UNIVERSITY OF TURKU

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ABSTRACT

Human pluripotent stem cells (hPSCs) hold great promise for future medicine. They can differentiate to all adult cell types and self-replicate virtually endlessly. These features would make hPSCs a valuable tool for applications of regenerative medicine. Pluripotency is controlled by extracellular cues which establish the correct culture conditions. Appropriate growth factors and extracellular matrix (ECM) composition must be provided for maintenance of viability, pluripotency and self-renewal *in vitro*. However, the stem cell field has overlooked many of the basic cell biological phenomena that could affect the regulation of pluripotency.

Focal adhesions (FAs) connect the ECM via integrin receptors to the cellular cytoskeleton. They are dynamic protein complexes responsible for broadcasting the information of composition and mechanical properties of the ECM to biochemical intracellular signalling cascades. Furthermore, they provide the physical anchoring points needed for cell adherence and movement. FAs have not been studied in the context of human pluripotency before.

In this thesis, I utilise high-resolution microscopy to describe for the first time the characteristics of FAs in hPSCs. We show that hPSCs have large cornerstone FAs connected via contractile actin stress fibres at colony periphery. We provide evidence that structures at the colony edge create traction forces needed for compaction of the cells. Also, we show that FAs function as signalling platforms. We employ 3D super-resolution microscopy and unveil unique ultrastructural features of large FAs and show that perturbation of the structure accelerates hPSC differentiation. Finally, we introduce a versatile, easy access method for implementation of fluctuation based super-resolution microscopy to measure cellular forces in nanoscale.

In summary, this thesis provides in detail characterisation of cornerstone FAs in hPSCs and highlights their role for morphological features of the colonies and pluripotency. In addition, it provides a new method for studying cellular forces exerted by the FAs.

KEYWORDS: Pluripotency, hPSC, Focal adhesions, Integrins, Super-resolution microscopy, Traction force microscopy

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TIIVISTELMÄ

Ihmisen erittäin monikykyiset kantasolut voivat mullistaa lääketieteen tulevaisuudessa. Kyseiset kantasolut voivat erilaistua kaikiksi aikuisen solutyypeiksi ja jakaantua lähes loputtomasti. Monikykyisyyttä säätelee joukko solun ulkoisia tekijöitä, joista on koostettu sopivat viljelyolosuhteet. Soluille täytyy tarjota kasvutekijöitä ja solunulkoinen matriksi, jotta ne voisivat selviytyä, pysyä monikykyisenä ja jakaantua soluviljelyolosuhteissa. Kantasolututkimuskenttä on ohittanut monia perussolubiologisia ilmiöitä, joilla voisi olla vaikutus monikykyisyyden säätelyyn.

Fokaaliadheesiot yhdistävät solunulkoisen väliaineen integriinireseptoreiden välityksellä solun tukirankaan. Fokaaliadheesiot ovat dynaamisia proteiinikomplekseja, joiden tehtävä on tunnistaa solunulkoisen väliaineen koostumus ja mekaaniset ominaisuudet sekä muuttaa tieto solunsisäisiksi signaaleiksi. Tämän lisäksi fokaaliadheesiot toimivat fyysisinä ankkurointipisteinä, joita solu tarvitsee kiinnittymiseen ja liikkumiseen. Fokaaliadheesiota ei ole ennen tutkittu ihmisen monikykyisyyden solujen näkökulmasta.

Tässä väitöskirjassa käytän edistynyttä valomikroskopiaa ihmisen monikykyisten kantasolujen fokaaliadheesioiden kuvailemiseen. Näytämme, että solupesäkkeiden laidalla on suuret fokaaliadheesiot, joita yhdistää voimaa tuottavat tukirangan säikeet. Todistamme myös, että näiden rakenteiden tuottamia voimia tarvitaan pesäkkeiden tiiveyden ylläpitoon ja signalointiin. Lisäksi käytämme 3D-superresoluutiomikroskoopiaa fokaaliadheesioden tarkan rakenteen selvittämiseen ja paljastamme niissä erityispiirteitä. Fokaaliadheesioiden rakenteen häiritseminen johtaa kantasolujen nopeampaan erilaistumiseen. Lopuksi esitelemme uudenlaisen tavan mitata solujen voimia nanomittakaavassa käyttäen säteittäisiin vaihteluihin perustuvaa superresoluutiomikroskopiaa.

Tämä väitöskirja kuvailee ihmisen monikykyisten kantasolujen fokaaliadheesioita ja alleviivaa niiden merkitystä solupesäkkeiden ulkomuodolle ja monikykyisyydelle. Lisäksi tämä kirja esittelee uuden työkalun fyysisten voimien tutkimiseen solutasolla.

AVAINSANAT: monikykyisyys, kantasolut, fokaaliadheesiot, integriinit, superresoluutiomikroskopia

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Abbreviations

Akt	protein kinase B
BMP4	bone morphogenetic protein 4
CRISPR	clustered, regularly interspersed short palindromic repeats
Cas9	native cas9 nucleases
CDK1	cyclin-dependent kinase 1
CDK2	cyclin-dependent kinase 2
CST	cell signalling technologies
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EMCCD	electron-multiplying charged coupled device
EMT	epithelial-mesenchymal transition
EpiSC	epiblast stem cell
ERK	extracellular signal-regulated kinase
ESC	embryonic stem cell
FA	focal adhesion
FAK	focal adhesion kinase
FBSR	fluctuation based super-resolution
FGF2	fibroblast growth factor 2
FTTC	Fourier transformation traction cytometry
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
hESC	human embryonic stem cell
hiPSC	human induced pluripotent stem cell
hPSC	human pluripotent stem cell
ILK	integrin-linked kinase
kank	kidney ankyrin repeat-containing protein
LIF	leukaemia inhibitory factor

LM	laminin
MAPK	mitogen-activated protein kinase
mESC	mouse embryonic stem cell
MET	mesenchymal-epithelial transition
miRNA	micro ribonucleic acid
Mn2+	manganese-ion
NA	numerical aperture
OSKM	oct4, sox2, klf4 and cmyc, Yamanaka factors
p130Cas	breast cancer anti-estrogen resistance protein 1
PAA	polyacrylamide
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PI3K	phosphatidylinositol-3-kinase
PIP2	phosphatidylinositol (4,5)-bisphosphate
RA	retinoic acid
RGD	arginyl glycyl aspartic acid
RI	reflective index
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
ROCK	rho-associated protein kinase
SACD	super-resolution method based on auto-correlation two-step deconvolution
SIM	structured illumination microscopy
sCMOS	scientific complementary metal-oxide-semiconductor
SDS	sodium dodecyl sulphate
SIM	structured illumination microscopy
siRNA	small interfering ribonucleic acid
SMAD	mothers against decapentaplegic homolog
SMLM	single molecule localisation microscope
SOFI	super-resolution optical fluctuation imaging
Src	src proto-oncogene, non-receptor tyrosine kinase
SRRF	super-resolution radial fluctuation
SSEA	surface-specific embryonic antigen
STED	stimulated emission depletion
TFM	traction force microscopy
TGF-β	transforming growth factor-beta

TIRF total internal reflection fluorescence

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Närvä, E., Stubb, A., Guzman, C., Blomqvist, M., Balboa, D., Lerche, M., Saari, M., Otonkoski, T. and Ivaska, J. A Strong Contractile Actin Fence and Large Adhesions Direct Human Pluripotent Colony Morphology and Adhesion. Stem cell reports, 2017, 9(1), pp. 67-76.

II Stubb, A*., Guzman, C*., Närvä, E., Aaron, J., Teng-leong, C., Saari, M., Miihkinen, M., Jacquemet, G. and Ivaska, J. Superresolution architecture of cornerstone focal adhesions in human pluripotent stem cells. Nature Communications, 2019, 10, pp. 4756

III Stubb, A., Laine, R., Guzman, C., Henriques, R., Jacquemet, G. and Ivaska, J., 2019. Fluctuation-Based Super-Resolution Traction Force Microscopy. bioRxiv, 2019, 772947

* equal contribution

1 Introduction

Human Pluripotent Stem Cells (hPSCs) have enormous potential for future medicine. Remarkably, pluripotent cells are capable of deriving almost all cell types of the human body. They are also able to self-replicate virtually endlessly. In the future, they might be used for the purposes of personalised regenerative medicine on a population scale. Still, the potential arising from the pluripotent cells has been speculated for many years, but utilisation in clinical work has been modest even if there has been a handful of relatively successful attempts. It seems that enthusiasm has not faded away and the vast possibilities are still widely recognised by the scientific community and clinical practitioners. We still lack in the perception of the underlying cellular mechanism guiding the complicated path of cellular differentiation and the resulted identity.

Understanding the essence of pluripotency took a significant step upon the introduction of induced pluripotency. By taking advantage of the technique generated by Professor Shinya Yamanaka, the science has advanced in modelling the disease and incorporation of genomic modifications in stem cell research. Generation of hPSCs from patient samples has opened an alleyway for studying how specific genotype translates into phenotype in tissue level. Compared to human embryonic stem cells isolated from a living blastocyst, induced stem cells are almost identical without the ethical dilemma of using human embryos for research purposes.

Studying the genomic landscape of pluripotent stem cells has gone a far distance. Our grasp of the transcriptional regulation of pluripotency is extensive and valuable. Still, we lack in observing many of the primary cell biological phenomena when it comes to human pluripotent stem cells. In detail, characterisation of adhesion qualities and the cellular cytoskeleton is missing from the field of stem cell research. Adhesion signalling and cell-ECM interaction play an essential role in many fundamental cell biological processes. Cells need the coordinates for spreading, migration and in some cases even in cell stage transitions. For sensing the composition and mechanical qualities of their environment, cells use a protein

complex called focal adhesion. This dynamic signalling platform broadcast the mechanobiological cues is also the physical link between the ECM and the force-generating actin cytoskeleton. Focal adhesions have not been studied in the context of human pluripotent stem cells before.

The objective of this study was to characterise the adhesion qualities of hPSCs and describe in nanoscale how the focal adhesion architecture differs from the cell types studied previously. I also aimed to develop tools to study the force transduction of the cells in nanoscale. To reach these goals, I decided to incorporate the highest quality light microscopy in my reach.

2 Review of the Literature

2.1 Stem cells

2.1.1 Potency in embryo development

There are over 200 different types of cells in the human body, and it is estimated that the adult human body consists of 37 trillion cells (Sender, Fuchs et al. 2016). Still, this the vast bulk of cells called an individual has originated from one cell from a mother (oocyte) and one cell from father (sperm). The resulted fertilised cell is called a zygote. Single fertilised "egg" or zygote contains all the necessary genomic information needed for generating the remaining cells types in the adult human body. This is a showcase of ultimate plasticity among the cells (Rossant, Patrick 2017). A zygote is considered as totipotent, which means that it has unlimited differentiation potential. Totipotent cells can form a complete individual and are thus capable of forming embryonic and extraembryonic tissues (De Los Angeles, Ferrari et al. 2015).

Pluripotency is the subsequent level of potency. Pluripotent cells can generate almost all cell types of the human body, with some exceptions. Pluripotency is distinguished from totipotency by the decreased ability to form extra-embryonic trophoblast layers, placenta and yolk sac derivates (De Los Angeles, Ferrari et al. 2015). However, pluripotent cells can differentiate to all three embryonic layers: ectoderm, endoderm and mesoderm and subsequently, all the cells derived from these layers (Yilmaz, Benvenisty 2019).

Upon zygote dividing into 16 cells, the embryo reaches the morula stage. In human embryo development, this stage occurs upon 3 days. Human cells isolated from this stage are considered pluripotent (Klimanskaya, Chung et al. 2006). After 4 to 5 days of embryo development, the cells form a structure called blastocyst, which consists of surrounding layer of trophoblast cells and pluripotent inner cell mass (Niakan, Eggan 2013). This is the stage where human embryonic cell lines are classically isolated (Figure 1.) (Thomson 1998).

2.1.2 Pluripotent Stem cells

2.1.2.1 Embryonic Stem Cells

Embryonic stem cells (ESCs) were first isolated from mouse blastocyst stage embryo (Evans, Kaufman 1981, Martin 1981) and recapitulated with human embryos sometime after (Thomson 1998). ESCs could be kept, in proper culture conditions, in the pluripotent stage virtually indefinitely. As a testament for pluripotency human embryonic stem cells (hESCs) were capable of forming teratomas, tumours made from several types of tissue, upon subcutaneous injection to mice.

Pluripotent stem cells can be used for modelling embryo development (Siggia, Warmflash 2018, Sozen, Amadei et al. 2018). Seemingly, the embryo has inherited capability pattern during embryogenesis to form functional tissues. To partially recapitulate the patterning during morphogenesis, human embryonic stem cells can form radial differentiation patterns in 2D conditions (Warmflash, Sorre et al. 2014) and mouse embryonic stem cells can be used to model embryo development with 3D multicellular organoids (Sozen, Amadei et al. 2018). Another, possibly even more valuable prospect of human embryonic stem cells is their utilisation in regenerative medicine (Trounson, DeWitt 2016). The indefinite ability to proliferate and the potential for generating tissues are perfect assets when the purpose is to create new tissues or organs. Nevertheless, there is a number of significant obstacles before large scale clinical implementation. The most publicly visible one has been the ethical considerations of using human embryos for research. Also, some religious point of views considers a zygote as a person which makes the usage of embryonic cells unethical and has caused a public debate on the matter. The other obstacles are more technical and related to the immune rejection of unfamiliar tissue (Boyd, Rodrigues et al. 2012). Also, genomic instability and heterogeneous cell populations have raised concerns and are seen as a risk for cancer ofter transplantation (Lee, Tang et al. 2013).

The state of pluripotency is maintained through tight transcriptional control (De Los Angeles, Ferrari et al. 2015). The transcriptional core regulatory network includes Oct4, Sox2 and Nanog, which are essential for human and mouse embryonic stem cells (Wang, Oron et al. 2012, Loh, Lim 2011). The regulatory network has been shown to govern itself via auto feedback loop where the produced proteins push their own expression (Boyer, Lee et al. 2005). Oct4, Sox2 and Nanog control the expression of most genes coding proteins, miRNAs and non-coding RNAs essential for pluripotent cells while simultaneously suppressing the differentiation lineage regulators (Kim, Chu et al. 2008).

In addition, to the differentiation potential and capability to self-replicate, hESCs possess some other unique features: (1) Accelerated cell cycle. In the pluripotent stage, a large part of the cell population is in S-phase of the cell cycle. Also, the G_1-phase is exceptionally short, and therefore the whole cell cycle only takes 15-16 hours, whereas the normal somatic cell usually divides in 24-36 hours (Becker, Ghule et al. 2006). (2) Unconventional epigenetic footprint. The genome of hESCs has high global methylation status and, according to some reports, cells from female origin have one epigenetically inactivated X-chromosome (Silva, Rowntree et al. 2008, Yilmaz, Benvenisty 2019). (3) Unique colony organisation. Cells grow in a tightly packed colony with well-defined edges which makes it possible to distinguish them from differentiated progenitor cells. The features of colony morphology are discussed more in detail in chapter 2.1.5.

2.1.2.2 Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) are generated from somatic or so-called terminal cell types. Transcription factors Oct4, Sox2, Klf4 and cMyc, are expressed in a given cell type to initiate the induction of pluripotency. The combination is known as "OSKM" or "Yamanaka factors". IPSCs can be generated from various cell types, but initially the protocol was implemented to mouse fibroblasts using a retroviral system (Takahashi, Yamanaka 2006) and subsequently also human fibroblasts were converted using either retro- and lentivirus systems (Takahashi, Okita et al. 2007, Yu, J., Vodyanik et al. 2007).

Human induced pluripotent stem cells (hiPSCs) closely resemble their embryonic counterparts (Mallon, Hamilton et al. 2014, Takahashi, Okita et al. 2007). The morphological characteristics, transcriptional footprint and the differentiation capabilities are virtually similar. The biggest advantage of hIPSCs over hESCs in regenerative medicine is their identical genome with the originator cells. For purposes of regenerative medicine, having the autologous donor cells would possibly overcome the immune rejection of transplants (de Almeida, Meyer et al. 2014). Furthermore, the ethical issues surrounding the use of hESCs are dissolved since the iPSCs are not directly derived from embryos.

The overexpression of OSKM reverts the transcriptional program to the embryonic stage. Epigenetically, reprogramming factors initiate a global stage of gene methylation and initiate the transcription program essential for pluripotency and self-renewal (Hurk, Kenis et al. 2016). However, it seems that there are multiple ways of completing the induction process and none of the transcription factors used is irreplaceable (Yilmaz, Benvenisty 2019). For example, the master regulators Oct4

and Sox2 can be used alone to induce the pluripotency in the presence of histone deacetylase inhibitor (Huangfu, Osafune et al. 2008). Also, Oct4 can be replaced with mesodermal lineage specifier (Gata3) and Sox2 with ectodermal lineage specifier (Znf521, Sox1 or Sox3) during reprogramming (Montserrat, Nivet et al. 2013).

The method for reprogramming is an essential consideration when aiming for clinical applications (Sullivan, Stacey et al. 2018). The use of retro- or lentiviruses is impossible since they integrate the coding sequence to the genome, which could yield a malignant tissue; hence, more precise reprogramming methods are needed. Somatic cell reprogramming can also be completed using chemicals (Zhao, Y., Zhao et al. 2015), episomal expression of transcription factors (Okita, Matsumura et al. 2011) or miRNA (Warren, Manos et al. 2010). IPSCs generated with these systems do not have additional DNA inserted to their genome. More recently, the invention of CRISPR/Cas9, a new method for genome modification has made it possible to only activate the needed promoter sites from DNA using CRISPRa which relies on catalytically inactivated Cas9 protein to control the transcription of endogenous loci (Weltner, Balboa et al. 2018). The endogenous activation of pluripotency related genes is a promising step forward in the optimisation of clinical-grade iPSC cultures.

2.1.2.3 Naive Pluripotency

Most remarkable advance in the field of pluripotency since the generation of iPSCs is the discovery of the naïve stage of pluripotency. Studies initially done in mouse cells suggest that there might be two stages of pluripotency. Mouse embryonic stem cells (mESCs), isolated from mouse blastocyst, present different features compared to post-implantation mouse epiblast stem cells (EpiSCs) (Nichols, Smith 2009). Both can be considered pluripotent, but the epiblast stem cells seem to have limited ability to form germ cells and are considered to be in a primed stage of pluripotency (Tesar, Chenoweth et al. 2007). In extensive transcriptome analysis, hESCs and hiPSCs seem to resemble more the mouse EpiSCs than the pre-implantation mESCs (Nakamura, Okamoto et al. 2016). The classical human embryonic stem cells (Thomson 1998) are therefore termed primed and pre-implantation cells naïve. However, it is still under debate if the naïve stage captured in laboratory conditions resembles the pre-implantation phase of actual human embryo development. The naïve stage is speculated to be present in a human embryo before the implantation to the uterine wall. Still, the human cells isolated from morula are in a primed pluripotent stage when cultured in vitro (Klimanskaya, Chung et al. 2006).

