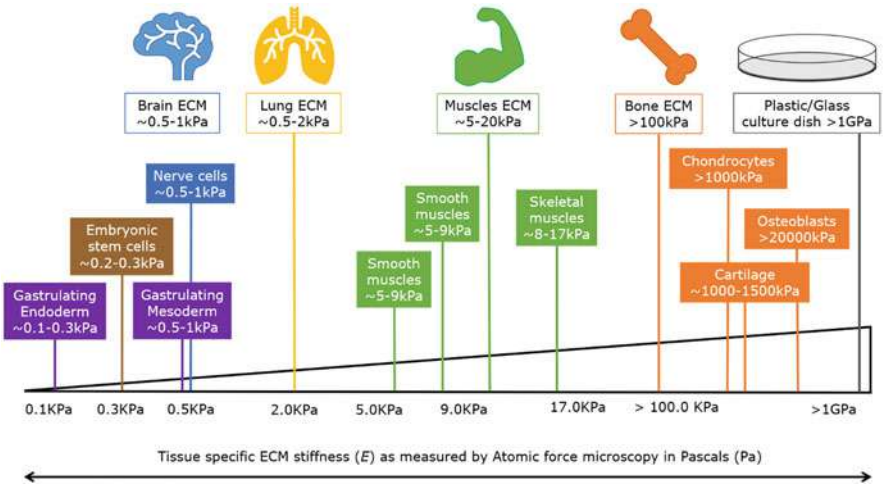

Fig. 15.1 Diagrammatic representation of the varied ECM stiffness range reported in different tissues measured by atomic force microscopy (AFM). The stiffness is defined in Young's modulus or elastic modulus (E) and measured in pascals (Pa)

2008; Budday et al. 2015). Such variations in tissue ECM have led researchers to develop scaffolds that mimic the biological ECM stiffness and properties.

Our understanding of how mechanical signals direct molecular signaling during embryo development and in in vitro differentiation is constantly evolving. The role of ECM in generating mechanical cues has been explored extensively, as the matrix is crucial in regulating cellular functions (Pelham and Wang 1997; Vining and Mooney 2017). Other than providing physical support for growth attachment, the ECM also regulates cell shape, growth, proliferation, differentiation, and migration. Numerous studies have reported that changing the mechanical properties of the matrix, such as stiffness, affects cell morphology, growth, differentiation, migration, and gene expression (Pelham and Wang 1997; Lo et al. 2000; Justin and Engler 2011; Toh et al. 2012, Ireland and Simmons 2015).

15.3 Biomaterials and Their Types

Traditionally used synthetic scaffolds from 2D polystyrene surfaces to 3D constructs provide only support to the cultured cells. Recent advances in tissue engineering have shown exciting results with various biomaterials of suitable physical and chemical properties in recreating complex in vivo microenvironment in the laboratory. Based on their source and properties, these biocompatible materials can be categorized as natural, semisynthetic, and synthetic biomaterials, with stiffness similar to the stiffness of the biological tissue (Virdi and Pethe 2021).

Natural biomaterials are synthesized using polymers such as chitin, agarose, collagen (Chevallay and Herbage 2000), alginate, and hyaluronic acid hydrogel (Toh et al. 2012) because of their similarity with native ECM. Another advantage is that they are highly biocompatible with binding sites for cells, thereby supporting cell growth. However, natural polymers are not consistent in composition, are not easy to modify, and have limited mechanical properties. To overcome these disadvantages of natural polymers, synthetic substrates have been synthesized using polyacrylamide (PA) gels (Engler et al. 2004), polydimethylsiloxane (PDMS) (Goffin et al. 2006), polyethylene glycol (PEG) hydrogel (Gilbert et al. 2010), and polyvinyl alcohol (Muduli et al. 2017), which provide better mechanical properties than natural biomaterials. The synthetic biomaterials provide a range of various stiffness similar to the stiffness of the biological tissue, have high reproducibility, and are well defined. However, synthetic polymers provide limited cell-ECM interactions as they lack the functional group to allow cells to attach.

