

MICROENVIRONMENTAL REGULATION OF HUMAN STEM CELL FATE VIA
INTEGRIN A6B1-FAK SIGNALING, IMPACT OF FEEDER-DERIVED
MITOCHONDRIAL TRANSFER AS A CULTURE CONFOUNDER, AND
DEVELOPMENT OF BIOENGINEERED SUBSTRATES FOR XENO-FREE CULTURE.

by

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DEDICATION

This thesis is dedicated to those who supported me throughout my education.

Thank you for helping me reach the end of this journey.

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Microenvironmental Regulation of Human Stem Cell Fate via Integrin $\alpha 6/\beta 1$ –FAK Signaling, Impact of Feeder-Derived Mitochondrial Transfer as a Culture Confounder, and Development of Bioengineered Substrates for Xeno Free Culture

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Abstract

Human pluripotent stem cells (hPSCs) and mesenchymal stem/stromal cells (hMSCs) are highly responsive to microenvironmental cues. Yet, the mechanisms linking extracellular matrix (ECM) signaling to nuclear gene regulation, fate stability, and culture reproducibility remain incompletely defined. In this dissertation, we investigate how microenvironmental regulation shapes stem cell behavior through three related contexts: ECM–cell interactions, cell–cell interactions, and ECM–soluble factor–cell interactions. To define ECM-dependent fate control in hPSCs, integrin $\beta 1$ was activated using manganese and a $\beta 1$ -integrin–activating antibody, followed by analyses of OCT4, SOX2, NANOG, pFAK Y397, and FAK-associated nuclear complexes. Integrin $\beta 1$ activation altered FAK localization, engaged the NT-FAK–MDM2–p53 axis, increased p53 and p21, and induced transcriptional changes consistent with early lineage priming. To examine cell–cell interactions in feeder-based culture, feeder-derived mitochondrial transfer was assessed using complementary imaging, molecular, and bioenergetic analyses. Mitotically inactivated mouse embryonic fibroblasts transferred mitochondrial material to hPSCs at high frequency, primarily through extracellular vesicle–enriched pathways. Mouse COX2 mtDNA persisted for three to four passages and was associated with altered membrane potential and reduced mitochondrial ATP output.

To investigate ECM–soluble factor–cell interactions in hMSCs, defined synthetic substrates and glycosaminoglycan-mimetic sulfonated polymer brushes were evaluated using molecular and functional potency assays. These biomaterials promoted laminin remodeling, increased hMSC expansion by 30–40%, preserved trilineage differentiation, and elevated FGFR1 and ITGA6 expression. Collectively, these findings establish a unified microenvironmental control framework in which integrin β 1-dependent nuclear FAK signaling regulates stem cell fate, feeder-derived mitochondrial transfer acts as a major bioenergetic confounder, and defined biomaterial strategies improve reproducibility and support scalable stem cell expansion.

Keywords: Integrin β 1–FAK, Nuclear FAK, Mitochondrial transfer, Defined biomaterials

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List of Abbreviations

Abbreviation	Definition	Abbreviation	Definition
AD-MIDAS	adjacent to the metal ion-dependent adhesion site	iMEF-CM	iMEF-conditioned medium
Agilent	Agilent Technologies	ITGA6	integrin alpha 6
AMBIC	ammonium bicarbonate	ITGA6/CD49f	integrin alpha 6 (CD49f)
APC	allophycocyanin	ITGB1	integrin beta 1
ARG-1	arginase 1	JC-1	MitoProbe JC-1 (mitochondrial membrane potential dye)
ARG2	arginase 2	KI67	marker of proliferation (Ki-67)
ARY003C	Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems)	KLF4	Kruppel-like factor 4
ATCC	American Type Culture Collection	LFQ	label-free quantification
ATP	adenosine triphosphate	MDM2	mouse double minute 2 homolog
ATRP	atom transfer radical polymerization	MEM	minimum essential medium
BCA	bicinchoninic acid assay	MEMA	ethylene glycol methyl ether methacrylate
bFGF	basic fibroblast growth factor	Mn ²⁺	manganese(II) ion
BiBB	α -bromoisobutyl bromide	MS	mass spectrometry
BMP4	bone morphogenetic protein 4	mt	mitochondria; mitochondrial
CCCP	carbonyl cyanide m-chlorophenyl hydrazone	mtATP	mitochondrial ATP
CDK2	cyclin-dependent kinase 2	mtDNA	mitochondrial DNA
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21)	NANOG	homeobox transcription factor NANOG
cDNA	complementary DNA	NEAA	non-essential amino acids
ChDM	chemically defined medium	NES	nuclear export signal
CHEK2	checkpoint kinase 2	NLS	nuclear localization signal
CM	conditioned medium	NT	N-terminal
CO ₂	carbon dioxide	NT-FAK	N-terminal focal adhesion kinase
COX2	cytochrome c oxidase subunit II (mitochondrial)	NTA	nanoparticle tracking analysis
CuBr ₂	copper(II) bromide	OCR	oxygen consumption rate
CUT&RUN	cleavage under targets and release using nuclease	OCT4	octamer-binding transcription factor 4 (POU5F1)
CUT&Tag	cleavage under targets and tagmentation	p21	cyclin-dependent kinase inhibitor p21
DAPI	4',6-diamidino-2-phenylindole	p53	tumor protein p53
DMEM	Dulbecco's modified Eagle medium	PBS	phosphate-buffered saline
DMEM/F-12	Dulbecco's modified Eagle medium/Nutrient Mixture F-12	pFAK	phosphorylated focal adhesion kinase
DMSO	dimethyl sulfoxide	pFAK Y397	FAK phosphorylated at tyrosine 397
DNA	deoxyribonucleic acid	PMDTA	N,N,N',N'',N''-pentamethyldiethylenetriamine

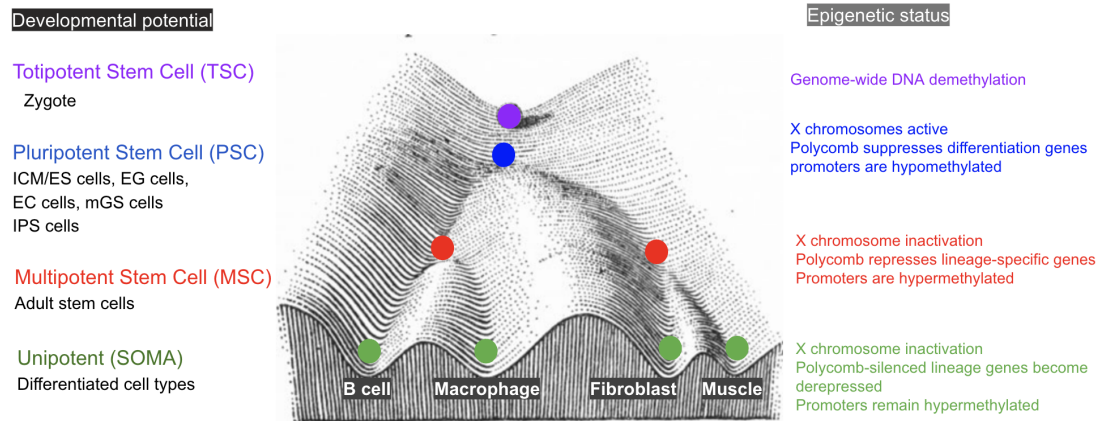
ECAR	extracellular acidification rate	PR	proline-rich
ECM	extracellular matrix	RNA	ribonucleic acid
EDTA	ethylenediaminetetraacetic acid	ROCK	Rho-associated protein kinase
ELISA	enzyme-linked immunosorbent assay	ROI	region of interest
EV	extracellular vesicles	RT	room temperature
EVOS	EVOS FL Cell Imaging System	RT-qPCR	reverse transcription quantitative PCR
FACS	fluorescence-activated cell sorting	SD	standard deviation
FAK	focal adhesion kinase	SEM	standard error of the mean
FBS	fetal bovine serum	SOX2	SRY-box transcription factor 2
FGF	fibroblast growth factor	SPMA	3-sulfopropyl methacrylate
FGFR	fibroblast growth factor receptor	TCP	tissue culture polystyrene
FTIR	Fourier-transform infrared spectroscopy	TE	trophectoderm
GAG	glycosaminoglycan	TMB	3,3',5,5'-tetramethylbenzidine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	TNT	tunneling nanotube
GFP	green fluorescent protein	TRIS	tris(hydroxymethyl)aminomethane
GlutaMAX	L-alanyl-L-glutamine	XF	Seahorse XF analyzer
hMSC	human mesenchymal stem/stromal cells	XPS	X-ray photoelectron spectroscopy
hPSC	human pluripotent stem cells	Y-27632	ROCK inhibitor Y-27632
HS	heparan sulfate	$\Delta\Psi_m$	mitochondrial membrane potential
iMEF	inactivated mouse embryonic fibroblasts	HSD	Honestly significant difference (Tukey's HSD)

Chapter 1. General Introduction

1.1 Pluripotent Stem Cells: Types, Self-Renewal, and Differentiation Regulation

Pluripotent stem cells (PSCs) are characterized by their ability to generate cells from all three germ layers: ectoderm, mesoderm, and endoderm (Thomson et al. 1998). Stem cell classification is determined by developmental potency, which underpins their applications in tissue regeneration, disease modeling, and cellular engineering (*Figure 1a*). Totipotent stem cells (TSCs), exemplified by the zygote and early blastomeres, possess the capacity to form both embryonic and extra-embryonic tissues, representing the highest degree of developmental potential (A. Smith 2006). In contrast, PSCs encompass embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), each capable of differentiating into any somatic lineage but unable to contribute to extra-embryonic structures. ESCs originate from the inner cell mass of the blastocyst and exhibit sustained self-renewal, whereas iPSCs are generated by reprogramming somatic cells with defined transcription factors such as OCT4, SOX2, KLF4, and c-MYC (Evans and Kaufman 1981, 1; Takahashi et al. 2007). Multipotent stem cells display more restricted lineage potential, giving rise to specific cell types within hematopoietic, mesenchymal, or neural lineages (Pittenger et al. 1999). A fine balance between self-renewal and differentiation defines stem cell function. Self-renewal maintains the stem cell pool, while differentiation enables specialization into distinct cell types (*Figure 1b*). This equilibrium is regulated by intrinsic transcriptional networks, including core regulators OCT4, SOX2, and NANOG, which maintain the pluripotent state (Boyer et al. 2005). Extrinsic signals from the stem cell niche, such as Wnt, Notch, and TGF- β signaling pathways, modulate fate decisions (Nusse et al. 2008). Disruptions in these regulatory pathways can lead to developmental defects or oncogenic transformation, underscoring the importance of understanding the molecular mechanisms that govern stem cell behavior (Reya et al. 2001).

a)



Hochedlinger and Plath 2009, p. 510

b)

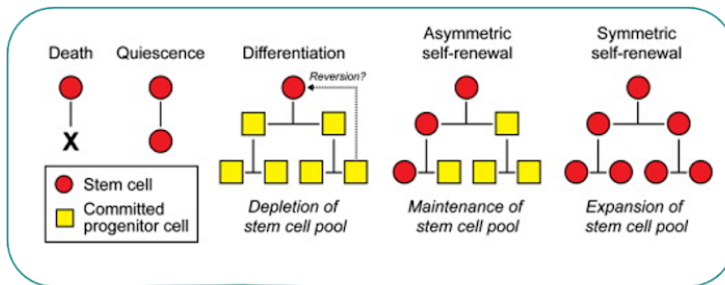


Figure 1: Human stem cells possess both potency and pluripotency, enabling them to differentiate into multiple specialized cell types.

a) The developmental potential and epigenetic states of cells at various developmental stages. It modifies C. H. Waddington's epigenetic landscape model to depict cell populations with distinct developmental potentials (left) and their corresponding epigenetic states (right). Developmental restrictions are illustrated by marbles rolling down a landscape into separate valleys, each representing a specific cell fate. Colored marbles indicate different differentiation states: purple for totipotent, blue for pluripotent, red for multipotent, and green for unipotent. Dashed arrows represent examples of reprogramming processes. Adapted, with permission, from Waddington (Waddington, 1957). **b)** Multiple facets to stem cell self-renewal. Schematic illustrating alternative stem cell fates—death, quiescence, differentiation, asymmetric self-renewal (maintenance of the stem cell pool), and symmetric self-renewal (expansion of the stem cell pool). Red circles denote stem cells and yellow squares denote committed progenitor cells.

1.2. hPSCs as microenvironment-responsive systems

hPSCs possess the ability to self-renew over the long term and the potential to differentiate into various specialized lineages, making them essential platforms for regenerative medicine, disease modeling, and cellular production. hPSC-derived cell types are being developed for tissue replacement and repair, while patient-specific models capture genetic background and disease phenotypes, improving the translational value of in vitro studies. Continued progress in defined, xeno-free, and fully synthetic culture systems is essential to transition hPSC-derived products toward clinical-grade workflows by enhancing reproducibility, safety, and standardization across laboratories and manufacturing pipelines ([Chaudhary and Villa-Diaz 2025a](#)). Simultaneously, hMSCs represent promising cell-based therapeutics for cancer, autoimmune diseases, and musculoskeletal disorders; however, their clinical translation is constrained by rapid declines in proliferative capacity and therapeutic potency during prolonged in vitro passaging, making large-scale expansion for clinical dosing a persistent challenge ([Nelson et al. 2026](#)).

Microenvironment control is essential for reproducibility, as the stem cell state is highly sensitive to external factors that can vary significantly across laboratories, researchers, and experimental batches. In human embryonic stem cell (hESC) and human pluripotent stem cell (hPSC) systems, even minor variations in extracellular matrix (ECM) composition, growth media formulation, substrate stiffness, and cell–cell interactions can alter integrin engagement and downstream signaling pathways. These changes can result in measurable differences in self-renewal capacity, lineage priming, and transcriptional outputs, even when the same cell line is utilized ([Ma et al. 2008](#)). Commonly used materials, such as Matrigel, are inherently complex and exhibit batch-to-batch variability, which complicates consistent cell attachment, differentiation efficiency, and experimental stability ([P. Zhou et al. 2022](#)). This variability has prompted a shift toward simpler, chemically defined, synthetic materials to reduce experimental uncertainty. Reproducibility is further complicated by the inherent variability of

stem cell types, such as MSCs, which can differ based on donor characteristics and tissue of origin. Even when standardized methods are employed, functional differences may persist (Maličev and Jazbec 2024). Therefore, rigorous control of the microenvironment—through standardization of matrix identity, ligand density, media chemistry, handling procedures, and defined biomaterial interfaces—serves as a quality control mechanism that minimizes uncontrolled variance and enhances interpretability, cross-study comparability, and scalability for manufacturing applications.

1.3 Integrins and Their Role in Stem Cell Regulation

The extracellular matrix (ECM) is a complex network of proteins that provides structural support and critical signals to cells, helping them adhere to surfaces, survive, grow, and differentiate into specific cell types. Integrins are key cell-surface receptors that mediate interactions between cells and the extracellular matrix, transmitting extracellular signals to intracellular pathways. They serve as critical linkers among extracellular components, the cytoskeleton, and intracellular signaling networks. Each integrin consists of two distinct subunits, designated α and β , and undergoes a conformational change from a bent, low-affinity state to an extended, high-affinity state to facilitate ligand binding and signal transduction (Luo and Springer 2006). In mammals, 24 unique integrins are formed from 18 α -chains and eight β -chains (Kawase and Nakatsuji 2023) (*Figure 2.a.*). The metal-ion-dependent adhesion site (MIDAS) within the integrin β subunit (e.g., ITG β 1) is essential for this process, coordinating divalent cations, typically Ca^{2+} , to stabilize ligand binding (G. Chen et al. 2011). Experimental studies demonstrate that Mn^{2+} significantly enhances integrin activation by stabilizing the extended conformation, even in the absence of extracellular matrix ligation. Specific integrin heterodimers exhibit distinct ligand preferences: RGD-binding integrins such as $\alpha\text{V}\beta$ 3 and $\alpha\text{V}\beta$ 5 bind fibronectin and vitronectin. At the same time, collagen receptors like α 1 β 1 and α 2 β 1 interact with fibrillar collagens and laminin (Barczyk

et al. 2010) (**Figure 2.b.**). Integrin activation initiates diverse signal transduction cascades that regulate cell differentiation, motility, polarity, proliferation, survival, apoptosis, and gene expression in a cell-type-specific manner. Due to their cell-surface localization, integrins also serve as valuable biomarkers for identifying distinct stem cell populations.

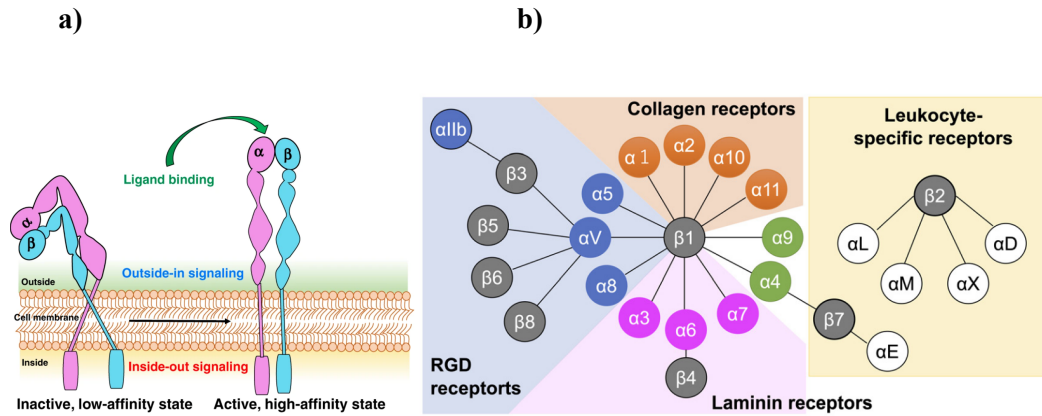


Figure 2: Integrins and laminins expressed in hPSCs

a) Schematic of integrin structure and activation, b) Characterization of integrins and laminins expressed in hPSCs (Ref. *Biomater. Sci.*, 2023, 11,2974)

Laminins are principal basement membrane ECM ligands. They deliver essential pro-self-renewal signals to hPSCs by engaging laminin-selective integrins, especially integrin α 6 (ITGA6; CD49f). In hPSCs, ITGA6 mainly associates with integrin β 1 (ITG β 1; CD29) to form the α 6 β 1 heterodimer (Luis G. Villa-Diaz et al. 2016). This serves as the primary laminin receptor in undifferentiated hPSCs. In contrast, pairing with alternative β subunits, such as β 4, is less common in pluripotent cells. Laminin- α 6 β 1 interactions enable strong cell adhesion and maintain colony integrity (Rodin et al. 2010). While certain laminin isoforms possess RGD motifs, these sequences are often hidden in the native laminin structure. Therefore, laminin-dependent adhesion typically relies on non-RGD domains (Tashiro et al. 1991). Peptide motifs such as YIGSR and IKVAV provide key recognition sites for laminin-selective integrins (Shenoy et al. 2025). Together, these features make laminins crucial regulators of the ECM (**Figure 3**). They connect the extracellular environment to integrin-mediated pathways that regulate hPSC self-renewal and cell-fate maintenance.

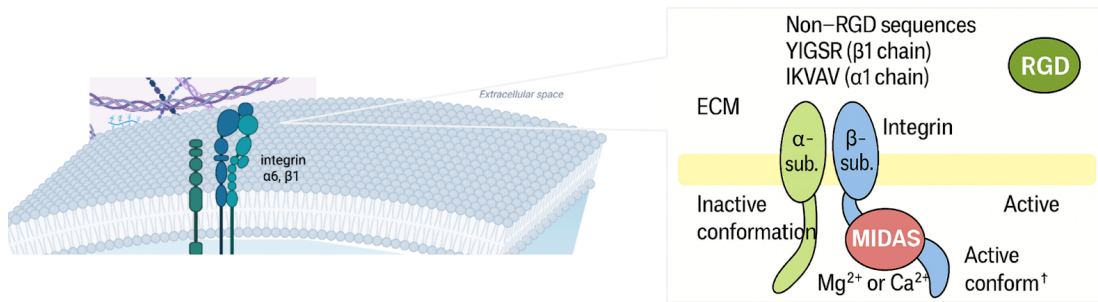


Figure 3: Integrin structure and activation

a) Schematic of integrin structure and activation, b) Characterization of integrins and laminins expressed in hPSCs (Ref. *Biomater. Sci.*, 2023, 11,2974)

hPSCs actively deposit and remodel their ECM, establishing an autocrine feedback loop that promotes self-renewal and phenotypic uniformity (Laperle et al. 2015a). Within defined culture systems, hPSCs synthesize basement membrane proteins, particularly $\alpha 5$ -containing laminins such as laminin-511 and laminin-521 (Rodin et al. 2010). These proteins facilitate cell adhesion through laminin-binding integrins, especially $\alpha 6\beta 1$. The interaction between laminin $\alpha 5$ and $\alpha 6\beta 1$ integrin in hPSCs is associated with tightly regulated, typically low or inactive, focal adhesion kinase (FAK) activity (Luis G. Villa-Diaz et al. 2016). This signaling state favors self-renewal over differentiation. Endogenous ECM remodeling enables hPSCs to create a microenvironment that reduces culture-induced variability and maintains long-term pluripotency.

1.4 Integrin $\alpha 6$ (ITGA6/CD49f)–Mediated Laminin Adhesion and Self-Renewal Signaling in Stem Cells

Integrin $\alpha 6$ (ITGA6/CD49f) is a laminin-binding integrin subunit that is broadly expressed in pluripotent, somatic, and cancer stem cell populations. Due to its widespread expression, ITGA6 serves as a reliable biomarker for identifying stem-like states. ITGA6 expression frequently distinguishes stem or progenitor cells from more differentiated lineages (Nieto-

[Nicolau et al. 2020](#)). (**Figure 4a**). ITGA6 mediates cell–extracellular matrix (ECM) adhesion by forming heterodimers, primarily with integrin $\beta 1$ (ITG $\beta 1$ /CD29) and, to a lesser extent, with integrin $\beta 4$ (ITGB4/CD104) ([Zheng et al. 2022](#)). These heterodimers function as receptors for specific laminin isoforms and regulate downstream signaling pathways associated with self-renewal and differentiation ([Lathia et al. 2010](#)). In pluripotent stem cells (PSCs), the ITGA6/ITG $\beta 1$ ($\alpha 6\beta 1$) heterodimer represents the predominant integrin complex. Conversely, ITGA6/ITGB4 expression remains low (less than 5%), consistent with reports indicating that ITGB4 is associated with more differentiated cell populations ([Krebsbach and Villa-Diaz 2017](#)). Upon engagement with laminin, $\alpha 6\beta 1$ is associated with a suppressed or low-baseline adhesion-signaling state in undifferentiated hPSCs, rather than robust activation that supports self-renewal. (**Figure 4b**).

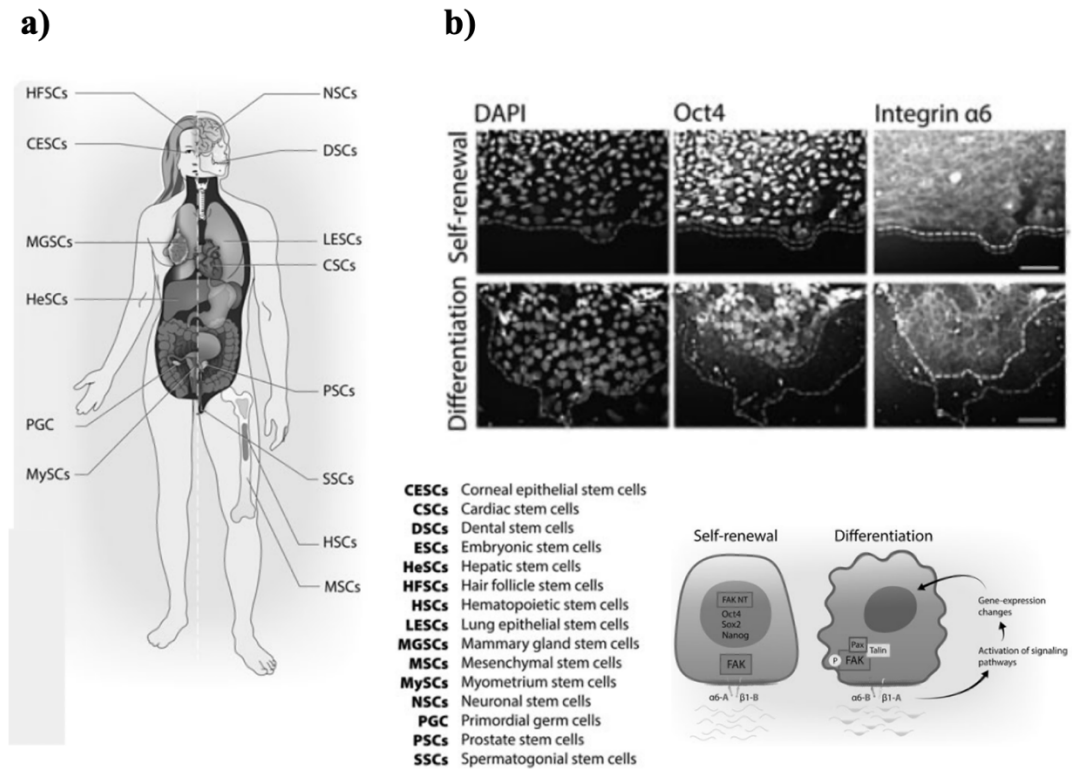


Figure 4: Integrin $\alpha 6$ (CD49f) is a conserved biomarker of stem cells involved in their self-renewal.

a) Illustration showing the presence of integrin $\alpha 6$ in diverse stem cell populations. **b)** Representative micrographs showing expression of integrin $\alpha 6$ with the pluripotent transcription factor Oct4 in undifferentiated (top panel) and differentiating (bottom panel) human ESC colonies. The drawing on the left depicts an undifferentiated ESC expressing pluripotency-related transcription factors Oct4, Sox2, and Nanog, as well as integrin $\alpha 6\beta 1$. (Ref. *Stem cells and development*, 26-15,)

1.5 Focal Adhesion Kinase as a Central Mediator of Adhesion-Dependent Gene Regulation: Linking Focal Adhesions to Nuclear Signaling

FAK (PTK2) functions as an integrin-proximal signaling hub that transduces ECM) adhesion into cytoskeletal remodeling and downstream signaling events (Guan 1997).

Canonical Focal adhesion kinase signaling

The canonical FAK–Src signaling axis controls focal adhesion turnover and actin cytoskeleton dynamics (**Figure 5**). Following integrin engagement and receptor clustering,

FAK is recruited to focal adhesions, where it undergoes autophosphorylation at tyrosine 397 (Y397), generating a binding site for Src-family kinases. This phosphorylation event promotes the assembly of multi-protein signaling complexes containing adaptor proteins such as paxillin and other focal adhesion constituents ([Aakriti et al. 2025](#)). Additionally, it activates key signaling pathways, including PI3K–AKT and MAPK/ERK, which modulate adhesion strength, cell survival, proliferation, polarity, and migration depending on cellular context ([M. A. Westhoff et al. 2004](#)).