Robust efforts by scientists working on the field of pluripotency have yielded multiple methods for reverting primed pluripotent cells back to naïve stage and maintaining them *in vitro* (Jacob Hanna, Albert W. Cheng et al. 2010, Ware, Nelson et al. 2014, Guo, von Meyenn et al. 2017, Gafni, Ohad, Weinberger et al. 2013, Chen, H., Aksoy et al. 2015, Chan, Göke et al. 2013, Duggal, Warrier et al. 2015, Theunissen, T. W., Powell et al. 2014). These studies have utilised stage-specific features observed from pre-implantation human embryos for the generation of culture systems supporting the naïve state (Yan, Yang et al. 2013, Stirparo, Boroviak et al. 2018). The inhibition of ERK- and WNT- signalling pathways using MAP2K and GSK3β inhibitors while supplementing the culture medium with leukocyte inhibitory factor (LIF) seems to be the consensus base medium (2i/LIF). However, different efforts pursuing the naïve state have resulted in the use of numerous different inhibitor combinations (Zimmerlin, Park et al. 2017). Certain naïve cell features are widely accepted among the scientific community while others are still under debate. To mention a few, human naïve stem cells have a status of global hypomethylation and distinct transcriptional profile when compared to primed hPSCs (Smith, Chan et al. 2014). Also, naïve-hPSCs grow in domed shape colonies, whereas the hPSCs grow in relatively flat colonies. Naïve cells also display better survival as a single cell compared to their primed counterparts that require cell-cell junctions or E-cadherin ligation for survival (Gafni, Ohad, Weinberger et al. 2013). The energy metabolism between the stages also seems to be different. The naïve cells rely on oxidative phosphorylation and the primed cells mainly rely on glycolysis (Takashima, Guo et al. 2014).

Naïve human stem cells could provide an important model for human development, and upon further study, they might be used for modelling the interaction between a human embryo and the uterine wall (Sagi, Benvenisty 2016). However, before establishing formidable models, some critical questions must be addressed. One problem surrounding the naïve cells is the increased genomic instability and accumulation of karyotypic abnormalities compared to the primed cells. This might be due to incomplete culture protocols or just an inherent feature of the naïve stage pluripotency (Theunissen, T. W., Powell et al. 2014, Liu, X., Nefzger et al. 2017). Furthermore, there is still an ongoing debate of the right cues for reverting the cells into naïve stage. Transcriptional studies show differences in the transcriptomes of cells derived with different protocols, and it has to be determined which methods recapture the essence of the preimplantation human embryo.

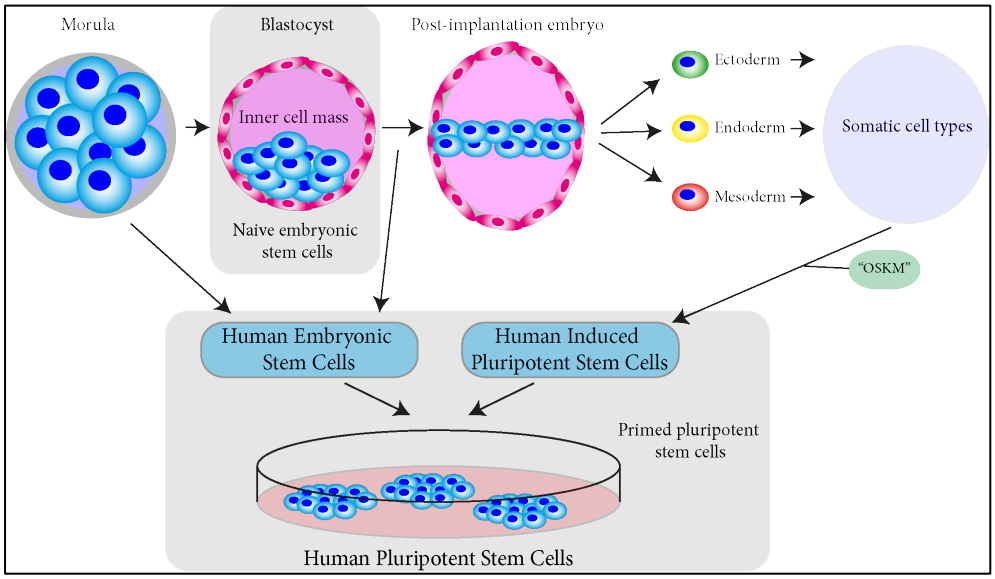

***Figure 1**. Origin of human pluripotent stem cells. Modified from (Yilmaz, Benvenisty 2019).*

2.1.3 Determining the pluripotent stage

Pluripotency can be assessed with functional assays. These are considered to be the baseline and only formidable way of determining pluripotency status. The least stringent functional assay is *in vitro* differentiation, where pluripotent cells are subjected to conditions where they differentiate. The differentiation is monitored with different lineage-specific indicators. In addition, a commonly used assay is teratoma formation where pluripotent cells are injected to immune-deficient mice (Nelakanti, Kooreman et al. 2015). If the cells are pluripotent, they generate a teratoma tumour. In the most stringent assays, pluripotent cells are incorporated to host blastocyst to form chimaera either as a cell population or as a single cell and observed as a blastocyst, developing embryo or adult mouse. Chimaera formation assays with human cells are considered as unethical, and therefore their use determining human pluripotency is limited. (De Los Angeles, Ferrari et al. 2015)

The status of pluripotency can also be determined by analysing the expression of core regulatory elements OCT4, SOX2 and NANOG and other pluripotency related genes (Boyer, Lee et al. 2005). There are robust microchip or RNA sequencing-based assays for analysing the transcriptome in a detailed manner surpassing the unethical teratoma or chimaera formation assays in more day to day experiments (Müller,

Schuldt et al. 2011, Bock, Kiskinis et al. 2011, Tsankov, Akopian et al. 2015, Bouma, van Iterson et al. 2017).

Studies comparing the naïve and primed states have also identified particular stage-specific factors that can be used to distinguish between the two different states (Theunissen, Thorold W, Friedli et al. 2016). The standard analysis of core regulatory elements is insufficient. OCT4, SOX2 and NANOG are shared regulators of both cell states, and even though they are expressed at higher levels in the naïve stage, they are cannot be considered as good indicators between states (Guo, von Meyenn et al. 2016). However, ZIC2, CD24 and SFRP2 have been shown to be formidable markers for primed cells and DNMT3L, DPPA3/5, GATA6, IL6ST, KLF4/5/17, and TBX3 for the naïve stage and can be used to distinguish between the naïve and primed cells (Messmer, von Meyenn et al. 2019, Yilmaz, Benvenisty 2019).

2.1.4 External signals governing primed pluripotency

Stable pluripotent cultures can be maintained in pluripotency-supporting culture conditions for long periods without genomic alterations or differentiation (Xu, C., Inokuma et al. 2001). The culture systems have come a long way from mouse fibroblast feeder cultures, and the hPSC lines can be maintained in entirely xeno-free environments (Rodin, Antonsson et al. 2014, Miyazaki, Futaki et al. 2012, Nakashima, Omasa 2016).

The classical system for hESC culture was the usage of feeder fibroblast layer under the epithelial type colonies to provide a surface to attach and necessary signalling for survival (Thomson 1998, Xu, C., Inokuma et al. 2001). Pluripotent stem cells can also be cultured with more modern xeno-free systems specially designed to yield cells for clinical purposes without contamination from objects unknown to the human body. Most minimalistic approach for hPSC culture is Essential 8 (E8) system, where the supplemented basal medium contains only eight essential components for pluripotency maintenance. Six of the components could be considered as typical culture medium supplements, but two growth factors are vital for maintaining the pluripotent status — fibroblast growth factor 2 (FGF2) and transforming growth factor-beta (TGF-β). (Chen, G., Gulbranson et al. 2011)

Maintenance of pluripotency and lineage-specific differentiation are reliant on an intriguing interplay between growth factor signalling derived from the study of embryos. The growth factors initiate signalling cascades, either supporting the pluripotency status or direct towards differentiation. TGF-β and FGF 2 have both been shown to be important for the maintenance of pluripotency (Vallier, Alexander

et al. 2005). Intracellularly, TGF-β receptor activation phosphorylates SMAD2/3, which then translocate to the nucleus activating translation pluripotency and self-renewal related genes (James, Levine et al. 2005). Inhibition of TGF-β signalling or SMAD2/3 phosphorylation leads to loss of expression of core regulatory elements and subsequent differentiation. FGF2 activates the mitogen-activated protein kinase (MAPK) and PI3K/Akt signalling pathways which both have been shown to have an essential role in cell survival and regulation of pluripotency (Vallier, Alexander et al. 2005, Eiselleova, Matulka et al. 2009).

2.1.5 Colony morphology

There are significant differences between the human and mouse embryonic stem cells (Ginis, Luo et al. 2004, Niakan, Eggan 2013). One feature, observable trough a tabletop microscope, is the difference in colony morphology. Human primed pluripotent stem cells grow as relatively flat monolayer colonies (Figure 2.), whereas the naïve stem cells display dome-shaped colonies with multiple layers of cells. Mouse embryonic stem cell colonies closely morphologically resemble naïve hPSCs (Hanna, Cheng et al. 2010, Gafni, O., Weinberger et al. 2013).

HPSCs cannot be cultured as single cells; they need mechanical support from the colony and E-cadherin mediated cell-cell junctions (Li, L., Bennett et al. 2012). The loss of E-cadherin junctions is sufficient to trigger RhoA hyperactivation leading to increased myosin II activity which over a prolonged period is enough to trigger apoptosis (Ohgushi, Masatoshi, Sasai 2011). Depletion of E-cadherin from cells is enough to prevent the induction of pluripotency (Li, D., Zhou et al. 2010). Furthermore, overexpression of E-cadherin in somatic cells enhances the reprogramming process (Chen, T., Yuan et al. 2010).

The dependence on neighbouring cells can be overcome with the use of ROCK inhibitor or by blebbistatin (Watanabe, Ueno et al. 2007, Chen, G., Hou et al. 2010). Both decrease actomyosin contraction by perturbing RhoA/ROCK/myosin II-signaling, prevent the apoptosis and increases clonal efficiency (Chen, G., Hou et al. 2010, Ohgushi, Masatoshi, Matsumura et al. 2010). However, it has been shown that the introduction of ROCK inhibitor disrupts colony morphology and causes a bias lineage specificity towards mesodermal lineage (Maldonado, Luu et al. 2016). Paradoxically, even though Rho hyperactivation causes apoptosis as a single cell, still, Rho activation is required for hESCs survival when growing in a colony. This discrepancy was explained by a study detecting that two different guanine nucleotide exchange factors (GEFs) regulate Rho/ROCK signalling in hESCs, one pro-apoptosis

(ABR) and one pro-survival (AKAP-Lbc) (Ohgushi, Masatoshi, Minaguchi et al. 2015).

Colony morphology is also dependent on the cell-ECM adhesion. A recent study demonstrated that morphologically different types of pluripotent stem cell colonies could be derived from the clones isolated from the same culture by modulating the adhesiveness of the ECM (Yu, L., Li et al. 2018). Low adhesion conditions constitute for a generation of flat monolayer colonies whereas high adhesion conditions yield more tightly packed dome-shaped colonies. Furthermore, the flat colonies had a higher tendency to differentiate towards ecto- and mesodermal lineages, whereas the domed colonies were likely to differentiate to the endodermal lineage. Both types of colonies expressed core pluripotency factors and could be maintained for multiple passages.

Cells in different parts of pluripotent stem cell colonies have different potential for differentiation. The cells at the colony edge, show more rapid response to bone morphogenetic protein 4 (BMP4), a known regulator of SMAD signalling. BMP4 initiates rapid cell differentiation at colony edge, whereas the cells in colony centre respond slower (Rosowski, Mertz et al. 2015). Also, the mechanical conditions inside the colony were shown to be different compared to highly contractile edges (Przybyla, Lakins, Sunyer et al. 2016). Indeed, the mechanical niche seems to be different in different parts of the colony. Supporting this notion, upon BMP4 introduction, the mechanosensitive transcription co-activator Yes-associated protein (YAP) was seen be predominantly nuclear at the edges whereas in the centre it was mainly cytoplasmic (Abagnale, Sechi et al. 2017). Also, distortion of the shape of the colony to more elongated by seeding them on nanolined patterns induced more rapid response BMP4. Colony morphology seems thus be closely connected to the differentiation potential of hPSCs (Abagnale, Sechi et al. 2017).

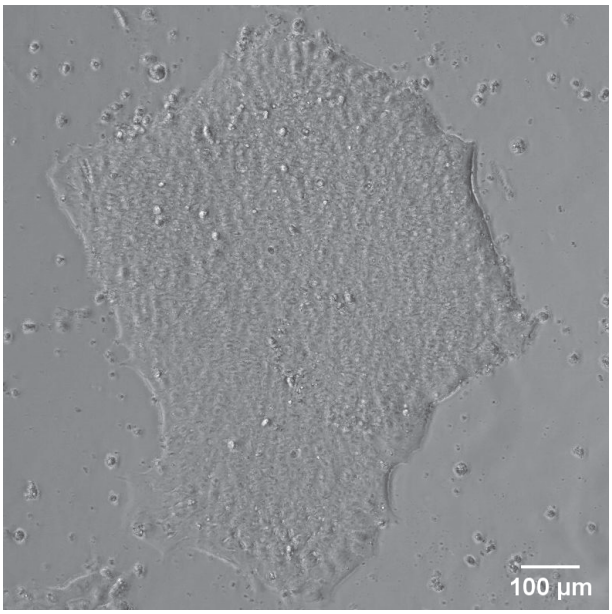

Figure 2. *Phase-contrast microscope image of a human iPSC colony.*

Human pluripotent stem cell colonies can be used to model the process of human gastrulation. Surprisingly, the tightly packed colonies can recapitulate the formation of the embryonic layers consistently (Heemskerk, Warmflash 2016). Since the morphology, size and cell density of the colony plays an important role in the guided differentiation process the colony features are controlled by seeding them on adhesion permitting round confining micropatterns (Warmflash, Sorre et al. 2014). The diameter of micropatterns used ranges from 500 µm to 1000 µm to recapitulate the number and density of cells in a gastrulating human embryo. Introduction of BMP4 initiates a process of spatially controlled differentiation where differentiated embryo layers are radially organized. One reason why the localisation of individual cells in the colony seems to have an essential role in the lineage determination might be the positioning of differentiation regulating surface receptors. It was shown recently that the cells in the centre, hide their TGF-β receptors laterally and become insensitive to morphogen BMP4. However, the cells at the edge did not undergo the lateralisation and remained sensitive to morphogen (Etoc, Metzger et al. 2016).

2.2 Cell-ECM interaction

There has been vast progress of culture conditions sufficient of maintaining pluripotency in vitro. Besides developing new growth factor combinations, advances in recombinant matrixes suitable stem cell culture have been achieved (Hayashi, Furue 2016). Establishing methods for xeno-free culture is highly important from the point of view of regenerative medicine where the possible elements causing immune rejection in tissue transplants have to be minimized. HPSCs can be seeded and maintained at least on embryonic stem cell grade matrigel (laminin rich ECM extract from mouse sarcoma) (Xu, C., Inokuma et al. 2001), human recombinant

vitronectin (Braam, Zeinstra et al. 2008), laminin 511 (Miyazaki, Futaki et al. 2012), laminin 521 (Rodin, Antonsson et al. 2014) and fibronectin (Hughes, Radan et al. 2011). The importance of ECM attachment for stable pluripotent stem cell culture has been established (Nakashima, Omasa 2016, Hayashi, Furue 2016, Kallas-Kivi, Trei et al. 2018), but the underlying mechanism of the interactions still remains nebulous. Based on a report systematically identifying barriers for somatic cell reprogramming to pluripotency, adhesions were seen as one of the major barriers (Qin, Diaz et al. 2014). According to a study, mouse pluripotency is connected to low adhesion signalling (Taleahmad, Mirzaei et al. 2017) and low adhesion strength can be used for enriching human pluripotent cells from cultures undergoing the induction process (Singh, Ankur, Suri et al. 2013). Still, stable human pluripotent stem cells grow as an adherent epithelial type colony. The peculiar adhesion qualities of hPSCs remain to be studied further.

2.2.1 Integrin receptors

Integrins are transmembrane receptors for recognising various ECM outside of the cells. An integrin heterodimer is composed of an alpha and a beta subunit. The N-terminal globular part of the integrins reaches outside from the cell followed up by transmembrane domain and finally the short cytoplasmic intracellular C- terminal tail. There are altogether 24 different known integrin heterodimers. To this date, we are aware of 18 different alpha units and 8 beta units. The functional heterodimers can be divided into 4 families by their extracellular ligand: collagen, laminin or RGD-binding integrins and a subfamily of leukocyte specific integrins. The classification is quite loose since there are multiple heterodimers capable of binding several ligands with variable affinity. Also, multiple heterodimers are able to bind the same ligand, likewise with variable affinity, but still may trigger different intracellular responses. The C-terminal intracellular tail is responsible for recruiting intracellular proteins such as kinases and adaptor proteins. The relatively short tail is covered by binding sites for regulatory proteins (Morse, Brahme et al. 2014). The role of integrins might be system dependent since all cell lines do not express the same subset of integrin subunits. Still, it is evident that integrins have a pivotal role in modulating cell spreading, migration, polarity, survival and proliferation. These events are important for many fundamental processes from development to homeostasis. (Richard O. Hynes 2002, Campbell, Humphries 2011)

The structural conformation of integrin heterodimers ranges from an extended open to a bend closed conformation. Integrin ligand binding affinity is dependent on the conformation and increases gradually upon extension and opening of the extracellular domain (Askari, Buckley et al. 2009). These steps of integrin activation

have been mainly established for the platelet integrin aIIbβ3 and many integrin β1-containing heterodimers but are also relevant for the β2-integrins in immune cells. The cues mediating the activity can come bi-directionally either from outside or inside of the cell. In the case of outside-in activation, the integrin heterodimer recognises different ECM components. The bound ECM is enough to trigger conformation change of extracellular domain and separation the cytoplasmic tails which subsequently enables the binding of the adaptors or signalling components to the integrin tails. Respectively during inside-out activation, an intracellular integrin activator such as talin and kindlin binds to the β-subunit cytoplasmic tail triggering the conformation change increasing the affinity to bound ECM. In either of the cases, the high-affinity conformation needs to be stabilised by forces from the outside (via binding to the ECM) and inside (via in-direct binding to actin cytoskeleton). (Figure 3.) (Sun, Zhiqi, Costell et al. 2019)

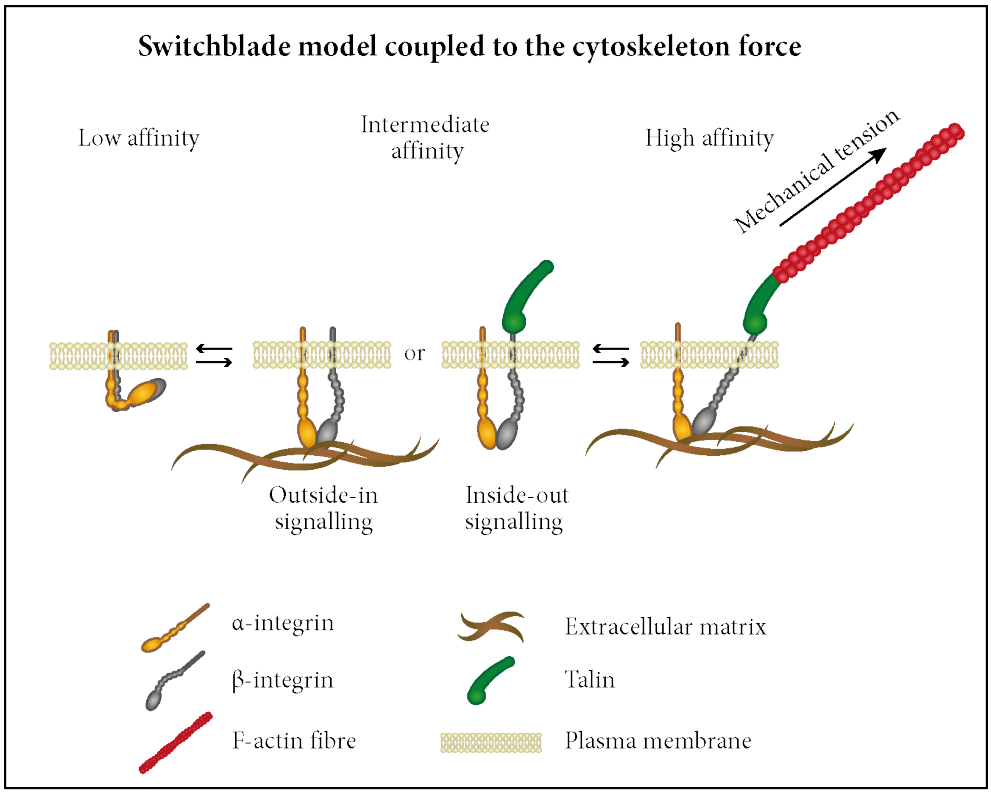

***Figure 3**. Simplistic illustration of integrin conformation change upon activation. Adapted from (Luo, Carman et al. 2007, Isomursu, Lerche et al. 2019)*

The model incorporating the three conformations of integrins is called switchblade model where the heterodimer can exist in bend, primed or extended conformation.