To overcome the drawbacks of natural polymers and synthetic biomaterials, a semisynthetic hydrogel, for example, gelatin methyl acrylate (GelMa) (Guilak et al. 2009), was designed, which has the biocompatibility of natural polymer and mechanical properties of synthetic biomaterials. To enhance the clinical application of scaffolds, it is important to achieve a xeno-free, chemically-defined system for stem cell culture other than hydrogels. In this regard, other scaffolds such as artificial nano- and micro-patterned substrates (Théry 2010), flexible micropillars (Halder et al. 2012), and electrospun nanofibers (Maldonado et al. 2015; Zhu et al. 2019)

have been synthesized to study the effect of substrate stiffness on stem cell growth, differentiation, and migration.

15.4 Immunomodulatory Biomaterials

As we have introduced above, biomaterials being a foreign material may provoke an immune response, which might hinder tissue repair and regeneration. To address this limitation, researchers are synthesizing new biomaterial designs, which incorporate immunosuppressive molecules or signaling molecules that facilitate activation of the desired phenotype within the host immune cells (Dziki and Badylak 2018). These types of biomaterials are known as immunomodulatory biomaterials. Specific and durable immunomodulation can be achieved by manipulating the surface property of the biomaterials such as topology, surface charge, and ligands; this can induce activation of a desired immune cell phenotype (Stabler et al. 2019). For instance, following the implantation in murine subcutaneous implant and volumetric muscle injury model, flow cytometry analysis identified macrophages ($F4/80^+$), $CD11c^+$ dendritic cells, $CD3^+$ T cells, and $CD19^+$ B cells within the microenvironment of the ECM bioscaffold (Sadtler et al. 2017). The authors have shown that the biomaterial microenvironment changes the polarization of the migrating immune cells upon implantation, causing them to alter the signals generated by microenvironment. This immunomodulatory effect of the biomaterial on the immune cells and the host tissue environment may help in improving the therapeutic capability of the biomaterials. Numerous similar studies that use ECM-based biomaterials show a dynamic interaction between a variety of the immune cells or between stem cells and immune cells, which promotes tissue repair (Brown et al. 2012; Sadtler et al. 2016; Dziki et al. 2018).

15.5 Biomaterials Influence Stem Cell Proliferation and Functionality

In order to design the biomaterial that allows stem cells to be transplanted for clinical use, it is important to study some key aspects such as (1) the traction forces exerted by the cells on the biomaterial, (2) stem cell growth and proliferation, and (3) the changes in the stem cell functionality and differentiation capacity when grown on biomaterial.

The synthetic hydrogel substrates are synthesized using one or more polymers, which forms an interconnecting network with the help of a cross-linking agent. The mechanical properties such as hydrogel substrates can be manipulated by changing concentrations of polymer and cross-linking agent. For example, in PA-gel substrates, altering the ratio of acrylamide to bis-acrylamide cross-linker allows variation in Young's modulus, which thereby affects cell behavior (Tse and Engler 2010). Human mesenchymal stem cells (hMSCs) cultured on stiff PA substrate with $E \sim 25$–40 kPa, which resembles bone ECM stiffness, differentiate toward osteoblast

lineage as indicated by the gene expression analysis, whereas, on soft PA substrate ($E \sim 0.1$–1 kPa) resembling brain ECM stiffness, the hMSCs differentiate toward neural lineage (García and Reyes 2005; Engler et al. 2006). Muscle stem cells self-renew when cultured on substrates mimicking the stiffness of muscle tissue ($E \sim 12$ kPa), and these cells contributed to muscle regeneration when transplanted in mice (Gilbert et al. 2010). Morphologically, stem cells appear flattened on the stiff substrate and spherical with reduced spreading and stress fiber formation on soft substrate (Deroanne et al. 2001; Engler et al. 2004). These studies reveal varying responses of stem cells toward their microenvironment, and substrate stiffness indicates an important role of substrate matrix in regulating cell behavior.

PA-gel substrate functionalized with glycosaminoglycan (GAG) peptides shows better cell attachment. Following this observation, the research group demonstrated that stiff PA-GAG substrate ($E \sim 10$ kPa) promotes pluripotency of human ESCs as evidently observed from the expression levels of pluripotency marker proteins octamer-binding transcription factor-4 (OCT4) and stage-specific embryonic antigen-4 (SSEA4) (Musah et al. 2012); however soft substrate ($E \sim 0.7$ kPa) selectively differentiated stem cells toward neuronal lineage. The same research group noted an interesting observation that even in the absence of neuronal inducing factor, hPSCs grown on softer substrate appeared neuronal-like phenotype and expressed high levels of tubulin beta 3 chain (TUJ1) protein, a neuronal specific-marker (Musah et al. 2014). A similar observation was reported by another group that used other biomaterials as well of different stiffness (Chen et al. 2020). These studies indicate that substrate stiffness alone can influence hPSC differentiation when cultured with an optimal mechanical microenvironment, independent of soluble signaling factors. Therefore, it can be said that the mechanical signals have a profound contribution on early embryo development and differentiation.