Non-Canonical Focal Adhesion Kinase Signaling. Beyond its membrane-associated roles, FAK can localize to the nucleus, where it functions as a non-canonical scaffold influencing gene regulation. In this nuclear context, FAK operates through mechanisms independent of its kinase activity, primarily mediated by its N-terminal FERM domain ([S. Lim 2013](#)). The FERM domain enhances MDM2-mediated ubiquitination and degradation of p53, thereby altering transcriptional programs that regulate cell-cycle progression and apoptosis. As a result, nuclear FAK directly modulates gene expression profiles and cell fate decisions ([M. Westhoff et al. 2017](#)). Collectively, these dual functions establish FAK as a spatially regulated integrator that couples extracellular microenvironmental signals to transcriptional regulation and cell state determination

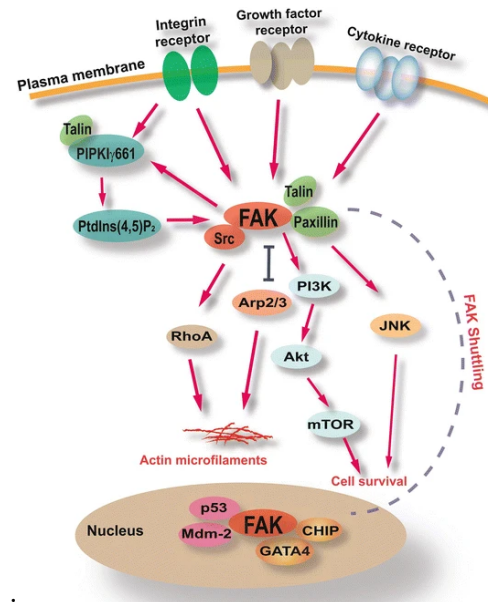


Figure 5: Model of canonical FAK signaling upon activation by integrins.

FAK signaling upon activation of growth factor receptors, and/or cytokine receptors. FAK, together with Src, paxillin, and talin, triggers downstream signaling pathways, including, but not limited to, PI3K/Akt/mTOR, JNK, and Rho GTPases. Disassociation of the Arp2/3 complex from FAK may induce actin turnover. FAK inhibition enhances its nuclear localization. (Ref. Choi, S. (eds) Encyclopedia of Signaling Molecules,)

1.6 Culture Confounders and Engineering Solutions

Stem cell culture systems are widely utilized; however, they frequently introduce confounding microenvironmental variables. Such variables complicate the interpretation of cell states, signaling pathways, and functional outcomes. Feeder-dependent workflows, in particular, expose hPSCs to numerous heterogeneous and poorly controlled inputs, including feeder-derived ECM, soluble factors, extracellular vesicles, and contact-dependent interactions. These factors can unintentionally influence transcriptional programs, cellular metabolism, and the propensity for differentiation. Recent studies indicate that feeder systems may facilitate unintended biological transfer, such as the exchange of cellular components.

Synthetic biomaterials address these limitations by providing chemically defined, reproducible, and scalable culture interfaces. These materials separate stem cell maintenance from xenogeneic variability (Jin et al. 2013). Engineered defined substrates enable precise control over ligand presentation, adhesion strength, and matrix remodeling. This strategy improves batch-to-batch consistency, supports xeno-free manufacturing, and enables robust expansion. It also preserves functional potency in both pluripotent and adult stem cell platforms (Hoang et al. 2021).

1.7 Dissertation objective and roadmap

The objective of this dissertation is to elucidate how the microenvironment regulates stem cell fate. This is achieved through an integrated framework connecting extracellular matrix (ECM)–integrin signaling, FAK/MDM2 spatial regulation, matrix remodeling, and engineered culture substrates. **Chapter 4** establishes the mechanistic basis by demonstrating that integrin $\beta 1$ –dependent adhesion regulates pluripotency, self-renewal, and gene expression via FAK signaling, including regulatory outputs associated with nuclear NT-FAK. **Chapter 5** addresses a significant culture-derived confounder. It characterizes mitochondrial exchange between feeder cells and hPSCs and determines how this unintended biological transfer alters cellular bioenergetics and influences fate-associated phenotypes. **Chapter 6** applies these microenvironment design principles to adult stem cells. It demonstrates that glycosaminoglycan-inspired polymer brush biomaterials enhance the expansion of human mesenchymal stem cells while preserving trilineage differentiation capacity.

Collectively, these chapters support a central model of stem cell microenvironmental regulation, in which ECM–cell interactions, cell–cell interactions, and ECM–soluble factor–cell interactions jointly govern stem cell behavior. Together, these findings provide actionable strategies to improve reproducibility and scalability in stem cell research and biomanufacturing.

Chapter 2. Literature Review / Background

2.1 Fate control in hPSCs

Fate control in hPSCs is a balance between self-renewal and differentiation competence. A single regulatory mechanism does not govern it. Instead, hPSC fate depends on an interconnected system that includes: **(i)** a core transcriptional circuitry (OCT4, SOX2, NANOG) sustaining pluripotency identity; **(ii)** upstream signaling pathways that modulate transcriptional states and lineage priming; and **(iii)** a metabolic program supporting rapid proliferation and providing regulatory metabolites that influence chromatin structure and gene expression (Z. Wang et al. 2012).

2.1.1 Core pluripotency network and its architectural logic

OCT4 (POU5F1), SOX2, and NANOG constitute the core of the pluripotency program in hESCs. Genome-scale mapping demonstrates that these factors frequently co-occupy target sites, forming a tightly regulated circuit (Boyer et al. 2005). This arrangement activates genes supporting pluripotency and maintains developmental regulators in a poised state. The architecture preserves cell identity through self-renewal genes, while differentiation genes remain silent, poised to respond to differentiation signals. NANOG contributes to the stabilization of the undifferentiated state. Expression-cloning studies identified NANOG as a key factor in maintaining undifferentiated embryonic stem cells (**Figure 6**), supporting the concept that pluripotency is an actively sustained state rather than a passive, default undifferentiated state (Chambers et al. 2003).

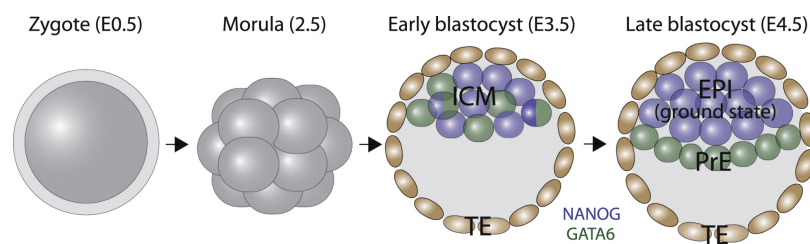


Figure 6. Establishment of the Pluripotent Ground State In Vivo

The zygote undergoes cleavage to form a morula and, by E3.5, a blastocyst comprising extraembryonic trophectoderm (TE) and the pluripotent inner cell mass (ICM). [Ref. Hackett et al. \(2014\). Cell stem cell, 15\(4\), 416-430.](#)

2.1.2 Upstream signaling pathways

hPSC signaling sensitivity: integrating pluripotency networks with fate control

hPSCs remain poised near developmental branch points because the core transcriptional circuitry both stabilizes pluripotency and serves as an information hub that integrates inputs from growth-factor and morphogen pathways ([Zorzan et al. 2020](#)). These signaling pathways, including TGF β /Activin, FGF, and WNT, modulate the transcriptional circuit, fine-tuning the output in a graded rather than binary manner (**Figure 7**). When a single pathway is partially inhibited, the crosstalk and compensation within the network dampen immediate differentiation. Instead, this network-level integration amplifies transcriptional noise and lineage priming, causing heterogeneity before overt phenotypic changes occur ([Yin et al. 2025](#)). Signaling sensitivity in hPSCs represents a key developmental characteristic.

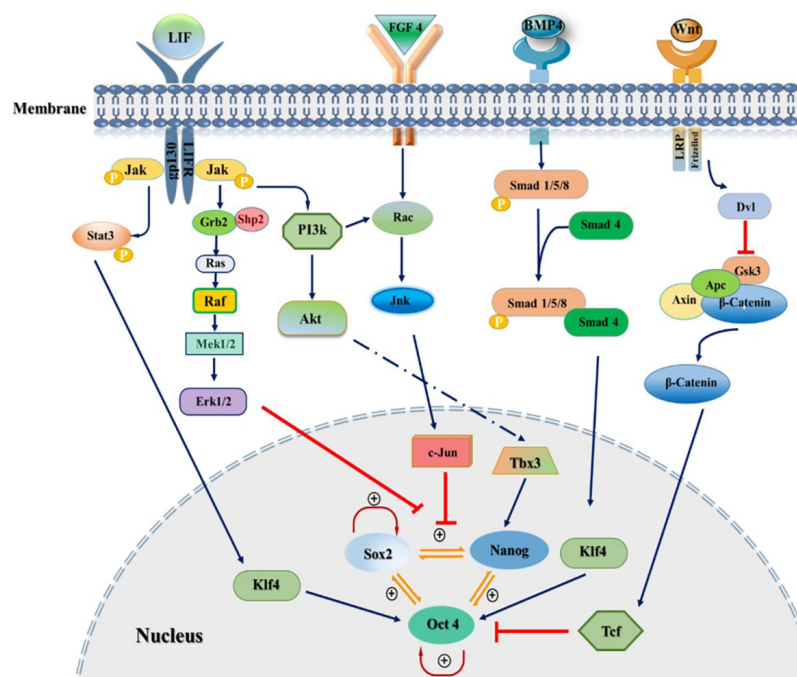


Figure 7. Integrated signaling control of pluripotency in PSCs.

Major upstream pathways (FGF/ERK–PI3K/AKT, Activin/Nodal–SMAD2/3, BMP–SMAD1/5/8, and WNT/β-catenin) converge on the OCT4/SOX2/NANOG transcriptional circuitry to stabilize or destabilize the pluripotent state in a context-dependent manner. Ref. Mossahebi-Mohammadi et al. (2020). Front Cell Dev Biol. 2020;8:79. doi:10.3389/fcell.2020.00079.

Activin/Nodal–FGF synergy as a major pluripotency-stabilizing axis

In hPSCs, Activin/Nodal signaling through SMAD2/3 is central. This signaling is required to maintain hESCs' pluripotency. FGF signaling works with Activin/Nodal to support pluripotency in defined conditions (Brown et al. 2011). This cooperation constitutes a division of labor: Activin/Nodal–SMAD2/3 sustains pluripotency programs and restrains differentiation, while FGF signaling fosters proliferation and inhibits destabilizing cues (Xiao et al. 2006).

Active suppression of differentiation: BMP signaling as a fate-driving pathway

BMP signaling controls cell-fate decisions in human ESCs. Xu and colleagues demonstrated that hESCs in unconditioned medium exhibit high BMP activity. To suppress BMP signaling, bFGF is utilized to preserve undifferentiated cell proliferation (Xu et al. 2005). hPSC self-renewal requires both activation of pro-pluripotency signals and active suppression of differentiation cues, notably BMP signaling in relevant contexts (Sarah Warsi et al. 2020). BMP functions as a differentiation driver; for instance, BMP4 exposure initiates trophoblast-like differentiation from hESCs.

WNT/β-catenin signaling context dependence and state-dependent outcomes

Canonical WNT signaling influences self-renewal and differentiation depending on the context. Sato and colleagues reported that WNT pathway activation via GSK-3 inhibition maintains self-renewal in both human and mouse ESCs. These observations support the perspective that WNT can stabilize pluripotency under specific conditions (Sato et al. 2004).

The effects of the WNT pathway depend on the underlying network state, the timing of activation, and interactions with pathways such as Activin/Nodal and FGF. WNT acts as a modulator, supporting self-renewal or promoting lineage specification, depending on specific circumstances.

Fate control as a systems integration problem

The OCT4/SOX2/NANOG network provides the foundation for pluripotency, but cell fate depends on its integration with multiple signaling pathways. Pluripotency is maintained as a metastable state through coordinated SMAD2/3–FGF input and active suppression of BMP signaling. When lineage-promoting signals overcome network reinforcement or the core network is weakened, differentiation occurs. This framework recognizes cell fate as an outcome of systems-level integration, spanning stable pluripotency to priming and differentiation, and reframes fate control as more than a simple binary switch.

2.2 Metabolic features linked to pluripotency and differentiation

PSCs demonstrate rapid proliferation and elevated biosynthetic demands. Distinct metabolic configurations sustain these demands by enabling energy production, macromolecular synthesis, and direct regulatory effects. While metabolism was once considered passively linked to cell fate, recent studies show it actively modulates gene regulation, influences epigenetic states, and shapes differentiation competence. Overall, metabolism acts as both a driver and a reinforcer of pluripotent identity by regulating ATP supply, redox balance, and the metabolites required by chromatin-modifying enzymes.

2.2.1 Metabolism is both a driver and reinforcer of pluripotent identity.

A glycolysis-dominant metabolic profile supports the growth and biosynthetic activities characteristic of pluripotency.

hPSCs typically display elevated glycolytic flux and increased lactate production (Gu et al. 2016). These include nucleotide and lipid biosynthesis. This metabolic setup supports rapid proliferation and provides intermediates for anabolic pathways. These include nucleotide and lipid biosynthesis. Zhang and colleagues emphasized that glycolysis supports not only energy generation but also fulfills biosynthetic and redox requirements (Folmes et al. 2012). These functions are essential for self-renewal and maintenance of pluripotency programs.

Metabolic remodeling occurs as a causal process accompanying differentiation, often leading to a shift toward oxidative metabolic programs.

During differentiation, hPSCs often develop increased mitochondrial oxidative phosphorylation (OXPHOS) and change mitochondrial morphology. These cells also exhibit enhanced respiratory function and altered redox homeostasis (Nit et al., 2021). Teslaa and Teitell identified these transitions as recurring hallmarks of pluripotency and lineage commitment (Teslaa and Teitell 2015). Mitochondrial development and reorganization are commonly associated with differentiation, and these metabolic changes function as adaptations. Differentiated cell types frequently rely on mitochondrial ATP production to sustain specialized functions and ensure long-term energy balance.

A critical consideration is whether changes in metabolic state causally drive fate transitions and cellular reprogramming, rather than merely accompanying them.

A key mechanistic insight is that metabolic alterations may precede transcriptional changes. Folmes et al. demonstrated that somatic cell reprogramming is characterized by an early metabolic shift from oxidative to glycolytic pathways. This alteration is associated with the acquisition of pluripotency. Stimulating glycolysis increased reprogramming efficiency, whereas inhibiting it reduced it, supporting a causal role for glycolytic remodeling in establishing pluripotency (Sekita et al. 2020).

Collectively, these observations support a general principle of fate control in hPSCs: metabolic rewiring causally modulates the effective barrier between transcriptional states, thereby influencing the probability that cells maintain pluripotency or undergo differentiation.

2.2.2 Metabolites that modulate chromatin-modifying enzymes.

Metabolite regulation of chromatin and transcription as a mechanistic bridge

The influence of metabolism on cell fate is partially mediated by metabolites that serve as substrates or cofactors for chromatin-modifying enzymes. For example, acetyl-CoA abundance can impact histone acetylation, chromatin structure, and transcriptional permissiveness. Yu et al. described how glycolysis-derived carbons promote cytosolic acetyl-CoA formation and histone acetylation, linking metabolic flux to epigenetic regulation in the maintenance of pluripotency (Yu et al. 2024). These programs are associated with the maintenance of pluripotency and illustrate the connection between metabolic flux and epigenetic regulation.

Metabolic regulation is heterogeneous and lineage dependent.

Although transitions from glycolysis to OXPHOS are widespread, metabolic adaptations linked to cell fate are not universal across differentiation pathways. Certain lineages have distinct metabolic needs, and some differentiation events proceed without requiring a specific metabolic transition. Appreciation of this variability is critical for accurate interpretation of experimental data. Changes in glycolytic and oxidative activities can serve as both causes and consequences of lineage specification, with the direction of these changes influenced by lineage identity, culture conditions, oxygen tension, and cellular stress.

2.2 ECM–integrin signaling in hPSCs

hPSCs are anchorage-dependent and interpret the ECM as both a structural scaffold and a signaling platform. In feeder-free systems, this ECM interface is especially instructive. The *identity* and *density* of matrix ligands determine which integrin heterodimers are engaged and specifically affect downstream processes, including cell adhesion strength, FAK-mediated survival signaling, generation of cytoskeletal tension via actomyosin activation, and maintenance of transcriptional stability (Maldonado and Leyton 2023). Fate control is not governed exclusively by soluble factors such as TGF β , FGF, and WNT; pluripotency stability also requires an adhesive microenvironment that minimizes stress-induced priming while permitting necessary signaling states (Lee et al. 2010a). Laminin-rich basement membrane matrices, especially those containing laminin $\alpha 5$ isoforms, consistently support stable hPSC self-renewal under chemically defined conditions.

2.2.1 Laminin isoforms (LN-511/521), ECM deposition, and adhesion strength

Laminin $\alpha 5$ –containing isoforms are dominant self-renewal substrates.

Laminins are heterotrimeric basement membrane proteins composed of α , β , and γ chains. In hPSC culture, laminins containing the $\alpha 5$ chain, particularly LN-511 and LN-521, are strongly associated with robust cell attachment, long-term genomic stability, and sustained pluripotency (Lee et al. 2010b). Rodin et al. demonstrated that recombinant human laminin-511 enables long-term feeder-free expansion of both hESCs and human hiPSCs (Rodin et al. 2010). This evidence indicates that a defined basement membrane ligand can replace xenogeneic, ill-defined matrices in supporting self-renewal. Additionally, LN-521 in combination with E-cadherin supports clonal survival and long-term maintenance in a defined, xeno-free environment. These findings highlight the synergistic roles of basement membrane adhesion and cell–cell adhesion in suppressing dissociation-induced stress responses and stabilizing self-renewal.

Adhesion strength is a biological control variable in hPSC stability.

In hPSCs, adhesion strength is a critical determinant of fate stability rather than a mere mechanical property. Weak or unstable attachment increases the risk of anoikis, elevates cytoskeletal stress signaling, and enhances the likelihood of differentiation-associated transcriptional drift ([Singh et al. 2013](#)). In contrast, LN-511/521 substrates typically promote uniform cell spreading and stable colony or monolayer architecture, thereby improving reproducibility across passages.

Laminin fragments (E8) reveal how ligand density and presentation control adhesion efficiency.

The use of laminin fragments, particularly E8 fragments, represents a significant advancement in hPSC culture. These fragments retain integrin-binding activity while enabling scalable, well-defined culture conditions. Miyazaki et al. demonstrated that laminin fragments can substantially accelerate hPSC adhesion and permit efficient attachment without the need for traditional precoating ([Miyazaki et al. 2012](#)). These findings indicate that ligand presentation during the earliest post-passaging period is a critical determinant of cell survival and stable outgrowth.

ECM deposition by hPSCs is an active component of the self-renewal program

An important finding from defined culture systems is that hPSCs can synthesize their own supportive ECM, making the initial substrate coating less relevant over time ([J. Zhang et al. 2022](#)). Laperle et al. identified endogenously produced LAMA5 as a key ECM component in undifferentiated hPSCs and demonstrated that LAMA5 promotes survival and self-renewal through both autocrine and paracrine mechanisms ([Laperle et al. 2015b](#)). Genetic depletion of LAMA5 led to increased apoptosis and reduced self-renewal, defects that exogenous LN-521 rescued. These results indicate that α 5-laminins constitute a functionally essential ECM axis

in pluripotent cultures. The partial rescue observed with blebbistatin or ROCK inhibition in this study suggests that laminin deposition stabilizes cell fate by reducing cytoskeletal tension and stress signaling. These findings demonstrate that ECM composition can mechanochemically regulate cell survival.

2.2.2 Integrin $\alpha 6$ signaling and self-renewal stability

Integrin $\alpha 6\beta 1$ is a principal laminin receptor in hPSC culture.

Integrins transduce ECM binding into intracellular signals through adhesion complexes, cytoskeletal linkages, and kinase activation ([Katoh 2025](#)). In hPSC systems, integrin $\alpha 6\beta 1$ (CD49f/CD29) functions as the primary laminin receptor and is essential for interpreting LN-511/521 signals ([Nakashima and Tsukahara 2022](#)). Structural studies by Arimori et al. resolved the structure of the $\alpha 6\beta 1$ integrin in complex with a laminin-511 fragment, providing a molecular explanation for the high specificity of $\alpha 6\beta 1$ for $\alpha 5$ -laminins in basement membranes ([Arimori et al. 2021](#)).

Pluripotency stability depends on *how* integrin signaling is routed, not just whether adhesion occurs.

A common assumption in adhesion biology is that increased integrin signaling universally supports stemness. However, hPSCs require a specific integrin signaling configuration that enables attachment and survival while preventing differentiation-associated adhesion signaling. [Villa-Diaz et al.](#) provided direct evidence by demonstrating that inhibition of focal adhesion kinase (FAK) signaling downstream of integrin $\alpha 6\beta 1$ supports hPSC self-renewal ([Villa-Diaz et al. 2016](#)). In this context, hPSCs maintain high $\alpha 6\beta 1$ expression while FAK activity remains low, as indicated by reduced Y397 phosphorylation. Differentiation is associated with decreased $\alpha 6$ expression and increased FAK activation.

Integrin $\alpha 6$ –laminin engagement interfaces with survival pathways and cytoskeletal stress control

Downstream of integrins, cell survival is primarily mediated by PI3K/AKT signaling, while differentiation and motility are associated with increased FAK/Src and RhoA–ROCK signaling. In practice, dissociation, sparse plating, and low-adhesion conditions elevate actomyosin contractility and promote apoptosis ([Jiang et al. 2017](#)). This mechanistic insight explains why LN-521-based systems frequently outperform weaker-binding substrates during cloning or gene editing. The clonal culture strategy developed by Rodin, which uses LN-521 and E-cadherin, demonstrates that hPSCs achieve optimal fate stability when both substrate adhesion and junctional integrity reduce stress signaling during reattachment ([Rodin et al. 2014](#)).

Integrin usage differs across defined substrates and can bias fate competence.

Although recombinant vitronectin was initially shown to support hESC self-renewal through $\alpha V\beta 5$ -mediated adhesion ([Braam et al. 2008](#)), subsequent studies demonstrated that hPSCs cultured on defined substrates deposit endogenous laminin, thereby shifting matrix engagement toward $\alpha 6\beta 1$ -dependent mechanisms that support self-renewal.

Summary

Current literature supports a model in which LN-511/521 substrates stabilize hPSC self-renewal through multiple mechanisms. First, high-fidelity adhesion via $\alpha 6\beta 1$ reduces detachment stress and enhances survival. Second, controlled adhesion signaling amplitude ensures that excessive focal adhesion activation is unnecessary and may otherwise destabilize pluripotency. Third, ECM self-reinforcement through laminin $\alpha 5$ deposition establishes a laminin-rich niche that supports self-renewal and reduces apoptosis. Fourth, context-dependent mechanochemical regulation occurs, with cytoskeletal contractility and dissociation stress acting as fate-modulating factors that $\alpha 5$ -laminin matrices and junction-

supportive strategies such as LN-521 and E-cadherin can mitigate. This framework positions ECM–integrin signaling as a central upstream regulator of fate control, operating in parallel with soluble growth factor pathways and influencing the noise tolerance of the OCT4/SOX2/NANOG network.

2.3 FAK signaling and spatial regulation

Focal adhesion kinase (FAK; PTK2) is a multidomain non-receptor tyrosine kinase that functions as a proximal integrin effector and serves as a central hub connecting ECM adhesion to survival, cytoskeletal remodeling, and transcriptional regulation. In hPSCs, this signaling module is particularly significant because it operates at the interface between the ECM–integrin system and growth factor pathways that govern pluripotency. The literature identifies two coupled regulatory layers as determinants of FAK output. The first is phosphorylation-state control, which regulates kinase activity and scaffolding interactions. The second is subcellular localization control, which dictates whether FAK acts within adhesion complexes or participates in nuclear gene-regulatory mechanisms. This dual regulatory mechanism provides a framework for understanding how similar upstream adhesion cues can generate distinct cell-state outcomes depending on cellular context.

2.3.1 Domain organization and activation architecture

FAK has three main regions: an N-terminal FERM domain, a central kinase domain, and a C-terminal region with proline-rich motifs and a focal adhesion targeting (FAT) domain (**Figure 8**). This arrangement supports both enzymatic signaling and multiprotein scaffolding. In its basal state, FERM and kinase domains interact to keep FAK autoinhibited. Activation usually occurs when integrin engagement and adhesion assembly change FAK's shape, exposing regulatory tyrosine for phosphorylation. The N-terminus also contains signals for nuclear localization and export, enabling compartment switching beyond adhesions ([Acebrón et al. 2020](#)).

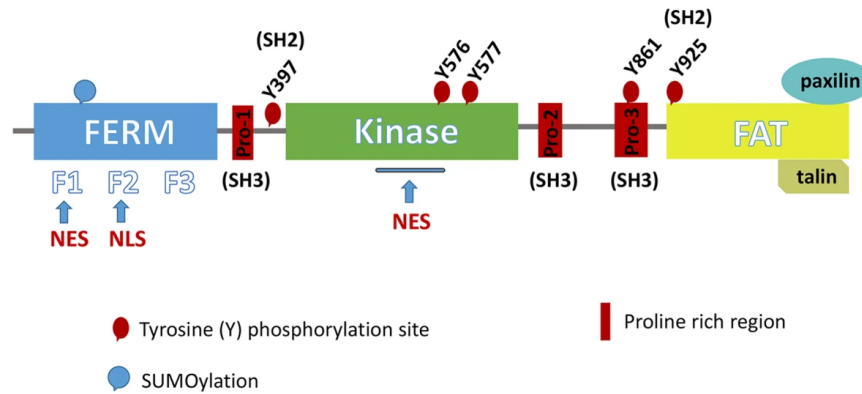


Figure 8. FAK domain structure.

FAK comprises an N-terminal FERM (F1–F3), central kinase, and C-terminal FAT domain; it contains NES/NLS motifs, proline-rich SH3-binding regions, and key phosphorylation sites including Y397 and Y925 (SH2-binding). *Ref. Zhou, J. et al. (2019). J Exp Clin Cancer Res 38, 250*

2.3.2 Canonical Focal Adhesion Kinase Signaling

The canonical initiating step in FAK activation is autophosphorylation at Y397, which generates a high-affinity SH2 docking site for Src-family kinases (**Figure 9**). This event is widely recognized as the molecular gateway linking integrin engagement to downstream kinase cascades, as it stabilizes the formation of the FAK–Src complex and enables both amplification and diversification of downstream signaling outputs ([Klinghoffer et al. 1999](#)).