In the bend form, the extracellular portion is bend towards the plasma membrane and closed from ligand having a low binding affinity. In the primed conformation, the extracellular portion is extended having still the ligand-binding domain closed harbouring an intermediate level of ECM affinity. In the extended conformation, the ligand-binding domain is open, and the extracellular portion is extended. This is the highest affinity conformation. (Figure 3.) (Luo, Carman et al. 2007)

The activity state of integrins can be detected using conformation-specific antibodies (Byron, Humphries et al. 2009, Su, Xia et al. 2016) of integrins or using 3D super-resolution microscopy to determine the distance of the N-terminal domain from the cell membrane (Moore, Aaron et al. 2018).

Another dimension of integrin regulation is their intracellular traffic. The integrin availability at the plasma membrane is controlled by clathrin-dependent and clathrin-independent endocytosis pathways. Depending on the integrin activity status, they are recycled at different rates with inactive β1-integrin undergoing rapid recycling and the active receptors recycling with slower kinetics (Arjonen, Alanko et al. 2012). Internalised active integrins are mostly recycled back to the cell membrane. Some are however directed to lysosomal compartments for degradation. Active integrins can contribute to signalling from the endosomal compartments, which have shown to contribute to cancer cell survival (Alanko, Mai et al. 2015). Usually, defects in integrin endocytosis lead to impaired migration but can also result in cytokinesis defects (Moreno-Layseca, Icha et al. 2019).

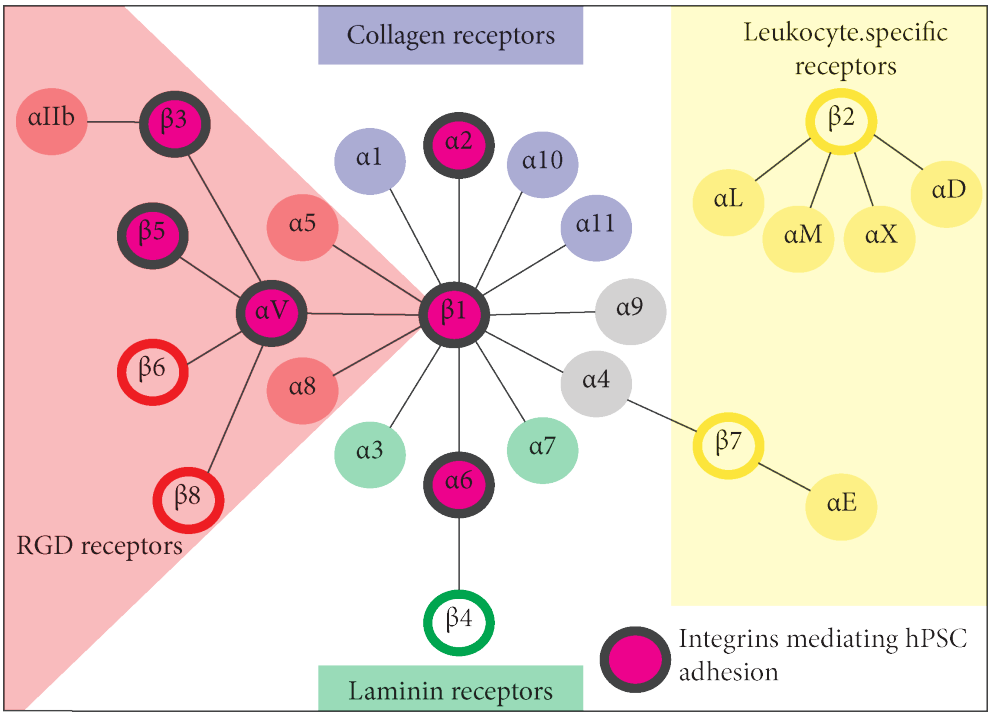

Figure 4. *Simplistic illustration of integrin α and β subunit pairing and ligand specificity. Integrins mediating hPSC adhesion are highlighted in the figure. Modified from (Hynes 2002)*

HPSCs express a broad spectrum of integrins (Xu, C., Inokuma et al. 2001). The main functional subunits are α2, α3, α6, αV, α5, β1 and β5 which mediate adhesion to their designated ECM and have shown to be present on the plasma membrane of hPSCs (Figure 4.) (Braam, Zeinstra et al. 2008). α6β1 attaches the cells to laminin and matrigel and α5β1 mediate adhesion to fibronectin which was shown in assays utilising integrin function-blocking antibodies (Braam, Zeinstra et al. 2008, Meng, Eshghi et al. 2010). Integrin β1 was also shown to be important for hPSCs proliferation on both matrigel and vitronectin and αVβ5 for attachment and proliferation on vitronectin (Rowland, Miller et al. 2010, Braam, Zeinstra et al. 2008). These results indicate that integrin β1 is essential for hPSCs in all the conditions mentioned.

2.2.2 Focal adhesions (FAs)

Cells independent of their origins constantly interact with their environment. The environment might consist of ECM, different or from the neighbouring cells. These interactions are critical for all cellular functions in the body. The lipid bilayer of the cell is covered with various receptors which are used for "sensing". The cell needs the information related to the consistency and physical qualities of the ECM to be able to function properly. Also, signals from surrounding cells are essential for many fundamental processes like development and tissue homeostasis. In some malignant conditions, a cell's capability to sense or respond to the environment has been hijacked and is used for unwanted migration and proliferation. This is true, for example in the case of migrating cancer cells (Conway, Jacquemet 2019).

FAs are integrin containing multimolecular complexes that via integrin binding to the ECM physically and biochemically connect the cell to its environment. The clustering of active integrin heterodimers initiates the formation of nascent adhesions. Upon build-up of mechanical force across the integrin-ECM linkage, the nascent adhesions grow in size and mature to FA. If there is no force-loading across the linkage, the adhesion loses its purpose and disassembles. During the *maturation* process, a nascent adhesion recruits FA component and turns into mature FAs. FAs come in variable sizes, compositions and states of activation, reflecting the different stages of maturation. (Riveline, Zamir et al. 2001, Oakes, Beckham et al. 2012)

FAs function as physical points of force transmission between the cell cytoskeleton and ECM and coordinate cytoskeletal reorganisation and cellular response. The forces from the ECM to the actin cytoskeleton are transmitted by integrins through the integrin tail- and actin-binding proteins talin and tensin. This is the simplistic view of the FA structural scaffold. In addition to the scaffold, the FA comprises of many different components and layers. The layers are named by their function as integrin signalling layer, force transduction layer and actin regulatory layer. (Sun, Zhiqi, Guo et al. 2016)

Besides physical force-transmitting, the FA also acts as a signalling hub. During the course of FA formation, maturation and growth, multiple proteins are recruited. The main force transmitting components do not have enzymatic activity, but there are many kinases, phosphatases, GTPases, adaptors and chaperones linked to the FA complex (Horton, Byron et al. 2015, Morse, Brahme et al. 2014).

The integrin-linked adhesion protein complex is called the adhesome (Zaidel-Bar, Itzkovitz et al. 2007) and it has been extensively studied using proteomics approaches (Byron, Humphries et al. 2011, Jones, Humphries et al. 2015). The adhesome consists

of all the proteins associated directly or indirectly with integrins at the plasma membrane in a variety of cell types on different ECMs and might include many contexts depended members. Furthermore, the isolated adhesome protein samples consists of all the proteins interacting with ECM trough plasma membrane and some of the components detected might not be part of the FA complexes (Jones, M. C., Humphries et al. 2015). There are thousands of proteins linked to integrin-mediated adhesion. The consensus adhesome, a curated list of adhesome components identified in multiple proteomic datasets from several differentiated cell types and isolated with different techniques, includes 60 components (Horton, Byron et al. 2015). The adhesome of pluripotent stem cell has not been studied.

Most of the non-transformed cells rely on their ECM adhesion and adhesion coupled forces to accurately align the mitotic spindles and for the cytokinesis to take place effectively (Chen, Y., Sibarita et al. 2005, Fink, Carpi et al. 2011). Before the entry to mitosis the FAs are however rapidly disassembled in most cell types. During the mitotic rounding up the cells are attached to the ECM via thin actin retraction fibres, which the daughter cells use as a template for the reattachment post mitosis (Mitchison, T. J. 1992, Fink, Carpi et al. 2011). Recently it was discovered that the FA size is dependent on the phase of the cell cycle and regulated by cyclin-dependent kinase 1 (CDK1) (Jones, Matthew C., Askari et al. 2018). The FAs are grown from G1- to S-phase where the maximal size is reached. Again, the size of FAs starts to decrease upon G2-phase and disassembled completely upon mitosis. Coinciding changes in cellular traction forces were detected during cell cycle (Vianay, Senger et al. 2018). Interestingly, another report showcases that the active β1 and αV integrins are still present at the ends of retraction fibres during mitosis even though the FA components such as paxillin, talin and zyxin are removed from the FAs (Dix, Matthews et al. 2018). Indeed, these linkages are needed for mitotic cell divisions in non-transformed cells. HPSCs have a shortened cell cycle as mentioned before. Especially the G1-phase is shortened and most of the cells in population are in S-phase (Becker, Ghule et al. 2006). In addition, the pluripotent cells are expressing high levels of CDK1 and CDK2 (Becker, Ghule et al. 2006, Neganova, Zhang et al. 2009). How the abnormal cell cycle of hPSCs and FAs are coupled in hPSCs remains to be studied further.

Recently a new class of adhesions named “reticular adhesions” has emerged (Lock, Jones et al. 2018). This new class of adhesions is closely linked to clathrin lattices present at sites of frustrated endocytosis (Baschieri, Dayot et al. 2018). Reticular adhesions are speculated to be involved in cell attachment during mitosis where the conventional FAs are disassembled, but the full picture of their biological role is still under debate (Lock, Baschieri et al. 2019). Flat clathrin-mediated adhesions have also

been described in cells adhering to collagen fibrils in 3D (Elkhatib, Bresteau et al. 2017).

In close proximity of FAs, there are proteins interacting directly with the FA scaffold. A good example is the Kank family proteins kank1 and kank2 that bind directly to the talin rod domain via their KN-domain. In the context of FAs, Kanks function as a mechanical link between the talin and microtubule ends. (Yu, M., Le et al. 2019). Kank binds to talin at the peripheral rim of FA and competes with the actin-binding site on talin thus reducing the force exerted on talin (Sun, Z., Tseng et al. 2016). Depletion of kank1 leads to dissociation of the microtubule tips from FAs (Bouchet, Gough et al. 2016). Kank2 is also known to increase the slipping of the integrin-mediated adhesion and thus to modulate the speed of migration (Sun, Z., Tseng et al. 2016). However, the function of kanks might be context-dependent since depletion of either kank1 or 2 has been shown to trigger local release of RhoA effector GEF-H1 and increasing myosin II activation leading to augmentation of FAs which has not been described in other reports (Rafiq, Nishimura et al. 2019).

In the context of mouse model systems, the loss of integrin β1, paxillin, talin, vinculin or FAK have been shown to be embryonically lethal showcasing the role of adhesion components during development (Stephens, Sutherland et al. 1995, Zaidel-Bar, Itzkovitz et al. 2007, Monkley, Zhou et al. 2000, Xu, W., Baribault et al. 1998). Recent multicellular *in vitro* models of developing mouse embryo have demonstrated that integrin β1 signalling is essential for epiblast cell polarisation and rosetta formation needed for lumenogenesis (Christodoulou, Kyprianou et al. 2018, Bedzhov, Zernicka-Goetz 2014). The role of integrin β1 and other adhesome components in human embryo remains to be studied.

2.2.3 FA signalling

Besides providing a mechanical anchoring point for the cells, the FAs operate as a signalling platform broadcasting extracellular information internally and regulating biochemical signalling cascades which control gene expression, cell proliferation and survival. FAs have been shown to coincide with a high amount of tyrosine phosphorylation (Nobis, McGhee et al. 2013). As described, before the adhesome contains proteins with enzymatic activity such as GTPases and kinases (Humphries, Chastney et al. 2019).

Focal adhesion kinase (FAK) is one of the most upstream kinases in the integrin signalling network. FAK is known to be recruited at the sites of integrin-mediated adhesion among the first proteins (Miyamoto, Teramoto et al. 1995). FAK has been

shown to bind to the integrin alpha tail via paxillin (Harburger, Calderwood 2009) and recruit talin to the newly formed adhesions (Lawson, Lim et al. 2012). Also, direct binding of the FAK to the integrin β-tail mimicking peptides has been reported (Schaller, Otey et al. 1995). However, the general consensus in the field is that FAK recruitment predominantly occurs through other FA components and not directly through the integrins (Legate, Fässler 2009). Upon auto-phosphorylation of tyrosine residue 397, FAK is able to interact with Src kinase which then further phosphorylates all other FAK tyrosine residues. Src binds to FAK directly, and together they are responsible for most of the signalling events originating from the FAs (Schaller, Hildebrand et al. 1994). For example, paxillin and p130Cas harbour a residue sensitive to FAK/Src activity and are recruited to the adhesions by them (Mitra, Schlaepfer 2006, Vitillo, Loriana, Kimber 2017). Upon depletion of FAK, FA turnover has been shown to be reduced leading to a decrease of migration and proliferation (Ilić, Furuta et al. 1995, Whitmore, Turner et al. 2004). The depletion of FAK also leads to reduced cell survival trough anoikis (Steven M. Frisch, Kristiina Vuori et al. 1996, Alanko, Mai et al. 2015).

Two canonical cascades originating from FAs are Ras-MAPK-Erk1/2- and PI3K/AKT- signalling pathways. The first one is implicated as a regulator of cell cycle and proliferation and the latter connected to the promotion of cell survival. (Harburger, Calderwood 2009)

Two FA linked proteins shown to be important for human pluripotency are FAK and integrin-linked kinase (ILK). FAK and ILK contribute mainly to activation of PI3K/AKT pathway preventing the apoptosis and maintaining the pluripotency status (Armstrong, Hughes et al. 2006, Hossini, Quast et al. 2016, Wrighton, Klim et al. 2014). Surprisingly, one study states that FAK signalling is not activated in hESCs while pluripotent (Villa-Diaz, Kim et al. 2016). They also observe a rapid reduction of OCT4 levels upon force activation of integrins with Manganese (Mn2+) or integrin β1 activating antibody. They also did not observe the presence of FAs in their normal culture conditions. However, the FAs also emerged upon integrin activation coupled to differentiation (Villa-Diaz, Kim et al. 2016). In contrasts, another report highlights the role of FAK in pluripotency maintenance and cell survival by promoting cell adhesion (Vitillo, Loriana, Baxter et al. 2016).

2.2.4 FA architecture and mechanosensing

The study of the three-dimensional organisation of the FA architecture has not been feasible for a long time since the FA protein complex is flat and not higher than a few hundred nanometres. Even though the horizontal resolution of the light microscopy

has improved considerably the substantial improvement in the vertical resolution has been relatively recent (Schermelleh, Ferrand et al. 2019). The emergence of 3D super-resolution microscopy (iPALM) made it possible to study the nanoscale architecture of the FA scaffold in detail (Shtengel, Galbraith et al. 2009). With iPALM it is possible to reach sub 20 nm resolution in horizontal and vertical axis. FAs are located to the plasma membrane linking the ECM to actin cytoskeleton. Different components of the scaffold are organised in distinct layers starting closest to the plasma membrane and ending up in the intersection with the cytoskeleton. The organisation of each component is found to be highly consistent throughout the across many cell types. The FA scaffold is divided into three layers which are named based on the function of its constituents (Figure 5.). The first layer comprises of the FA signalling components and important adaptor proteins paxillin and FAK. Also, integrin tails are included in this layer. The integrin signalling layer spans from the plasma membrane to approximately 50 nm above the ECM. The second layer is called the force transduction layer based on its function. This layer contains talin and vinculin, the mechanosensitive and force transmitting elements of FA scaffold ranging from ~50 nm to the actin engagement at ~ 90nm. The third and highest layer is called actin regulatory layer which consists of actin and actin modulators α-actinin, zyxin and vasodilator-stimulated phosphoprotein (VASP). (Kanchanawong, Shtengel et al. 2010)

The organisation of FA architecture has not been studied in human pluripotent stem cells. However, a recent report illustrates the conserved stratification of FA scaffold in mouse embryonic stem cells. The integrin signalling layer, force transmission layer and actin regulatory layer were all identified from the cells. Interestingly, the authors had studied the molecular architecture in cells plated on different substrates and noticed a peculiar difference on laminin linked adhesions compared to FA on fibronectin and gelatin. The mechanosensitive force enhancer vinculin was oriented head above tail, which opposes the conventional model where the vinculin C-