As explained above, when mimicking various physiological stiffness like neural ($E \sim 1$ kPa), muscle ($E \sim 12$ kPa), and bone ($E \sim 30$ kPa) tissues, substrates can induce respective lineage-specific differentiation of MSCs. In addition to cellular function, substrate stiffness also influence cell migration. Cell migration is important in numerous physiological processes such as wound healing, organogenesis, immune response, tumor metastasis, and morphogenesis; thus, it is crucial in regeneration tissue engineering and cancer therapy. Many studies have demonstrated stem cells migrate toward the stiff substrate, whereas neurons show a preference for softer regions (Tse and Engler 2010; Vincent et al. 2013; Flanagan et al. 2002; Hadden et al. 2017). The mechanical properties of the ECM influence the factors known to regulate cell migration, such as the integrin-cytoskeletal interaction and cytoskeletal stiffness. The cells sense the change in the matrix through an active tactile exploration mechanism and respond by exerting contractile forces (Lo et al. 2000). To understand the migration of stem cells on matrix stiffness, MSCs were treated with focal adhesion kinases (FAK) inhibitor and siRNA targeting transcriptional factor Yes-associated protein (YAP) gene. They observed reduced cellular motility of treated cells compared to untreated cells, indicating that FAK and YAP control the movement of cells from the soft region toward the stiff region (Wang et al. 2001; Hadden et al. 2017; Lachowski et al. 2018).

15.6 Mechanobiology: Mechanism of Interactions (Molecular Mechanisms)

Mechanobiology is the study of the relationship between a cell and its microenvironment. The interactions between the cell and the microenvironment mainly occur at the interface. The properties of biomaterials such as hydrophilicity, surface charge, roughness, softness, and chemical composition affect the transplantation success. To improve the interaction between cell and scaffold, the physical, chemical, and biological properties of the biomaterials need to be optimized according to the cell type. Before seeding the cells onto a scaffold, surface modification is necessary to facilitate cell adhesion and growth. Surface modification can be either coating the surface with extracellular membrane protein or modifying the surface using functional moieties, hydrophobic or hydrophilic molecules (Shi et al. 2015; Elosegui-Artola et al. 2017).

A cell senses its external environment via membrane-bound receptors, focal adhesions to the ECM, adhesion junctions between neighboring cells, and gap junctions. The perturbation of protein conformation by mechanical forces influences the cytoskeletal organization, which triggers a series of intracellular signaling pathway resulting in inactivation or inhibition of gene expression, morphology, and motility (Discher et al. 2005; Guilak et al. 2009). Integrin-based adhesion complexes are one of the key molecular players closely associated with actin filaments. Focal adhesion complex, Ras homologous (Rho) GTPases, myosin light chain kinases, and Rho-associated kinases (ROCK) form a link between integrins and actin filaments. The activated focal adhesion complex comprises talin, vinculin, paxillin, alpha-actinin, p130cas, FAK, and SRC formed near cell surface integrin receptor (Geiger et al. 2009). The cells are able to sense the substrate stiffness, topology, surface area, and dimensionality of the scaffold by means of integrin molecules and focal adhesion complexes (del Rio et al. 2009; Amano et al. 2010; Donato et al. 2010; Ciobanasu et al. 2013; Janoštiak et al. 2014; Elosegui-Artola et al. 2017).