After Src recruitment, FAK is phosphorylated at additional sites, including Y576 and Y577 in the kinase domain, which promotes full catalytic activity. Additional phosphorylation in the C-terminal region, such as at Y861 and Y925, maintains signaling and supports adaptor protein recruitment, broadening FAK’s signaling capacity ([Tan et al. 2023](#)).

Phosphorylation serves functions beyond a simple on/off switch; it contributes to the dynamic assembly and disassembly of focal adhesions. As a result, the duration and magnitude of signaling are influenced by adhesion turnover. Y397 phosphorylation is particularly critical for these dynamics, suggesting that phospho-FAK regulates adhesion remodeling ([Rondas et al. 2011](#)).

PI3K–AKT signaling (survival and buffering against adhesion stress)

A main role of integrin–FAK signaling is to protect against detachment-induced apoptosis (anoikis). It helps maintain survival when cells depend on anchorage. FAK–Src signaling often converges on PI3K–AKT signaling, which boosts cell survival and stress resistance. In hPSC culture, dissociation and replating cause cytoskeletal and membrane stress (Vitillo and Kimber 2017). This pathway is thus crucial for maintaining cell viability and preventing stress-driven secondary differentiation.

MAPK/ERK signaling (proliferative outputs and context-dependent fate bias)

FAK signaling activates MAPK/ERK via adaptor-mediated complex assembly at adhesion sites. While ERK can promote proliferation, in hPSC contexts, ERK often modulates cell fate. Prolonged or elevated ERK activity coincides with lineage priming, while restrained ERK activity supports self-renewal. FAK-to-ERK coupling yields state-dependent outcomes: identical ERK outputs may differ based on transcriptional circuitry and morphogen environment (Vitillo et al. 2016).

mTOR network coupling (growth, translation, and metabolic allocation)

FAK-dependent PI3K/AKT activation functionally intersects with mTOR signaling, thereby linking the adhesive state to biosynthetic capacity and cell growth programs. Although specific molecular interactions may vary across contexts, a consistent theme is that FAK operates upstream of multiple growth modules that affect cell-state stability through gradual biosynthetic outputs rather than solely through acute cytoskeletal effects.

2.3.3 Non-canonical Focal adhesion kinase signaling

Mechanistic basis for nuclear–cytoplasmic shuttling

FAK has trafficking motifs that let it move between focal adhesions and the nucleus.

Ossovskeya et al. showed that FAK has two nuclear export sequences (NES), one in the FERM domain and one in the kinase domain. This confirms that nuclear partitioning is controlled rather than accidental ([Ossovskeya et al. 2008a](#)). Other reviews identified nuclear localization signals (NLS) within the FERM domain of FAK. They stress that shuttling is a conserved feature among FAK-family kinases ([Ossovskeya et al. 2008b](#)).

Nuclear FAK as a gene-regulatory scaffold (kinase-independent signaling)

In the nucleus, FAK can regulate transcription through scaffold functions that do not require its kinase activity. Lim et al. found that nuclear-targeted FAK encourages proliferation and survival. It does this by helping Mdm2-dependent p53 degradation, using the FERM domain as a scaffold to increase p53 turnover ([S.-T. Lim et al. 2008](#)). This mechanism aligns with broader principles of p53 regulation by ubiquitination pathways, in which Mdm2 is a major E3 ligase controlling p53 stability ([Kubbutat et al. 1997](#)).

Nuclear FAK as an active transcriptional regulator (kinase-dependent outputs)

Nuclear FAK also directly affects transcription. Serrels et al. showed that nuclear FAK kinase activity regulates chemokine transcription networks. These networks shape how cells interact with the immune microenvironment. This demonstrates that nuclear FAK can be a transcriptional regulator with clear physiological effects ([Serrels et al. 2015](#)).

Although these findings were developed primarily in cancer models, they establish the general principle that nuclear FAK can modulate gene expression programs through mechanisms distinct from adhesion-site signaling. This supports the broader conceptual distinction between adhesion-localized mechanochemical outputs and nuclear gene-regulatory outputs.

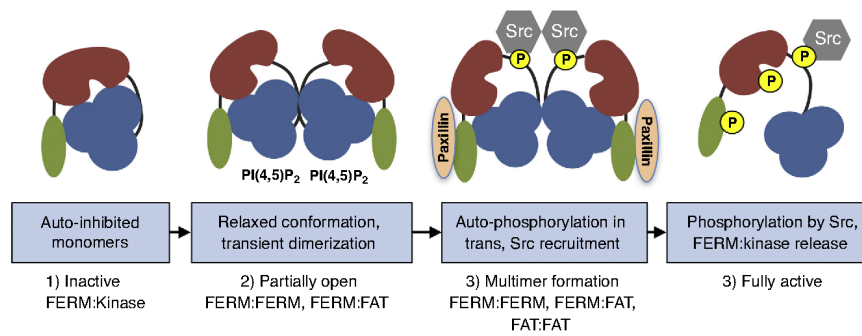


Figure 9. Schematic model of FAK activation.

In the inactive state, FAK exists as an autoinhibited monomer stabilized by intramolecular interactions between the FERM (blue) and kinase (red) domains; upon FERM binding to phosphatidylinositol-4,5-bisphosphate [PI(4,5)P₂], FAK adopts a more open conformation that promotes clustering and transient FERM: FERM and FAT: FAT dimerization, enabling trans-autophosphorylation at Y397 (yellow). At focal adhesions, paxillin can reinforce FERM–FAT association (FAT, green), and pY397 creates an SH2-dependent docking site for Src, which subsequently phosphorylates additional tyrosine residues to drive a conformational transition that releases FERM–kinase autoinhibition and yields full FAK activation. [Ref. Kleinschmidt EG, et. Al. \(2017\), Curr Opin Cell Biol. 45:24-30.](#)

2.3.4 Conceptual model: phosphorylation + localization define “FAK state” in fate control

In pluripotent stem cell systems, the 'FAK state' integrates two regulatory axes. The first, the activation axis, is a phosphorylation cascade that begins with autophosphorylation at Y397. Src recruitment follows, then Y576/Y577 activation-loop phosphorylation, and later events (like at Y861 and Y925) that broaden scaffold capacity and diversify downstream pathways. However, in hPSCs, this canonical adhesion-associated FAK activation program is generally suppressed or maintained at a low basal state under self-renewing conditions. The second, the spatial axis, describes FAK's subcellular routing. Adhesion-localized FAK mainly supports rapid cytoskeletal and survival signaling. Nuclear FAK enables longer-term transcriptional control via scaffold-dependent and kinase-dependent mechanisms. This two-dimensional framework matters in hPSCs because pluripotency destabilizes gradually—through stress-

induced priming and cell heterogeneity—rather than abruptly. Therefore, even subtle release of canonical FAK suppression, or changes in nuclear partitioning, can alter network stability and bias cell fate, even when canonical pluripotency markers remain present.

2.3 Microenvironment confounders

In hPSC culture, the microenvironment extends beyond the defined medium. Cells encounter matrix-derived cues, soluble factors, and in situ-produced particulate cargo (*Figure 10*).

Confounding occurs when these components fluctuate independently of the experimental variable, altering baseline signaling, cellular stress responses, or lineage priming and thereby complicating causal interpretation. Such issues are particularly evident in xenogeneic feeder-based workflows and systems that use animal-derived matrices, which have incompletely defined and variable biological compositions across different lots and over time.

2.3.1 MEF-derived paracrine and matrix cues as confounders

Xenogeneic feeder layers, such as mouse embryonic fibroblasts (MEFs), establish an active cellular niche rather than serving as a passive substrate. These feeders secrete a range of factors and provide ECM ligands, influencing integrin engagement and downstream signaling pathways. Proteomic analyses demonstrate that supportive and non-supportive feeders secrete distinct protein profiles. Certain secreted factors can partially substitute for conditioned media and help maintain pluripotency markers ([Chin et al. 2007](#)). These findings indicate that feeder output is both significant and variable between preparations. Insufficient control of feeder parameters contributes to increased experimental variability.

Feeder and Matrix Preparation Variables that Shift Baseline Signaling

Operational variability in the preparation and use of feeder layers represents a significant confounding factor. Key variables include feeder strain or source, passage number, inactivation method, plating density, time elapsed since plating, and media exchange

schedules. Each of these parameters influences feeder-derived signals and ECM deposition. Consequently, two cultures described as 'MEF-supported' may experience markedly different niche environments, even when maintained in identical basal media. These differences can affect cell adhesion, proliferation, passaging efficiency, stress sensitivity, and baseline lineage priming before changes in OCT4, SOX2, or NANOG expression. The use of xenogeneic matrices such as Matrigel further increases variability, as Matrigel is poorly defined, exhibits lot-to-lot variation, and contains residual growth factors. These characteristics complicate the reproducibility of differentiation outcomes.

Microbial Contamination and Culture Adaptation as Hidden Confounders

Xenogeneic systems introduce significant biosafety and translational risks that may compromise phenotypic analyses. Microbial contamination, particularly by mycoplasma, is prevalent and can alter host cell biology and gene expression. Large-scale studies indicate that such contamination is common in current datasets ([Nagano et al. 2005](#)). Subclinical infections can undermine reproducibility even in the absence of overt symptoms. Prolonged culture under stressful or fluctuating conditions may select for genomic abnormalities in hPSCs. Reviews of genomic integrity frequently report recurrent chromosomal alterations during cell expansion ([Ross et al. 2011](#)). Therefore, rigorous monitoring is essential, especially when stressful conditions or feeder variability may promote genetic changes. Unstable microenvironments impose stress and selection pressures that accelerate culture adaptation.

Potential hazards	Effects on hPSCs
 Zoonotic pathogen transmission	▶ Viruses, mycoplasma, or prion contamination
 Batch variability of feeder cells or serum	▶ Unpredictable growth kinetics; uncontrolled differentiation
 Genomic instability	▶ Long-term culture increases risk of chromosomal abnormalities
 Immune rejection	▶ Xeno-antigens trigger immune responses in human recipients
 Spontaneous or undesired differentiation	▶ Residual feeder-cell cues bias lineages differentiation
 Gene expression interference	▶ Feeder-cell secretions alter the transcriptome and signaling
 Non-compliance with clinical standards	▶ Unsuitable for therapeutic-grade manufacturing

hPSC, human pluripotent stem cells; GMP, Good Manufacturing Practice.

Figure 10. Risks associated with feeder cells and xenogenic components in hPSC culture. *Ref. Chaudhary, N., & Villa-Diaz, L. G. (2025). Frontiers in Toxicology, 7, 1667573.*

Nonhuman Epitope Incorporation and Viral Contamination Risks

Biochemical xeno-imprinting of nonhuman epitopes represents an additional artifact. hESCs cultured with animal-derived substances can incorporate the nonhuman sialic acid Neu5Gc into their surface glycoconjugates, resulting in immunogenic features absent in humans (Martin et al. 2005). Although Neu5Gc contamination is largely reversible upon cessation of exposure, it can still bias analyses during the period of contact. Murine-derived matrices present similar risks. For instance, certain Matrigel batches have tested positive for lactate dehydrogenase–elevating virus (LDEV) in defined cultures (Nagaoka et al. 2010). These findings underscore the risk of infection associated with murine biologics and highlight the necessity of rigorous testing of xenogeneic reagents.

2.3.2 Mitochondrial Exchange and Its Implications for Mitochondrial Phenotyping

Intercellular mitochondrial exchange is a well-documented phenomenon in which mitochondria or their components are transferred from donor to recipient cells. This process can modify the recipient cell's bioenergetics, redox homeostasis, and stress resilience. Early

functional studies demonstrated that mitochondrial transfer can restore aerobic respiration in metabolically compromised recipient cells, establishing that this process has physiological consequences rather than representing a tracer artifact ([Spees et al. 2006a](#)). In vivo studies further confirm the biological relevance of mitochondrial transfer. For example, bone marrow–derived stromal cells deliver mitochondria-containing vesicles to alveolar epithelial cells, which improves outcomes in acute lung injury models ([Islam et al. 2012a](#)). These findings support a protective role for mitochondrial donation during cellular stress.

Mechanisms of mitochondrial transfer include both contact-dependent and contact-independent pathways.

Contact-dependent trafficking occurs via tunneling nanotubes, which are membranous conduits that facilitate organelle transport between physically connected cells ([Vignais et al. 2017](#)). *Contact-independent transfer* is mediated by extracellular vesicles (EVs) that carry mitochondrial cargo. For example, neural stem cells have been shown to deliver functional mitochondria via EVs, indicating that vesicle-mediated delivery is a regulated intercellular pathway that influences recipient mitochondrial function ([Peruzzotti-Jametti et al. 2021](#)). These transfer routes are not mutually exclusive and may be enhanced by microenvironmental stress, elevated cell density, inflammatory signals, or alterations in cytoskeletal dynamics. Such factors can increase vesicle production, uptake, or the formation of tunneling nanotubes ([Roballo et al. 2019](#)).

Intercellular mitochondrial transfer as an unrecognized confounding factor in mitochondrial phenotyping

Intercellular mitochondrial exchange introduces a significant confounder in mitochondrial phenotyping, as the measured mitochondrial state may reflect both endogenous recipient mitochondria and newly acquired donor material ([Li et al. 2024](#)). This donor material can include intact mitochondria, mitochondrial fragments, proteins, or mitochondrial DNA

(mtDNA) (Sercel et al. 2021). Functional measurements over short time scales, such as oxygen consumption rate (OCR) and ATP production, are particularly susceptible to this confounder because they reflect both intrinsic cellular metabolism and rapid changes driven by external factors. For example, donor mitochondria can enhance respiration or ATP output in recipient cells (Frankenberg Garcia et al. 2022). This may lead to misattributing metabolic flux changes to intrinsic remodeling rather than to mitochondrial acquisition, highlighting the need to interpret assay results carefully in these contexts. Fluorescence-based assays, including those using $\Delta\Psi_m$ dyes or mitochondrial content imaging, have specific vulnerabilities. For example, mitochondria-containing vesicles may enter recipient cells and appear as discrete puncta before full integration with the host mitochondrial network. These occurrences can artificially elevate measured mitochondrial abundance or exaggerate $\Delta\Psi_m$ heterogeneity in microscopy-based analyses, thereby introducing assay-specific bias (Vianello et al. 2023). Molecular assays are similarly vulnerable because exogenous mtDNA can persist in recipient cells or become co-isolated during sample preparation, potentially distorting measurements of mtDNA copy number and variant signals.

2.4 Bioengineered substrates

Bioengineered substrates enable reproducible, scalable, and interpretable hPSC culture. They serve as defined alternatives to traditional matrices. These materials feature customizable ligand identities, surface densities, mechanical properties, and degradability. This enables precise modulation of integrin and growth factor pathways that govern self-renewal and lineage priming. This regulation is especially important for hPSCs, which depend on tightly controlled signals from their microenvironment. Even minor changes in adhesion, cytoskeletal tension, or paracrine signaling can disrupt key molecular pathways, leading to loss of pluripotency. Bioengineered substrates also provide experimental platforms for ECM–integrin signaling studies. They minimize culture variability and support xeno-free, Good

Manufacturing Practice (GMP)-compliant workflows for downstream differentiation and therapeutic applications.

2.4.1 Synthetic surfaces controlling ECM deposition and lineage bias

Bioengineered culture substrates replace matrices like Matrigel with controlled interfaces. They separate what the cell senses—ligand identity, density, mechanics, and adsorbed proteins—from uncontrolled xenogeneic variability. These materials work by presenting defined adhesive ligands, such as recombinant ECM fragments or short peptides. They also tailor surface chemistry so that selected medium proteins reliably adsorb and assemble into an organized adhesion layer. This process supports self-renewal by consistently presenting appropriate binding cues for hPSCs. High-throughput studies show that subtle changes in surface chemistry shift attachment, colony cohesion, and differentiation ([P.-Y. Wang et al. 2013](#)). This suggests fate can be encoded via material–protein–integrin coupling rather than solely by soluble factors.

Synthetic polymer coatings support long-term hPSC propagation in defined conditions.

PMEDSAH coatings sustain long-term growth of human ESCs across various media ([Luis G. Villa-Diaz et al., 2010](#)). This shows polymer chemistry can substitute for biological coatings if the interface favors stable adhesion and colony expansion. Even with PMEDSAH, changes in gel architecture—such as thickness or hydration—can affect propagation efficiency, underscoring that interfacial physicochemistry—not just ligand identity—can limit scalable expansion. Other polymers, like PMVE-alt-MA, also support hPSC attachment and self-renewal ([Brafman et al. 2010](#)). Multiple chemistries can enable a permissive state by balancing adsorption and adhesion while preserving pluripotency markers and the karyotype. The APMAAm hydrogel exemplifies how polymer media interactions govern self-renewal.

Long-term self-renewal in a defined medium required specific binding between BSA and the polymer surface. BSA serves as a molecular bridge mediating cell adhesion in this system (Irwin et al. 2011). Thus, even synthetic substrates function through a dynamic, medium-dependent adsorbed protein corona. Changes in the medium may alter the protein layer's composition or structure, enabling the same polymer to either maintain self-renewal or induce differentiation.

Peptide-functionalized synthetic substrates offer another defined approach.

They reduce batch variability and engage integrins via a known sequence. Vitronectin-derived peptide surfaces, such as Synthemax-like formats, support expansion and can be used for directed differentiation in a xeno-free manner. Peptide-acrylate surfaces also support long-term self-renewal and differentiation. These findings show engineered substrates support stable maintenance and predictable morphogen responsiveness. Bioengineered substrates not only replace attachment but also tune adhesion strength, cytoskeletal organization, and ECM deposition. This modulation of substrate features alters the balance of intracellular signals, shifting the point at which external pro-differentiation cues can override the network that maintains pluripotency. In this way, tuning substrate properties biases the likelihood of specific lineage outcomes.

2.4.2 Polymer brush strategies for expansion without potency loss

Polymer brushes are densely tethered polymer chains grown from a surface. They give precise control over interfacial hydration, charge, and ligand presentation. They can also suppress nonspecific protein adsorption if desired. Brush architectures are modular cell culture platforms. They allow separate tuning of nonfouling properties and bioactivity. This is key when expansion must preserve potency rather than move toward lineage commitment.

Brush design principles: hydration, charge, and ligand modularity

Nonfouling chemistries, often zwitterionic, minimize unintended differentiation by specifically blocking nonspecific integrin binding and restricting signal activation from random protein adsorption. These limits signal cascades that could otherwise push cells toward unplanned fates. In MSCs, a poly(carboxybetaine) hydrogel inhibited differentiation and preserved multipotency, even under differentiation-promoting conditions ([Bai et al. 2014](#)). This shows that reducing nonspecific interactions helps maintain stem-like states. Potency-preserving materials are not inert. They can be designed to provide instructive cues during controlled 'fouling.' Ligands such as RGD can be added to guide adhesion and differentiation as required.

Brush-enabled scalable expansion through localized mitogen presentation

For scalable expansion, brush systems can sequester and stabilize mitogenic factors locally without causing differentiation. For example, glycosaminoglycan-inspired brushes promoted human MSC proliferation and maintained trilineage potential, addressing the trade-off between expansion and potency in manufacturing ([Nelson et al. 2026](#)). Zwitterionic microgel and hydrogel platforms maintain multipotency and reduce nonspecific differentiation. Highly hydrated, dynamic networks preserve cell state during long-term culture by resisting unwanted protein adsorption and supporting hydration.

Brush platforms as xeno-free, tunable substrates for fate control

For hPSCs, brush-based strategies have clear translational value. They offer reproducibility across lots and sites. They are compatible with xeno-free workflows and support tunable integrin coupling. This is achieved by presenting defined ligand densities, such as laminin- or vitronectin-mimetics. Such features support substrate-centric fate control and stabilize the baseline cell state by reducing uncontrolled signaling ([Eroshenko et al. 2013](#)). Deliberate changes to ligands and soluble factors then trigger differentiation. This approach improves experimental interpretation and reduces lineage bias introduced by culture conditions.

2.5. Synthesis and Knowledge Gap: Linking ECM–Integrin Signaling to FAK Spatial Routing and Mitochondrial Readouts

The current literature demonstrates that the fate of hPSCs is governed by an integrated regulatory network rather than a single pathway (Jeong et al. 2025). The OCT4–SOX2–NANOG circuit establishes pluripotent identity. Yet, this state is dynamically modulated by ECM interactions, integrin signaling, cytoskeletal tension, survival pathways, metabolic state, and metabolite-dependent chromatin regulation (Shi and Jin 2010). These factors collectively influence cellular adhesion, mechanical tension, and survival. Extracellular cues converge on focal adhesion signaling, with FAK serving as a central integrator. Notably, FAK function is determined not only by its phosphorylation status but also by its subcellular localization between adhesion sites and the nucleus (Wu et al. 2004). This spatial distribution enables environmental conditions to influence gene expression outcomes. Nevertheless, investigating these complex networks presents significant experimental challenges.

Confounding variables frequently constrain causal inference in cell culture experiments. For instance, xenogeneic feeder layers and animal-derived matrices introduce additional factors, including paracrine signals, matrix ligands, and particulate cargo. Furthermore, extracellular vesicles and mitochondrial material can independently modify baseline signaling, bioenergetic states, and lineage-specific readouts, irrespective of the intended experimental manipulation. These complexities underscore the importance of defined, engineered substrates as reproducible experimental platforms. However, even under 'defined' conditions, robust models are required to connect adhesion receptor engagement with downstream signaling and transcriptional regulation. Addressing these confounding factors is critical for advancing from correlative findings to mechanistic insights.

Despite advances in characterizing individual components, a significant knowledge gap persists. The field lacks a comprehensive framework that connects specific ECM–integrin

interactions to discrete FAK signaling states, encompassing differential phosphorylation, spatial distribution, and downstream gene regulatory outcomes in hPSCs. Experimental designs must also account for xenogeneic microenvironmental variability and intercellular mitochondrial transfer, as these factors can influence mitochondrial phenotyping and obscure cell-intrinsic mechanisms of fate regulation. Consequently, distinguishing intrinsic regulatory mechanisms from culture-induced microenvironmental effects remains challenging.

Addressing this gap will enable more precise analysis of how ECM–integrin cues, FAK dynamics, and mitochondrial responses collectively govern hPSC fate.

Bioengineered substrates reduce confounding variables and facilitate causal analysis of adhesion-mediated regulatory mechanisms. These materials provide defined ligand identities and presentations, such as recombinant LN-511/521 or peptide motifs, combined with controlled surface chemistry and tunable mechanical properties. Engineered substrates promote consistent integrin engagement, including $\alpha 6 \beta 1$, allowing direct evaluation of how specific ECM–integrin interactions affect FAK phosphorylation, spatial signaling pathways, and subsequent cellular outcomes. Moreover, these platforms minimize batch-to-batch variability, support xeno-free and GMP-compliant workflows, and provide a reproducible, translationally relevant foundation for elucidating the microenvironmental regulation of hPSC fate.

Chapter 3. Central Hypothesis and Specific Aims

3.1 Central Hypothesis

Microenvironmental cues direct human stem cell fate decisions between self-renewal and differentiation

3.2 Specific Aim 1

Integrin $\beta 1$ -activation disturbs integrin-nuclear FAK signaling to regulate cell fate.

Elucidate the mechanisms by which Integrin $\beta 1$ activation disturbs nuclear FAK–dependent transcriptional programs that destabilize pluripotency and promote lineage-priming gene expression.

Rationale

hPSCs exhibit high sensitivity to microenvironmental adhesion cues. Although integrin-mediated adhesion is known to influence the hPSC state, the specific fate consequences of $\beta 1$ -integrin activation remain unresolved. A key gap was the lack of a clear framework that links $\beta 1$ activation to the FAK signaling state, subcellular routing, and fate-associated transcriptional control in hPSCs.

Working hypothesis

Integrin $\beta 1$ activation disrupts nuclear FAK–dependent transcriptional programs, destabilizing pluripotency and promoting lineage-priming gene expression.

Approach

1. Integrin $\beta 1$ signaling was perturbed under defined hPSC culture conditions using controlled activation-based experimental treatments, as outlined in Chapter 4.

2. The downstream effects were assessed at several levels, including integrin-proximal signaling, FAK-associated regulatory characteristics, and subcellular localization, with a focus on nuclear FAK-related mechanisms.
3. These molecular and signaling data were analyzed alongside transcriptional profiles relevant to pluripotency maintenance and the initiation of lineage-specific programs.

Expected outcomes

This objective aims to elucidate how activation of integrin β 1 influences hPSC fate by characterizing its relationship with the FAK-MDM2-p53 axis. Additionally, the study seeks to determine how these signaling states correspond to transcriptional programs that determine cell fate, maintain pluripotency, or initiate lineage priming.

3.3 Specific Aim 2

Feeder-derived mitochondrial transfer acts as a microenvironmental confounder that biases cell fate.

Assess whether mitochondrial exchange originating from feeder cells alters hPSC fate-associated phenotypes.

Rationale

Feeder-dependent culture systems introduce microenvironmental variables beyond soluble factors and ECM support, including particulate cargo and the potential for intercellular transfer. Mitochondrial transfer, in particular, constitutes a plausible confounding factor, as donor-derived mitochondrial material can modify recipient cell bioenergetics, stress responses, and fate-associated outcomes. An unresolved question is whether conventional feeder-based hESC culture allows mitochondrial transfer at levels sufficient to influence mitochondrial phenotyping and the interpretation of metabolism-linked cell fate.

Working hypothesis

Feeder-derived xenogeneic mitochondrial transfer into human pluripotent stem cells confounds microenvironmental inputs, impairs mitochondrial function, and biases fate-associated phenotypes.

Approach

1. Mitochondrial transfer within feeder-based hESC cultures will be evaluated using complementary detection methods to quantify its extent and characterize its dynamics.
2. Potential routes of mitochondrial transfer will be investigated by comparing feeder co-culture with extracellular vesicle-associated exposure models.
3. The biological significance will be assessed by correlating transfer-associated burden with mitochondrial phenotyping and cell-state readouts related to pluripotency, cellular stress, and lineage priming.

Expected outcomes

This aim is anticipated to determine whether feeder-derived mitochondrial transfer constitutes a significant microenvironmental confounder in hESC culture. Additionally, it is expected to clarify how such a transfer may affect the interpretation of mitochondrial phenotyping and metabolism-associated fate readouts in feeder-based versus defined culture systems.

3.4 Specific Aim 3

Bioengineered substrates (GAG-Inspired Polymer Brushes) enhance microenvironmental bFGF presentation to regulate cell fate.

Glycosaminoglycan (GAG)-inspired polymer brush substrates establish a defined microenvironment that increases hMSC proliferation while preserving trilineage differentiation potential.