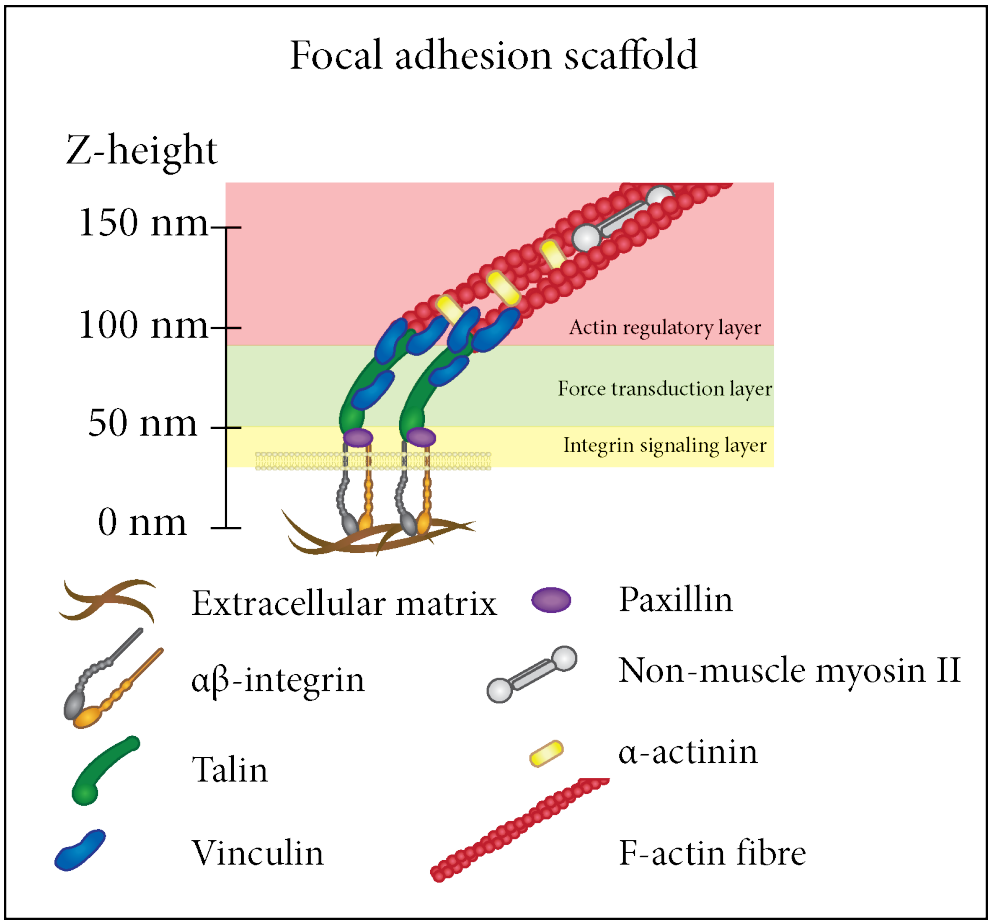

Figure 5. *Simplistic overview of focal adhesion scaffold. Adapted from (Isomursu, Lerche et al. 2019, Kanchanawong, Shtengel et al. 2010)*

terminal head is binding to actin and N- terminal tail is connected to talin. Authors suggest that vinculin might be a variable component of the molecular clutch even though evidence for the mechanism remains insufficient. (Xia, Yim et al. 2019)

FAs and actin are mechanically linked together. The interaction happens through multiple adaptor proteins linking the integrin tails to the actin cytoskeleton. FAs regulate the actin cytoskeleton through Rho-family small GTPases. FA linked tyrosine phosphorylation drives GEF activation and stimulates RhoA, which then drives myosin II activity subsequently driving the FA assembly (Guilluy, Swaminathan et al. 2011). The mechanical link between the ECM bound integrin tails and the force-producing actin cytoskeleton is generally referred to as a molecular clutch. The molecular clutch model was initially prosed as a model coupling the actin generated forces to ECM and named by its mechanical counterpart (Mitchison, T., Kirschner 1988). Integrins do not directly bind to actin and therefore intermediates in between are needed. The clutch components bind to integrin cytoplasmic tails and/or actin engaging the forces from actin retrograde flow and myosin II contraction to ECM (Sun, Guo et al. 2016). The most noteworthy clutch components are talin (Klapholz, Brown 2017) and vinculin (Atherton, Stutchbury et al. 2015), but also kindlins (Bledzka, Bialkowska et al. 2016) and α-actinin (Roca-Cusachs, Rio et al. 2013) can be considered acting a part in the machinery. When the clutch is not engaged, the traction forces are not exerted to the ECM and vice versa. The engagement of the clutch leads to cell movement by pulling from the integrin-mediated adhesions and by pushing the plasma membrane form cell leading edge (Case, Waterman 2015). The molecular clutch is capable of sensing different stiffnesses of the ECM and adapt to them (Elosegui-Artola, Oria et al. 2016). On a soft surface, lower forces are employed compared to the stiff environments. The discrepancy is caused by faster mechanical loading rates on talin which opens binding sites for clutch reinforcing vinculin whereas on soft ECM mechanical loading is slower, talin remains unopened and not reinforced by vinculin. This is due to the dynamics of the clutch components which are dependent on the force load and rupture of the mechanical bonds as was proposed in initial *in silico* modelling experiments (Chan, Odde 2008).

Mechanotransduction is an event where the extracellular mechanobiological conditions are transferred to biochemical signalling. One of the major hubs for initiating mechanotransduction are FAs (Sun, Zhiqi, Guo et al. 2016, Kechagia, Ivaska et al. 2019). The fact that cells respond to forces has been evident for years. It is also known that FAs respond to forces and have structural components that are sensitive to changes in forces. FAs tend to be more abundant on rigid surfaces and are diminished when interacting with soft surfaces (Wozniak, Desai et al. 2003). However, the size of FAs cannot be considered as a direct reflection of traction force exerted to the adhesion site (Burridge, Guilluy 2016).

Upon initiation of integrin clustering, paxillin is one of the first components recruited to the adhesion site, and it is recruited through FAK-Src mediated phosphorylation (Hu, Lu et al. 2014). Paxillin is considered as scaffold and adaptor protein and it also participates in many signalling events by binding a multitude of adhesome components (Turner 2001). Physically, paxillin is located with FAK and integrin tails in close proximity of the cell membrane in a layer called integrin signalling layer (Kanchanawong, Shtengel et al. 2010).

Talin is a key mechanosensitive component of the FA scaffold (Klapholz, Brown 2017). Talins 1 and 2 share close homology and they are known to have compensatory effects upon depletion of one. Talin is the main physical link between the integrin tails and actin cytoskeleton (Sun, Zhiqi, Guo et al. 2016). It is considered to be the core structure of the force transduction layer spanning from integrin tails to the FA and cytoskeleton interface (Kanchanawong, Shtengel et al. 2010). Mutation in talin cause reorganisation of FA architecture which shows that the scaffold is dependent on talin. Talin is a large protein (270 kDa) and in the FA scaffold it has been shown to be approximately 97 nm long and have an incline of 15° from C- to N- terminus (Liu, J., Wang et al. 2015). In addition to the mechanical support, talin acts as an inside out activator of integrins and increases the ECM binding affinity (Anthis, Wegener et al. 2009). Talin is known to bind many adhesome components, and throughout the protein, there are 11 vinculin binding sites, two integrin binding sites and three actin-binding sites among many others (Sun, Zhiqi, Guo et al. 2016). Upon mechanical tension, talin is known to act as a spring opening various places enabling interaction with vinculin (del Rio, Perez-Jimenez et al. 2009, Yao, Goult et al. 2016). Talin is unfolded from its autoinhibitory conformation upon binding to integrin tails and making contacts with the plasma membrane negatively charged phospholipids. The unfolding might help to recruit additional integrins to form FAs utilising the additional integrin binding sites. (Klapholz, Brown 2017)

Vinculin is known to be important for many FA functions and it reinforces the link between FA scaffold and actin cytoskeleton (Carisey, Ballestrem 2011, Thievessen, Thompson et al. 2013). Talin, paxillin, F-actin and contractile forces are needed for recruitment of vinculin to FA (Carisey, Tsang et al. 2013, Pasapera, Schneider et al. 2010). In some systems, vinculin depletion leads to increased cellular migration because of amplified FAK and paxillin phosphorylation (Mierke, Kollmannsberger et al. 2010). Vinculin has 19 different direct binding partners and in context of FA it is known to interact with talin, actin, VASP, paxillin, vinexin, membrane phospholipid PIP2, and α actinin (Carisey, Ballestrem 2011). Vinculin displays high affinity for autoinhibition where the head and tail domains bind together inhibiting the function of the protein (Ziegler, Liddington et al. 2006). It is thought so that

vinculin activation happens most probably when two factors bind the protein in combination since the autoinhibitory affinity between the head- and tail-domain is so strong (Carisey, Ballestrem 2011). Furthermore, some of the protein binding sites in vinculin overlap making it impossible to bind PIP2 and F-actin simultaneously (Steimle, Hoffert et al. 1999). In the context of FA architecture vinculin starts in autoinhibited conformation close to the plasma membrane by binding to phosphorylated paxillin (Zaidel-Bar, Milo et al. 2007). The talin binding is required to activate vinculin and the activated form locates to the force transduction layer. Finally, the force build-up and the maturation of FA leads to higher localisation of vinculin to actin regulatory layer respectively (Case, Baird et al. 2015). Vinculin is thought to stabilise the Talin "spring" locking it to extended conformation (Yao, Goult et al. 2016). The orientation of vinculin is thought to be C- terminal tail domain above the N- terminal head (Case, Baird et al. 2015). This model would support the idea of extended vinculin molecule binding to talin and F-actin in mature FA.

The actin regulatory layer consists of barbed end F-actin bundles connected to the FA, actin-bundling and adaptor proteins like VASP, α actinin and zyxin (Kanchanawong, Shtengel et al. 2010). VASP and zyxin have more transient interaction with actin (Yoshigi, Hoffman et al. 2005), whereas the α actinin constitutes for more formative crosslinking action (Sjöblom, Salmazo et al. 2008).

FAs are organised into functionally different vertical nanodomains. However, studying how the organisation affects cell behaviour remains to be complicated. This is mainly because of the technical limitations of 3D super-resolution microscopy. For example, live imaging experiments are hard to conduct since the acquisition time of the images is long whereas the nature of the FA complex is highly dynamic. Furthermore, perturbing the nanodomains without disturbing the overall scaffold is hard but can be achieved by using talin mutant constructs (Liu, Wang et al. 2015). Talin is one of the key regulators of the FA architecture. The clearest example of how the three-dimensional organisation plays a role in cell function is the spatiotemporal localisation of vinculin (Case, Baird et al. 2015). As mentioned before, vinculin can bind many FA scaffold components. When vinculin is located at the lowest integrin signalling layer it is most likely to be recruited by phosphorylated paxillin in an autoinhibited conformation. Once the force load grows and the FA matures the force-sensitive talin "spring" opens and enables vinculin binding to higher z-planes which further enforces the mechanical linkage. Thus, the position of vinculin in the FA scaffold plays a role in its function in reinforcing the mechanical linkage. In addition to vertical stratification some FA components are organised into horizontal nanodomains. Integrin β1 and β3 have distinct nanoscale organisation inside

individual FA (Rossier, Octeau et al. 2012). The engaged β3 is less dynamic while β1 moves rearwards with the actin flow which means that transmitting actin motion to ECM might happen via specific integrin units inside of FAs. Integrin β1 has also been shown to spatially segregate into nanoclusters based on the activity (Spiess, Hernandez-Varas et al. 2018). This implies that there might be a coordinated mechanism for integrin activation that would take place in a synchronized manner inside the nanoclusters.

FAs are the main mechanosensors of the cells. Indeed, the mechanobiological conditions seem to have an effect also on pluripotent stem cell differentiation. On stiff polyacrylamide gels increased integrin β1 activity driven Src activation inhibits priming to mesodermal differentiation, whereas the decreased Src activity on soft surfaces enables more rapid mesodermal differentiation (Przybyla, Lakins, and Weaver 2016). Furthermore, Src-dependent protection of pluripotent stage is highlighted by a study reporting that transient depletion of Scr leads to more efficient differentiation to ecto- meso- and endodermal lineages (Chetty, Engquist et al. 2015).

2.3 Optical microscopy

2.3.1 Conventional light microscopy

Optical microscopy often called light microscopy is a method of visualising small objects using a set of lenses and mirrors to capture a picture of visible light from the sample. A simple microscope could be considered to be one magnifying lens such as magnifying glass, but the microscopes used in modern biological research are much more complicated consisting of at least a light source, objective, condenser and detector or ocular.

Resolution depicts the ability to separate two objects close to each other. Resolution of a standard light microscope is in optimal case limited to 200 nm laterally and to 600 nm axially, but in practice, the resolution is also dependent on other factors affecting the optimal travel of light which might be irregularities in materials used and slight misalignment of light paths. However, the theoretical limit is due to the diffraction limit, which arises from the wavelength of visible light. The limitations in the resolution of optical microscopes were first proposed by Ernest Abbe in 1873. Systems superseding the Abbe's theoretical limits are considered to be super-resolution (SR) microscopes.

Widely used fluorescence microscopy modalities in biological research today are widefield (WF) microscopy, confocal microscopy and total internal reflection microscopy (TIRF). Each modality comes with its advantages and disadvantages. Widefield microscope illuminates the whole field of a sample using either normal visible light or a more specific wavelength of light emitted by modern light-emitting diodes (LEDs). These microscopes are considered to be easy to use, and they are relatively cheap to purchase. Widefield images can be enhanced *in silico* by implementing deconvolution or fluctuation-based enhancements (Dertinger, Colyer et al. 2009, Gustafsson, Culley et al. 2016, Schermelleh, Ferrand et al. 2019). This microscopy modality is best suited on thin samples, in movement or with dim fluorescence. Widefield microscopy is not well suited in the cases where the sample is thick or when the excellent axial resolution or 3D reconstructions are needed. (Sanderson, Smith et al. 2014)

Confocal microscope utilises a component called pinhole to filter out scattered light and therefore improving the resolution considerably. Microscopes are equipped with lasers exiting only a small portion (point illumination) of the sample, which intensifies the emitting signal and decreases the out-of-focus light gathered by the camera or detector (Thorn 2016). Confocal microscopes are often equipped with laser scanning or spinning disk unit. Confocal microscope with spinning disk unit is well suited for live imaging or rapid 3D optical sectioning (Fischer, Wu et al. 2011), whereas the laser scanning unit yields the best achievable resolution of confocal microscopy by doing multiple scans per pixel unit. (Sanderson, Smith et al. 2014)

Total internal reflection (TIRF) microscopy is best suited to observe structures on the cell membrane or near to it (Axelrod 1981). TIRF microscopy takes advantage of the steep gap in refractive indexes of glass and water to generate a thin excitation field near to the glass. The depth of the excitation field or evanescence field can be as thin as 100 nm giving the TIRF excellent axial resolution. TIRF can be used to track single fluorescent molecules near the cell membrane and the excitation method also considerably lowers the phototoxicity experienced by the samples. (Fish 2009)

2.3.2 Super-resolution microscopy

The concept of super-resolution microscopy incorporates the microscopy modalities surpassing the theoretical resolution limit described by Abbe. For the past decades, scientists have tried successfully to overcome this limit in the area of optical microscopy using different approaches. Today such SR modalities can be acquired commercially, but they are still considered expensive and complicated to use.

Current SR modalities are based on TIRF-, WF or confocal setups. The most noteworthy commercially available SR techniques to this date are Stimulated Emission Depletion microscopy (STED), Structured Illumination Microscopy (SIM), and modalities of Single-Molecule Localization Microscopies (SMLM). Also, there are new *in silico*-based methods that can be used for surpassing the 200 nm resolution limit by using radial fluctuation-based image enhancement algorithms. (Schermelleh, Ferrand et al. 2019)

SMLM uses excitation laser to either activate or switch off dyes for an extended period to acquire data from a small group of molecules at a time. The events are collected during a long timespan by taking thousands of individual images. The data from localisations of individual molecules are merged creating a binary super-resolved image of the imaging plane (Small, Stahlheber 2014). SMLM techniques can be implemented by using camera-based WF setups. The resolution of SMLM methods is highly dependent on sample labelling, but in good samples, up to 20 nm lateral and 50 nm axial resolution can be achieved in optimal cases. The different SMLM techniques differ in their approach to achieve the blinking of the dyes. (Schermelleh, Ferrand et al. 2019)

Interferometric Photoactivated Localisation Microscopy (iPALM) is a SMLM technique capable of enhancing the vertical resolution even further down to sub 20 nm level (Betzig, Patterson et al. 2006, Shtengel, Galbraith et al. 2009). IPALM can provide 3D information of ultrastructures of cellular protein complexes or cytoskeleton close to cell membrane. The vertical resolution improvement was gained by using two separate light paths were the emitted photons simultaneously travels. The paths are subsequently connected together allowing interference of the photon with itself. The difference in path length of the photon directly reflects the position of the emitter. iPALM provides quantitative data of 3D localisations of photoactivatable molecules, and it is well suited for observing the nanoscale structure of plasma membrane, cell-ECM adhesions and cell cytoskeleton (Kanchanawong, Shtengel et al. 2010, Xia, Yim et al. 2019).

In STED microscopy the excitation beam is overlaid with a doughnut-shaped depletion laser. The doughnut-shaped depletion laser inhibits the emission from the edges leaving the doughnut hole sized emission spot (Hell, Wichmann 1994, Klar, Jakobs et al. 2000). 50 nm resolution can be achieved with a finely aligned system in an optimal situation. STED uses high power lasers to achieve SR quality, and special dyes are needed for ideal performance. (Schermelleh, Ferrand et al. 2019)

SIM microscope is a WF based system that illuminates samples with a striped pattern where the distance between the stripes is close to the resolution limit (Heintzmann, Cremer 1999). Patterns are then rotated and phase-shifted while multiple images are taken. Otherwise, unresolvable sample features are enhanced by interference. Raw images are then decoded and reconstructed to generate a super-resolved image. SIM is able to reach a resolution of 100 nm laterally and 300 nm axially. This can be further enhanced by for example using ultra-high numerical aperture objective and TIRF lasers. SIM can be considered a relatively gentle SR modality and can be used for live imaging samples. (Schermelleh, Ferrand et al. 2019)

Recently, fluctuation-based analysis methods have opened a new convenient way to access SR imaging. Super-Resolution Radial Fluctuations (SRRF) (Gustafsson, Culley et al. 2016) and Super-Resolution Optical Imaging (SOFI) (Dertinger, Colyer et al. 2009) are both methods that can be employed to enhance imaging dataset generated by normal camera-based WF, TIRF or confocal microscopes. Both in SRRF and SOFI, tens or hundreds of images from the same position from the fluorescent sample are taken. Algorithms are based on detecting the temporal fluctuations between the pixels and implementing high-order statistical analysis to reconstruct SR image. Fluctuation based methods have reduced levels of phototoxicity compared to other SR modalities and are well suited well for live imaging of cells (Geissbuehler, Sharipov et al. 2014, Culley, Tosheva et al. 2018).

All the mentioned SR techniques use deconvolution or other heavy computational processing to achieve super-resolved images. Furthermore, the modalities utilising complicated optical setups are susceptible to misalignments in the optical path (Wegel, Göhler et al. 2016). For these reasons, there is a high possibility for artefacts in SR images which in the worst case leads to misinterpretation of biological data. In an optimal situation, the quality of each SR image should be ensured by imaging the same sample with a microscopy modality with even better resolution, but this is often impossible. A recently introduced method provides a software-based approach quantitatively estimating the quality of SR images. (Culley, Albrecht et al. 2018)

2.3.3 Traction force microscopy

Cells exert forces on their environment. Cellular forces are derived from actin treadmilling and myosin II-dependent actomyosin contraction. The forces are transmitted to the extracellular matrix through the FA scaffold. Currently, we know that cells use physical forces for sensing their environment and in response modulate their behaviour accordingly (Kechagia, Ivaska et al. 2019). Cells direct their migration (Sunyer, Conte et al. 2016), morphogenesis (Bosveld, Bonnet et al. 2012)

and initiate transcriptional programs (Farge 2003) based on the force probing of their environment. Also, mesenchymal stem cells are known to select a path of differentiation according to their environmental mechanical conditions (Isomursu, Lerche et al. 2019). Furthermore, in malignant conditions cancer cells respond to changed mechanical cues (Lee, Han et al. 2019). Recent evidence showcases that the mechanisms by which cells mediate their responses are based on the cross-talk between the biochemical signalling and cell mechanics (Schwarz, Gardel 2012). Therefore, understanding the cell mechanics in addition to the biochemistry is important for generating the full picture of the cell behaviour. Consequently, there is a demand for different tools for measuring forces on cellular level on many spatiotemporal accuracies.