In brief, integrins are transmembrane ECM proteins and mechanoreceptors as they sense the change in the ECM, thereby mediating the mechanotransduction by focal adhesions, which link integrins to cytoskeleton (Hynes 2020). A traction force is generated in the actin cytoskeleton, which activates the downstream signaling and translocates the signal into the nucleus. These traction forces are also exerted on the integrins and focal adhesions, thus maintaining them in the isometric tension (Bershadsky et al. 2003). External stresses generate a mechanics-based positive feedback loop by increasing tension on the cell surface receptor and activating G protein Rho and its target ROCK. Stiff substrate results in an increase in kinase activities of ROCK, FAK, and extracellular signal-related kinases (ERK1/2), causing osteogenic differentiation of MSCs. Inhibition of ROCK and FAK leads to downregulation of osteogenic markers during osteogenic induction (Shih et al. 2011). Taken together, this implies that stiff substrates affect the regulation of

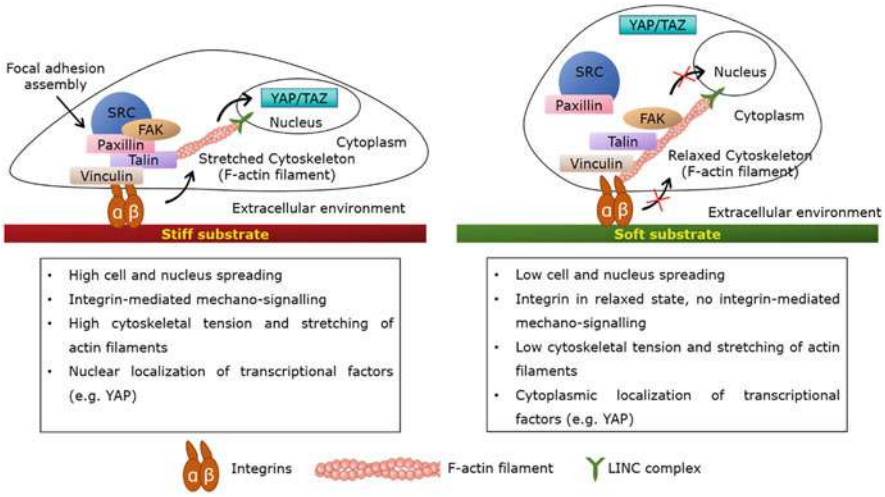

Fig. 15.2 Schematic representation of the effect of stiff and soft substrate on cell morphology and function via integrin-mediated mechano-signaling. On stiff substrate, a cell receives biophysical cues from integrin-based focal adhesion complex, which increases the cytoskeletal stress via stretching of F-actin filaments. The stretching of LINC complex due to stiff substrate and stretched F-actin causes nuclear localization of transcriptional factors such as YAP. Conversely, on the soft substrate in the absence of less integrin activity, the focal adhesion complex is not formed, leading to less cytoskeletal tension and less stretching of actin filaments, thereby leading to cytoplasmic localization and no substrate-dependent nuclear localization of the transcriptional factors

ROCK-mediated FAK and ERK1/2, and these signals regulate the transcriptional factors, thereby determining the fate of MSCs.

The mechano-sensitive transcriptional coactivators such as myocardin-related transcription factor (MRTF) (Speight et al. 2016), nuclear factor kappa B (NF-κB) (Kumar and Boriek 2003), nuclear factor erythroid 2-related factor 2 (NRF2) (Escoll et al. 2020), YAP, and beta-catenin (Gumbiner 1995; Huber et al. 1996) bind to their respective DNA-binding proteins and activate specific genes. The nuclear or cytoplasmic localization of these transcriptional factors is controlled by nuclear envelope receptor—linker of nucleoskeleton and cytoskeleton (LINC) complex (Guilluy et al. 2014; Driscoll et al. 2015) (Fig. 15.2). Apart from integrin-ligand binding, several studies have suggested that the cells produce nano-length projections that sense the surface for optimum spreading. Thus, different nano-topographical features guide cell migration and spreading on the scaffold with different topographies. The fact that cellular orientation and alignment can be controlled by topographical cues was demonstrated as early as 1911 by Robert Harrison (1911). To date, the biomaterial-based scaffold has undergone many surface modifications and alternations and has emerged as a powerful tool for mimicking in vivo microenvironment.