Rationale

A xeno-free, cost-effective, and well-defined hMSC culture substrate that closely replicates the regulatory role of native glycosaminoglycans in growth factor signaling is needed. Polymer brushes inspired by GAGs may fulfill this requirement by absorbing growth factors, such as FGF, from the medium and presenting them to cells, thereby establishing a more biomimetic interface for stem cell expansion. However, it remains unclear whether these materials can enhance hMSC proliferation while preserving cellular potency.

Working hypothesis

Glycosaminoglycan (GAG)-inspired polymer brush substrates create a defined microenvironment that enhances human mesenchymal stem cell (hMSC) proliferation while maintaining the capacity for trilineage differentiation.

Approach

1. Engineer the brush compositions and architectures described in Chapter 6 and characterize their relevant interfacial properties-growth factor binding, surface hydration, and cell interaction.
2. Quantify expansion performance by measuring growth kinetics, population doublings, and stress or senescence indicators across multiple donors and passages.
3. Assess functional potency following expansion using standardized trilineage differentiation assays and lineage-specific marker panels.

Expected outcomes

This aim is anticipated to establish a xeno-free, cost-effective, and scalable substrate platform for hMSC culture. Additionally, it will determine whether biomaterial-mediated growth factor sequestration and presentation can enhance expansion efficiency while maintaining trilineage differentiation potential.

3.5 Summary of integration across aims and chapters

This dissertation addresses how microenvironmental cues regulate human stem cell fate decisions through three major levels of interaction: ECM–cell interactions, cell–cell interactions, and ECM–soluble factor–cell interactions.

Aim 1 focuses on cell–ECM interactions, asking how the activation of specific integrin $\beta 1$, beyond the previously established role of integrin $\alpha 6$, influences adhesion-linked signaling and cell-fate programs in hPSCs (Chapter 4).

Aim 2 addresses cell–cell interactions by testing whether widely used feeder-dependent culture introduces xenogeneic mitochondrial transfer, which may confound mitochondrial readouts and fate-associated phenotypes (Chapter 5).

Aim 3 examines soluble factor–cell interactions by developing a xeno-free engineered substrate that absorbs growth factors from the culture medium and presents them to stem cells in a biomimetic manner, thereby supporting expansion under defined conditions (Chapter 6).

Together, these studies define how extracellular adhesion, intercellular transfer, and biomaterial-mediated presentation of growth factors each contribute to the microenvironmental control of stem cell fate.

Chapter 4. Integrin β 1-Dependent Control of Pluripotency and Gene Regulation via Nuclear NT-FAK

Core Question

How does β 1-integrin-mediated signaling regulate hPSC fate decisions-self-renewal vs early lineage priming?

4.1 Introduction

Human pluripotent stem cells (hPSCs) are regulated by a multiscale control architecture wherein extracellular matrix (ECM) ligands, integrin-mediated adhesion, and cytoskeletal dynamics converge on nuclear transcriptional programs that promote self-renewal and suppress differentiation. Among the integrin subunits expressed in hPSCs, $\alpha 6$ and $\beta 1$ predominate in laminin-rich culture systems. In these environments, $\alpha 6\beta 1$ functions as the principal laminin receptor and is widely associated with the undifferentiated state ([Miyazaki et al. 2012](#)). Therefore, integrin-dependent adhesion acts not only as a permissive factor for hPSC growth but also as an active regulator of pluripotent cell identity. Because $\alpha 6$ and $\beta 1$ function as a heterodimeric receptor complex, the $\beta 1$ subunit serves not only as a structural partner of $\alpha 6$ but also as a critical signaling component that regulates ligand affinity, receptor clustering, and downstream outside-in signaling ([Luo et al. 2007](#)). Consequently, $\beta 1$ -integrin activation represents a mechanistically significant intervention point for investigating how changes in adhesion state are linked to FAK-dependent regulation of pluripotency.

Unlike many somatic cell types, canonical integrin–focal adhesion kinase (FAK/PTK2) signaling is attenuated in self-renewing hPSCs, as indicated by low basal phosphorylation of FAK at Y397. In contrast, differentiation or forced integrin activation leads to increased pFAK^{Y397} ([Luis G. Villa-Diaz et al. 2016](#)). Previous studies have demonstrated that integrin $\alpha 6$ expression parallels OCT4 and SOX2 in undifferentiated colonies, decreases during BMP4-induced differentiation, and increases during somatic reprogramming. Furthermore, depletion of integrin $\alpha 6$ has been shown to reduce OCT4, SOX2, and NANOG expression, supporting an instructive role for $\alpha 6$ -associated adhesion in maintaining the pluripotent state. In parallel, FAK has been detected in hPSC nuclei, where its N-terminal domain associates with OCT4 and SOX2 ([Luis G. Villa-Diaz et al. 2016](#)). This observation suggests that hPSCs maintain robust adhesion while limiting classical focal-adhesion signaling outputs through a non-canonical nuclear FAK mechanism.

This study advances the integrin $\alpha 6$ -centered framework by directly examining the integrin $\beta 1$ arm of the integrin $\alpha 6\beta 1$ receptor complex. While integrin $\alpha 6\beta 1$ -mediated adhesion has been implicated in hPSC maintenance (*Figure 11,12*), it remains unclear whether selective activation of integrin $\beta 1$ is sufficient to modify the FAK regulatory state, disrupt nuclear NT-FAK-associated signaling, and initiate downstream transcriptional programs associated with loss of pluripotency and early lineage priming (*Figure 13,14*). To address this question, this study utilizes two mechanistically distinct strategies to increase integrin activity. Manganese ions (Mn^{2+}) serve as broad integrin activators by binding near the β -subunit metal ion-dependent adhesion site (MIDAS/AD-MIDAS region), which stabilizes the extended high-affinity conformation and enhances ligand binding across multiple integrin heterodimers. In contrast, a $\beta 1$ -integrin-activating antibody selectively targets ITGB1, promoting $\beta 1$ activation and receptor clustering to initiate more receptor-restricted outside-in signaling (*Figure 15*). Comparing these approaches allows distinguishing between global integrin activation and $\beta 1$ -specific activation, thereby clarifying whether the $\beta 1$ subunit alone is sufficient to reconfigure FAK localization and downstream signaling associated with fate in hPSCs.

Therefore, this chapter evaluates the hypothesis that activation of integrin $\beta 1$ shifts the integrin $\alpha 6\beta 1$ –FAK axis from a self-renewal-supportive state to a signaling state that destabilizes pluripotency and promotes fate-associated gene regulation. Specifically, the proposed model predicts that increased $\beta 1$ activation will elevate FAK activation, facilitate its redistribution from a primarily nuclear FAK-associated state to focal-adhesion-linked signaling, and consequently modify transcriptional programs that maintain pluripotency.

Main findings from our team's previous research

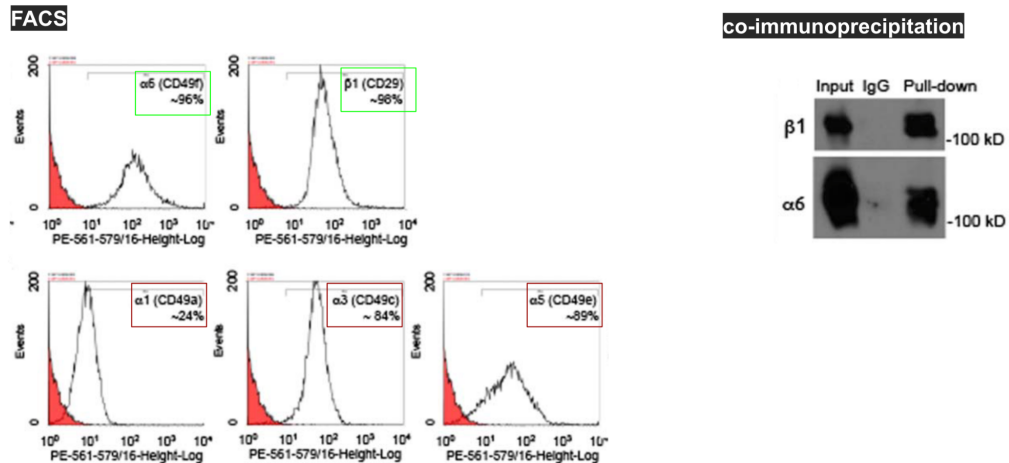


Figure 11. Integrin $\alpha 6$ and $\beta 1$ are the predominant integrin isoforms in hPSCs and physically associate to form a heterodimer.

Flow cytometry analysis indicates higher surface expression of integrins $\alpha 6$ (CD49f) and $\beta 1$ (CD29) than of other α -subunits in hPSCs. Co-immunoprecipitation confirms the interaction between $\alpha 6$ and $\beta 1$, supporting $\alpha 6\beta 1$ as the principal laminin-binding integrin complex in the pluripotent state. *Adapted from Villa-Diaz et al., Stem Cells (2016) 34(7):1753–1764.*

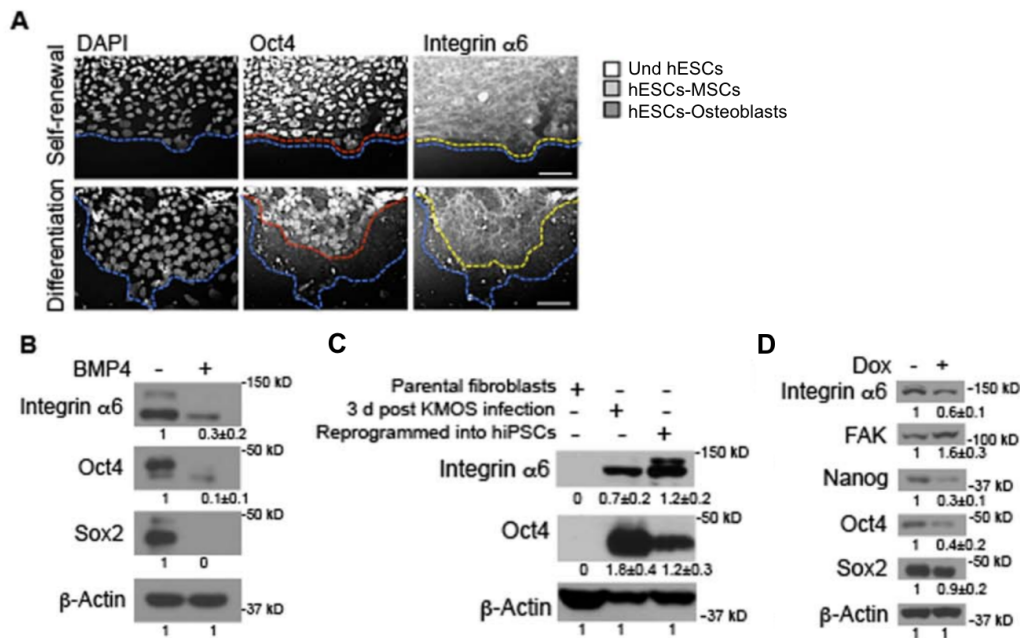


Figure 12. Integrin $\alpha 6$ expression is associated with the pluripotent state and is required to maintain core pluripotency factor expression.

a) Representative immunofluorescence images of hPSC colonies cultured under self-renewal (top) or differentiation (bottom) conditions, stained for nuclei (DAPI), OCT4, and integrin $\alpha 6$. Dashed boundaries indicate colony edges and illustrate the spatial distribution of OCT4⁺ and integrin $\alpha 6$ ⁺ regions. Peripheral cells undergoing differentiation display reduced OCT4 and integrin $\alpha 6$ relative to undifferentiated regions. **b)** Immunoblot analysis of ITGA6 and pluripotency factors following BMP4 treatment shows reduced OCT4 and SOX2 levels, along with decreased integrin $\alpha 6$. β -actin is used as the loading control. **c)** Immunoblot analysis across reprogramming stages indicates induction of integrin $\alpha 6$ during KMOS-mediated reprogramming. ITGA6 is undetectable in parental fibroblasts, becomes detectable at 3 days post-infection, and is elevated in fully reprogrammed hiPSCs. OCT4 serves as a marker of reprogramming and pluripotency. β -actin is used as the loading control. **(d)** Doxycycline-inducible knockdown of integrin $\alpha 6$ in hhPSCs leads to reduced integrin $\alpha 6$ levels and decreased expression of NANOG, OCT4, and SOX2 proteins. β -actin is used as the loading control. Where indicated, band intensities are normalized to the loading control and expressed relative to the corresponding untreated or control condition. [Reference-Krebsbach, P. H., & Villa-Diaz, L. G. \(2017\). Stem cells and development, 26\(15\), 1090-1099.](#)

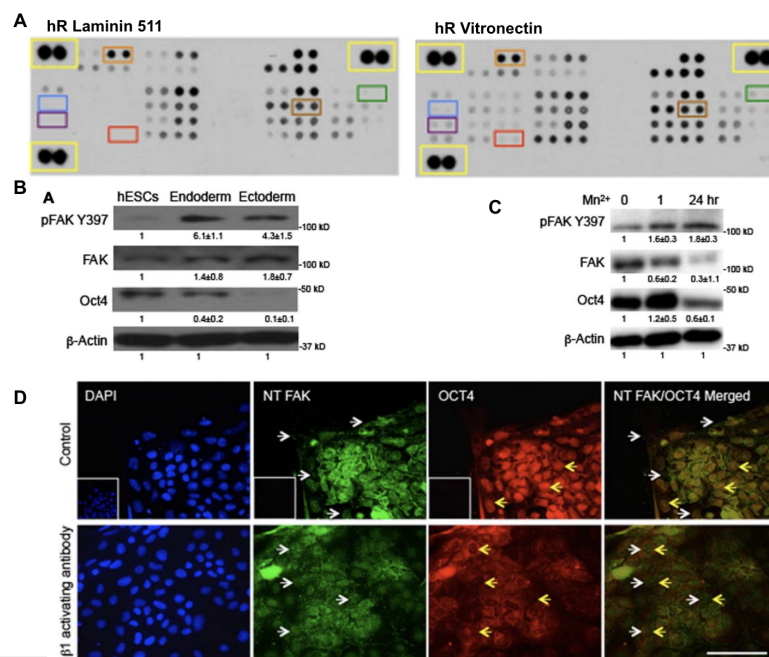


Figure 13. FAK activation is diminished in undifferentiated hPSCs and increases during cellular differentiation or following integrin activation.

a) Phospho-kinase array analysis of undifferentiated hPSCs cultured on hR laminin-511 or vitronectin reveals robust MAPK phosphorylation (positive control) and minimal phosphorylation of focal-adhesion proteins, including FAK, paxillin, Src, and Fyn. **b)** pFAK(Y397) levels are increased in differentiated endoderm and ectoderm relative to undifferentiated hPSCs, which coincides with reduced OCT4 expression. **c)** Treatment with Mn^{2+} ($MnCl_2$), an integrin activator that stabilizes integrins in a high-affinity state, for 1–24 hours increases pFAK(Y397) and decreases OCT4 expression relative to control. β -actin is used as a loading control; densitometry data are presented as mean $\pm$ SEM ($n = 3$). **d)** Activation of integrin $\beta 1$ using a $\beta 1$ -activating antibody increases focal-adhesion-like NT-FAK signal (white arrows) and reduces OCT4 staining (yellow arrows) compared to control. DAPI labels nuclei; scale bar, 100 μm . The inset shows the no-primary-antibody control.

Reference-Villa-Diaz, L. G., et al.(2016). *Stem Cells*, 34(7), 1753-1764.

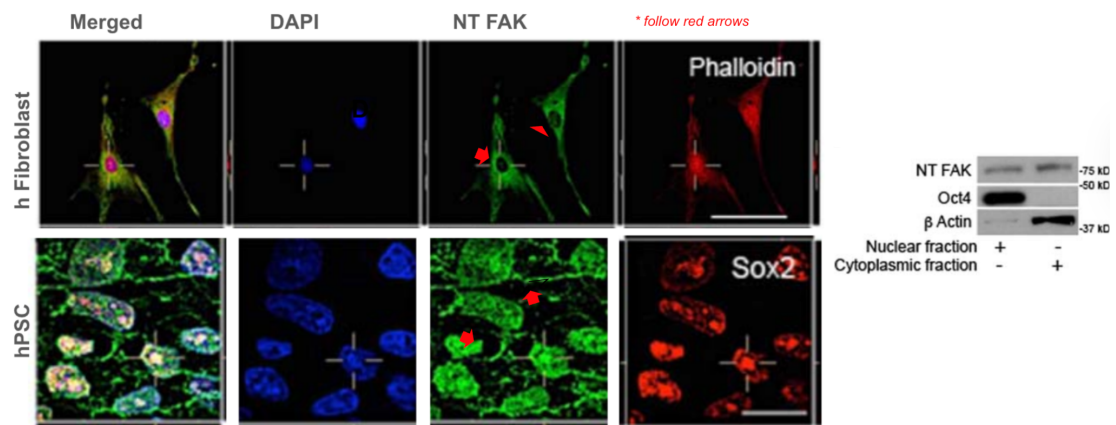


Figure 14. The N-terminal (NT) domain of FAK localizes to the nucleus in hPSCs and associates with pluripotency transcription factors.

a) Confocal immunofluorescence comparing human fibroblasts and hPSCs shows NT-FAK signal in both cytoplasm and nuclei of hPSCs, whereas fibroblasts display predominantly cytoplasmic NT-FAK. Nuclei are labeled with DAPI; actin is labeled with phalloidin (fibroblasts); SOX2 marks the undifferentiated hPSC state. Merged images illustrate nuclear overlap between NT-FAK and SOX2. Scale bars, 25 μm (fibroblasts) and 100 μm (hPSCs). **b)** Immunoblot of nuclear and cytoplasmic fractions from hPSCs detects NT-FAK in both compartments. OCT4 is used as a nuclear/pluripotency marker, and β -actin as a cytoplasmic marker/loading control. Reference-Villa-Diaz, L. G., et al.(2016). *Stem Cells*, 34(7), 1753-1764.

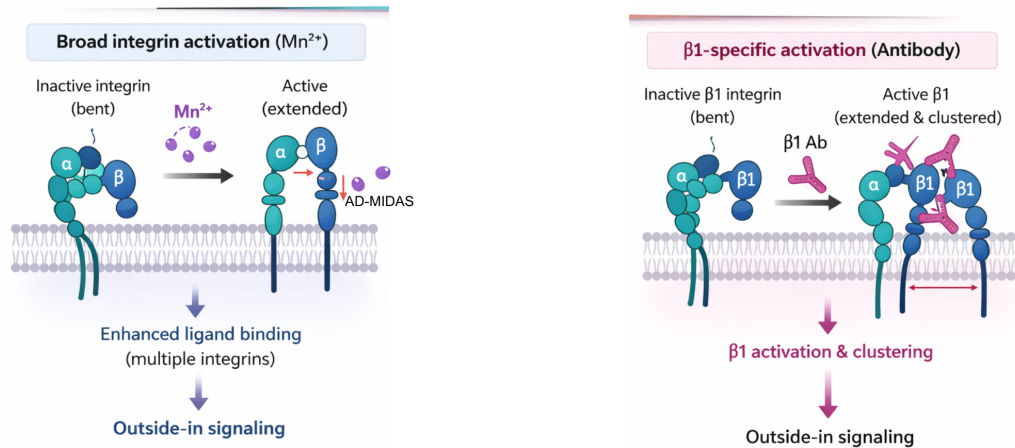


Figure 15. Two mechanistically distinct integrin activation strategies are illustrated.

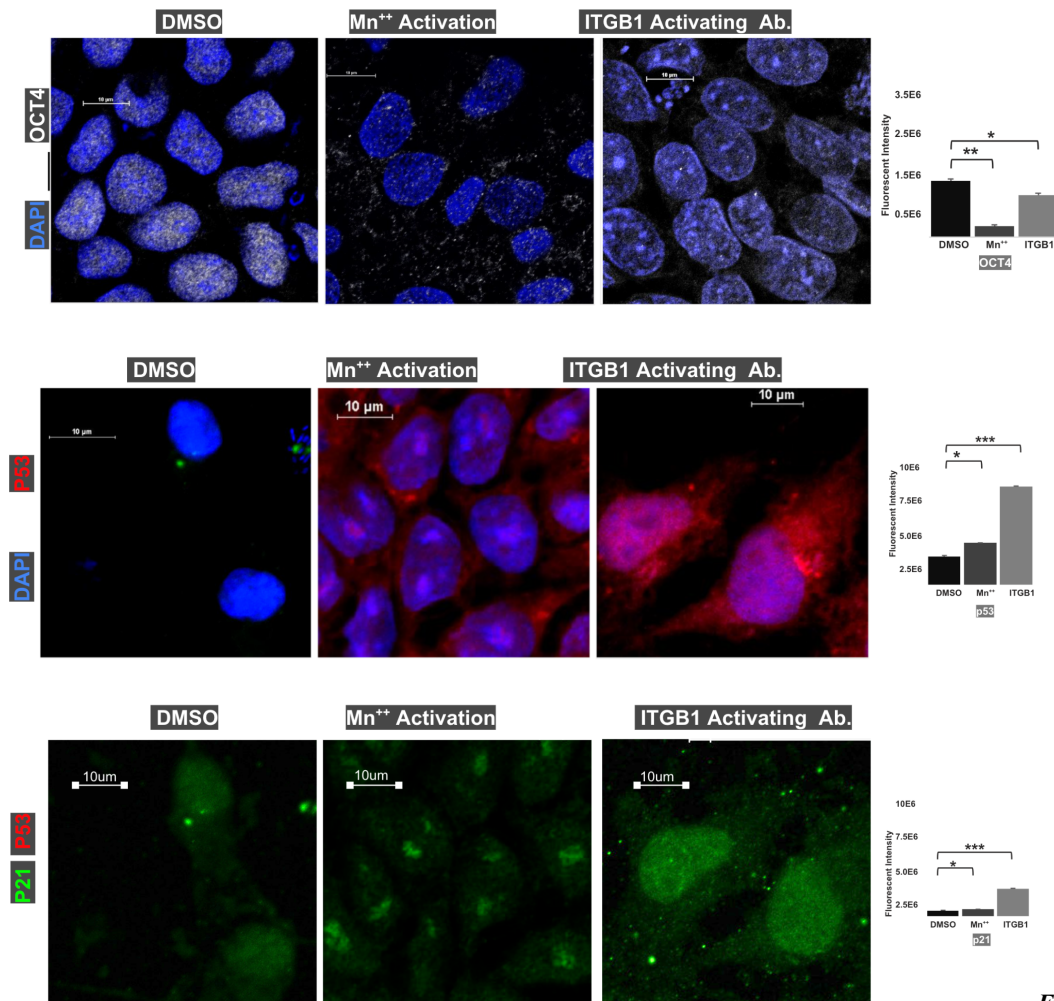
On the left, **Mn²⁺** induces broad, cation-dependent integrin activation by binding the β -subunit adjacent to the MIDAS (AD-MIDAS) region, which stabilizes the extended high-affinity conformation and enhances ligand binding, thereby promoting outside-in signaling across multiple integrin types.

On the right, a **β 1-activating antibody** selectively engages ITG β 1, facilitating β 1 activation and receptor clustering to initiate β 1-specific outside-in signaling without altering overall divalent-cation concentrations.

4.2 Results

4.2.1 Integrin β 1 activation reduces OCT4 and induces p53-p21 signaling

Under control conditions, the OCT4 signal was robust and predominantly nuclear, consistent with maintenance of pluripotency. Following β 1-integrin activation, both Mn²⁺ treatment and exposure to an integrin ITG β 1-activating antibody reduced OCT4 intensity. Control cultures exhibited minimal p53 and p21 signals, consistent with low basal stress signaling. In contrast, both Mn²⁺ and ITG β 1-activating antibody conditions resulted in stronger p53 staining and increased p21 signal, indicating activation of downstream p53 transcriptional targets (**Figure 16**).



Figure

16. $\beta 1$ -integrin activation reduces *OCT4* and increases *p53*-associated *p21* readout in hPSCs.

Representative confocal images of hPSCs treated with vehicle, Mn^{2+} , or an ITG $\beta 1$ -activating antibody. The top row shows the *OCT4* signal overlaid on DAPI, illustrating the distribution of the pluripotency marker and nuclear identity. The middle row shows *p53* (red) with DAPI (blue). The bottom row presents *p21* (green), visualizing induction of a downstream *p53* effector in response to integrin activation. Scale bars, 10 μm .

4.2.2 Integrin $\beta 1$ activation redistributes NT-FAK and alters *p53*–MDM2 spatial organization

In control cells, NT-FAK exhibited a cytoplasmic and peripheral distribution with nuclear enrichment. Upon integrin activation, NT-FAK localization was remodeled, with increased perinuclear and nuclear-adjacent signal and altered cytoplasmic organization compared to control (**Figure 17**). Concurrently, *p53* and MDM2 staining patterns shifted relative to control, with activation conditions displaying stronger *p53* signals and reduced MDM2 within

the same fields.

Confocal Imaging

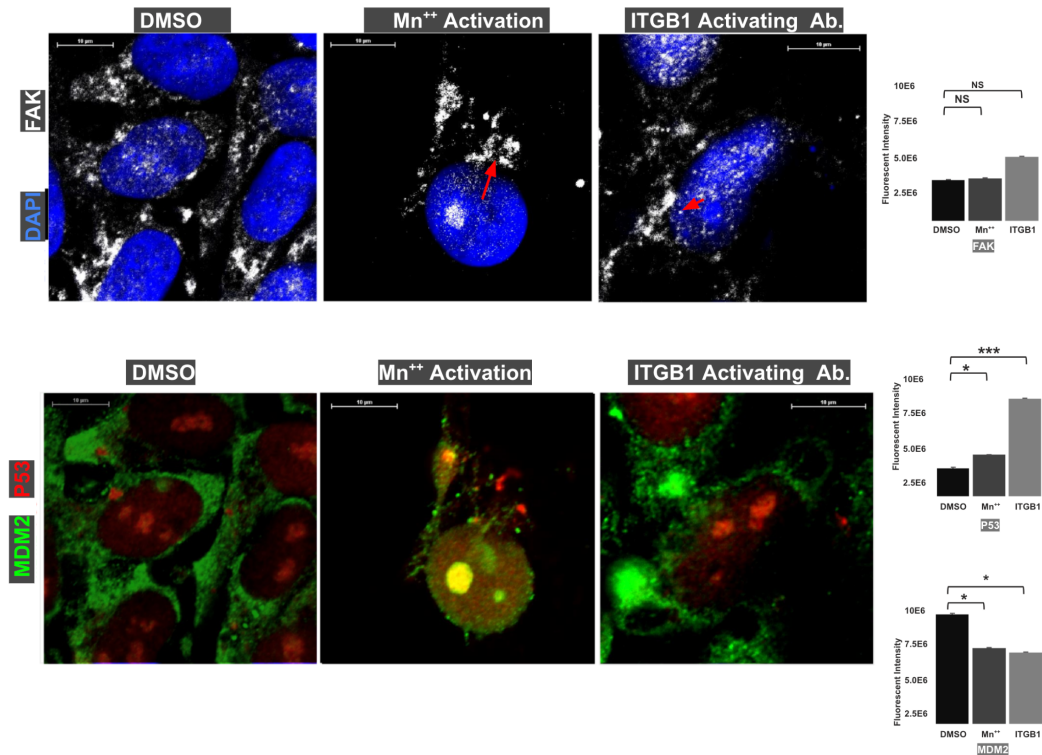


Figure 17. Integrin activation is associated with the redistribution of NT-FAK and altered spatial organization of p53–MDM2.