The direct measurement of the cellular forces is challenging and can currently only be achieved at the level of single molecules using, for example, DNA-based force probes (Blakely, Dumelin et al. 2014) or for discrete cell adhesion receptor patches by pulling with ECM-coated AFM cantilevers (Taubenberger, Hutmacher et al. 2014, Bufi, Durand-Smet et al. 2015) or beads (with optical/magnetic tweezers) (Sugimura, Lenne et al. 2016). However, to evaluate forces in the cell scale, we can observe deformations of an elastic material such as poly acryl amide (PAA) gel. Traction force microscopy (TFM) is the earliest method for measuring forces at the cell level. In a TFM experiment, cells are usually plated on hydrogels, with known elastic moduli, embedded with fluorescent beads. Using fluorescence microscopy, an image of the beads is taken while under traction stress implemented by the cells (Figure 6.). Subsequently another image of the gel in a relaxed stage is taken after detaching the cells. The movement of individual beads is tracked using an *in silico* algorithm to generate so-called displacement maps which reflect directly the movement of the beads. (Roca-Cusachs, Conte et al. 2017) The displacement map can then be converted into traction stress (force/area) by utilising a proper mathematical framework like Fourier Transformation Traction Cytometry (FTTC) (Tolic-Norrelykke, Fabry et al. 2002) and analysis algorithm.

TFM suffers from poor sampling density trough the optical limits of conventional microscopes. Most often TFM experiments are performed with confocal microscopes. In other words, it is impossible to track beads over smaller distances than the resolution allows. Usually, forces applied by the cells are in ranges of a few nano newtons and conventional TFM is best suited to observe forces in micro-scale. The TFM analysis algorithms have been extensively developed significantly improving conventional TFM accuracy and sensitivity (Schwarz, Balaban et al. 2002, Soiné, Brand et al. 2015, Han, Oak et al. 2015). However, the new analysis methods are computationally heavy and need a high degree of programming capability from

the user and the algorithms make assumptions based on the localisation of either F-actin bundles or FAs. Also, there are fundamental limits of improving the TFM quality by applying more elaborate analysis methods, if the images lack sufficient information of deformations (Roca-Cusachs, Conte et al. 2017).

The accuracy and sensitivity of TFM can also be improved by increasing the number of trackable beads either by using a more powerful microscopy modality or either by using two separate colours of the beads and merging together the displacement vectors. The first alternative was successfully done by using STED microscope for the TFM imaging (Colin-York, H., Shrestha et al. 2016) and the latter by implementing a complicated physics approach to sum together the displacement vectors (Plotnikov, Sabass et al. 2014). Both attempts significantly improved the conventional TFM method. However, access to super-resolution microscopes or implementation of complicated physics makes the improved methods hard to access for normal cell biology laboratories.

In addition to measuring forces in 2D surfaces, TFM can be performed by measuring the displacements in 3D on a 2D surface (often referred as 2.5D) using the same principles as described before (Hur, Zhao et al. 2009, Toyjanova, Bar-Kochba et al. 2014). Also, the 2.5D TFM was recently reinvented by using 3D SIM to observe deformations in x,y and z directions (Colin-York, Huw, Javanmardi et al. 2019).

TFM remains to be the go-to method for measuring mechanical forces exerted by the cells because of the straightforwardness and accessibility of hard- and software. However, this is only the case with the simplest application of TFM. Because of the recent advancements in the area of mechanobiology, there is a clear demand for easy access methods that can measure traction forces at the FA level.

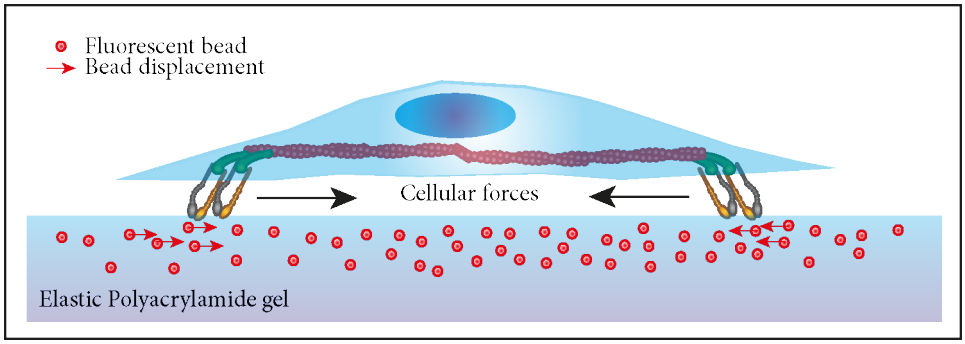

Figure 6. *Schematic illustration of traction force microscopy.*

3 Aims

Human pluripotent stem cells hold great promise for future medicine. Their capability to differentiate and self-renew are characteristics that would be valuable for tissue generation and disease modelling. However, the field of regenerative medicine is facing obstacles in issues related to safety and reasonable efficiency for large scale medical purposes. Many of the obstacles can be directly linked to the fact that we do not know in detail what are all the cues affecting the lineage specification of differentiating stem cells. Cell-ECM adhesion and the possible signalling related to the cell-ECM contacts has been insufficiently studied in the context of human pluripotent stem cells even though we know that mechanical qualities of the ECM have an effect on lineage selection in adult stem cells and cell survival in pluripotent stem cells. We still lack basic research describing a key property of the cells: to adhere and sense their surroundings. FAs are structures mediating the physical contact between the cellular cytoskeleton and the ECM. Through FAs, cells "sense" the molecular composition and biomechanical properties of the ECM and broadcast the information as biochemical signalling cascades that govern the other functions of the cells. The overall purpose of this work was to characterise the structure of FAs in hPSCs and to describe how the biochemical and mechanical signalling components of FAs are positioned as part of the structural scaffold.

Specific aims:

1. Determine the adhesive and cytoskeletal features of pluripotent stem cells and how they differ from differentiated cells
2. Employ super-resolution imaging to decipher the nanoscale architecture of iPSC adhesions
3. Develop a method to detect cell traction forces with nanoscale resolution.

3.1 Aim 1 (Original publication I)

Human pluripotent stem cells grow as a tightly packed colony with low adhesive strength (Thomson 1998, Singh, A., Suri et al. 2013). Cell morphology and adhesiveness significantly differ from originator cells used for induction of pluripotency and cell adhesion have been described as a barrier for reprogramming (Qin, Diaz et al. 2014). Substantial remodelling of the adhesion qualities is therefore needed for acquiring the pluripotent stage. Still, cell-ECM adhesion is a prerequisite for human pluripotent cells to survive (Chen, K. G., Mallon et al. 2014). The purpose of this study was to define the morphological landmarks of FAs and actin cytoskeleton in hPSCs compared to originator fibroblasts. Furthermore, we wanted to see if FAs take part in signalling and force transmission. Finally, we aimed to test which features were specific for the pluripotent stage by differentiating the cells.

3.2 Aim 2 (Original publication II)

Large 'cornestone' FAs at the peripheral edge of the colony were established as a characteristic of hPSCs (Original publication I). However, the information of the dynamics, composition and structural organisation of hPSC FAs was still lacking. Recent advances in superresolution microscopy have enabled in a detailed study of the nanoscale architecture of FA scaffolds and revealed that FAs are organised in three functionally distinct layers (Kanchanawong, Shtengel et al. 2010, Case, Baird et al. 2015, Liu, J., Wang et al. 2015). The purpose of this project was to study the nanoscale molecular organisation of cornerstone FAs in hPSCs using 3D super-resolution microscopy. Also, we wanted to discover if the FAs at the centre and the edge are displaying different dynamics and do a structural disturbance of large edge FAs an effect to the pluripotency maintenance.

3.3 Aim 3 (Original publication III)

The ability of cells to exert forces to their surroundings is a central regulator of tissue modelling and morphogenesis (Heisenberg, Bellaïche 2013, Wickström, Niessen 2018). Dysfunctions of mechanobiological features of the cells and tissues can be linked to malignancies and ageing (Broders-Bondon, Nguyen Ho-Bouldoires et al. 2018, Segel, Neumann et al. 2019). Upon discovery of new mechanosensitive functions of the cells, there is a requirement for enhanced techniques to detect cellular forces with high accuracy. Traction force microscopy (TFM) can be considered as the most straightforward method for measuring forces at the cellular level (Roca-Cusachs, Conte et al. 2017). However, the classical TFM suffers from poor sampling density due to the resolution limits of standard microscopy and is thus unable to detect forces at sub-cellular level (Colin-York, Huw, Shrestha et al.

2016). The spatial resolution of TFM can be enhanced by implementing heavy mathematical algorithms or by using super-resolution microscopy (Plotnikov, Sabass et al. 2014, Colin-York, Huw, Shrestha et al. 2016). However, the methods for enhancement are not easy to access. The purpose of this study was to implement new open access fluctuation based super-resolution imaging to TFM pipeline and estimate if this super-resolution modality could enhance the spatial resolution of TFM experiments.

4 Materials and Methods

4.1 Cell culture (I, II, III)

HPSC lines were a generous gift from Professor Timo Otonkoski (University of Helsinki). HEL 11.4 (Mikkola, Toivonen et al. 2013), HEL 24.3 (Trokovic, Weltner et al. 2015) and H9 cells were grown either on human recombinant vitronectin (5µg/ml) (Life Technologies, A14700) or Matrigel (Corning, 354277) using Essential 8 (E8) (Life Technologies, A15169-01) culture medium. Culture medium was changed daily, and the cells were passaged using 0.5 mM EDTA (Life Technologies, 15575-038) for 3 minutes in room temperature. ARPE-19 retinal epithelial cells were a gift from Professor Olav Andersen (University of Aarhus) and cultured in DMEM-F12 (Life Technologies, 10565-018) supplemented with 10% fetal bovine serum (Biowest, S1860). U2OS osteosarcoma cells were acquired from DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig DE, ACC 785) and grown on either vitronectin (5µg/ml) at E8 medium (II) or on fibronectin (10µg/ml) at DMEM-F12 supplemented with 10% fetal bovine serum (III). U-251 glioma cells were a generous gift from Professor Michael Odde (University of Minnesota) on fibronectin at DMEM-F12 supplemented with 10% fetal bovine serum. All cell lines were cultured in a humidified incubator at +37°C and 5%CO_2.

Transient RNAi

To deplete the expression of kank1 and kank2 from hPSCs Accell siRNA pools were used (Dharmacon, kank1 E-012879-00-0005; kank2 E-027345-00-0005). According to the manufacturer's protocol, the siRNAs were diluted into the culture medium (final concentration 1µM). As a control non-targeting siRNA pool was used (Dharmacon, D-001910-10-20). New siRNA was added daily for 48 hours before seeding the cells on vitronectin coated culture wear for immune fluoresces or protein sample collection.

4.2 HPSC differentiation

Retinoic Acid (I)

HPSCs growing on vitronectin or matrigel were differentiated by introducing 10μM retinoic acid (Sigma, R2625) to the culture medium for 72 hours. Culture medium was changed daily.

BMP4 (II)

To initiate differentiation cell culture medium Essential 6 (Gibco, A1516401) was supplemented with morphogen BMP4 100 ng/ml BMP-4 (R&D systems, 314-BP) and subsequently differentiated up to 6 days. Samples were collected on days 1,3 and 6 for the time-series experiment. Culture medium was changed daily.

Spontaneous differentiation (II)

To initiate spontaneous differentiation cells were cultured in Essential 6 basal medium (Gibco, A1516401) on vitronectin (5μg/ml) coated surfaces (either uniform surface [normal cell culture dishes], or nanogrooved surface [Nanosurface Biomedical]). Cells were differentiated up to 6 days and samples collected on days 1, 3 and 6 for the time-series experiment. Culture medium was changed daily.

4.3 Western blotting (I, II)

Protein samples were collected in denaturing buffer and run on SDS-PAGE gels for separation. Subsequently, samples were transferred to nitrocellulose membranes. Membranes were then blocked using LI-COR blocking solution (LI-COR bioscience) for 1 hour in room temperature. Primary antibody was diluted into the blocking buffer and incubated with the membrane for overnight (+4◦C) or 2 hours (room temperature). The unbound primary antibody was washed away with TBS-Tween 20 (0.5% tween). Secondary antibodies diluted in blocking buffer were incubated with the membranes for 2 hours in room temperature. Finally, membrane washed with TBST and scanned with Odyssey infrared scanner (LI-COR Biosystems). The protein band intensities were determined using ImageJ distribution Fiji (Schindelin, Arganda-Carreras et al. 2012, Rueden, Schindelin et al. 2017). Antibodies used are listed in table 1.

4.4 Microscopy

Sample preparation (I, II)

In the original publication I, for immunofluorescence, the cells were grown of vitronectin coated culture dishes (MatTek, P35GC-1.5-14-C) for 2- 24h depending on the experiment. Samples were fixed with 4% (v/v) PFA warmed to +37°C (16% (v/v) PFA diluted in culture medium) for 15 minutes. Subsequently, the samples were permeabilised with 0.5% Triton-X-100 for 15min. Primary antibody was then diluted to 30 % horse serum and samples stained for overnight in +4°C or in room temperature for 2 hours. Subsequently, samples were washed with PBS and stained with secondary antibody for 2 hours in room temperature and again washed with PBS before imaging. Antibodies and dilutions used are presented in table 1.

In the original publication II, for immunofluorescence, the cells were grown of vitronectin coated glass-bottomed culture dishes for 20 to 24 hours prior fixation. Samples were fixed and permeabilised with 4% (v/v) PFA and 0.3 % Triton-X-100 for 15 minutes. Subsequently, samples were quenched for 10 minutes using 0.1 M Glycine in PBS. Primary antibody was then diluted to 1 % BSA (Bovine serum albumin) stained for overnight in +4°C or in room temperature for 2 hours. Next, samples were washed with PBS and stained with secondary antibody for 2 hours in room temperature and again washed with 0.2 % PBS-Tween 20 for 15 minutes, followed by a wash with PBS prior to imaging. Antibodies and dilutions used are resented in table 1 and 2. Actin dyes used are listed in table 3.

For imaging of Eos-tagged proteins, cells were transfected DNA plasmids (Table 4) using transfection reagent DNA-in (MTI-Global Stem) according to the manufacturer's protocol. The ration of DNA in micrograms and reagent in micro litters was 1:3. After 20-24 hours of transfection cells were fixed and imaged.

TIRF (I)

Carl Zeiss Laser-TIRF3 (Carl Zeiss GmbH, Jena, Germany) equipped with EMCCD camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) was used for TIRF imaging. All the TIRF imaging was performed using 63x oil immersion objective (NA 1.46, Plan Apochromat, DIC). The laser angles were adjusted according to the channel imaged. Nuclear markers were imaged from the middle plane with direct laser angle (Nanog) or using LED (Dapi).

Spinning Disk (I, II, III)

Mariana Spinning Disk microscope with a scanning unit (Yokogawa CSU-W1) installed in Zeiss Axio Observer Z1 frame was used. Images were taken with either sCMOS (Orca Flash4, Hamamatsu Photonics) or EMCCD (Evolve, Photometrics) camera using 20x air (NA 0.8, Plan Apochromat, DIC), 63x oil (NA 1.4, Plan-Apochromat), 63x water (NA 1.15 water, LD C-Apochromat) or 100x oil (NA 1.4 oil, Plan-Apochromat) objective depending on the sample.

Airyscan (II)

Zeiss LSM880 with Airyscan detector (Carl Zeiss GmbH, Jena, Germany) was used as for Airyscan imaging. 40x water (NA 1.2, Plan-Apochromat) and 63 oil (NA 1.4, Plan-Apochromat) objectives were used. Imaging was done using standard airyscan super-resolution settings.

SIM (II)

DeltaVision structured illumination microscope (OMX v4, GE Healthcare) was used for SIM imaging with structured illumination (3 rotations x 5 phases). 60x (NA 1.42, Plan-Apochroamat) with immersion oil (RI 1.514) was used. Images were taken using sCMOS (pco.edge, PCO AG) camera.

EVOSfl (I)

The colony compaction and morphology were observed using EVOSfl tabletop cell culture microscope (Advanced microscopy group) with 20x air objective and Dapi filter.

iPALM (II)

For iPALM imaging, that was carried out in HHMI Janelia Campus (Virginia, USA), the hPSCs were transfected with Eos-tagged constructs. Cells were seeded on vitronectin coated gold fiducial markers containing cell culture coverslips (Nanopartz, Inc.) (Kanchanawong, Shtengel et al. 2010). Fixed samples submerged in STORM buffer (Dempsey, Vaughan et al. 2011) were exited with 561 nm laser and photoconverted using 405 nm laser. Images (40 000-80 000) were taken and processed subsequently with Peak Detector software (HHMI Janelia Campus).

Image analysis

Microscopy images were analysed using ImageJ Fiji distribution (NIH) (Schindelin, Arganda-Carreras et al. 2012, Rueden, Schindelin et al. 2017). IPALM images were analysed using PeakSelector software (HHMI Janelia Campus) (Kanchanawong, Shtengel et al. 2010). In detailed description of the image analysis is presented in original publications (I-III).

4.5 Hydrogel preparation (I, III)

For hydrogel attachment cell culture dishes (MatTek, P35G-1.0-14-C [I], Cellvis, D35-14-1N [III]) were treated with Bind-Silane (GE Healthcare, Silane A-174) for 15 minutes and subsequently washed with absolute ethanol (95%) and mQH2O. A gel pre-mix was prepared from PBS, 40% acrylamide (Sigma, A4058) and 2% Methylenebisacrylamide (Sigma, M1533). The recipes yielding specific stiffnesses can be found from the original publication I and II. If the gels were made for traction force microscopy fluorescent beads were added to the pre-mixture (200nm beads [Life Technologies, F881]). The mixture was then vortexed and Temed 1:500 (Sigma, T9281) and 10% ammonium per sulphate 1:100 (APS) were added to initiate the polymerisation. Quickly, 11.8 µl drop was added on top of the culture glass and 13 mm coverslip was placed carefully on the drop to press the gel flat. The gels were let to polymerise for 1 hour in room temperature before carefully removing the top coverslip with forceps. The gel was then agitated in mixture of EDC (2mg/ml, Sigma, 03450) and Sulfo-SANPAH (0.2 mg/ml, Thermo Scientific, 22589) diluted in 50nM HEPES (Sigma-Aldrich, H0887) for 30 minutes. Subsequently, the gel was activated with UV-radiation for 10 minutes in UV chamber (Jelight Company Inc., UVO CLEANER, 342-220). After three washes with PBS gels were coated with vitronectin 5µg/ml (I) or fibronectin 10µg/ml (Merck-Millipore, 341631) (II).