15.7 Biomaterials as Promising Tools for Tissue Engineering and Regenerative Medicine

From the aforementioned considerations, it can be evident that mechanobiological processes in stem cells will impact the development of innovative therapeutic methods for tissue engineering and, eventually, regenerative medicine applications. The successful outcome of any stem cell-based regenerative medicine critically depends on cell survival after transplantation and to maintain tissue homeostasis mainly by differentiating into the respective lineage. To attain this, it is crucial to maintain optimal physiologically similar culture conditions in vitro for stem cell maintenance, proliferation, and quick differentiation when required. For instance, culturing the resident liver stem cells (RLSC) on polyacrylamide gel substrate having a stiffness of 0.4 kPa has shown to help in differentiation of RLSC into hepatocytes within 24 h, whereas RLSC cultured on a stiff substrate of stiffness 80 kPa resulted in only initial hepatocyte-specific transcriptional activity (Cozzolino et al. 2016). This variation in differentiation potential is due to culturing cells on soft stiffness—which is similar to healthy liver tissue stiffness (0.3–6 kPa)—rather than using normal stiff TCP. Similarly, instead of 2D culture system, Schoonjans and colleagues developed a synthetic 3D culture system using polyethylene glycol (PEG) hydrogels with a matrix stiffness of 1.3 kPa. This 3D culture system mimicking physiological liver stiffness provided better efficiency of live organoid derivation from mouse and human hepatic progenitors (Sorrentino et al. 2020). These studies show that clinically relevant human progenitor/stem cells cultured in physiologically relevant mechanical environments open perspectives for liver organoid-based clinical applications.

An interesting study focused on regenerating complex neural tissue such as motor neurons through modulating substrate stiffness because during embryo development, biophysical cues from the surrounding microenvironment along with soluble morphogens like sonic hedgehog (SHH) and retinoic acid (RA) play an important role in morphogenesis. Sun et al. (2014) and colleagues synthesized a system with PDMS with a stiffness range of $E = 1.0$–1200 kPa for generating motor neurons (MN) derived from hPSCs. Their findings suggest that soft substrate ($E = 1$ kPa) support early MN differentiation of hPSCs compared to stiff substrate ($E = 1200$ kPa). In addition, the yield and purity of functional MNs improve four- to tenfold on soft substrate compared to stiff substrate (Sun et al. 2014). Thus, culturing hPSCs on a synthetic cell culture surface with controlled mechanical properties (such as substrate stiffness) improved the efficiency of hPSC differentiation into motor neurons. Such advances open new doors in the therapeutics of motor neuron-associated neurodegenerative (Sun and Fu 2014).

An electrospun nanofibrous vascular scaffold made up of poly(L-lactide) (PLLA) was embedded within PA hydrogel on the outer surface. This nanofibrous polymer system had stiffer matrix near the polymer and was less stiff away from the polymer and was used as a graft for cell regeneration in vivo. Multipotent neural crest stem cells (NCSCs) generated from hiPSCs were embedded within the graft and implanted in rat carotid arteries. The stiffer matrix of the polymer scaffold with

$E = 50$ kPa or higher supported the differentiation of NCSCs into smooth muscle cells (SMCs). The soft matrix area of the scaffold with $E = 15$ kPa supported the differentiation into glial cells. The results suggests that the mechanical properties of substrate play a significant role in designing biomaterials for tissue engineering (Zhu et al. 2019).

hiPSCs are traditionally generated by genetic reprogramming of adult somatic cells using biochemical signals (Takahashi and Yamanaka 2006). Fascinatingly, Grãos and colleagues demonstrate that MSCs can be reprogrammed into iPSCs by biophysical cues alone. They showed that human umbilical cord MSCs (huMSCs) exhibit PSC phenotype when cultured on soft PDMS substrate with $E = 1.5$ kPa and 15 kPa compared to stiff TCP ($E \sim 1$ GPa). huMSCs undergo chromatin modeling and show enhanced expression of pluripotency-related markers *OCT4*, *SOX2*, and *NANOG* in response to the soft substrate. Soft substrate allowed huMSCs to acquire relaxed nuclei, small FA, fewer stress fibers, and high euchromatic and lower heterochromatic content and expression of pluripotency specific genes. In short, their results suggest that substrate stiffness influences several phenotypic features of iPSCs and colonies and that soft substrate favors iPSC reprogramming (Gerardo et al. 2019). Such milestone studies indicate that substrate stiffness is a critical biophysical cue that influences stem cell differentiation into the specific lineage. Such studies also highlight the importance of biomaterials in tissue engineering and a promising platform for improving tissue engineering and regenerative applications.