The top row shows the OCT4 signal overlaid on DAPI, illustrating the distribution of the pluripotency marker and nuclear identity. The bottom row presents merged p53 (red) and MDM2 (green) with DAPI (blue), enabling assessment of changes in the stress-response regulatory axis under each condition. The bottom row shows merged images of p53 (red) and MDM2 (green) from matched fields, illustrating how changes in NT-FAK localization relate to concurrent remodeling of the p53–MDM2 module. Scale bars, 10 μm.

Across imaging readouts, enforced β1 activation (Mn²⁺ or ITGβ1 antibody) correlated with reduced OCT4 intensity, remodeling of NT-FAK distribution, and a coordinated shift in the p53–MDM2 axis, including induction of the downstream effector p21. These findings support the central model that integrin β1 activation state, rather than integrin expression alone, governs the transition from a “pluripotency-supportive” NT-FAK nuclear module to a p53-

active state. This provides a mechanistic link between $\beta 1$ activation, FAK spatial routing, and the loss of pluripotency stability, which is further examined in subsequent subsections.

4.2.3 FAK-MDM2-p53 Protein-protein interactions in hPSC

To elucidate the mechanistic basis for integrin-dependent regulation of pluripotency via nuclear NT-FAK, the physical association between NT-FAK, core pluripotency transcription factors, and p53 pathway components in hPSCs was examined (**Figure 18**). Co-immunoprecipitation of NT-FAK recovered OCT4 and SOX2 compared to IgG controls, indicating the presence of NT-FAK-containing nuclear regulatory complexes associated with pluripotency circuitry. Concurrently, immunoblotting for MDM2 and p53 in the corresponding fractions demonstrated the involvement of NT-FAK-associated complexes with the MDM2–p53 axis. These findings support a model in which NT-FAK integrates adhesion-dependent signaling with nuclear protein networks to modulate p53 regulation and stem cell state.

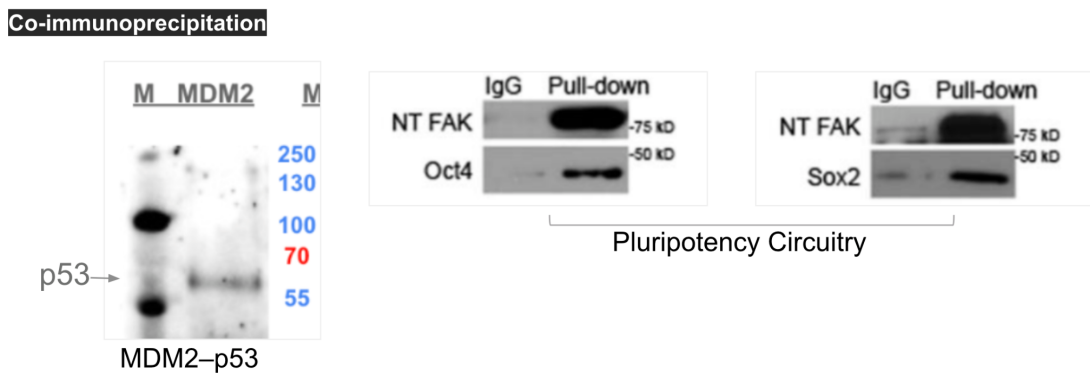


Figure 18. NT-FAK associates with pluripotency transcription factors and MDM2–p53 pathway components in hPSCs.

a) Immunoblot detection of MDM2 and p53 in the corresponding FAK-associated fractions indicates that FAK complexes overlap with components of the MDM2–p53 axis. M, molecular weight marker. **b)** Co-immunoprecipitation (Co-IP) of FAK from hPSC nuclear lysates enriches OCT4 and SOX2 relative to IgG control pull-downs, supporting FAK-containing nuclear complexes linked to pluripotency circuitry.

4.2.4 Integrin $\beta 1$ activation suppresses self-renewal signaling and promotes stress-response kinase activity

To characterize the early signaling consequences of integrin activation, kinase phosphorylation was profiled in hPSCs following Mn^{2+} treatment (6 hours) or exposure to an ITG $\beta 1$ -activating antibody (24 hours), relative to DMSO controls, using a phosphorylation antibody array (**Figure 19**). Mn^{2+} treatment resulted in a pronounced increase in stress-associated kinase activity, particularly Chk2 and c-Jun, which is consistent with activation of DNA damage and stress response pathways. Concurrently, Mn^{2+} reduced phosphorylation across pathways associated with hPSC self-renewal and growth, including mTOR signaling (p70S6K sites), STAT signaling (such as STAT3), and receptor-associated signaling (such as EGFR). Notably, Mn^{2+} exposure was associated with reduced p53 phosphorylation at S392, suggesting altered regulation of genome-surveillance signaling under acute Mn^{2+} exposure. In contrast, ITG $\beta 1$ antibody activation induced a more modest and selective remodeling of these signaling nodes, generally exhibiting intermediate effects compared to Mn^{2+} . Collectively, these phosphorylation profiles indicate that Mn^{2+} induces a stronger stress-biased signaling state than antibody-mediated ITG $\beta 1$ activation, providing a rationale for subsequent proteomic mapping of FAK-associated nuclear and cytoplasmic complexes under each condition.

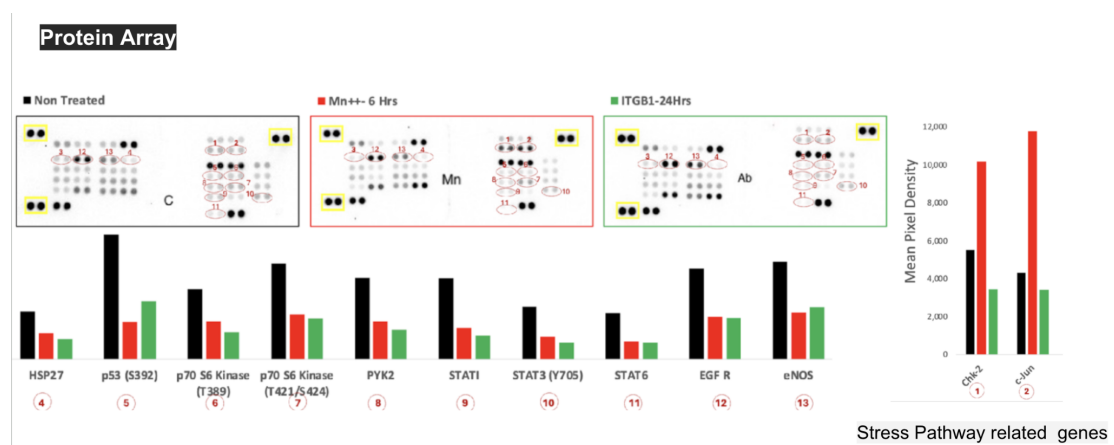


Figure 19. Mn^{2+} inhibits self-renewal-associated signaling pathways and promotes phosphorylation of stress-related kinases.

hPSCs were treated with DMSO (control), $MnCl_2$ (Mn^{2+} ; 6 hours), or an ITG β 1-activating antibody (24 hours) and analyzed using a kinase phosphorylation antibody array. Representative membranes are presented for each condition, with each target detected in duplicate and quantified by mean pixel density. Yellow boxed reference spots serve as positive controls for incubation and detection (streptavidin-HRP). Bar graphs display normalized signal intensities for selected targets, emphasizing kinases associated with stress, DNA damage, differentiation (e.g., Chk2, c-Jun, Fgr), and self-renewal pathways (e.g., p70S6K, STAT3, EGFR), as indicated.

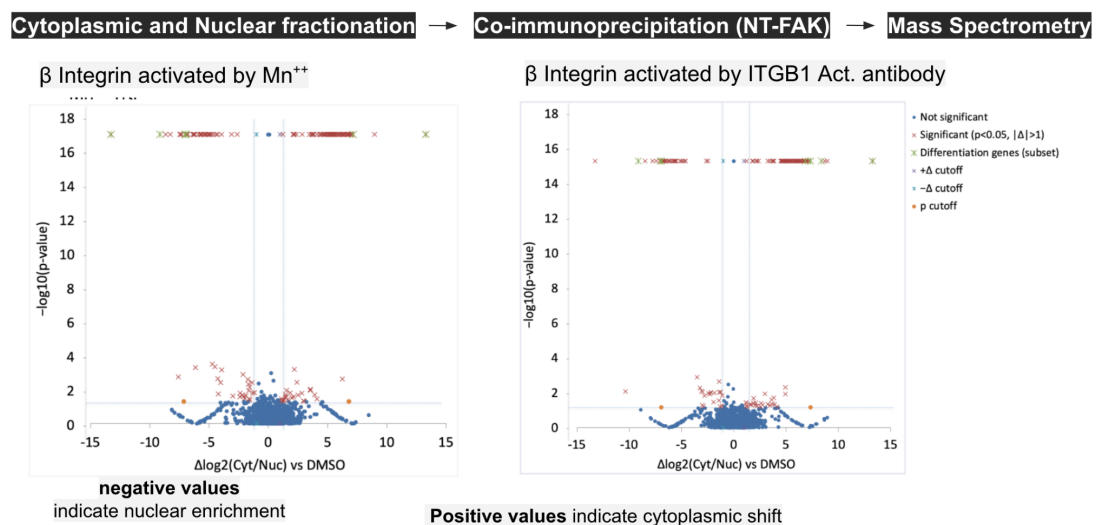
4.2.5 Integrin β 1 activation alters NT-FAK-associated nuclear–cytoplasmic protein distribution

To assess whether integrin activation modifies FAK-associated protein complexes in hPSCs, FAK co-immunoprecipitation was conducted using matched nuclear and cytoplasmic fractions from cells untreated, Mn^{2+} , or an ITG β 1-activating antibody. Beads-only samples served as controls. Co-purifying proteins were analyzed by label-free quantitative mass spectrometry. Protein redistribution was quantified as $\Delta\log_2(\text{Cyt/Nuc})$ relative to untreated, where positive values indicate a relative cytoplasmic shift and negative values indicate relative nuclear enrichment (**Figure 20a**). Applying thresholds of $p < 0.05$ and $|\Delta\log_2| > 1$, both Mn^{2+} and ITG β 1-activating antibody treatments resulted in significant changes in the nuclear–cytoplasmic distribution of FAK-associated proteins.

To identify the most pronounced redistribution events, proteins were ranked by $|\Delta\log_2(\text{Cyt/Nuc})|$, and the top 50 proteins for each treatment condition were plotted relative to the control (**Figure 20b**). The resulting heatmaps revealed distinct redistribution patterns in response to Mn^{2+} and ITG β 1-activating antibody treatments. Functional classification of the top 50 proteins indicated that gene-regulation factors associated with RNA or chromatin accounted for 12% of the redistributed set in both conditions. Differentiation- and development-related proteins accounted for 18% of the Mn^{2+} top 50 set and 24% of the

ITGβ1-activating antibody top 50 set. The remaining proteins were classified as other (70% for Mn²⁺ and 64% for ITGβ1 activation).

Among differentiation and development-associated proteins identified in the Mn²⁺ group included CBX2, BRD3, CECR2, STAG2, FMR1, METTL3, ZFP91, YTHDC1, and NCKAP1. In the ITGβ1-activating antibody group, this category comprised CBX2, BRD3, CECR2, STAG2, FMR1, METTL3, ZFP91, YTHDC1, NCKAP1, RAB35, UFM1, and PSPH. The heatmaps also demonstrated treatment-dependent differences in both the magnitude and direction of redistribution among individual proteins within these sets. Overall, compartment-resolved co-immunoprecipitation mass spectrometry demonstrated that both Mn²⁺ and ITGβ1 activation altered the nuclear–cytoplasmic distribution of FAK–associated proteins, with distinct top-ranked redistribution signatures and different proportions of differentiation/development-associated factors in the two treatment conditions.



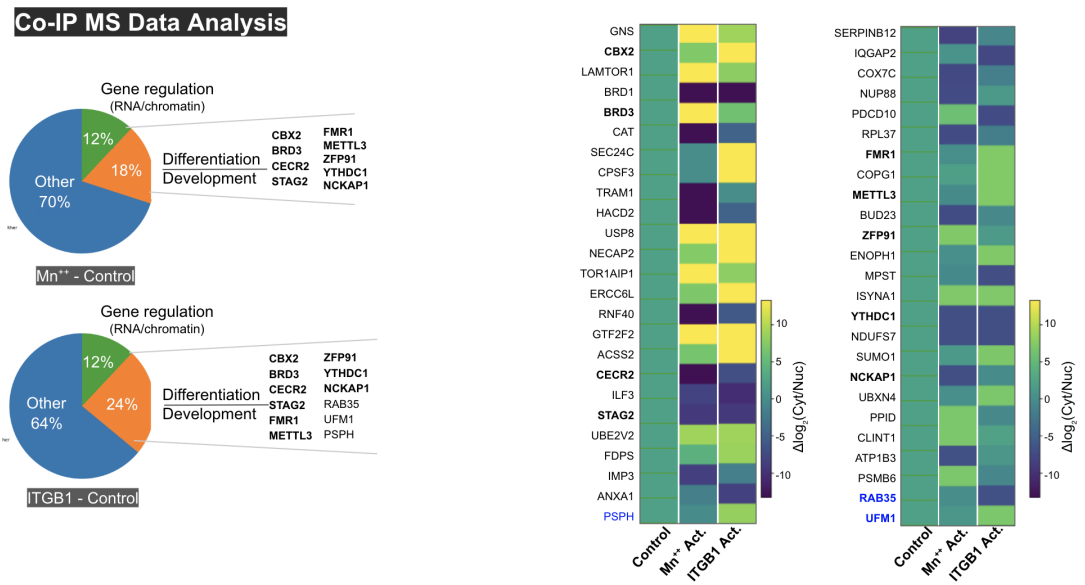


Figure 20. Integrin $\beta 1$ activation alters the distribution of FAK-associated nuclear-cytoplasmic proteins and enriches for differentiation-linked regulators.

a) Volcano plots of $\Delta\log_2(\text{Cyt/Nuc})$ relative to DMSO for Mn^{2+} treatment (left) and ITGB1-activating antibody (right). Positive values indicate cytoplasmic shift; negative values indicate nuclear enrichment. Dashed lines mark $|\Delta| = 1$ and $p = 0.05$. Blue, not significant; red, significant ($p < 0.05$, $|\Delta| > 1$); green, differentiation/development genes (subset). **b)** Left: heatmap of the top 50 proteins ranked by $\Delta\log_2(\text{Cyt/Nuc})$ for ITGB1 and Mn^{2+} conditions. Right: functional breakdown of top 50 proteins (RNA/chromatin, differentiation/development, other); gene lists indicate differentiation/development-associated proteins in each condition.

4.2.6 Integrin $\beta 1$ activation remodels the stem cell pluripotency transcriptional network

To assess the impact of integrin $\beta 1$ activation on transcriptional programs related to pluripotency and lineage specification, a TaqMan Human Stem Cell Pluripotency Array was employed to compare untreated control iPSCs with cells treated with either Mn^{2+} or an ITGB1-activating antibody (**Figure 21**). Gene expression changes were categorized as upregulated (≥ 2 -fold), downregulated (≤ 0.5 -fold), or unchanged (0.5–2-fold relative to control). Heatmaps illustrate relative expression changes across array targets, with red indicating increased expression and green decreased expression. Mn^{2+} -mediated integrin $\beta 1$ activation resulted in at least a 2-fold change in 41 of 89 targets, while 48 targets remained unchanged. Upregulated genes in the Mn^{2+} group included *SOX2*, *AFP*, *CDX2*, *MYOD1*,

DDX4, *KRT1*, *DES*, *XIST*, and *GATA4*. Genes downregulated to ≤ 0.5 -fold comprised *NPPA*, *LIFR*, *COMMD3*, *FGF5*, *WT1*, and *IGF2BP2*. The corresponding heatmap showed both increased and decreased signals across pluripotency- and lineage-associated targets.

In contrast, antibody-mediated integrin $\beta 1$ activation resulted in at least a 2-fold change in 57 of 89 targets, with 32 targets remaining unchanged. Upregulated genes in the ITG $\beta 1$ -activating antibody condition included *PTF1A*, *MYOD1*, *GCM1*, *AFP*, *PAX4*, *XIST*, *GJB*, *CD9*, *LT6ST*, and *TERT*. Downregulated transcripts (≤ 0.5 -fold) included *UTF1*, *FGF5*, *TNS*, *T*, *EOMES*, *LEFTY2*, *NODAL*, *ZFP42/REX1*, *PAX6*, and *PTEN*. The heatmap for the ITG $\beta 1$ -activating antibody condition showed a greater number of altered targets across the array than in the Mn^{2+} condition. In summary, analysis of the pluripotency array demonstrated that both Mn^{2+} -mediated and antibody-mediated integrin $\beta 1$ activation altered the expression of targets in the stem cell pluripotency array. The ITG $\beta 1$ -activating antibody condition affected more genes than Mn^{2+} treatment.

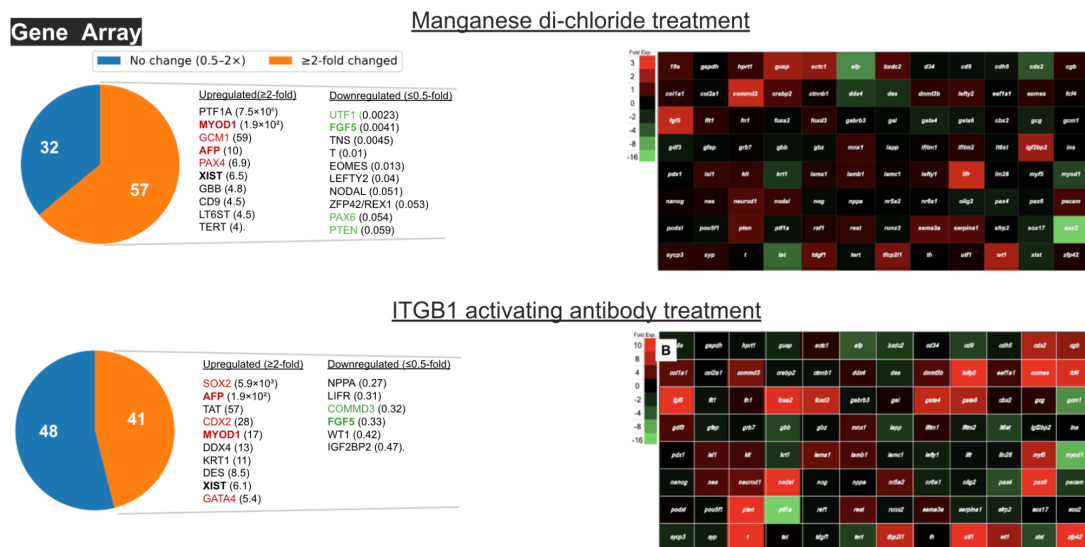


Figure 21. The context of integrin activation leads to distinct remodeling of the pluripotency network in iPSCs.

TaqMan Human Stem Cell Pluripotency Array heatmaps and pie charts compare untreated control iPSCs to those treated with Mn^{2+} (manganese dichloride; top, representing global integrin activation characterized by FAK phosphorylation and OCT4 reduction) and to $\beta 1$ -integrin activation using an ITG $\beta 1$ -activating antibody (bottom).

Heatmaps display relative expression changes, with red indicating increased and green indicating decreased expression. Pie charts categorize targets as “No change” ($0.5\text{--}2\times$) or “ ≥ 2 -fold changed” ($\geq 2\times$ or $\leq 0.5\times$), and list representative genes with ≥ 2 -fold up- or down-regulation. Mn^{2+} treatment resulted in 41 of 89 targets exhibiting ≥ 2 -fold change (48 unchanged), whereas *ITG β 1* activation affected 57 of 89 targets (32 unchanged), demonstrating a broader transcriptional response with $\beta 1$ activation. Bars indicate relative expression normalized to control (mean $\pm$ SEM). Statistical significance compared to control is indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Ω

4.2.7 Integrin $\beta 1$ activation remodels the p53 signaling network

A TaqMan p53 Signaling Array was employed to assess whether integrin $\beta 1$ activation alters transcriptional components of the p53 pathway by comparing untreated control iPSCs with Mn^{2+} -treated cells (**Figure 22**). Gene expression changes were categorized as upregulated (≥ 2 -fold), downregulated (≤ 0.5 -fold), or unchanged ($0.5\text{--}2$ -fold relative to control). The resulting heatmap shows relative expression changes across p53 pathway targets, with red indicating increased expression and green decreased expression. After Mn^{2+} -mediated integrin $\beta 1$ activation, 87 out of 92 targets demonstrated at least a 2-fold change in expression, while 5 targets remained unchanged. Significantly upregulated genes included *PRKDC*, *GADD45A*, *CHEK2*, *CDK2*, *E2F3*, *FAS*, *SIRT1*, *UBC*, *HDAC1*, *HDAC5*, and *EP300*. These upregulated genes included DNA damage and checkpoint regulators (*PRKDC*, *GADD45A*, *CHEK2*, *CDK2*) and chromatin regulatory factors (*HDAC1*, *HDAC5*, *EP300*). Additional upregulated targets included *UBC*, *E2F3*, *FAS*, and *SIRT1*. Significantly downregulated transcripts included *E2F2*, *MAPK10*, *HDAC11*, *CASP4*, and *SIRT3*. *HDAC11* was identified as a chromatin-associated regulatory factor, while *E2F2*, *MAPK10*, *CASP4*, and *SIRT3* represented other p53-pathway-associated targets that were reduced under the same conditions. The corresponding heatmap showed that most p53 pathway components deviated from baseline expression, with only a small subset remaining unchanged. In summary, Mn^{2+} -mediated integrin $\beta 1$ activation extensively remodeled the p53 signaling network, as

evidenced by increased expression of p53 and stress-response regulatory genes and suppression of apoptosis-associated effector genes.

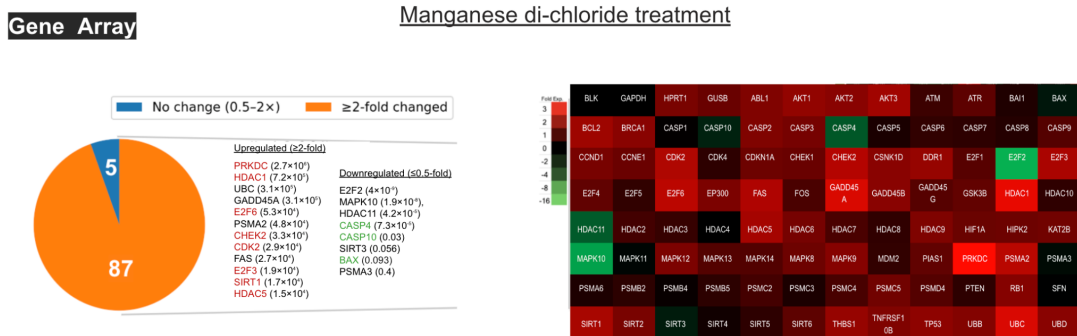


Figure 22. The context of integrin activation leads to distinct remodeling of the p53 pathway in iPSCs.

The TaqMan p53 Signaling Array for Mn²⁺-treated iPSCs revealed a predominantly “≥2-fold changed” profile (87 of 92 targets; 5 unchanged), with representative induced and suppressed p53-network components listed adjacent to the pie chart. Bars indicate relative expression normalized to control (mean ± SEM). Statistical significance compared to control is indicated as follows: *P<0.05, **P<0.01, ***P<0.001.

4.2.8 Integrin β1 activation induces time-dependent RT-qPCR changes in pluripotency- and lineage-associated transcripts

Transcriptional changes following integrin β1 activation were quantified using targeted RT-qPCR in hPSCs treated with control, Mn²⁺ for 6 hours, or an ITGβ1-activating antibody for 24 or 48 hours (**Figure 23**). Transcript levels were assessed for integrins (*ITGA6*, *ITGB1*), pluripotency markers (*OCT4/POU5F1*, *NANOG*, *SOX2*), ectoderm-associated genes (*PAX6*, *NES*, *NEUROD1*), mesoderm-associated genes (*RUNX2*, *EOMES*, *PECAMI*), endoderm-associated genes (*NODAL*, *FOXA2*, *GATA4*), and laminin (*LAM5*). ITGA6 and ITGβ1 transcript levels remained within a narrow range across all treatment conditions compared to the control. Among pluripotency-associated genes, *OCT4* and *NANOG* showed only modest variation, whereas *SOX2* showed the largest increase in response to antibody-mediated activation of integrin β1 at 24 and 48 hours. *LAM5* expression increased following Mn²⁺

treatment but was reduced under ITGβ1-activating antibody treatment at both 24 and 48 hours compared to control. NES expression increased in antibody-mediated integrin β1 activation, with higher levels at 24 and 48 hours than in control- and Mn²⁺-treated cells. PAX6 exhibited a modest increase at 24 hours under antibody treatment, while *NEUROD1* showed minimal changes across conditions. Within the mesoderm-associated panel, *RUNX2* showed the largest increase, peaking at 48 hours under antibody-mediated integrin β1 activation. *EOMES* and *PECAM1* displayed smaller, condition-dependent changes. In the endoderm-associated panel, *NODAL* showed the greatest increase at 24 hours under antibody-mediated integrin β1 activation, whereas *FOXA2* and *GATA4* remained near baseline, with comparatively minor differences across treatment groups. Mn²⁺-mediated and antibody-mediated integrin β1 activation produced distinct RT-qPCR response patterns across pluripotency, lineage-associated, and laminin-related transcripts. The ITGβ1-activating antibody condition demonstrated the most pronounced time-dependent increases in NES, *RUNX2*, and *NODAL*, while changes in core pluripotency marker expression remained limited. These findings indicate minimal remodeling of pluripotency marker expression but increased lineage-associated and early differentiation-related transcriptional signals upon integrin β1 activation.