4.6 Traction force microscopy

In the traction force microscopy, the cells were plated on hydrogels prepared as described before. In the classical TFM the 200 nm fluorescent beads were embedded inside the gel (I). However, in the fluctuation base super-resolution (FBSR) TFM the fluorescent beads were attached to the 13 mm coverslip before casting the gel (detailed protocol described in original publication II). Fluorescent beads were imaged using confocal microscopy when the cells were attached to generate pre-image. Subsequently, cells were detached either by using trypsin or 20% SDS and the beads were imaged again to generate post-image. In the case on normal TFM individual frame was taken (I) whereas in the FBSR TFM 100 frames from the same

position were acquired. Pre- and post-images were then aligned in Fiji software and analysed using either Fiji PIV and FTTC plugins (Tseng, Duchemin-Pelletier et al. 2012) or MATLAB software (MathWorks) with TFM analysis algorithm (Betz, Koch et al. 2011, Han, Oak et al. 2015). In detail description of TFM analysis can be found from original publication III.

Table 1. Primary antibodies.

Primary antibodies						
Antigen	**Species**	**Catalog no.**	**Manufacturer**	**Application**	**Concentration**	**Original publication**
Nanog	goat	AF1997	R&D	IF/WB	1:50 / 1:5000	I, II
E-cadherin	mouse	ab1416	Abcam	IF	1:100	I, II
SSEA-1	mouse	MAB4301	Millipore	IF	1:100	I, II
α-actinin 4	rabbit	ALX-210-356-C050	Enzo	IF	1:200	I
pMLC	rabbit	3674	CST	IF	1:100	I
MYOII	rabbit	909801	Biolegend	IF	1:100	I
Active Integrin β1 (12G10)	mouse		In house production	IF	1:100	I, II
Integrin αVβ5	mouse	MAB2019Z	Millipore	IF	1:250	I, II
Total integrin β1 (K20)	mouse	sc-18887	Santa cruz	IF	1:100	I
α-tubulin	mouse		In house production	IF	1:100	I
paxillin	mouse	612405	BD	IF/WB	1:100 / 1:1000	I, II
paxillin	rabbit	sc-5574	Santa Cruz	IF	1:100	I, II
pPaxillin Y118	rabbit	2541	CST	IF	1:100	I
pSRC Y416	rabbit	2101	CST	IF/WB	1:100 / 1:1000	I
pFAK Y397	rabbit	8556	CST	IF	1:100	I
SRC	rabbit	2101S	CST	WB	1:5000	I
pFAK Y397	rabbit	8556	CST	WB	1:1000	I
FAK	mouse	610088	BD	WB	1:5000	I
vinculin	mouse	V9131	Sigma	WB	1:1000	I, II
Oct 3/4	rabbit	sc-9081	Santa cruz	IF/WB	1:100 / 1:1000	I, II
GAPDH	mouse	5G4MaB6C5	Hytest	WB	1:5000	I, II
β-actin	mouse	A1978	Sigma	WB	1:5000	II
talin (8D4)	mouse	T3287	Sigma	IF/WB	1:100 / 1:1000	II
Total integrin β1 (P5D2)	mouse		In house production	IF	1:100	II
Integrin β3	mouse	MCA728	AbD Serotec	IF	1:100	II
Integrin αV (L230)	mouse		In house production	IF	1:100	II
paxillin	mouse	sc-365379	Santa cruz	IF	1:100	II

α-actinin	mouse	A5044	Sigma	IF/WB	1:100 / 1:1000	II
kank1	rabbit	A301-882A	Bethyl	IF/WB	1:100 / 1:1000	II
kank2	rabbit	HPA015643	Sigma	WB	1:1000	II
Integrin β5	rabbit	3629	CST	IF/WB	1:100 / 1:1000	II
E-cadherin	rabbit	3195S	CST	WB	1:1000	II
Sox2	mouse	MAB2018	R&D	WB	1:1000	II

Table 2. Secondary antibodies.

Secondary antibodies					
Dye	**Species**	**Manufacturer**	**Application**	**Concentration**	**Original publication**
Alexa Fluor 488 anti-rabbit IgG	donkey	Thermo Fischer	IF	1:300	I, II
Alexa Fluor 488 anti-mouse IgG	donkey	Thermo Fischer	IF	1:300	I, II
Alexa Fluor 568 anti-rabbit IgG	donkey	Thermo Fischer	IF	1:300	I, II
Alexa Fluor 568 anti-mouse IgG	donkey	Thermo Fischer	IF	1:300	I, II
Alexa Fluor 647 anti-goat IgG	donkey	Thermo Fischer	IF	1:300	I, II
Alexa Fluor 647 anti-mouse IgG	goat	Thermo Fischer	IF	1:300	I, II

Table 3. Actin dyes.

Actin dyes				
Dye	**Manufacturer**	**Application**	**Concentration**	**Original publication**
Atto 405 phalloidin	Thermo Fischer	IF	1:300	II
Alexa Fluor 488 phalloidin	Thermo Fischer	IF	1:300	II
Atto 647N phalloidin1:200	Sigma	IF	1:200	I
Sir-Actin	Cytoskeleton Inc.	IF	1:5000	II

Table 4. DNA-plasmids.

Plasmids			
Plasmid	**Addgene number**	**Application**	**Original publication**
tdEos-Talin-18	57672	iPALM	II
tdEos-Talin-N-22	57673	iPALM	II
tdEos-Vinculin-14	57691	iPALM	II
mEos2-Vinculin-N-21	57439	iPALM	II
tdEos-Lifeact-7	54527	iPALM	II
mEos2-Actin-7	57339	iPALM	II
mEos2-Alpha-Actinin-19	57346	iPALM	II
tdEos-Paxillin-22	57653	iPALM	II

5 Results

5.1 Human pluripotent stem cell colonies are encircled by thick actin stress fibres and large cornerstone FAs (I)

5.1.1 Large FAs localise to the edges of the hPSC colonies

At the time when this project was initiated, very few studies had investigated adhesion and cytoskeletal features of iPSC. There was even a notion that pluripotent stem cells would not have FAs in their undifferentiated stage (Baxter, Camarasa et al. 2009, Vitillo, Loriana, Baxter et al. 2016). Therefore, we set out to investigate this in detail. Using high-resolution TIRF and spinning-disc confocal microscopy, it became immediately obvious that iPSC are characterized by a unique cytoskeleton. HiPSC colonies plated on vitronectin for 24 hours displayed large paxillin positive FAs located at the colony edges connected by thick F-actin bundles. In contrast, the FAs and actin in parental fibroblasts (the cells used for reprogramming to generate the hPSC) displayed remarkably different F- actin and FA morphological characteristics (I, Fig. 1A) Where the FAs in fibroblast were relatively homogenous in size and distributed uniformly, the FAs in hiPSC colonies predominantly located at the edge and were larger than the ones in colony centre. In fibroblasts, FAs were covering a characteristic area of 1.7 μm2, had coverage of 2.5% of the cell area and density 0.012/μm2. These FA characteristics were comparable to the small adhesion in hPSC colony centre (1.9 μm, 2.3%, 0.017/μm2). In contrast, the FAs at the colony were larger, covered a larger area and were more densely packed (4.2 μm2, 12.3%, 0.022/μm2) (I, Fig. 1D). We named the large FAs at the colony periphery as "cornerstone adhesions". Similar adhesions and F-actin bundles were also present in hESCs cultured on VTN and hiPSCs cultured on different ECMs (MG and LM-521) (I, Fig. S1B). These results imply that the attachment of hPSC colonies might be mediated by the colony edge rather than the centre.

To study if the large adhesions had a role beyond physical anchoring, we immunostained the colonies with antibodies recognising active FA signalling

components (Mitra, Schlaepfer 2006). The cornerstone adhesions were positive for both p-paxillin (Y118) and focal adhesion kinase pFAK (Y397) (I, Fig. S1C and 1C) suggesting a role in active signalling. Furthermore, the cornerstone FAs were assembled already 2 hours after seeding the colonies (I, Fig. S2A) and were growing in size up to 20 hours (I, Fig. S2B) while the number of FAs remained constant (I, Fig. S2B). Also, when cells were plated on hydrogel surfaces with varying stiffness's or glass (3kPa, 10 kPa, 30kPa and glass) the FA size and number reduced in the function of stiffness (I, S2D). This indicates that the FAs in human pluripotent stem cells are assembled close after the cell-ECM interaction and that they are sensitive for mechanobiological conditions.

5.1.2 Thick ventral stress fibres circle the hPSC colony

Ventral stress fibres (VSFs) can be characterised by the presence of actin cross-linker α-actinin and force-generating motor protein myosin II (Naumanen, Lappalainen et al. 2008). Immunofluorescence staining showed that the thick F-actin bundles at the colony periphery were positive for with both of these markers. This indicates that the colony circling F-actin bundles are contractile VSFs. Remarkably, the actin fibres seemed to be linked by E-cadherin mediated cell-cell junctions (I, Fig 1B) as well as cornerstone adhesion. Given that E-cadherin, as well as ECM adhesions, are essential for hPSCs survival (Li, L., Bennett et al. 2012), these data suggest that coupling of both adhesion systems to VSFs could play an important role for hPSC biology.

Next, we decided to study the mechanical forces exerted by the colony by using traction force microscopy, as large FAs have previously been linked to high traction stress. We seeded the cell colonies on VTN-coated 10 kPa hydrogel to obtain an optimal balance between gel deformation capability and FA formation. The mechanical forces in hPSCs located mainly to the edges of the colony and were present at the ends of VSFs directed parallel to the colony edge (I, Fig 2D). This implies that the forces might help to maintain the colony compaction and that the traction stresses are mainly exerted trough cornerstone FAs. It also seems that the cells in the colony centre exist in different mechanical "niche" compared to the ones at the colony edge.

Inhibition of myosin heavy chain ATP binding (Blebbistatin) or Rho kinase (ROCKi, Y-27632) leads to a decrease in actomyosin contraction and subsequent disassembly of FAs (Katoh, Kano et al. 2010). The treatment of hiPSCs with either of the inhibitors resulted in rapid loss VSFs circling the colonies and cornerstone FAs as anticipated (I, Fig. 3A, S3A). Also, both inhibitor treatments increase of area covered

by an individual cell as a part of the colony (I, Fig. 3C, 3D). These results further show that cornerstone FAs are dependent on forces originating from actomyosin contraction that controls colony compaction.

Next, we sought out possible functional roles for the contractile actin ring. From our multiple microscopy images, it was obvious that mitotic cells at the colony edge were always associated with a localised disruption of the VSFs. To study this in more detail, we quantified the orientation of mitotic spindles from colony edge and centre. Cells dividing in the colony periphery had their mitotic spindles predominantly oriented parallel to colony edge such that a dividing edge cell would give rise to two daughter cells (and not and edge and a colony centre cell). In contrast, at the colony centre, the orientation of mitotic spindles was random. Introduction of blebbistatin to the culture medium randomised the orientations also at the colony edge (I, Fig. 3F). These data indicate that the orientation of mitotic spindles is dependent on the actomyosin mediated forces or/and the FAs. The orientation of mitotic spindles at the colony edge might also help cells maintain their identity as edge cells.

5.1.3 Cornerstone adhesions participate in active signalling

hPSCs express several integrins on the protein level. The most prominent ones are α3, α5, α6, αv, β1, and β5 (Meng, Eshghi et al. 2010, Rowland, Miller et al. 2010, Vuoristo, Virtanen et al. 2009, Xu, C., Inokuma et al. 2001). Since integrin heterodimer αvβ5 and integrin β1 are known to be important for hPSCs adhesion and proliferation on vitronectin coated surfaces (Braam, Zeinstra et al. 2008, Rowland, Miller et al. 2010), we decided to stain them with antibodies for imaging. We found that integrin αvβ5 closely overlapped with the paxillin positive cornerstone FAs (I, Fig 3G). Also, integrin β1 could be detected at colony edges (I, Fig 3H). These results indicate that both integrins might play a role as initiators of FA signalling in hiPSCs.

To further study the signalling cascade, we set out to measure on the protein level, which signalling components are expressed and active in hiPSCs (I, Fig. 3I, S3D). Clear tyrosine 397 phosphorylation of FAK and tyrosine 416 phosphorylation of Src was detected. However, compared to the originator fibroblasts, only phosphorylation of Src was proportionally higher in hPSC implying the importance of Src activity for hPSCs. Furthermore, pSrc was high in hiPSC compared to fibroblast FAs (I, Fig. 3J). To investigate the functional significance of these adhesion linked kinases, we treated the cells with FAK- and Src family kinase inhibitor (I, Fig S3E, S3F, S3G). Both inhibitor treatments resulted in the rapid detachment of the

cells. These experiments suggest that hiPSCs have active FA signalling which is needed for the cell-ECM attachment.

5.1.4 Differentiation of hPSC alters the adhesion linked features and colony morphology

Introduction of retinoic acid (RA) to hiPSC culture leads to a rapid differentiation and loss of the typical colony morphology (I, Fig. S4C). We validated the differentiation process by measuring the protein level of Nanog and Sox2 (I, Fig. S4A). Surprisingly, differentiation coincided with the loss of the cornerstone FAs and led to a more disorganised actin phenotype (I, Fig. 4A). The characteristic area of FAs was also reduced upon differentiation further validating the connection between the cornerstone FAs and pluripotency (I, Fig. 4B).

The differentiation and disorganisation of contractile actin structures (I, Fig. 4D) also led to an apparent reduction and redistribution of traction forces (I, Fig. S4D, S4E) when compared the pluripotent cells where the traction forces were concentrated to the colony edges (I, Fig. 2D). The differentiated cells also occupied a larger area per cell and were flatter (I, Fig. 4F, S4H), indicative of increased cell spreading upon differentiation. Thus, tight colony compaction and prominent traction forces at the colony periphery were unique characteristics of pluripotent cells colonies, resulting in distinct 3D organisation of pluripotent- and differentiated cells (I, Fig. 4H).

Next, we wanted to test if the differentiation process affected the FA signalling. Since β1 integrins have been shown to be important for hPSCs on many ECMs we used β1 activation specific antibody (12G10) to detect changes in integrin activation upon differentiation (I, Fig. 4F). The overall activation of β1 integrin seemed to be higher in originator fibroblasts and differentiated cells compared to pluripotent cells (I, Fig. 4G, S4K). Also, the active β1 integrin colocalised more accurately with FA in the differentiated cells. Conversely, Src phosphorylation displayed significant reduction upon differentiation (I, Fig. 4E, S4I). High levels of pSrc and low integrin beta1 activity seemed both be features of pluripotent stem cells. This inverse correlation is intriguing since Src-mediated phosphorylation of β1 integrins has been suggested to correlate with integrin inactivation (Hirst, Horwitz et al. 1986, Anthis, Haling et al. 2009).

5.2 Nanoscale architecture of cornerstone FAs (II)

5.2.1 Cornerstone FAs are dynamically stable

We establish large cornerstone adhesions and thick F-actin bundles as hallmark structure of hPSCs plated on pluripotency supporting ECM (original publication I). To investigate the specificity of these structures to hPSC, we imaged normal human epithelial cell colonies. Importantly, the large adhesions at the colony periphery were not detected in retinal epithelial cells (ARPE-19), grown on vitronectin and stained with paxillin (II, Fig. S1A). Our initial imaging of the cornerstone adhesions was performed with TIRF microscopy and spinning disk confocal. To study the structure of large adhesions in hPSCs in more detail, we used Structural illumination microscopy to observe the structures with better spatial resolution than previously. Fascinatingly, it seemed that individual cornerstone adhesion is composed of many smaller adhesions clustered tightly together (II, Fig. 1B). In addition, we performed live-cell imaging experiments with hiPSC line with GFP tagged endogenous paxillin to study the dynamics of FAs. Detail quantification of FA dynamics showed that the FAs at the edges of the colony are more stable than the adhesion in the colony centre. In addition, even though cornerstone adhesions are relatively low in number they occupy more area than the FAs at the colony centre (II, Fig. 1D, 1E and 1F). These data from live microscopy indicated that the FAs at the edge are more stable and mature than the ones in the centre.

5.2.2 Spatial segregation of cornerstone FA facilitates spontaneous differentiation

To further investigate the biological role of large adhesion structures for pluripotency, we plated the cells on nanogrooves surfaces coated with vitronectin. The nanopatterns segregate the surface to ridges (800 nm wide) and grooves (800 nm wide, 600 nm deep) (II, Fig. 2C) and we speculated that the cell adhesions could only be formed on top of the ridge. As expected, the FAs were segregated into smaller subunits that were aligned along the direction of the ridges. The nanogrooved surface decreased the characteristic size of individual FA from 4.2 μm^2 into 1.8 μm^2 (II, Fig. 2A, 2B). In addition, the classical round colony morphology was distorted and became more elongated (II, Fig. 2A, 2B). Importantly, we did not observe changes in E-cadherin protein level (II, Fig. S2B, S2C) or capability to constitute for cell-cell junctions (II, Fig. S2D) after 24 hours of culture on nanogrooves. It seemed that the FAs had a direct consequence in colony morphology either by realigning the actin cytoskeleton or using another mechanism still unknown to us.

Next, we analysed the protein level of pluripotency core regulatory factors from spontaneous differentiation time series. The differentiation was initiated by removing the TGF-β and FGF2 from the culture conditions. We compared differentiating cells cultured on uniform VTN-coated surface and nanogrooved VTN coated surface. Cells cultured on uniform VTN-coated surface in the presence of differentiation-inducing morphogen BMP4 were included as a positive control for differentiation. The nanogrooved surfaces facilitated somewhat faster differentiation. Levels of pluripotency factor Oct4 declined significantly more rapidly on day 1 and day 3 in cells cultured on nanogrooves compared to uniform surface cultured cells. In addition, Sox2 was significantly reduced on day 6 on the protein level in nanogroove plated cells compared to the uniform surface (II, Fig. 2D, 2E). Congruently, surface-specific embryonic antigen 1 (SSEA1), a surface marker for differentiation, was also detected in higher level on nanogrooved surface after 3 days of culture (II, Fig. 2F).

Furthermore, the effect on differentiation seemed not to be transient since pluripotency markers did not recover after the introduction of normal culture conditions (II, Fig. S2E, S2F and S2G). These data implicate that modification of the FA structural features has a functional role in pluripotency maintenance. However, to prove direct causality, more accurate information about the mechanism must be unveiled.

5.2.3 Setting up the iPALM imaging

To further investigate the architecture of cornerstone adhesions we decided to use the iPALM to resolve the 3D ultrastructure of the FA scaffold, since the microscope enables sub 20 nm resolution axially and vertically (Shtengel, Galbraith et al. 2009). Previous studies using iPALM have identified that FAs are formed from three functionally different layers (Kanchanawong, Shtengel et al. 2010). We selected components from each layer to investigate if the vertical stratification in cornerstone adhesion resembles the previously studied FAs. From the integrin signalling layer, we selected paxillin and VTN adhesion mediating integrins β5 and αV. From the force transduction layer, we selected key mechanosensors talin1 and vinculin. Finally, from the actin regulatory layer, we selected actin-crosslinking protein α-actinin and actin. In addition, we wanted to elucidate the location of two new FA proximal proteins kank1 and kank2 described to localise at the proximal belt of FAs in differentiated cells (Bouchet, Gough et al. 2016, Sun, Z., Tseng et al. 2016).