15.8 Mechanobiology in Cancer Cells

Mechanobiology is one of the driving forces in guiding cell motility and tissue development during embryonic development. This cellular and tissue mechanobiology approach has been used by many researchers in understanding cancer development and tumor invasion. One of the key mechanisms by which cancer cells evade therapy is metastasis, and it has been hypothesized that the tumor cells might rely on mechanical forces for invasion and migration. The tumor microenvironment is an aggregation of cancer-associated fibroblasts (CAFs), vascular cells, immune cells, an abundance of extracellular matrix proteins, and hypoxic conditions (Choudhury et al. 2019; Sahai et al. 2020). Hypoxia and hypervascularization are directly and indirectly associated with ECM realignment and shear stress (Wang et al. 2017).

The ECM is a fundamentally essential component of the tumor microenvironment that interacts closely with cancer cells. Apart from providing necessary growth factors for tumor growth (Briquez et al. 2015), the ECM also helps in transmitting signals integrins (Canel et al. 2013). Additionally, upregulation of ECM remodeling molecules, such as transforming growth factor-beta (TGF-β), is linked to the development of desmoplasia in tumors (Papageorgis and Stylianopoulos 2015). Desmoplasia is the development of dense fibrous and connective tissue around tumor growth, usually characterized by increased synthesis of total collagen, fibronectin, glycoproteins, mainly tenascin C, proteoglycans, and a sizeable stromal cell

population that amasses within the tumor. The increased production of tumorigenic and inflammatory growth factors transforms a large number of fibroblasts into CAFs. It has been proposed that the multifunctional cytokine TGF-β activates the transformation of fibroblasts into CAFs, which produces more ECM fibers, eventually causing desmoplasia (Papageorgis and Stylianopoulos 2015). The ECM stiffness of the fibrotic/cancer tissue is around 1.08–68 kPa (Kawano et al. 2015) and has shown to upregulate alpha-smooth muscle actin (α-SMA) expression, a proven CAF marker. Another known transcriptional factor that facilitates CAF generation and maintenance is YAP/TAZ, which activates only during high actomyosin contractility and high stiffness (Goffin et al. 2006; Calvo et al. 2013). YAP has been shown to regulate the expression of specific cytoskeletal proteins, including anillin actin-binding protein, myosin regulatory light polypeptide 9, and diaphanous related formin 3, which induces CAF (Calvo et al. 2013).

During cancer progression, uncontrolled cell proliferation results in an increase in tumor mass. This leads to a difference between the ECM stiffness of tumorous tissue and normal tissue. For instance, Samani et al. (2007) reported that the mean Young's modulus of normal breast tissue is 1.9 kPa, whereas that of fibroadenoma was 11.42 kPa and that of invasive ductal carcinoma was 22.55 kPa. Multiple in vitro reports show that the stiffness of the tumor tissue and matrix directly correlates with tumorigenesis and metastasis (Zaman et al. 2006; Tilghman et al. 2010; Gkretsi and Stylianopoulos 2018; Jang et al. 2020). A breakthrough study published by Weaver and colleagues proves the hypothesis that mechanical signals mediate malignant transformation. They showed that culturing non-tumorigenic mammary epithelial cells on stiffness mimicking tumor-like stiffness induces cell proliferation, dysplasia and activates oncogenic epithelial signaling pathways. They also found that transformed cells maintain a functional link between integrins and Rho-dependent cytoskeletal tension, and in the presence of ROCK or integrin adhesion pharmaceutical inhibitors the malignant behavior of tumors was tempered (Paszek et al. 2005).

Cancer stem cells (CSCs) have been shown to reside within the tumor, and these cells have the ability to self-renew and differentiate into several cell types, which proliferate uncontrollably. Thus, CSCs sustain the growth of cancerous mass. The cancer stem cells are hard to eliminate due to their efficient DNA repair mechanisms, relative slow growth rate, and the high number of channel proteins to efflux drugs out (Turdo et al. 2019; Hirschmann et al. 2004; Fujiwara et al. 2021). Cancer stem cells lead to relapse of cancers after treatment (Eyler et al. 2008), and hence, it is necessary to investigate these cells including their mechanobiology machinery. In summary, understanding how cancer cells sense the mechanical signals and converted them into biochemical pathway may usher in new ways to control cancers. Given the similarities between the biology of stem cells and cancer cells (Shackleton 2010; Rahman et al. 2016), researchers are exploring the functional and mechanistic similarities between stem cell mechanobiology and cancer mechanobiology, with the aim of understanding the former using the latter as a guide (Fig. 15.3).