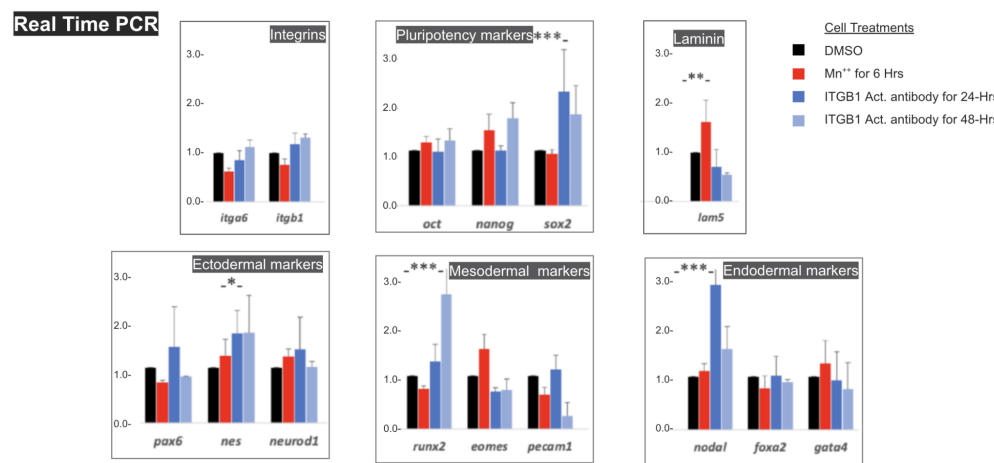


Figure 23. Targeted RT-qPCR analysis was performed to assess the kinetics of integrin-, pluripotency-, and lineage-associated transcripts following Mn^{2+} or ITG β 1-selective activation.

hPSCs were treated with vehicle control (DMSO), Mn^{2+} for 6 hours, or a β 1-integrin-activating antibody for 24 and 48 hours. Transcript levels for integrins (ITGA6, ITG β 1), pluripotency markers (OCT4/POU5F1, NANOG, SOX2), ectodermal markers (PAX6, NES, NEUROD1), mesodermal markers (RUNX2, EOMES, PECAM1), endodermal markers (NODAL, FOXA2, GATA4), and laminin (LAM5) were quantified using TaqMan RT-qPCR, normalized to internal reference genes, and expressed relative to control. Data are presented as mean $\pm$ SEM, with statistical significance versus control indicated (* P <0.05, ** P <0.01, *** P <0.001).

4.3 Discussion

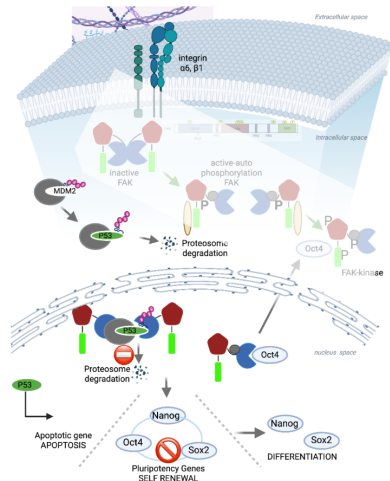
The collective data support a model in which integrin β 1 activation reprograms the hPSC state by shifting cells from a low-basal, pluripotency-supportive adhesion configuration to an activated signaling state associated with stress-responsive and lineage-priming outputs. This interpretation aligns with previous studies demonstrating that α 6 β 1 is the primary laminin-binding integrin in hPSCs and that canonical FAK signaling is typically restrained in self-renewing cultures. At the same time, disruption of this state correlates with loss of pluripotency (Luis G. Villa-Diaz et al. 2016). The present findings build upon the α 6 β 1 framework by demonstrating that direct β 1 activation is sufficient to perturb the FAK regulatory state and remodel downstream transcriptional programs. Imaging data reveal that reduced OCT4, redistribution of NT-FAK, altered p53–MDM2 organization, and increased p21. These results are consistent with the work of Lim et al., who demonstrated that nuclear FAK promotes p53 turnover via MDM2-dependent ubiquitination, and that disruption of this scaffolding function increases p53/p21 signaling (S. Lim 2013). Our data collectively suggest that this spatial reorganization indicates that β 1 activation does not merely enhance adhesion. Instead, it alters the interpretation of adhesion-derived signals within the nucleus. OCT4 and SOX2 are essential components of the pluripotency transcriptional network, and even modest disruptions in this network can destabilize stem cell identity. Therefore, the observed reduction in OCT4, along with the emergence of a p53/p21-associated response, suggests an

early exit from the pluripotent steady state rather than immediate terminal differentiation. A key observation is that the two $\beta 1$ -activating conditions produced distinct responses. Manganese (Mn^{2+}) induced a broader, more acute, and stress-weighted response, characterized by widespread remodeling of the p53 array and stronger suppression of apoptosis-associated effector genes.

In contrast, the ITG $\beta 1$ -activating antibody elicited a more selective, time-dependent response, with greater enrichment for lineage-associated and chromatin or RNA-regulatory factors. This difference likely reflects underlying mechanisms: Mn^{2+} broadly stabilizes high-affinity integrin conformations, whereas antibody treatment promotes receptor-selective clustering of $\beta 1$. Proteomic analyses support this interpretation, as FAK-associated complexes shifted differently under the two activation modes, with antibody-mediated $\beta 1$ activation enriching for chromatin regulators, RNA-associated proteins, and differentiation-linked factors. Transcriptional data indicate that $\beta 1$ activation initially remodels lineage-associated genes more robustly than core pluripotency genes. In the RT-qPCR dataset, integrin transcript levels remained relatively stable, whereas NES, RUNX2, and NODAL were more strongly induced than OCT4 or NANOG.

The proteomic analysis provides additional insights. Following antibody-mediated $\beta 1$ activation, FAK-associated complexes were enriched with chromatin and RNA/epitranscriptomic regulators, including METTL3 and YTHDC1, as well as factors associated with differentiation and development. These findings are consistent with studies demonstrating that METTL3-dependent m6A regulation facilitates exit from pluripotency and that YTHDC1 is essential for stem cell self-renewal and differentiation potential through nuclear RNA- and chromatin-associated functions ([Geula et al. 2015](#); [C. Chen et al. 2021](#)). In this dataset, these regulators were identified within the redistributed FAK-associated network, coinciding with a more pronounced increase in lineage-associated transcripts compared to core pluripotency transcripts. Collectively, these results support the interpretation that integrin

$\beta 1$ activation promotes an early lineage-primed state by redirecting FAK-associated complexes toward chromatin and RNA-regulatory nodes that influence cell fate competence (Figure 24).



Integrin $\beta 1$ activation in hPSCs

- ☐ shifts cells from a low baseline to active adhesion-signaling state
- ☐ disrupts nuclear FAK signaling through the FAK-MDM2-p53 pathway
- ☐ increases p53/p21 signaling
- ☐ reduces pluripotency gene network
- ☐ promotes early differentiation signals

Figure 24. Proposed model of integrin $\beta 1$ activation-dependent fate remodeling in hPSCs.

In self-renewing hPSCs, the $\alpha 5 \beta 1$ integrin axis maintains low basal canonical FAK activation and a nuclear FAK state that sustains the pluripotency gene network. Activation of integrin $\beta 1$ shifts cells from a low-basal adhesion-signaling state to an activated signaling state, marked by disruption of the nuclear FAK-MDM2-p53 regulatory module, increased p53/p21 signaling, reduced expression of pluripotency-associated genes, and induction of early differentiation signals. This model synthesizes imaging, phospho-signaling, transcriptional, and proteomic data presented in this chapter.

Chapter 5: Impaired mitochondrial function of human pluripotent stem cells after xenogeneic mitochondrial transfer from feeder cells.

Core Question

Do feeder cells transfer mitochondria/mtDNA to hPSCs, and does this alter fate-relevant phenotypes?

5.1 Introduction

Human pluripotent stem cells (hPSCs) play foundational roles in research on development, disease modeling, toxicology, and regenerative medicine. However, their usefulness depends on culture conditions that maintain phenotypic stability and ensure experimental reproducibility ([Chaudhary and Villa-Diaz 2025b](#)). These fundamental advances have enabled scalable access to self-renewing and developmentally adaptable human cell states. As hPSC-based assays increasingly inform mechanistic biology and translational decisions, even minor, unrecognized culture-derived variables can introduce systematic bias into assessments of pluripotency, lineage priming, and metabolic fitness ([L. Smith et al. 2025](#)).

A key characteristic of pluripotent state regulation is its strong association with mitochondrial (mt) biology and cellular metabolism ([K. Liu et al. 2020](#)). During reprogramming, somatic cells fundamentally shift their energy production from oxidative phosphorylation toward glycolysis, a metabolic hallmark of pluripotency ([W. Liu and Chen 2021](#)). Direct manipulation of glycolytic pathways markedly improves reprogramming efficiency, underscoring the essential role of metabolic remodeling in attaining pluripotency ([Folmes et al. 2011](#)). In established hPSCs, glycolytic regulators such as HIF1 α and UCP2 actively regulate metabolism and link mitochondrial function to cell-fate decisions. Disruption of this metabolic regulation impairs differentiation potential, demonstrating that mt status functions not as a passive indicator but as an active determinant of cellular fate competence ([Mathieu and Ruohola-Baker 2017](#); [R. Zhang et al. 2013](#)). Consequently, any process that may alter mt content or function during standard culture requires careful examination and consideration.

Intercellular mt transfer has emerged as a significant biological phenomenon that can alter the recipient cell's bioenergetics and stress responses ([Z. Liu et al. 2022](#)). Early studies demonstrated that mt and mtDNA can be transferred between cells, and that this process can restore aerobic respiration in dysfunctional cells, providing proof of concept for functional

recovery via horizontal mt exchange ([Spees et al. 2006b](#)). Subsequent in vivo research revealed that microvesicle- and exosome-mediated mt delivery protects tissues, illustrating the physiological value of contact-independent transfer pathways ([Hayakawa et al., 2016](#); [Islam et al., 2012b](#)). However, a critical knowledge gap remains whether xenogeneic feeder-based workflows—standard in hPSC maintenance—facilitate the transfer of feeder-derived mt into hPSCs, and whether this exposure meaningfully impacts mt function and pluripotency assessment. Addressing these questions is essential, as even transient xenogeneic mt contamination could compromise the integrity of mt functional assays, metabolic profiling, and conclusions about cell state.

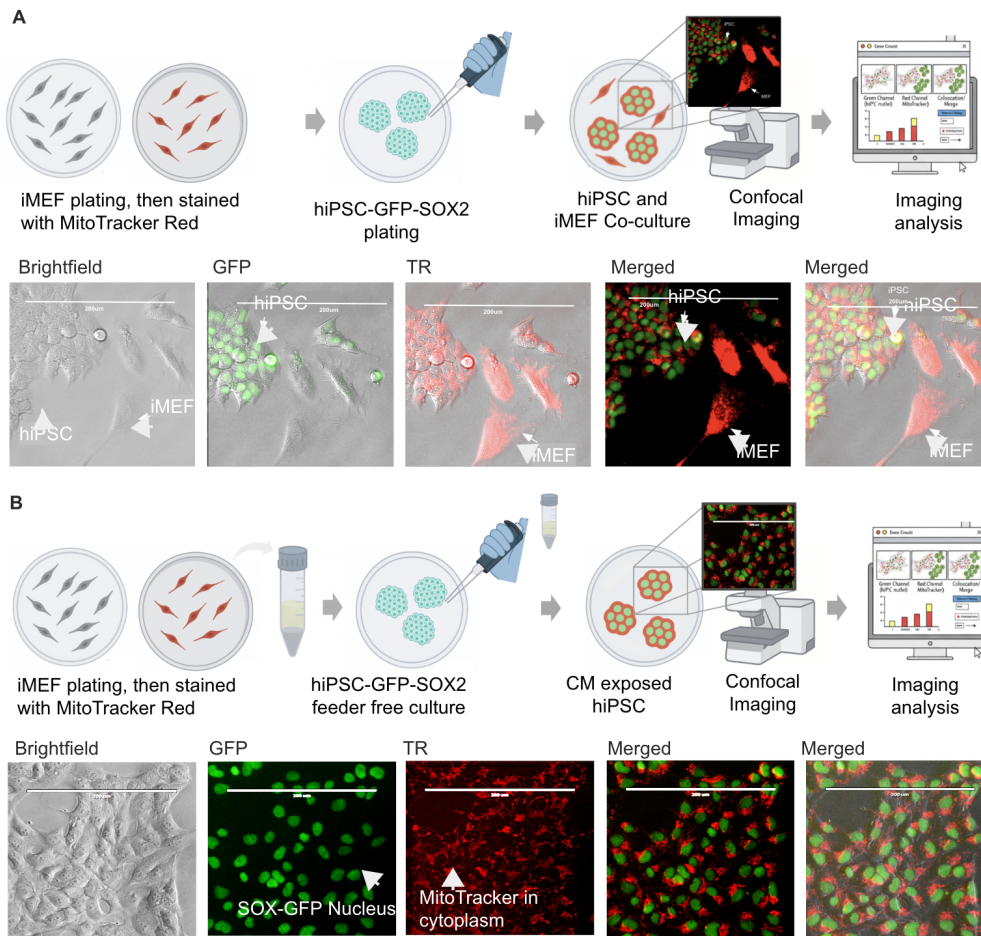
This study examines whether irradiated mouse embryonic fibroblasts (iMEFs) convey mitochondria (mt) material to hPSCs via both contact-dependent and contact-independent mechanisms, and whether this transfer compromises mt function in the recipient cells. We used MitoTracker-labeled iMEFs as donors and detected mt signals in hiPSCs after both direct co-culture and exposure to iMEF-conditioned medium (iMEF-CM). This shows that mt uptake happens without cell-to-cell contact. Flow cytometry showed a MitoTracker⁺ hPSC population within hours of exposure to iMEF-CM. Species-specific PCR confirmed the presence of mouse *COX2* amplicons in the exposed hPSCs but not in untreated controls, supporting xenogeneic mtDNA carryover. Live-cell imaging revealed TNT-like intercellular connections that displayed robust mt and vesicular fluorescence. At the same time, nanoparticle tracking analysis of iMEF-CM identified a distinct population of EVs with diameters of 200–250 nm. Pharmacological perturbation validated pathway involvement: GW4869 slightly decreased uptake, whereas desipramine markedly enhanced intracellular signaling. Following mt exposure, pluripotent recipient cells exhibited a compromised mt membrane potential, as demonstrated by the JC-1 assay. They diminished ATP production, as measured by Seahorse XF real-time ATP rate profiling.

The dataset presented here provides quantitative evidence for xenogeneic mitochondrial transfer and mtDNA dynamics, evaluates potential transfer mechanisms through tunneling nano tubes (TNT)-like structures versus extracellular vesicle-enriched conditioned media, and characterizes the resulting mitochondrial functional outcomes in hPSC recipients.

5.2 Results

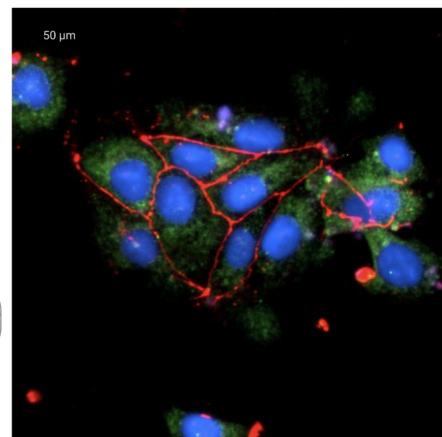
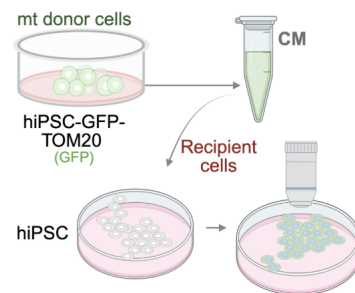
5.2.1 Feeder cells-derived mitochondria (mt) are transferred to hPSCs, increasing the recipient cell's mt signal.

We first asked whether hPSCs acquire mt from iMEFs under co-culture conditions, and then under feeder-free culture conditions using CM collected from iMEFs. For this, iMEFs were labeled with MitoTracker™ Red and used as mt donors either by direct co-culture or via CM collected from labeled iMEFs (*Figure 25*). In direct co-culture, MitoTracker Red-positive puncta were detected in all hPSCs tested: H1, H9, hiPSC-GFP-SOX2, and hiPSC-GFP-TOM20, indicating entry of donor-derived mt material into recipient cells. Results from the hiPSC GFP-SOX2 cell line are used as a representative example of the consistent results across all hPSCs tested (*Figure 25a*). In the absence of cell-cell contact, hPSCs exposed to iMEF-CM exhibited a similar intracellular MitoTracker Red signal (*Figure 25b*), indicating contact-independent mitochondrial transfer. To test whether hPSCs also secreted EV-associated mt and to verify that the mt signal detected in recipient cells is not an artefact of the Mitotracker staining, CM was collected from hiPSC-GFP-TOM20 cells and used during feeder-free culture of hESC H9 cells. Consistently, hESCs exposed to CM from hiPSC-GFP-TOM20 acquired mt from the donor cells, as shown by the intracellular GFP signal (*Figure 25c*). Mitochondrial (mt) transfer between iMEFs and human foreskin fibroblasts (hFFs) in co-culture. (*Figure 25d*).



c)

S3)



d)

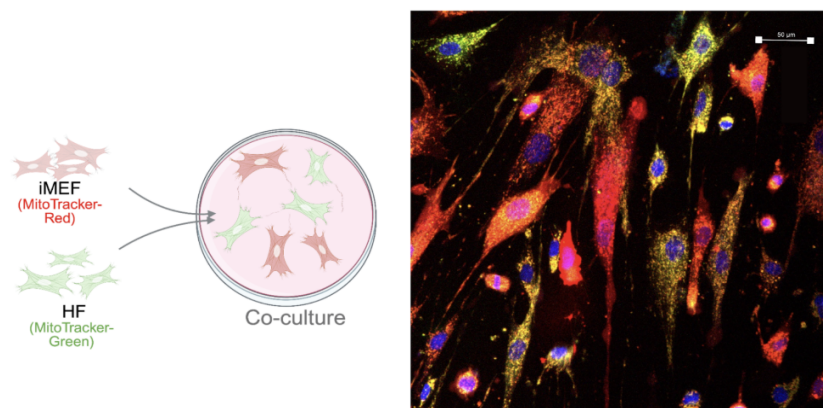


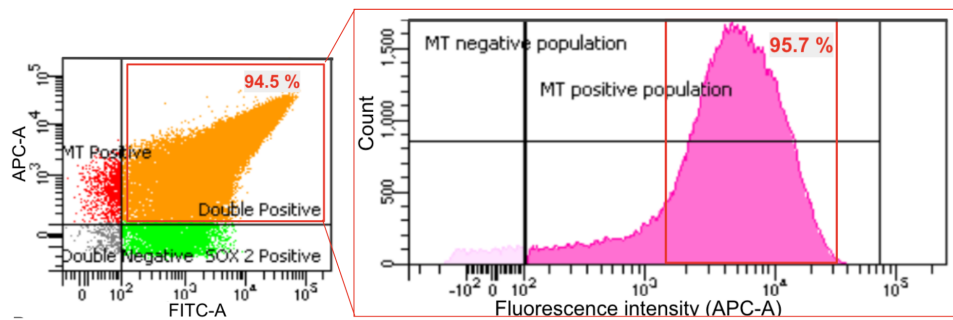
Figure 25. Xenogeneic mitochondrial (mt) transfer between cells.

a) Representative micrograph demonstrates direct mitochondrial transfer to hiPSC-GFP-SOX2 cells during co-culture with pre-MitoTracker Red-labelled iMEFs. Scale bars = 200 μ m. **b)** Representative micrograph showing pre-labelled mt (MitoTracker Red) transferred to hiPSC-GFP-SOX2 cells cultured in feeder-free conditions with iMEF-CM, indicating uptake of extracellular mt material. **c)** Representative fluorescence micrographs are shown for (top) untreated feeder-free hPSCs (GFP-negative), (middle) donor iPSCs stably expressing TOM20-GFP (mitochondrial outer-membrane marker; positive control), and (bottom) feeder-free hPSCs after 24 hours of exposure to donor conditioned medium (CM-TOM20), demonstrating acquisition of the TOM20-GFP signal. Cell borders are stained with anti-ZO-1 (red), nuclei with DAPI (blue), and TOM20-GFP is visualized in green. All images were acquired using identical exposure and laser settings. Scale bar: 50 μ m. **d)** Representative micrograph showing transfer of pre-labelled mt from MitoTracker Red-stained iMEFs to MitoTracker Green-stained hFFs during co-culture, indicating intercellular exchange of mt material. DAPI (blue) labels nuclei. Scale bar, 50 μ m

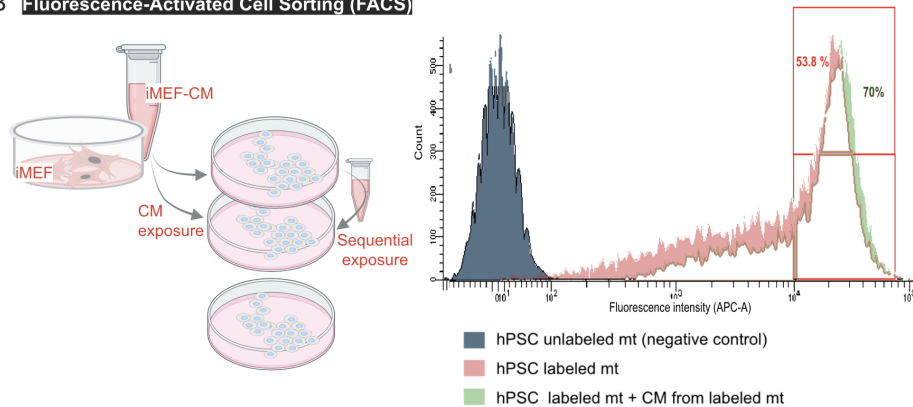
Flow cytometry analysis confirmed that mt transfer occurred at a high frequency following 24-hour exposure to iMEF-CM. Quadrant analysis (FITC = GFP-SOX2; APC = MitoTracker) showed ~95% GFP+/APC+ double-positive population (**Figure 26a**). To quantify the addition of exogenous mt into recipient cells, hPSCs with pre-labelled mt and cultured under feeder-free conditions were exposed to CM from pre-labelled mt iMEFs for 6 h (**Figure 26b**). Flow cytometry analysis shows an increase of mt signaling -from ~54% to ~78% in the gated area-, indicating increased accumulation of mt in the hPSCs exposed to iMEF-CM. Species-specific PCR analysis provided orthogonal evidence of xenogeneic

mtDNA carryover in hPSCs exposed to iMEF-CM. Mouse *COX2* DNA was detected in hPSCs exposed to iMEF-CM and in iMEFs; however, it was not detected in untreated hPSCs, while human *COX2* DNA was observed in all hPSC samples and not detected in the iMEF sample (**Figure 26c**). Collectively, these complementary approaches demonstrate that iMEF-derived mt material is transferred to hPSCs via exposure to CM, thereby increasing mt-associated signals in the recipient cells.

A Fluorescence-Activated Cell Sorting (FACS)



B Fluorescence-Activated Cell Sorting (FACS)



C Species-specific PCR genotyping

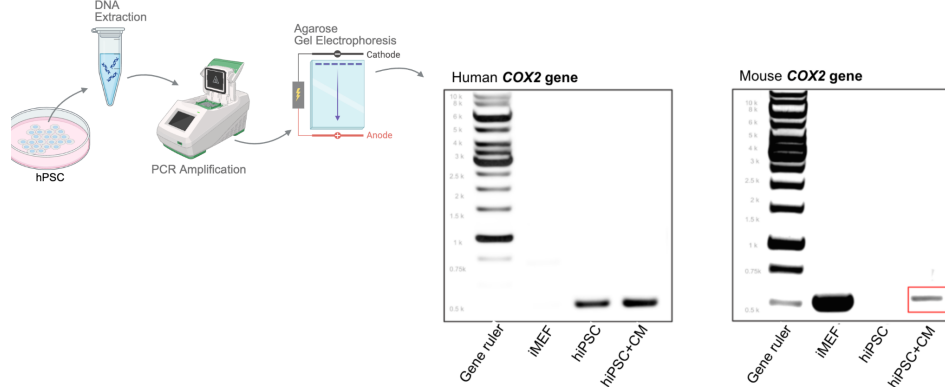


Figure 26. Contact-independent mitochondrial (mt) material transfer to hPSCs via iMEF CM.

a) Representative histogram of hiPSC-GFP-SOX2 cells exposed for 24 h to iMEF-CM pre-labeled with MitoTracker-Red. The FITC channel was used to detect the endogenous GFP signal of hiPSC-GFP-SOX2 cells, while the APC channel was used to detect the signal from the iMEF's mt labelled with MitoTracker (transferred signal). Quadrant analysis (10,000 events) reveals that GFP⁺/APC⁺ (double positive) accounts for ~95 % of the cells, while the APC⁺/GFP⁻ and double negative population were 0.9% and 0.3%, respectively. **b)** Overlay of histograms of MitoTracker™ Red labelled mt fluorescence intensity signal detected with APC. Unstained hPSC control (dark blue; negative control), hPSCs with mt labelled (pink histogram; ~54% of high-intensity gated area), and hPSCs with mt labelled and cultured with iMEF-CM containing labeled mt (green histogram; ~70 of high-intensity gated area). A rightward shift and increased fraction of events within the high-intensity gate suggest an elevated mt-associated fluorescence signal in hPSCs exposed to iMEF-CM compared to non-iMEF-CM-exposed cells. Acquisition and gating were performed identically across all samples. Data were acquired and analyzed using consistent instrument settings and gating across all conditions. **c)** Genomic DNA from iMEFs, hPSCs, and hPSCs exposed to iMEF-CM (hPSC+CM) was amplified with human and mouse-specific COX2 primers. Representative agarose gels showing the expression of human COX2 (left side): ~500 bp band in hPSCs and hPSCs+CM, while absent in iMEFs. Representative agarose gel on the right showing the expression of mouse COX2 in iMEFs and hPSC+CM, while absent in hPSCs not exposed to iMEF-CM.

5.2.2 Intercellular mitochondrial (mt) transfer occurs via contact-dependent and extracellular (EV-enriched) routes.

We investigated cellular routes mediating mt transfer, focusing on tunneling nanotube (TNT)-like structures for direct cell-cell mt transfer and extracellular microvesicles (EVs) that carry mt and are associated with transport from donor-derived CM to recipient cells (**Figure 27**). Live-cell confocal imaging revealed thin TNT-like intercellular connections spanning tens to hundreds of micrometers, with mt labeled with Mitotracker present within these structures (**Figure 27a**), consistent with direct cytoplasmic conduit-mediated transport. Treatment with Cytochalasin D, a known inhibitor of TNT formation through actin disruption, markedly shortened TNT-like intercellular connections, supporting an actin-dependent basis for these structures (**Figure 27b**). However, mt-labeled donor signal remained detectable in recipient

PSCs despite Cytochalasin D treatment, indicating that TNT-like conduits do not exclusively mediate mt transfer and can also occur via TNT-independent mechanisms.