All FA scaffold components selected were found to be endogenously expressed in hiPSCs on protein level (II, Fig. S2). Furthermore, the endogenous proteins localised to the cornerstone FAs or to their near proximity (II, Fig. S3). To enable the iPALM microscopy, the proteins of interest needed to be expressed in combination with photoactivatable fluorescent protein Eos. We decided to transiently express the Eos-tagged constructs in hiPSCs and observed using confocal microscopy if there were effects on colony morphology or decline of pluripotency regulator Oct4, neither were detected. In addition, the Eos-tagged proteins localised to cornerstone FAs, as anticipated.

5.2.4 Talin-1 and integrin β5 accumulate at the edges of FAs

First, the lateral distributions of the FA components respective to the functional layers were determined from the iPALM imaging data (II, Supplementary table 1). The integrin subunits and paxillin in cornerstone adhesions were found to vertically position close to the plasma membrane to the integrin signalling layer, in line with previous publications (II, Fig. 3E, 3F). However, integrin β5 and talin-1 displayed unconventional horizontal distribution. Both proteins were enriched at the edges of FAs (II, Fig. 3A, 3B and 3C) whereas the known integrin β5 partner αV was homogeneously covering the FA (II, Fig. S6A). These observations were validated using antibodies recognising the endogenous proteins and imaging with confocal microscopy (II, Fig. 3D, S6, S7). In contrast to hPSC, the ring-like distributions were not detected in normal retinal epithelial cells (II, Fig. S5E, S5F). Furthermore, total and active integrin β1 localised in the middle of the FA rather than to the edges when stained with antibodies (II, Fig. 3D, S7A, S7B). Again, the normal ARPE-19 cells did not display similar segregation of different integrins inside adhesion, but the FAs were predominantly separated into β1- or αVβ5 dominant individual adhesions (II, Fig. S6E). These results indicate that the integrin signalling layer in cornerstone adhesions is spatially separated into subdomains by integrin subunits.

5.2.5 Force transduction layer components display peculiar features

Talin-1 and vinculin both displayed higher vertical position in cornerstone adhesion compared to previously described (II, Fig. 4A, 4B, Supplementary Table 1). Talin can exist in various conformations as a part of the FA scaffold (Yao, Goult et al. 2016) and we decided to image both C- and N-terminally tagged talin-1 constructs to investigate if the talin is fully extended and what is the angle of incline as a part of cornerstone adhesion scaffold. In spite of the overall higher vertical talin positioning, the vertical distance between the C- and N- termini was in line with the earlier

publications. Furthermore, the angle of incline ($\theta \approx 17°$), calculated from the vertical positions of the protein heads and the overall length of talin, supports the notion of fully extended talin molecule described previously (Case, Baird et al. 2015, Liu, J., Wang et al. 2015). The data suggest that talin is fully extended in cornerstone adhesions and clusters with higher density to FA edges.

Vinculin is a known enforcer of FAs (Atherton, P., Stutchbury et al. 2016). It is also known to interact with various FA scaffold components, especially with extended talin, which has multiple vinculin binding sites (Yao, Goult et al. 2016). We detected vinculin to be on higher z-plane of FA scaffold compared to the previously reported (II, Fig. 4A, 4C, Supplementary Table 1). The high vertical localisation of vinculin has been linked to mature FAs and vinculin activation (Case, Baird et al. 2015). The measurements indicated that vinculin might be active and the cornerstone FAs could be considered mature.

Moreover, we decided to investigate the orientation of vinculin molecule by employing the same strategy as with talin. We measured the z-distance of both N- and C- terminal ends of the molecule from the cover glass and interestingly, we observed a head above tail orientation of vinculin (II, Fig. 4C, Supplementary Table 1). In the classical model of the FA scaffold in somatic cells, vinculin is orientated tail above head and assumed to bind with talin with its head-domain and actin with the tail domain when in its active conformation (Case, Baird et al. 2015). To further validate the observed unexpected inverted orientation of vinculin, we performed dual colour imaging where we used Alexa 647-conjugated antibodies to detect paxillin from the integrin signalling layer in combination with N- terminal vinculin tail (II, Fig. 4D). The dual coloured imaging from the same cornerstone adhesions revealed partial overlap with paxillin and vinculin tail suggesting interaction with integrin signalling layer and vinculin. The head above tail orientation of vinculin was also recently observed in mouse embryonic stem cells seeded on lamin, but not on gelatin or fibronectin (Xia, Yim et al. 2019). These data imply that the inversed orientation of vinculin might be a feature of FAs present in pluripotent cells on specific matrixes.

5.2.6 FA proximal scaffold components kank1 and kank2 locate on higher z-plane in when connected to the FAs

Kank1 and kank2 are evolutionarily conserved scaffold proteins that locate at the so-called “proximal belt” of the FA and form a mechanical link between microtubule and FA scaffold (Bouchet, Gough et al. 2016, Sun, Z., Tseng et al. 2016). Kanks are known to bind talin directly at the FA border (Yu, M., Le et al. 2019). Since there are

clear distinctions between the edges and centres of cornerstone FAs, we decided to perform dual colour iPALM imaging to precisely investigate the 3D localisation of kank1 and kank2 (II, Fig. 6A-6D). As shown before, the Eos linked kank1 localises to FA edges and a few molecules were also detected at the FA centre (II, Fig. 6A, 6B). In the proximity of FAs, Kank2 however, was always positioned at the outer rim accumulating to the distal end, but occasionally following the along the stress fibres (II, Fig. 6C). Vertical localisation of kank1 and kank2 overlapped with the force transduction layer when the structures were analysed within FAs (based on paxillin signal with 2-colour iPALM) (II, Fig. 6D, Supplementary Table 1). However, when the kank structures were not in immediate proximity of the FA scaffold, the vertical positions of kank1 and kank2 were substantially lower residing closer to the plasma membrane (II, Fig. 6A, 6C, 6D, Supplementary Table 1).

Furthermore, as both kanks localised at the border of cornerstone adhesions, we wanted to investigate if they functionally influenced the properties of the large adhesions. Depletion of kanks with siRNAs (II, Fig. 6E) led to decrease of FA size at the hiPSC colony edge whereas the FAs at the colony centre grew in size (II, Fig. 6F, S10A). These results suggest that kanks have a role in the regulation of cornerstone adhesions. Interestingly, kanks might, however, regulate differently the FAs at the colony centre compared to the colony edge.

5.3 Fluctuation-based super-resolution TFM (III)

5.3.1 SRRF enhances detection of beads from PAA gels

The accuracy of traction force microscopy can be enhanced by implementing enhanced computationally heavy mathematical algorithms to force analysis or by increasing detection of the fluorescent beads from the deformable elastic gels (Han, Oak et al. 2015, Colin-York, Huw, Shrestha et al. 2016, Plotnikov, Sabass et al. 2014). However, each method that enhances the quality comes with drawbacks. The advantages and disadvantages are presented in Table 1 (III, Table 1). We decided to test if fluctuation-based super-resolution (FBSR) microscopy could be applied to enhance bead detection and circumvent the problems arisen from the more conventional super-resolution modalities (III, Fig. 1A). FBSR utilises pixel to pixel fluctuation in camera-based images to generate super-resolved images from tens or hundreds of confocal, tirf or widefield frames (Gustafsson, Culley et al. 2016, Zhao, W., Liu et al. 2018).

Classically traction force microscopy is performed using 200 nm beads embedded within the polyacrylamide (PAA) gels (Style, Boltyanskiy et al. 2014). We set out to

streamline the gel-making protocol where we would be able to use smaller 40 nm beads embedded only on top of the gel to reduce out of focus light and to enable the use of widefield microscopes (III, Fig. 1B). This was achieved by coating the glass coverslip used for flattening the polyacrylamide gel in casting phase, with 40 nm fluorescent beads suitable for super-resolved TFM (III, Fig S1A).

To test if we were able to resolve the 40 nm beads from the top of the PAA gel, we imaged the same position using spinning disk confocal- and widefield microscopy (III, Fig. 1C, 1D). Excitingly, the FBSR algorithms, liveSRRF and SACD, substantially enhanced our ability to detect beads (III, Fig. 1C, 1D). The use of classical TFM by our group has yielded bead densities ranging from 0.2 to 0.5 beads per square micron which could be considered as baseline values. The use of FBSR enabled us to detect 1.2 beads per square micron. This substantial enhancement was achieved with both analysis algorithms and microscopy modalities (III, Fig. 1F). The density of the beads detected was comparable with the TFM performed with SIM (1.0 μm^{-2}) but considerably lower to the density reported with STED (2.2 μm^{-2}) (III, Table 1).

Interestingly, the FBSR processing performed especially well when images were taken with widefield microscopy yielding a homogenous field of view. However, the confocal microscopy and SACD particularly seemed to be more sensitive for out of focus light (III, Fig. 1C, 1D).

To further asses the quality of our FBSR-processed images and to ensure that no artefacts were introduced by the algorithms we analysed the processed frames with NanoJ SQUIRREL to measure the resolution scaled error and person's correlation (RSE and RSP) (III, Fig. 1E) (Culley, Albrecht et al. 2018). Moreover, we decided to implement the SQUIRRLE analysis as part of the FBSR optimisation pipeline since the quantitative metrics can be used to assess the parameters used by the fluctuation processing algorithm. To establish the best possible parameters for the processing, we also took into consideration the number of trackable beads and as an eliminating factor, the presence of reconstruction artefacts (III, Fig. S1B, S1C).

5.3.2 Better bead detection yields enhanced displacement fields and traction maps

Next, we wanted to implement the FBSR as a part of a TFM experiment. The TFM pipeline incorporating FBSR is presented in Figure 2A. The workflow is similar to classical TFM with additional steps of FBSR processing and quality insurance (Style, Boltyanskiy et al. 2014). To assess if FBSR processing enhances the quality of TFM displacement maps or force maps generated, we used the classical method in

combination with the new protocol (III, Fig. 1B). This was achieved by embedding the 200 nm beads (green) into the gels but also casting the 40 nm beads (dark red) onto the surface as described earlier. We were able to analyse the data generated from the same cell with the classical TFM and FBSR TFM respectively (III, Fig. 2B-2F, S2A, S2B). Using the bigger 200 nm beads and standard spinning disk confocal imaging, we were able to track 8253 beads from a single field of view (III, Fig. 2C, S2A, S2B). Whereas utilising the same imaging tactics and analysing the same position we could trace 11328 40 nm beads (III, Fig. 2D, S2A, S2B). However, after FBSR processing of the 40 nm images (100 frames with liveSRRF and 50 frames with SACD) we were able to tract 20799 beads from liveSRRF images (III, Fig. 2E, S2A, S2B) and 22908 beads from SACD images (III, Fig. 2F, S2A, S2B) again using the same field of view as before.

FBSR enhanced the quality of displacement and traction maps considerably. From the liveSRRF and SACD enhanced maps, we were able to detect multiple clustered displacements and forces from underneath the cell (III, Fig. 2B, 2E, 2F). The clustered areas closely resembled structures that could represent FAs, the major force transmitting units of the cells. From the maps generated with the conventional TFM method, we could only see a few individual displacement or force clusters within the cell area (III, Fig. 2C). Importantly, the use of 40 nm beads alone did not improve the quality of TFM images (III, Fig. 2D).

5.3.3 FBSR TFM can be used in a variety of cell biological experiments

To assess, if the FBSR TFM would be suitable for cell biology purposes we set out to conduct experiments which commonly employ TFM to illustrate the versatility of the improved method. TFM is commonly used to measure how the cellular traction forces are affected by a specific protein or a drug treatment (Hakanpaa, Kiss et al. 2018, Jacquemet, Stubb et al. 2019, Narva, Stubb et al. 2017). We treated the cells for 15 minutes either with myosin II inhibitor blebbistatin or DMSO to cause mild relaxation of acto-myosin contractility without completely perturbing the FAs (III, Fig. 4A, 4B). The imaging was performed before and after the treatment to measure the force changes per cell. The treatment was enough to trigger an apparent reduction of forces, but surprisingly, we were not able to detect a significant reduction of force or strain energy in population-level (III, Fig. 4C). This was due to the large variations in traction forces between individual cells in blebbistatin-treated population and DMSO treated control populations. However, when we measured the fold change in force and strain energy in each cell between the time point zero and 15 minutes, we detected significant reduction recapitulating the observations

from the traction maps (III, Fig. 4D). This experiment highlighted the importance of measuring the forces from the same cell before and after perturbation. Furthermore, the FBSR TFM was capable of detecting changes in the forces even before the disassembly of FAs which is usually associated with loss of actomyosin contraction.

To illustrate the capability of extended live cell imaging with FBSR, we performed an extended period live-imaging experiment where we measured the traction forces from migrating U-251 glioma with endogenously GFP-tagged paxillin (III, Fig. 4E, S4A). We performed FBSR imaging in 5-minute intervals for over 100 minutes using our enhanced pipeline. Importantly, the cell was not visibly influenced by the microscopy and we could detect modulation of forces under the leading edge while the cell was moving. Furthermore, we performed a similar live imaging experiment within our experience more photosensitive hiPSCs (III, Fig. S4B). These results indicate that FBSR TFM could be used for live measuring of cellular forces.

6 Discussion (I-III)

The cell-ECM interaction and the mechanical forces have been linked to a multitude of biological processes, and mechanobiology is suggested as a new frontier for studying pluripotency (Sun, Yubing, Fu 2013). The FAs and actin cytoskeleton are important for adhesion, migration and subsequently for transcription (Conway, Jacquemet 2019). Moreover, the physical features of ECM play a role in cell lineage specification (Vining, Mooney 2017). Importantly, the absence of various FA components has been shown to be embryo-lethal in mice (Stephens, Sutherland et al. 1995, Zaidel-Bar, Itzkovitz et al. 2007, Monkley, Zhou et al. 2000, Xu, W., Baribault et al. 1998). Since the field of regenerative medicine is constantly actively looking for cues that could enhance the lineage-specific differentiation, we find it intriguing that nobody has turned their eyes on FAs and investigated them thoroughly in context of human pluripotency. Our results indicate that FAs in hPSCs are large and stable and participate in active signalling. We think that FAs in hPSCs take part in sensing the extracellular space, as in other cell types and might be important for lineage-specific differentiation. Also, the large FAs and stress fibres at colony periphery create a mechanical niche that seems to be necessary for the right response to morphogens. Fine-tuning the FA signalling might prove out to be a non-invasive tool for modulating the lineage selection. Besides, the ultrastructure of hPSCs is distinctively different from the somatic cells (Kanchanawong, Shtengel et al. 2010). The structural differences might the key to understanding the responses of hPSCs to the physical stiffness and biochemical composition. We hope that this detailed characterisation of FAs would provide insights that could be used for purposes of regenerative medicine and further understanding the complex process of human embryo development.

6.1 Original publication I

The hPSC colonies are often described as epithelial type colonies with sharp lined defined edges (Thomson 1998, Ginis, Luo et al. 2004). The edges and centre of hPSCs display distinct features. The edge of the colony is known to respond more rapidly to morphogen BMP4 and differentiate faster (Warmflash, Sorre et al. 2014, Rosowski, Mertz et al. 2015). Also, the mechanical niche, in which the cells exist

appears to be different at the edge compared to the centre (Przybyla, Lakins, Sunyer et al. 2016, Rosowski, Mertz et al. 2015). However, to the best of our knowledge, ours is the first study to demonstrate structural differences between the edge and centre of the pluripotent cell colonies that could constitute these differences. The large FAs that we termed as cornerstone FAs are clearly implemented by the cells at the colony edge (I, Fig. 1A, 1D, S1B). This surprised us since it was previously thought that hPSCs would not have assembled FAs. However, our observations clearly prove this notion to be otherwise (Baxter, Camarasa et al. 2009, Vitillo, Kimber 2017). Cells in the colony centre have FA, but they are considerably smaller (I, Fig. 1D). During induction of pluripotency pluripotent cells can be enriched using their low overall adhesion (Singh, Suri et al. 2013). It is tempting to speculate that maybe the adhesion itself or adhesion mediated signalling could constitute for the functional differences and cause the weaker attachment of pluripotent cells. Furthermore, when we compared the FAs in hPSC to those of originator fibroblast cells or differentiated cells the morphological characteristics were remarkably different (I, Fig. 1D, 4A, 4B). Cell-ECM adhesion has been recognised as a barrier for the induction of pluripotency (Qin, Diaz et al. 2014) and our data further highlights the differences between hPSCs and originator somatic cells. In the future, it would be exciting to systematically modulate the environment stiffens and composition to see if more rapid reprogramming could be achieved.

The large cornerstone FAs are connected by thick force-generating VSFs (I, Fig 2A, 2B, 2C) which is in accordance with the concept of force build-up leading to increase in FA size (Goffin, Pittet et al. 2006). Inhibition of myosin II activity diminishes the actin bundles, and FAs respectively leading to colony flattening (I, Fig 3A-3D). Furthermore, in TFM experiments we detect accumulations of traction forces at the periphery of the hPSCs colonies localising at the ends of F-actin bundles (I, Fig. 2D). The forces aligned according to the direction of ventral stress fibres. This is not a common feature of epithelial monolayers (Chen, T., Saw et al. 2018). Typically, the cells at the free edge of epithelium generate finger-like protrusions lead by lamellipodium (Poujade, Grasland-Mongrain et al. 2007, Reffay, Parrini et al. 2014). However, we did not detect such structures in hPSCs. Also, the differentiation of cells leads to flattening of the colony and the reduction of traction forces coinciding with the more common free edge phenotype of epithelial cells (I, Fig. S4F, 4D) (Chen, T., Saw et al. 2018). The signalling interplay between the FAs and actin is thought to occur through the Rho-ROCK-myosin II signalling cascade (Katoh, Kano et al. 2010). Our observations indicate that inhibition of this cascade leads to randomisation of the orientation of mitotic spindles of cells dividing at colony periphery (I, Fig. 3F). We think that VSFs and related forces are important for maintaining colony compaction, organisation and in generation of the mechanical,

pluripotency supporting niche. Mechanical forces have also been connected to the maturation of FAs (Burridge, Guilluy 2016). The fact that we observe large FAs indicate that the increased size of FAs at the colony periphery might be caused by the mechanical tension. FAs are also known to grow in size during maturation process. We observed cornerstone adhesions 2 hours after seeding the colonies and saw them growing in size until 20 hours. This might indicate that the FAs reach their mature state upon 20 hours. It would be intriguing to study further what is the reason behind the increased forces at the colony periphery compared to the centre. In addition, detailed analysis of expression/activity of RhoA activators and inactivators (GEFs and GAPs) in cells at colony periphery might unveil yet another distinction between the edge and centre of the colonies.

We also detect an accumulation of E-cadherin-mediated junctions at the edge zone of the colonies (I, Fig. 1B). E-cadherin has been shown to be an essential signalling cascade initiator and mechanical supporter that prevents overtly high contractile forces applied on individual cells and thus hPSCs cannot survive as single cell (Li, L., Bennett et al. 2012, Ohgushi, M., Matsumura et al. 2010). We think that the cell-cell junctions take part in distributing the forces and enabling the colony compaction. The thick ventral stress fibres might be also connected to the cadherin junctions since cadherin clusters to the proximity of the VSFs and cornerstone FA at the low focal plane. One report already connected the FAs and increased beta-catenin signalling from cell-cell junctions by showing that hESCs cultured on soft environment are directed to mesenchymal lineage when less Scr activity was present (Przybyla, Lakins, and Weaver 2016). However, the interplay between the FA and cadherin-mediated junctions is still to be studied further in the context of pluripotency and differentiation.