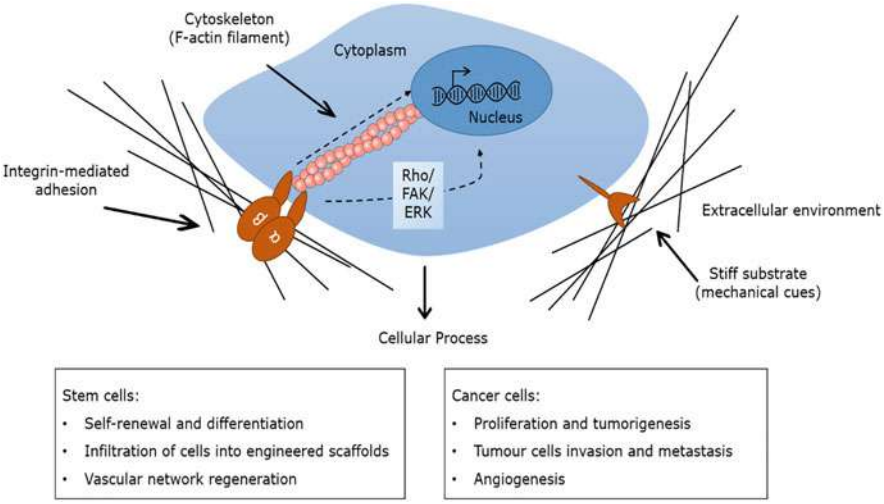

Fig. 15.3 The similarities between the ECM-cell mechanobiology of stem cells and cancer cells

15.9 Concluding Remarks and Perspectives

Many advances in fabricating biomaterials for regenerative medicine have been reported in recent decades. Fundamental properties of biomaterials and of cell responses to biochemical and biophysical cues have been described via structural and functional studies. In this chapter, we have briefly described various properties of biomaterials and their impact on cellular behavior. For detailed information on the physical, chemical, and functional properties of the biomaterials, the authors recommend some extensively detailed reviews by Amani et al. (2019) and Cun and Hosta-Rigau (2020). The existing knowledge on ECM-cell interactions has been mainly derived from 2D in vitro studies. Although the 2D culturing system is convenient and has uncovered several crucial aspects about mechanobiology and biomaterials in cell migration, adhesion, proliferation, and differentiation, it does not mimic the in vivo microenvironment, which is 3D. It is becoming increasingly evident that the cells have a distinct behavior in the 3D microenvironment than that seen in 2D microenvironment. These facts have led to the use of a 3D culture system to mimic the physiological environment required for stem cell differentiation and the generation of organoids (Pepelanova et al. 2018; Bailey et al. 2019). hPSCs cultured on 3D scaffold have already been used to develop neuronal (Levenberg et al. 2003), liver (Baharvand et al. 2004), and cartilage (Hwang et al. 2006; Bai et al. 2010) tissue equivalents, along with rudimentary vascular networks (Ferreira et al. 2007).

Other than 3D culture, 3D bioprinting can be used to fabricate well-organized cell-laden scaffolds, which can be used to repair or regenerate damaged tissue

(Antich et al. 2020; Jeong et al. 2020). Further advancement is organ-on-a-chip technology, which helps in generating self-organizing miniature organs from stem cells that replicate the functional and structural characteristics of cells present in in vivo microenvironment (Park et al. 2019). This organ-on-a-chip method has been employed in cancer cells to understand the disease progression and predict drug-induced responses (Sun et al. 2019). The studies discussed herein demonstrate the significance of the extracellular microenvironment in determining cellular behavior. They also highlight the importance of developing novel biomaterials to provide cells with biophysical cues which will help in cell-based therapies and regenerative medicine. Although much is yet to be unraveled about the influence of mechanobiology on stem cells, the newer discoveries give us insight into a promising future but also raised certain fundamental questions, such as the following: How much of the mechanical information is needed for the desired response from stem cells to form complex tissues? Can the biomaterials transplanted cause uncontrolled proliferation of the surrounding tissue? How cells generate their own mechanical forces during embryogenesis? With such diverse materials and methods for synthesizing biomaterials, it becomes crucial to understand how much of the material complexity is required for the desired stem cell response. We envision that the current research will help pave the way in understanding mechanobiological influence on stem cells and have major implications on tissue engineering and regeneration approaches.

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