On the other hand, nanoparticle tracking analysis (NTA) of iMEF-CM revealed an EV-enriched population with a number-weighted median size of ~228 nm (**Figure 27c**). In contrast, the volume-weighted profile was shifted toward larger diameters, with a volume-weighted median of ~334 nm, indicating that although most particles clustered near ~200–250 nm by count, larger vesicles contributed the majority of total vesicular volume. To probe the functional involvement of EV-associated pathways and/or the recipient's handling of transferred material, pharmacologic perturbation was performed. iMEFs were treated with Desipramine and GW4869 to modulate EV release. Desipramine treatment significantly increased intracellular MitoTracker signal in hPSCs exposed to iMEF-CM, whereas GW4869 caused a non-significant increase in MitoTracker signal relative to vehicle (**Figure 27d**).

Together, these data support a model in which mt transfer can occur via both contact-dependent routes (TNT-like connections) and contact-independent routes mediated by EV-enriched CM.

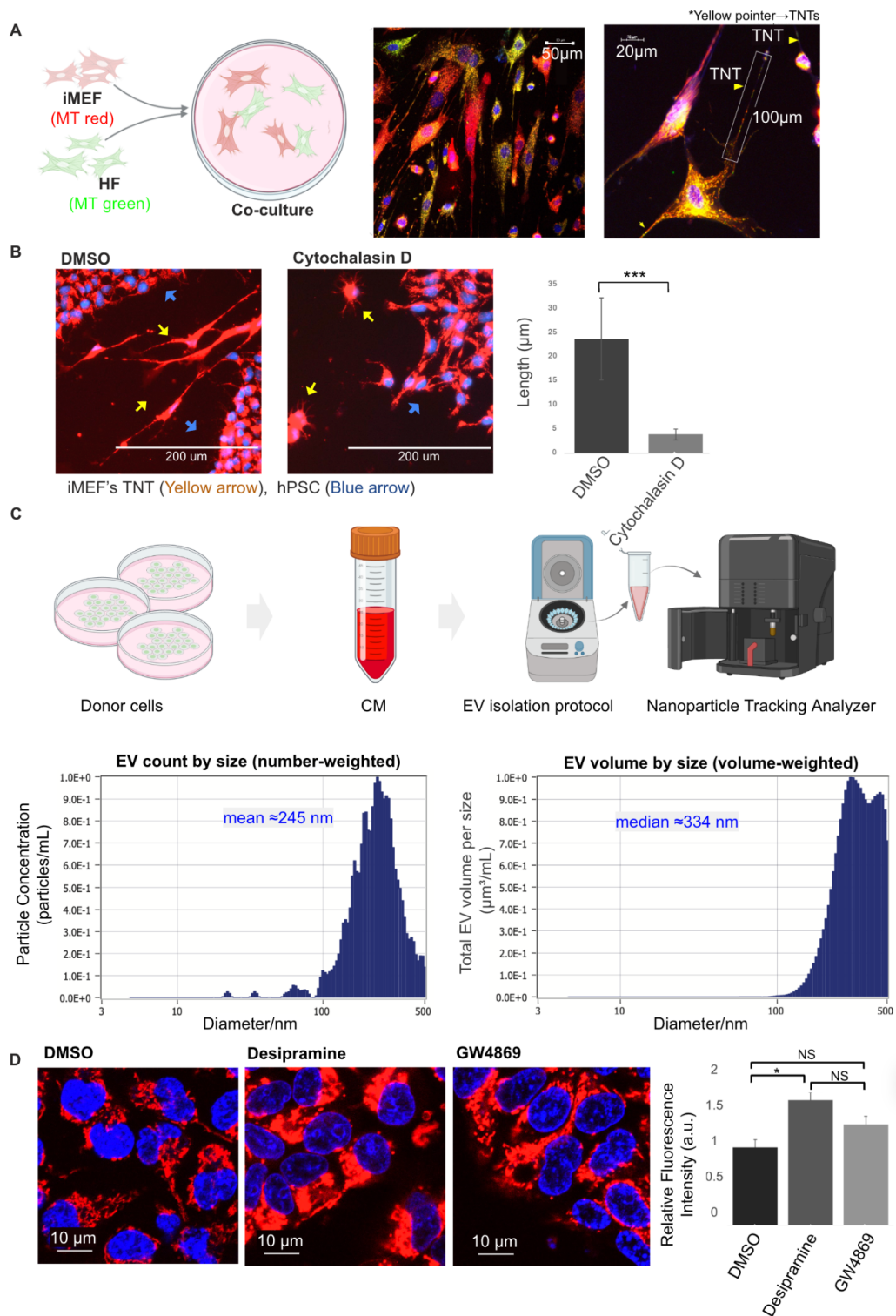


Figure 27. Mechanisms of contact-dependent and contact-independent mitochondrial (mt) transfer—pathways include tunneling nanotubes (TNTs) and extracellular vesicles (EVs).

a) Live-cell TNT-mediated mt exchange during co-culture between iMEFs and human fibroblasts. The two fibroblast populations were separately labeled with mt dyes (MitoTracker Red CMXRos or MitoTracker Green),

lifted, mixed at a 1:1 ratio, and co-cultured for 24 h. Live-cell confocal imaging reveals tunneling nanotubes (TNTs)—elongated, thin intercellular bridges (~10–200 μm) connecting the fibroblasts. Magnified insets show red and green mt within TNTs. Imaging settings were matched across panels, as indicated by scale bars. **b)** Cytochalasin D shortens TNT-like connections. Representative iMEF–hPSCs co-culture images, and quantification show reduced TNT-like connection length with Cytochalasin D versus DMSO treatment (mean $\pm$ SEM; *** $P < 0.001$). Donor MitoTracker signal remains detectable in PSCs. Scale bar sizes are indicated in each micrograph. **c)** EV size and distribution characterization from CM by nanoparticle tracking analysis (NTA). Schematic of the procedure (top panel). Isolated EVs were analyzed by NTA (bottom panel), showing size distributions by particle count (number-weighted; left bottom panel) and by particle volume (volume-weighted; right bottom panel). The sample contained $\sim 2.5 \times 10^{11}$ particles/mL in the original CM (after correcting for a 3,200-fold dilution). By particle count, most EVs were ~140–360 nm (median ~228 nm); by volume, larger particles contributed more (median ~334 nm). Measurements were acquired in PBS at 27.6 °C (174 particles/frame; 349 tracks). **d)** Donor EV inhibition vs recipient retention in mt transfer. Recipient hPSCs were exposed for 24 h to EV-rich CM from MitoTracker Red–labeled MEFs. Representative confocal fields (Hoechst nuclei, blue; mitochondrial signal, red) are shown for DMSO, desipramine (ASM/FIASMA), and GW4869 (nSMase2 inhibitor); scale bars, 10 μm . Right, quantification of relative MitoTracker-Red fluorescence (a.u.) normalized to DMSO (mean $\pm$ SEM, per-field integrated intensity). Desipramine increased the red signal consistent with endolysosomal retention of particulate cargo, whereas GW4869 produced a modest reduction.

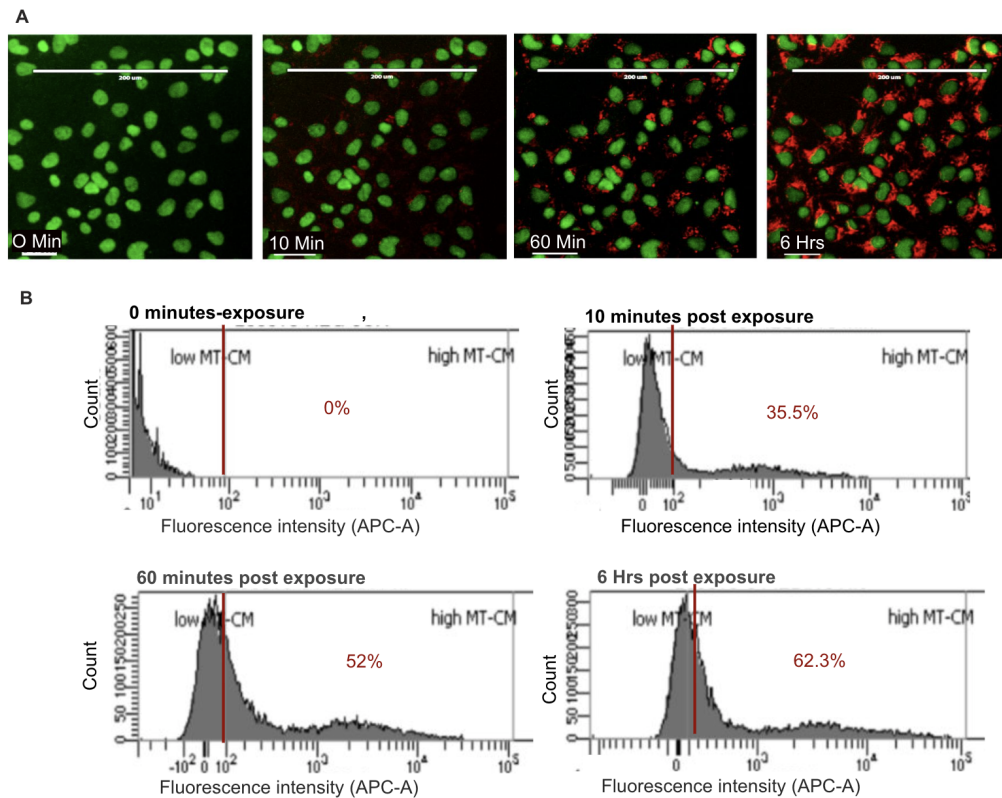


Figure 28. Temporal dynamics of mitochondrial (mt) uptake in hPSCs

a) Time-resolved uptake and quantification. Live confocal imaging of hPSCs incubated with iMEF-CM from MitoTracker-Red-labeled donors shows progressive accumulation of mt signal at 0, 10, 60 min, 6 h, and 24 h. The GFP signal indicates the nuclei of hiPSC-GFP-SOX2 cells, while MitoTracker-Red marks donor-derived mt material. Scale bars equal to 200 μm. **b)** Flow cytometry of hiPSC-GFP-SOX2 cells exposed to iMEF-CM with MitoTracker-Red labeled mt. APC = MitoTracker.

Having established that mt transfer occurs during co-culture of iMEF with hPSCs and during feeder-free hPSC culture using iMEF-CM, we quantified the temporal dynamics of uptake, evaluated the persistence of xenogeneic mtDNA across passages, and assessed pluripotency-associated phenotypes following xenogeneic mt exposure (**Figure 29**). These experiments were designed to distinguish early uptake from longer-term maintenance of xenogeneic mt material in the recipient population. Time-lapse imaging and time-resolved flow cytometry demonstrated a progressive accumulation of iMEF's mt-MitoTracker signal in recipient hPSCs over minutes to hours, reaching maximal labeling by 24 hours.

In parallel, we directly tested the persistence of xenogeneic mtDNA acquired through iMEF-CM exposure and subsequent serial passage under feeder-free, xeno-free, chemically defined conditions. Species-specific primers detected a mouse *COX2* amplicon at early passages after exposure to iMEF-CM, which progressively decreased with continued passaging and became undetectable at later passages. In contrast, the human control amplicon (*hGAPDH*) remained robust across all passages (**Figure 29a**). These findings suggest that xenogeneic mtDNA introduced by iMEF-CM is transient and becomes diluted or lost during subsequent feeder-free expansion. Up to this point, all experiments were performed on hESC (H1 and H9) and hiPSC (hiPSC-GFP-SOX2 and hiPSC-GFP-TOM20) lines, which were derived and cultured in iMEFs. Thus, we derived new isogenic hiPSC lines from hFFs in two conditions: 1) hiPSCs exposed to xenogenic mt (three clones were exposed to iMEFs during the derivation), and 2) hiPSCs not exposed to xenogenic mt (three isogenic clones were derived in feeder-free cultures with chemically defined medium). After their derivation, the individual hiPSC cell lines were subcultured under feeder-free conditions in a chemically defined medium. To assess the potential xenogeneic mt presence and persistence in the newly derived hiPSCs in the presence of iMEFs, and sub-cultured in feeder-free conditions with xeno-free chemical-defined medium. PCR analysis showed a mouse *COX2* signal in these hiPSC lines during the first two consecutive passages, but it was no longer detected in the third passage. Mouse *COX2* signal was not observed in hiPSC lines derived in feeder-free conditions and never exposed to iMEFs or iMEF-CM (**Figure 29b**). Exposure to iMEFs did not significantly alter *SOX2* or *POU5F1/OCT4* transcript levels compared to feeder-free derived hiPSCs, although *NANOG* expression was modestly reduced (**Figure 29c**). In contrast, expression of lineage-associated transcripts was selectively modified, *SOX17* (endoderm) and *EOMES* (mesoderm) showing significant induction in hiPSCs derived in iMEF compared to hiPSCs derived in feeder-free conditions.

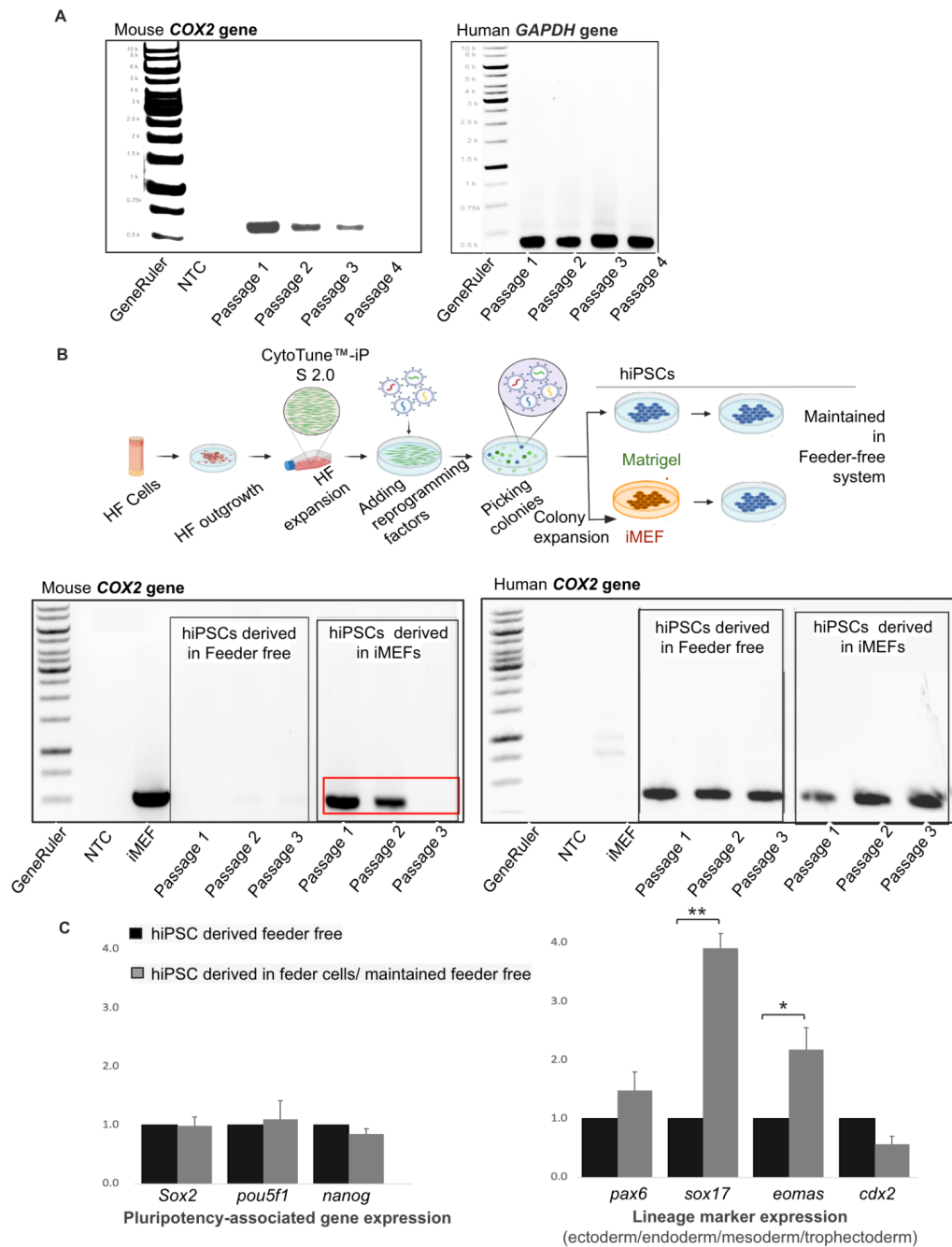


Figure 29. Post-transfer outcomes in hPSCs following exposure to iMEF-derived mitochondrial material.

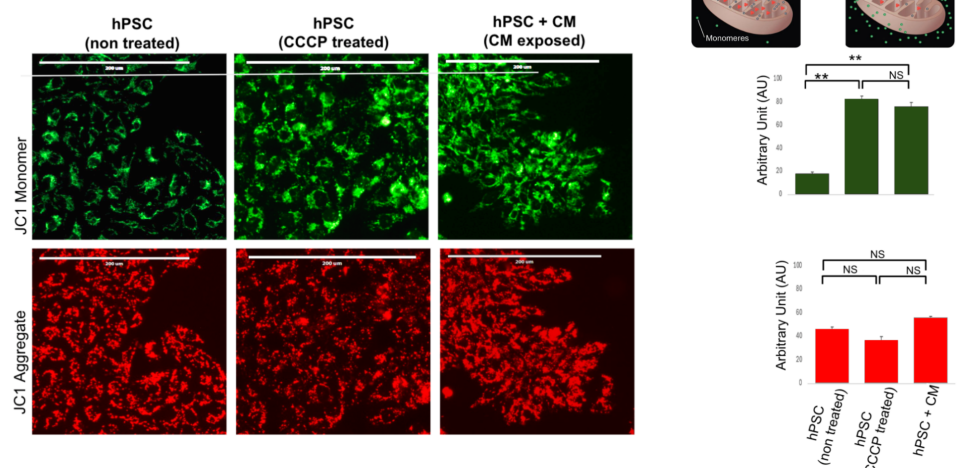
a) Species-specific PCR was used to track the persistence of xenogeneic mitochondrial DNA (mtDNA) following exposure to CM. hESCs; H1 and H9 were exposed to mt from iMEFs via iMEF-CM, then subcultured under feeder-free, xeno-free, chemically defined conditions. PCR with mouse-specific (mCOX2) and human-specific (hGAPDH) primers was performed across sequential passages to assess xenogeneic mtDNA persistence. **b)** Parallel workflows for derivation and maintenance, as well as comparative PCR analysis, were conducted in

*hiPSCs generated with or without feeder exposure. The schematic illustrates fibroblast reprogramming and subsequent maintenance under parallel conditions: feeder-free on Matrigel or iMEF-supported culture and/or exposure to feeder-derived CM. The lower gels show mouse and human COX2 PCR results from hiPSCs maintained on feeders or exposed to iMEF-CM, then passaged feeder-free. The iMEF lane serves as a positive control for mouse COX2, NTC is the negative control, and GeneRuler ladders are shown on the left. c) Transcriptional profiles following feeder exposure history. RT-qPCR analysis compared hiPSCs derived and maintained under feeder-free conditions with those derived in feeder conditions and subsequently maintained feeder-free. Pluripotency-associated transcripts (SOX2, POU5F1/OCT4, NANOG) and lineage-associated markers (PAX6, SOX17, EOMES, CDX2) were analyzed. Data were normalized to housekeeping genes and expressed relative to feeder-free controls (mean $\pm$ SEM; biological N $\geq$ 3). Statistical significance is indicated on the plot (*P < 0.05, **P < 0.01).*

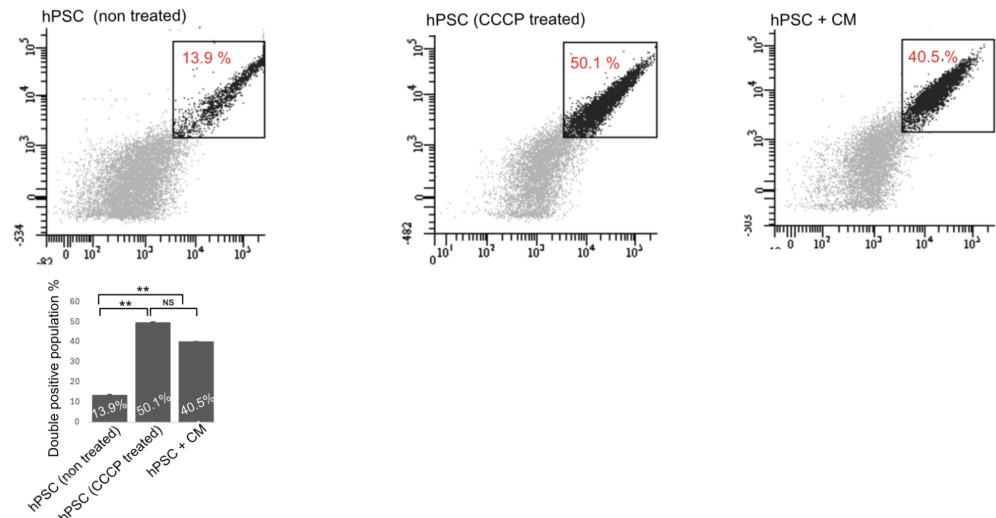
5.2.3 Xenogeneic mitochondrial (mt) transfer is associated with altered mt functional state and reduced mt ATP output in recipient hPSCs.

We next asked whether the acquisition of xenogeneic mt material is accompanied by functional perturbations in the recipient cells. Mitochondrial membrane potential ($\Delta\Psi_m$) was evaluated using the JC-1 aggregate (red) versus monomer (green) signals, quantified by live-cell imaging and flow cytometry, with appropriate depolarization/hyperpolarization controls. Compared to hPSCs non-exposed to iMEF-CM, a significant increase in JC1 monomer concentration was observed in hPSCs containing exogenous mt after iMEF-CM exposure, comparable to hPSCs treated with CCCP to induce mt stress (**Figure 30a**). Bioenergetic profiling using the Seahorse XF Real-Time ATP Rate Assay quantified mt ATP production, glycolytic ATP production, and derived indices from OCR/ECAR measurements, showing different profiles between hPSC exposed or not to iMEF-CM (**Figure 30b**).

MitoProbe™ JC-1 analysis-Confocal imaging



MitoProbe™ JC-1 analysis-FACS



Seahorse XF real-time ATP rate assay

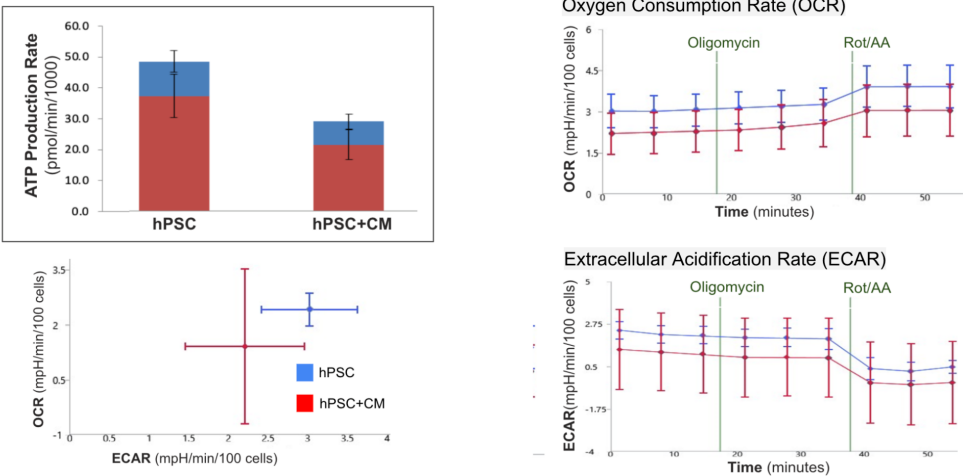


Figure 30. Altered mitochondrial (mt) function in hPSCs containing xenogenic mitochondria.

a) The mt membrane potential ($\Delta\Psi_m$) of hPSC recipients of xenogeneic mt after exposure to iMEF-CM was assayed using MitoProbe™ JC-1 (5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide). Representative live confocal micrographs (top) show red JC1-aggregates (high $\Delta\Psi_m$) versus green monomers (low $\Delta\Psi_m$), with the red/green ratio computed per cell (fixed LUTs, identical exposure). FITC = JC-1 monomers; PE or PE-TxRed = JC1-aggregates. hPSCs not exposed to xenogeneic mt were used as a negative control group, while hPSCs treated with CCCP were used as a positive control to be compared to hPSCs containing iMEF's mt. Graphs show the quantification of JC-1 monomers and aggregates by group. Flow cytometry (bottom) was used to quantify $\Delta\Psi_m$ at the population level. **b)** Graphs showing results from comparative analysis of ATP production using Seahorse XF real-time ATP rate assay between hPSC not exposed to xenogeneic mt and hPSCs containing iMEF's mt. Graphs indicating OCR and ECAR measurements in hPSC not exposed to xenogeneic mt and hPSCs containing iMEF's mt. After oligomycin and rotenone/antimycin A injections, the mt ATP production rate, glycolytic ATP production rate, total ATP rate, basal respiration, ATP-linked respiration, proton leak, and spare respiratory capacity were computed. Data were normalized to cell number and presented as mean $\pm$ SEM ($N \geq 3$).

5.3 Discussion

The present study demonstrates that iMEFs transfer mt material to hPSCs via both direct co-culture and contact-independent exposure to iMEF-CM. MitoTracker-positive puncta were observed across hPSC lines, flow cytometry revealed high-frequency acquisition in hiPSC recipients, and species-specific PCR confirmed xenogeneic carryover by detecting mouse COX2 DNA following CM exposure. Mechanistic investigations using live imaging identified TNT-like connections consistent with conduit-mediated trafficking (Rustom et al. 2004) and compatible with TNT-linked mitochondrial exchange (Zamberlan and Semenzato 2025), including the capacity to transfer larger mitochondrial cargo beyond typical vesicular constraints (Nawaz and Fatima 2017). Concurrently, nanoparticle tracking analysis revealed abundant particles ~200–250 nm, consistent with EV-enriched CM (Comfort et al. 2021), although definitive EV attribution requires multi-parameter characterization (Théry et al. 2018). Pharmacologic perturbations supported partial EV involvement: GW4869 modestly

reduced uptake (Ozaki et al. 2024; Théry et al. 2018), whereas desipramine increased intracellular signaling, likely reflecting altered endolysosomal processing and increased intracellular persistence rather than enhanced transfer flux (Trajkovic et al. 2008; T. S. Wang et al. 2020). These findings underscore the importance of distinguishing transfer rate from retention when interpreting fluorescence intensity (Berridge et al. 2018). Xenogeneic mtDNA was transient, as mouse COX2 declined across passages during subsequent feeder-free culture, consistent with dilution, selective clearance, and potential Mito nuclear incompatibility (Dong et al. 2023; Piccinini et al. 2021). In summary, these data support a model in which iMEFs transfer mitochondrial material to hPSCs via both TNT-like, contact-dependent conduits and EV-enriched CM, resulting in a high-frequency transfer phenotype with detectable xenogeneic mtDNA that is subsequently lost over successive passages (**Figure 31**). Notably, acquisition is associated with reduced mitochondrial ATP production and altered $\Delta\Psi_m$ in recipient hPSCs, as well as the selective induction of lineage-associated transcripts, such as SOX17 (R. Zhang et al. 2013; W. Zhou et al. 2012). Collectively, these findings indicate that feeder-derived mitochondrial transfer is a biologically significant modifier of stem cell state and bioenergetic phenotype, with important implications for experimental interpretation, disease modeling, and the development of reproducible, clinically relevant hPSC culture workflows (Li et al. 2024; Sercel et al. 2021; Frankenberg Garcia et al. 2022; Vianello et al. 2023; Nakamura et al. 2025).