Integrin-mediated adhesion has been shown to be important for hPSCs attachment and proliferation (Rowland, Miller et al. 2010, Meng, Eshghi et al. 2010). In our immunostainings, we observe that αVβ5 and integrin β1 are localised to cornerstone adhesion or to their proximity (I, Fig. 3G, 3H). Also, we observed changes in integrin β1 activation level upon differentiation (I, Fig. S4J). Moreover, the active β1 integrin localises weakly into the cornerstone adhesions when cells were grown on VTN coated surfaces (I, Fig. 4H). This coincides with a recent report depicting differentiation upon forced activation of integrins with manganese (Mn^{2+}) (Villa-Diaz, Kim et al. 2016). The influential role of integrin β1 in embryo development has been highlighted using *in vitro* embryo models (Bedzhov, Zernicka-Goetz 2014, Christodoulou, Kyprianou et al. 2018). Also, integrin β1 knock out from mouse embryo causes embryo lethality (Stephens, Sutherland et al. 1995). However, it remains uncertain if the integrin β1 is needed for the maintenance of pluripotency

or the differentiation-related processes. Integrin β1 facilitates the adhesion with variable pluripotency supporting ECMs (Meng, Eshghi et al. 2010). Yet, integrin β1 it is not a known receptor for vitronectin in any heterodimer combination. Still, we know that vitronectin supports pluripotency and hPSC proliferation (Braam, Zeinstra et al. 2008). Surprisingly, we observe integrin β1 at cell membrane in the periphery of hPSC colonies cultured on vitronectin (I, Fig. 3H,) and detect secreted laminin-α5, an ECM produced by the hPSCs (Vuoristo, Virtanen et al. 2009) suggesting that hPSC cultures seeded on vitronectin and also adhering to their endogenous secreted laminin-matrix through integrin β1.

We found that cornerstone adhesions were positive for phosphorylated paxillin (Y118) (I, Fig. 1C) FAK (Y397)(I, Fig, 1C) and Src (Y416) (I, Fig. 3J). Also, the inhibition of either FAK or Src family kinases led to rapid detachment of the cells (I, Fig. S3E). FAK signalling is known to play a role in pluripotent stem cell survival via PI3K/Akt pathway (Hossini, Quast et al. 2016). Also, it has been suggested that inhibition of FAK leads to anoikis or differentiation (Vitillo, L., Baxter et al. 2016). Interestingly one report positions FAK to the nucleus of hPSCs and demonstrated FAK association with Oct4 and Nanog, the core regulatory network members suggesting that FAK might have a role beyond FA signalling in hPSCs (Villa-Diaz, Kim et al. 2016). Together these results imply that FAK is important in the context of pluripotency. Our results, however, position autophosphorylated FAK directly to cornerstone FA suggesting that FAK is actively functioning in adhesions, possibly recruiting signalling kinases such as Src. The role of FAK as kinase phosphorylating downstream substrates is debatable, but the role of FAK autophosphorylation and subsequent recruitment of other kinases like Src is undisputed (Mitra, Schlaepfer 2006). Furthermore, we see that activated Src (Y416) is also located to cornerstone FAs and when the total protein levels are compared with fibroblast or differentiated cells, the ratio between pSrc (Y416) and total Src is significantly higher in pluripotent cells (I, Fig. 3I, 4E). Taking into consideration that Scr is important for pluripotency maintenance (Annerén, Cowan et al. 2004) and cell cycle regulation (Chetty, Engquist et al. 2015), we think that high Src activity is a feature of hPSCs and that hPSCs have active FA signalling.

6.2 Original publication II

Detailed characterisation of FAs dynamics of hPSCs, using live microscopy, revealed that the cornerstone FAs are remarkably stable compared to the small adhesions at the colony centre (II, Fig. 1C-1F). The physical lifetime of FA complexes can be connected to their maturation stage (Burridge, Guilluy 2016). This led us to speculate that the cornerstone adhesions might be in a stage of ultimate maturation. In

addition, we provide evidence that the cornerstone adhesions are not present in epithelial cell types cultured on vitronectin (II, Fig. S1A).

In the original publication I, we identified large cornerstone adhesions as hallmark morphological feature hPSCs. However, we did not investigate whether FA size itself plays a role in pluripotency maintenance. To investigate this, we decided to dissect the cornerstone adhesion to smaller substructures by seeding cells onto nanogrooved surfaces (II, Fig. 2A-2F). When cultured in differentiation enabling medium, the nanogrooved surface-induced more rapid differentiation of hPSCs than the uniform substrate (II, Fig. 2D-2F). However, it is worth pointing out that morphologies of the colonies also changed upon culture on nanogrooves and the spatial organisation of the colony is known to dictate differentiation patterning (Warmflash, Sorre et al. 2014). We believe that the uniformly aligned nanogrooves are responsible for the alignment of the colonies trough reorganisation of FAs. We did not observe changes in E-cadherin localisation to cell-cell junctions or in total protein level after 24 hours of culture (II, Fig. S1B-S1D) whereas significant decline of Oct4 was already observed (II, Fig. 2D, 2E) indicating that the FAs are the first structures perturbated in these assays. Moreover, we did not see recovery of the pluripotency factors when cells were returned into normal culture condition, important notion, since there is a report of Nanog expression returning after long periods of depletion (II, Fig. S1E-S1G) (Chambers, Silva et al. 2007). Similar experiments were performed on nanopatterned lines by another group and their observations of more rapid differentiation in response of BMP4 are fully in line with ours (Abagnale, Sechi et al. 2017). E-cadherin is known to be important for pluripotent cells by supporting hPSC colonies mechanically (Chen, G., Hou et al. 2010, Ohgushi, Matsumura et al. 2010) and as an initiator of essential biochemical signalling (Li, Bennett et al. 2012). We cannot experimentally exclude E-cadherin as a mediator of FA signalling in hPSCs since it is essential for survival of the cells and depletion of the protein would lead to differentiation or apoptosis of the cells. Therefore, the morphological characteristics of FAs seem to play role in pluripotency maintenance. However, E-cadherin might be important intermediate of the signalling cascade. Furthermore, we do not know the exact molecular mechanism of this phenomenon. Additional investigations should be conducted, for example, on micropatterns where we would have more control over the size and spatial organisation of adherent dots. It would be intriguing to study if certain sized FAs would constitute for lineage-specific differentiation.

Three-dimensional superresolution imaging of FA complexes has revealed that their protein scaffold is organised to three functionally different strata's (Kanchanawong, Shtengel et al. 2010). These include the integrin signalling layer containing integrins and paxillin, the force transduction layer containing talin and vinculin as key

mechanosensitive elements, and finally the actin regulatory layer containing actin and actin crosslinker α-actin (Liu, J., Wang et al. 2015, Case, Baird et al. 2015). We set out to use iPALM microscopy (Shtengel, Galbraith et al. 2009) to determine if cornerstone FAs in hPSCs display unique structural features in three-dimensional space by using the scaffold components prementioned. We observed a peculiar spatial organization of integrin β5 and talin1. Both proteins clustered more at the peripheral edge of FAs (II, Fig. 3A-3D). In addition, integrin β1 (active and total) localized to the centre of the cornerstone adhesion structures (II, Fig. 3D, S7). The spatial clustering and segregation of integrin heterodimers have been described before in the context of single-molecule dynamics (Rossier, Octeau et al. 2012) and activation stage (Spiess, Hernandez-Varas et al. 2018) and it might be possible that the spatial segregation of cornerstone adhesion would be due to the same reasons, this, however, needs to be investigated further. To the best of our knowledge, our data are the first to demonstrate spatial clustering of talin1. We speculate that talin localisation might be a consequence of the exceptionally large size of cornerstone FAs or maybe the FA proximal scaffolding proteins kank1 and kank2 enable the clustering via their direct talin binding sites (Bouchet, Gough et al. 2016, Sun, Z., Tseng et al. 2016). However, it seems evident that cornerstone adhesion edges display different properties compared to the centres. The biological role of these structural differences remains to be studied further.

The cornerstone adhesions display clear stratification to the three functional layers described before (Kanchanawong, Shtengel et al. 2010). However, we detect higher z-localization of talin1 and vinculin, whereas paxillin and integrins were positioned close to the plasma membrane as described before in somatic cell types (II, Supplementary table 1). The high vertical position of vinculin has been previously connected to a mature stage of FAs (Case, Baird et al. 2015). This observation further supports the notion that the cornerstone FA might be in a stage of ultimate maturation together with stable dynamics. Another peculiar quality of cornerstone FA is the inverted orientation of vinculin (II, Fig. 4C) which has not been observed in classical FAs. We speculate that the head above tail orientation could be a cause of high traction forces since vinculin has been shown to disassociate from talin upon enough pulling force (Yao, Goult et al. 2014). Another contributing factor could be vinculin interaction with other proteins than the classical vinculin-head-talin and vinculin tail-actin bonds. The disassociation of the vinculin head from talin might, for example, enable binding to another scaffold partner such as α-actinin. Another possibility would be that vinculin tail is interacting with a component of integrin regulatory layer such as paxillin as we detected association with vinculin tail and the integrin signalling layer (II, Fig. 4D). However, vinculin is known to interact with a multitude of other binding partners and have a high affinity for autoinhibition which

both might cause a change in its orientation and z localisation (Atherton, Paul, Stutchbury et al. 2016). We do not have sufficient evidence to concretely back up either of these suggestions. Similar inverted vinculin orientation was recently seen in FAs of mESCs while the cells were seeded on laminin, but not on fibronectin or gelatin (Xia, Yim et al. 2019). The biological role of the inverted vinculin still remains unknown, but it is tempting to speculate that it would be linked to pluripotent state.

Kank1 and kank2 were recently identified as a part of FA proximal scaffold (Bouchet, Gough et al. 2016, Sun, Z., Tseng et al. 2016, Yu, M., Le et al. 2019). They form a mechanical link between the talin and microtubule ends. However, the nanoscale localisation of either kank1 or kank2 has not been described before. In cornerstone adhesions, both kanks were laterally distributed around the paxillin positive area, forming a vertical wall (II, Fig. 6A-6D). When we analysed the z-positions of kanks, we noticed that both localise on a higher plane close to the force transduction layer when in close proximity of paxillin positive cornerstone adhesions (II, Fig. 6D, Supplementary Table 1). This coincides with previous reports describing the direct interactions between talin and kank1 and kank2 (Bouchet, Gough et al. 2016, Sun, Z., Tseng et al. 2016, Yu, M., Le et al. 2019). However, when we measured the z-distribution of kank structures further away from FAs we noticed that they were on clearly lower, highlighting the important role of direct talin interaction (II, Fig. 6D, Supplementary Table 1). Furthermore, we decided to study the biological role of kanks by transiently depleting them from hPSCs (II, Fig. 6E). We observed a slight reduction of FAs size at the edges of the colony whereas the FAs at the colony centre grew in size (II, Fig. 6F, S10). These data furthermore highlight the fact that the FAs at the colony edge differ from the ones in the centre. Kanks might indeed be part of the regulatory network maintaining the structural differences between the centre and edge FAs. However, the full details of this regulatory action need to be investigated further.

6.3 Original publication III

Fluctuation-based super-resolution (FBSR) imaging is a relatively novel approach for generating super-resolved microscopy images. The FBSR methods take advantage of pixel to pixel fluctuations present in camera-based microscopy images (Gustafsson, Culley et al. 2016, Zhao, W., Liu et al. 2018). Multiple frames of the same position are imaged and processed with FBSR algorithm to generate super-resolution images. We took an approach where we implemented FBSR imaging as a part of the traction force microscopy pipeline to gain more spatial resolution to our displacement and traction force maps (III, Fig 1A).

First, we decided to streamline the protocol for making SR capable PAA gels. The protocol for embedding the 40 nm fluorescent beads only on the top layer was has been described in detail (Colin-York, H., Shrestha et al. 2016, Colin-York, H., Eggeling et al. 2017). We took inspiration from this previous work and simplified the process by removing some steps we felt redundant. Making the gels with a new streamlined protocol does not take longer than the classical TFM gel preparation. Furthermore, our protocol is suitable for glass-bottomed culture dishes (III, Fig. 1B, S1A).

Accuracy and sensitivity of TFM depend on trackable fluorescent beads (Style, Boltyanskiy et al. 2014). Moreover, the spatial resolution of TFM can be improved by enhancing bead detection. We decided to illustrate the enhanced bead recognition capability of FBSR by imaging a sample from the same position using spinning confocal and widefield microscopy (III, Fig 1C, 1D). Subsequently, the images were processed with two different FBSR algorithms, liveSRRF and SACD. Both algorithms were capable of significantly improving the bead images (III, Fig 1C, 1D). However, from the full field of view images it is apparent that SACD is more sensitive for having exactly right focus plane. Surprisingly, widefield microscopy performed especially well and could easily be implemented as part of TFM pipeline if there is no need for detecting planar structures like FAs. Widefield microscopy yielded more homogenous full-field images of the gels (III, Fig 1D). To associate how much the implementation of FBSR improved the bead density we compared the densities obtained in the previous publication of our group (III, Fig 1F). Classical TFM in combination with spinning disk confocal microscopy yielded bead densities close to 0.5 beads per square micron. The number coincides with other published work (Colin-York, H., Shrestha et al. 2016). With FBSR we were able to detect 1.2 beads per square micron, a considerable improvement to the classical method, but under the 2.2 beads per square micron reported using STED microscope (Colin-York, H., Shrestha et al. 2016). However, utilisation of FBSR circumvents some of the drawbacks which STED microscopy suffers from. FBSR have reduced phototoxicity and is cable of imaging large field of view, which are not features that STED microscopy is known (Schermelleh, Ferrand et al. 2019).

Super-resolution microscopy is known for artefacts caused by heavy in silico processing of the images (Wegel, Göhler et al. 2016). We wanted to make sure we are not detecting artefacts, so we decided to implement NanoJSQUIRREL a software method for detecting SR artefacts (Culley, Albrecht et al. 2018), as part of our experimental pipeline (III, Fig 1E, S1B, S1C). Furthermore, apart from FBSR processing and quality control steps, FBSR TFM pipeline is similar to the classical TFM experimental pipeline (III, Fig 2A). To compare the performance of FBSR TFM

and classical TFM we performed experiments with both methods from the same position and observed remarkable improvement in spatial resolution when using FBSR TFM (III, Fig 2b-2F). Both FBSR algorithms performed better compared to the classical TFM method. The improvement was probably caused by the ability to track more beads over more pixel units since FBSR methods artificially expand the images over a higher number of pixels. As part of FBSR TFM experimental pipeline, we use standard open-access methods for the analysis (Han, Oak et al. 2015, Tseng, Duchemin-Pelletier et al. 2012) and mathematical framework called Fourier Transformation Traction Cytometry. In principle, we should be able to improve the spatial resolution of our method even further if more mathematically heavy algorithms would be implemented (Soiné, Brand et al. 2015, Schwarz, Balaban et al. 2002).

Finally, we test FBSR in cell biological experiments to provide further insight into the versatility of the method (III, Fig 4A-4E, S4B). FBSR can be implemented to standard experiments where TFM is used to study the function of protein or effect of drug treatment with high accuracy. The reduced phototoxicity also makes FBSR TFM suitable for long-term live measurements of cellular forces (Culley, Tosheva et al. 2018). These experiments highlight the potential utility of FBSR TFM for the field of basic cell biological research and mechanobiology.

In the context of FA in hPSCs, FBSR TFM could be used for detecting the force changes during cell differentiation in live-imaging setup since the mechanobiological properties are known to influence the stem cell faith (Engler, Sen et al. 2006). It would also be intriguing to study if the structural differences between the FA edges and centres would translate to differences in forces exerted by individual FAs in hPSCs. However, to achieve this, the protocol would need additional optimisation to improve the spatial resolution further. The improvement could be attained by utilising two different coloured beads (Plotnikov, Sabass et al. 2014) to increase the density of detectable units and more mathematically heavy analysis pipeline (Han, Oak et al. 2015).

7 Conclusions

The purpose of the work in this thesis was to increase the knowledge of basic cell biological features of hPSCs. We think that there are significant gaps in the research when it comes to the adhesion mediated signalling and FA biology. To our knowledge this is the first time that somebody characterises the structural features of FAs and actin cytoskeleton in hPSCs We hope that the data derived from carefully done experiments and in detail analysis provided by this book will prove out to be useful in the future and offer additional perspectives for understanding the complicated signalling cascades regulating the pluripotency of hPSCs. Two publications included in this thesis book showcase the peculiar FAs in human induced pluripotent stem cells. The last original publication provides a method for high accuracy force measurement at the cellular level.

7.1 FAs in hiPSCs (I)

We illustrate the adhesion and actomyosin contraction linked differences between the colony edge and centre. Pluripotent stem cell colony is known to be functionally different at the edge compared to the centre. For the first time, we provide evidence of structural differences. We describe in detail large cornerstone adhesions at the colony edge which significantly differ from FAs at the centre. We also show that the cornerstone adhesions participate in active signalling and have a high amount of active Src. The cornerstone adhesions are connected to each other via ventral stress fibres that encircle the colony. The forces generated by the actin bundles constitute for colony compaction and organisation. Importantly, we show that all the features prementioned are diminished upon differentiation. We hope that the characterisation of these features will provide new ways of studying the induction of pluripotency and lineage-specific differentiation.

7.2 Super-resolution architecture of cornerstone FAs (II)

By using 3D super-resolution microscopy, we unveil the nanoscale architecture of cornerstone FAs in human pluripotent stem cells. Cornerstone FAs display unusual features in the vertical and horizontal organisation of the scaffold. Talin1 and integrin β5 cluster at the edges of FAs whereas the integrin β1 is located at the middle. We also showcase an inverted orientation of vinculin, a feature seemingly unique for pluripotent cells. Finally, we describe for the first time the nanoscale localisation of FA proximal scaffold components kank1 and kank2. Our results indicate that the cornerstone FA edge is structurally different from the centre.

7.3 Fluctuation based superresolution TFM (III)

We introduce a fluctuation based super-resolution (FBSR) modalities as a part of the traction force microscopy (TFM) experimental pipeline. The new enhanced method improves the spatial resolution of classical traction force microscopy substantially. The method utilises easy to access hardware and open access software to gain advancement. Other SR microscopy modalities that can be used as a part of TFM experiment require investments on hardware whereas our pipeline can be performed with equipment commonly available. FBSR TFM can be used for addressing the same mechanobiological questions that the classical method but with improved sensitivity and accuracy. Measuring the forces can be done near to FA level. Since the method reduces the phototoxicity, it can be used for measuring the traction forces in live-imaging experiments or from multiple timepoints without compromising the viability of the cells. We show that measuring traction forces from multiple timepoints with our improved method can be used for detecting slight differences in forces that would be undetectable when measured from one timepoint as is usually done in the classical TFM experiment. Therefore, novel factors affecting the cells capability to produce forces could be found by implementing our protocol. Furthermore, we demonstrate that the method can be sufficiently conducted with widefield microscope and an air-objective which enables implementation in large-scale screening of factors and compounds affecting the mechanobiological properties of the cells. We believe this method can quickly be adopted by the field of mechanobiology to be used where accurate measurement of cellular forces is needed.

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Aki Stubb

9 References

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