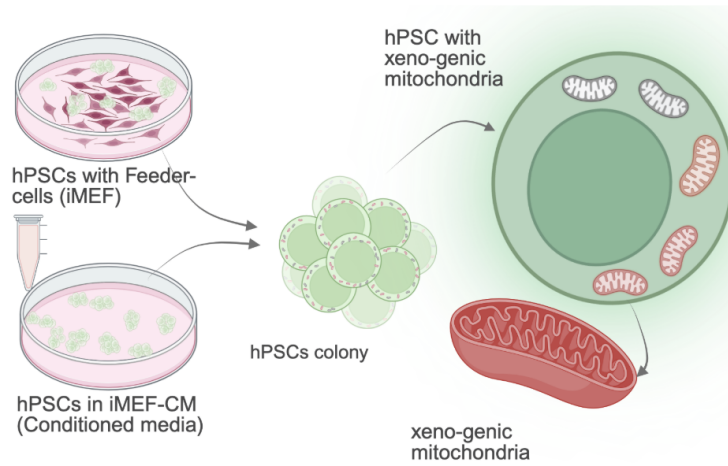


Figure 31. Schematic model of feeder-derived xenogeneic mitochondrial transfer to hPSCs.

hPSCs cultured either directly on iMEF feeder cells or iMEF-CM are exposed to feeder-derived biological cargo. This model illustrates the transfer of xenogeneic mitochondrial material from feeder-associated sources into hPSC colonies, resulting in recipient hPSCs containing non-human mitochondria. The schematic summarizes the proposed feeder-to-hPSC mitochondrial transfer pathway examined in this study.

Chapter 6 GAG-Inspired Polymer Brushes Promote human mesenchymal stem/signaling cells (hMSC) Proliferation While Retaining Trilineage Differentiation.

Core Question

Can bioengineered polymer brushes enhance MSC expansion while maintaining potency?

6.1 Introduction

Human mesenchymal stem/signaling cells (hMSCs) are widely investigated as cell-based therapeutics for cancer, autoimmune disorders, and musculoskeletal repair ([Olsen et al. 2018](#)). However, clinical translation is frequently constrained by manufacturing requirements, including the need for very large cell doses and the associated costs and batch-to-batch variability in product quality ([Aijaz et al. 2018a](#)). hMSCs can be isolated from adult tissues or generated from pluripotent stem cells and expanded in scalable culture systems, but extended passaging typically reduces proliferation and functional potency, with additional variability introduced by donor age, tissue source, and culture protocols ([Serra et al. 2018](#); [L.G. Villa-Diaz et al. 2012](#), [Zaim et al. 2012](#)). These limitations motivate the development of chemically defined, scalable culture platforms that increase yield while preserving trilineage differentiation capacity and therapeutic function.

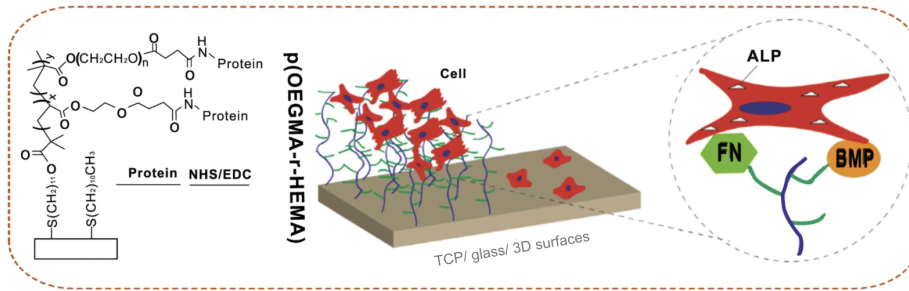
Because hMSC fate and potency are microenvironment-sensitive, substrate design has emerged as a practical strategy to control expansion. Nano topographical surfaces can enhance proliferation by reorganizing the cytoskeleton and focal adhesions, but the complexity of fabrication can hinder scalability ([McMurray et al. 2011](#)). Three-dimensional scaffolds and hydrogels more closely mimic the extracellular matrix architecture but often offer limited chemical tunability to control growth factor interactions and adsorption-mediated signaling ([Heo et al. 2018](#)). Polymer brushes provide a modular, ultrathin coating platform that can be grafted onto planar substrates and scalable formats such as microcarriers and three-dimensional constructs (**Figure 32a**). By tuning brush chemistry, grafting density, microstructure, and thickness, polymer brushes can regulate hydration, protein adsorption, and cell–material interactions ([Qian et al. 2014](#); [Kumar et al. 2017](#)). Importantly for translation, this approach enables “defined” microenvironment control without relying on costly recombinant matrices as the primary instructive cue.

In this chapter, we apply a glycosaminoglycan (GAG)-inspired strategy using sulfonated polymer brushes to enhance hMSC expansion while retaining trilineage potential. Sulfonated brushes present a high density of negatively charged sulfonate groups (SO_3^-), which can electrostatically attract and sequester positively charged, heparin-binding growth factors from defined media. This approach is motivated by the central role of basic fibroblast growth factor (bFGF) in promoting hMSC self-renewal via FGFR signaling (Schmidt et al., 2006) and by the practical limitation that soluble bFGF is expensive and labile in standard workflows (Chen et al., 2012). To overcome diffusional loss and rapid depletion, sulfonated brush interfaces are designed to capture and stabilize bFGF, presenting it multivalently at the cell–material interface (**Figure 32b**), thereby prolonging effective receptor engagement and downstream proliferative signaling (Zehe et al., 2006). Because highly hydrated or super hydrophilic surfaces can impair adhesion, hydrophilicity is treated as an adjustable design variable to maintain robust attachment and expansion (Ayala et al., 2011; Qian et al., 2014). Consistent with prior observations that sulfonated brushes can enhance proliferation in other systems; this work extends these principles to hMSC manufacturing contexts and explicitly tests whether potency is preserved (Marchena et al. 2024). Finally, aligned with the dissertation’s microenvironmental control framework, this chapter evaluates whether combining fully defined media with chemically defined, GAG-mimetic brush interfaces can reduce batch variability associated with serum and biologically derived GAGs and avoid limitations of heterogeneous heparan sulfate supplementation, while improving expansion efficiency without loss of functional potency (Mende et al., 2016; Ling et al., 2016).

a)

Recombinant materials- Polymer brushes

Provide a physical substrate



b)

FGF-FGFR signaling pathway

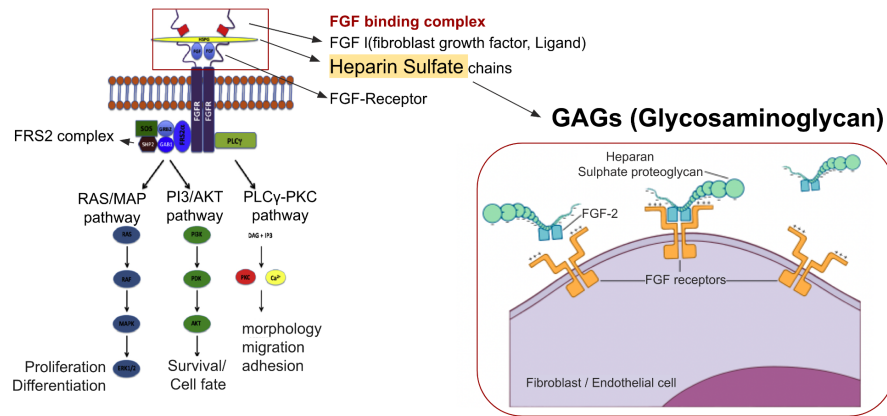


Figure 32. Model of GAG-mimetic bFGF presentation to support hMSC expansion.

(a) In native tissues, heparan sulfate proteoglycans bind and concentrate fibroblast growth factor 2 (FGF-2/bFGF) at the cell surface, facilitating FGFR engagement and signaling. *Ref. Ren, X., & Wei, S. (2011). Langmuir, 27(19), 12069-12073.*

(b) Sulfonated (SO_3^-) polymer brushes mimic GAG function by sequestering bFGF at the interface, enabling localized presentation of growth factors to adherent hMSCs and sustained FGFR activation. *Ref. Schultz, G. S., & Wysocki, A. (2009). Wound repair and regeneration, 17(2), 153-162.*

6.2 Results

6.2.1 Design and Characterization of GAG-Mimetic Sulfonated Polymer Brushes

Sulfonated polymer brushes were synthesized using ARGET SI-ATRP to produce homopolymer controls (H, M, S) and binary or ternary copolymers (S25H75, S50H50,

S75H25, S75M25, S33H33M34), with nomenclature reflecting monomer feed ratios (**Figure 33a**). Formulations rich in M and low in S content exhibited optical defects and were therefore excluded from further analysis. Reaction times were optimized to achieve consistent brush thicknesses (approximately 21–33 nm) across all compositions, thereby minimizing effects attributable to thickness variations (**Figure 33b**).

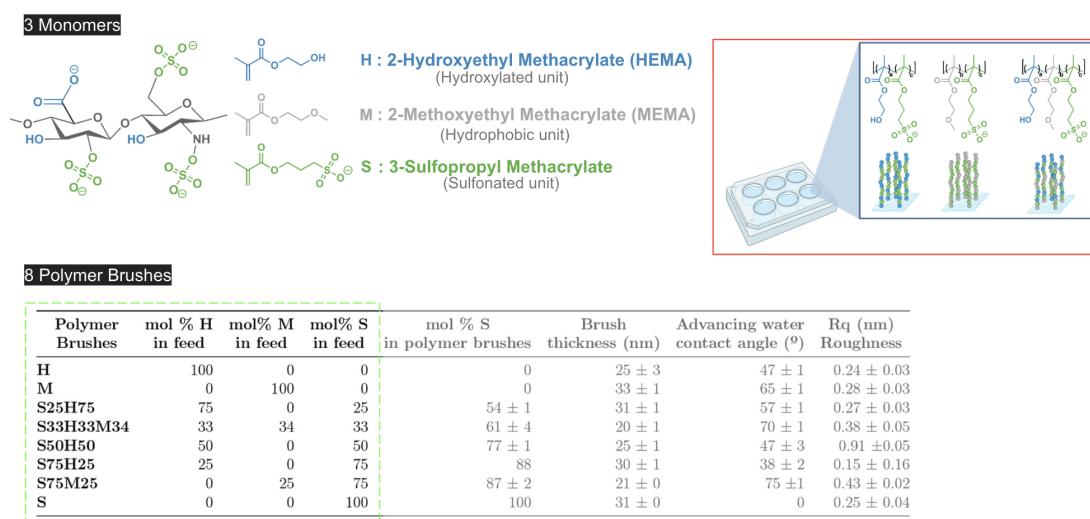


Figure 33. Polymer brushes encoding key structural features of glycosaminoglycans: multivalent brush architecture, sulfonation, and hydroxylation.

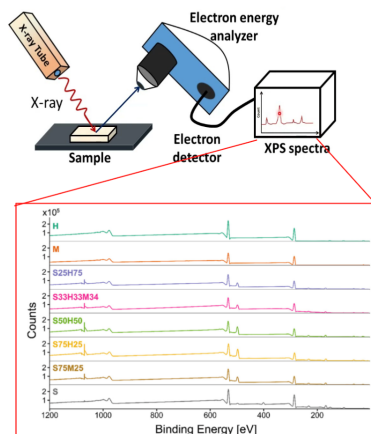
a) synthesized binary and ternary copolymer brushes comprising different ratios of sulfonated (3-sulfopropyl methacrylate or S), hydroxylated (2-hydroxy ethyl methacrylate or H), and hydrophobic (2-methoxy methyl methacrylate or M) repeat units. **b)** Table summarizing polymer brush characterization data.

6.2.2 Surface Chemistry Characterization: Sulfonation Degree and Sequence Bias

Sulfonation levels were quantified by X-ray photoelectron spectroscopy (XPS) using the S2p signal and S/O ratios, referenced to fully sulfonated S brushes (**Figure 34a**). Mayo–Lewis fitting revealed that S exhibited greater reactivity than H and M, which accounts for the observed sulfonation levels consistently exceeding the intended feed ratios (**Figure 34b**). Fourier-transform infrared spectroscopy (FTIR) provided qualitative confirmation, as increasing SO_3^- bands correlated with higher S content (**Figure 34c**).

a)

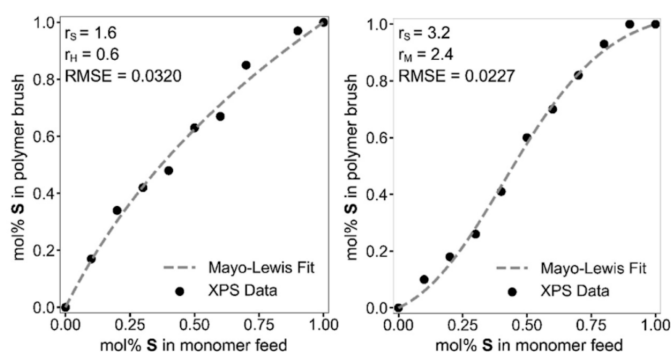
XPS (X-Ray Photoelectron Spectroscopy)



Polymer	S at %	O at %	C at %	Na at %
H	0.0 ± 0.0	25.6 ± 0.2	70.9 ± 0.2	0.0 ± 0.0
M	0.0 ± 0.0	29.0 ± 0.2	74.4 ± 0.2	0.0 ± 0.0
S25H75	3.1 ± 0.0	28.4 ± 0.3	61.8 ± 1.6	6.7 ± 1.8
S33H33M34	3.3 ± 0.3	27.2 ± 0.2	64.9 ± 0.7	4.6 ± 0.9
S50H50	4.4 ± 0.4	28.5 ± 1.2	58.7 ± 1.0	8.4 ± 1.3
S75H25	5.2 ± 0.2	29.7 ± 0.6	54.8 ± 1.3	10.3 ± 2.0
S75M25	5.1 ± 0.2	29.3 ± 0.4	58.0 ± 2.1	7.6 ± 1.6
S	5.1 ± 0.5	25.9 ± 0.5	68.1 ± 0.7	14.0 ± 1.0

$$\text{Degree of Sulfonation (DS, \%)} = \frac{(S/O)_{\text{copolymer}}}{(S/O)_{\text{homopolymer}}} \times 100$$

b)



c)

Fourier Transformed Infrared Spectroscopy (FTIR) Analysis

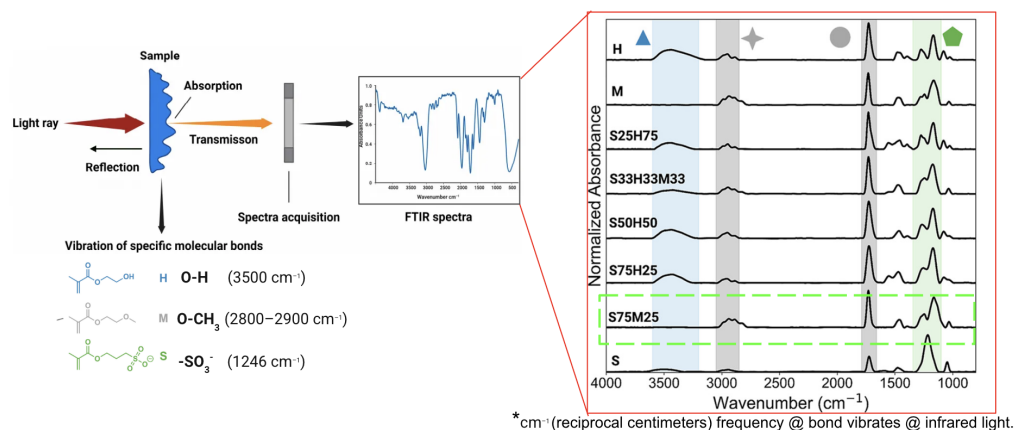


Figure 34. Quantification of the degree of brush sulfonation and reactivity ratio analysis.

a) XPS quantified the incorporation of sulfonated monomers in copolymer brushes. b) XPS aided reactivity ratio analysis. c) Fourier-transformed infrared spectra

6.2.3 Influence of Sulfonation on polymer brush wettability and polymer brush swelling response.

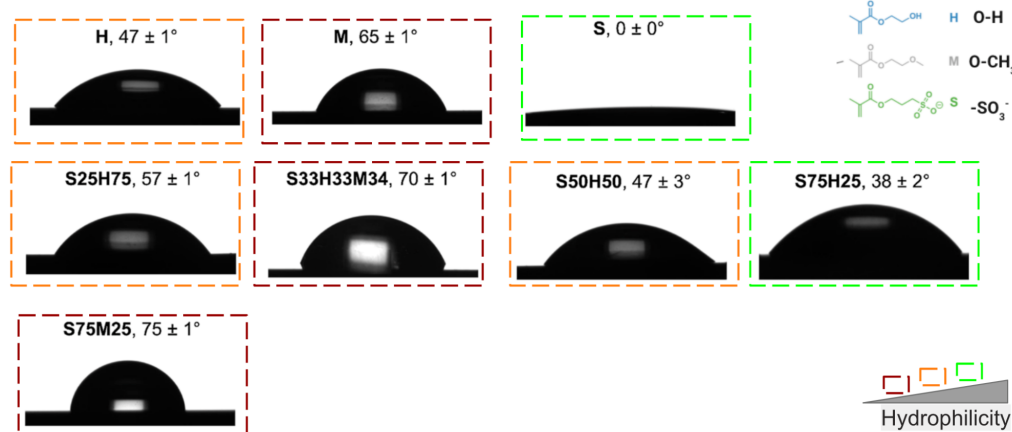
The influence of brush chemistry on interfacial water interactions relevant to hMSC culture was evaluated by measuring advancing water contact angle (wettability) and brush swelling in both water and phosphate-buffered saline (PBS) (**Figure 35**). As surface roughness was consistent across all coatings ($R_q < 1$ nm), observed differences in wettability were attributed to variations in chemical composition.

Wettability, S brushes exhibited complete wetting (0°), M brushes were hydrophobic (approximately 65°), and H brushes demonstrated intermediate wettability (approximately 47°) (**Figure 35a**). In S–H copolymers, increased sulfonation corresponded to lower contact angles. In contrast, copolymers containing M segments remained relatively hydrophobic even at higher sulfonation levels, consistent with surface rearrangement exposing M-rich domains.

Swelling, as measured by in situ ellipsometry, increased with sulfonation in water, which is consistent with charge-driven chain expansion. H brushes also exhibited greater swelling due to their inherent hydrophilicity (**Figure 35a**). Swelling was reduced in PBS because the presence of salt screens electrostatic repulsion, and H brushes swelled less due to decreased solubility. With the exception of the S homopolymer, most brushes exhibited similar swelling in PBS, indicating partially extended conformations under culture-relevant ionic strength with accessible interfacial groups.

a)

Brush wettability- Contact Angle Measurement



b)

Brush Swelling in water/PBS -in situ ellipsometry

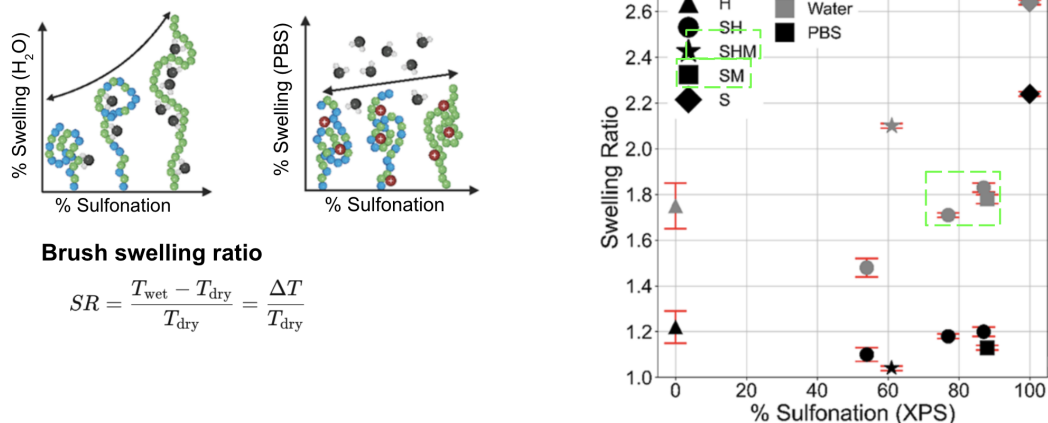


Figure 35. Sulfonation governs the wettability and swelling response of polymer brushes.

a) We compared **brush wettability** by measuring the advancing contact angle. b) **Brush swelling** responses in ultrapure water and phosphate-buffered saline (PBS) were investigated via in situ ellipsometry.

6.2.4 Comparison of bFGF Adsorption on GAG-Mimetic Polymer Brushes and Heparan Sulfate

The adsorption of bFGF to the brush coatings was quantified using ELISA to assess whether sulfonation or surface wettability predicts growth-factor sequestration (**Figure 36**). Coatings containing M were excluded from ELISA analysis because of insufficient optical transparency. Tissue-culture polystyrene (TCP) and heparan sulfate (HS) served as negative

and positive controls, respectively. All polymer brush coatings bound measurable amounts of bFGF, and several exhibited mean values comparable to or exceeding those of HS. However, most differences among the brushes compared with HS were not statistically significant. Overall, bFGF adsorption correlates with the degree of sulfonation. Notably, S75M25 and S75H25 sulfonated brushes have significantly higher bFGF levels as compared to non-sulfonated brushes.

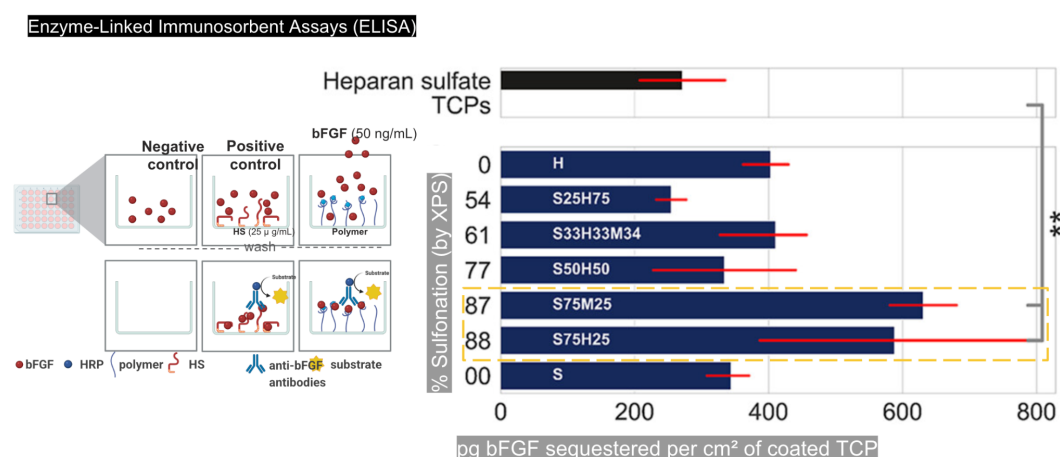


Figure 36. Comparing the sequestration of bFGF by polymer brushes.

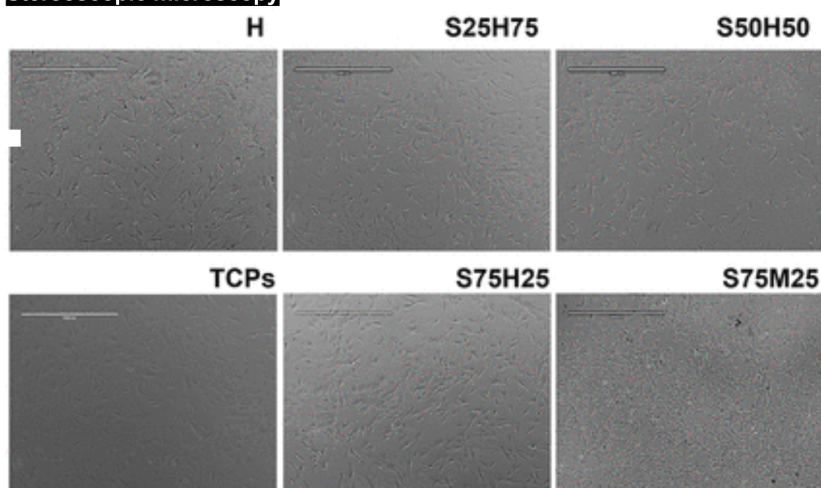
Quantification of bFGF adsorption via enzyme-linked immunosorbent assays (ELISAs). (** indicates $p < 0.01$, Tukey's honestly significant differences (HSD) post hoc test).

6.2.5 Sulfonated polymer brushes promote enhanced hMSC proliferation compared to TCP

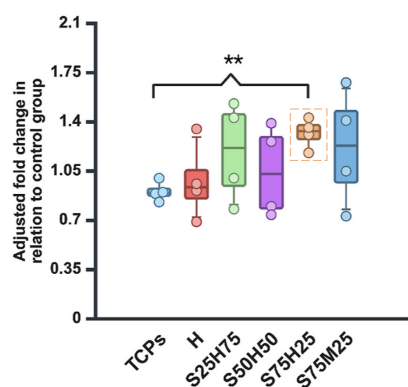
hESC-derived hMSCs (passages 3–4) were cultured in serum-free medium on polymer brush coatings and TCP to compare cell adhesion and proliferation (**Figure 37**). Coatings M-rich were excluded due to insufficient optical clarity. Additionally, S and S33H33M34 coatings were omitted because they did not support adequate cell growth and induced a stressed cell morphology. hMSCs exhibited comparable adhesion and retained fibroblastic morphology on the remaining polymer brushes (H, S25H75, S50H50, S75H25, S75M25) and on TCP (**Figure 37a**). Expression levels of CDK2 and KI67 transcripts were higher in S75H25 than in TCP, although these differences did not reach statistical significance (**Figure 37b**). After

seven days, all tested polymer brushes produced higher cell yields than TCP, with S75H25 showing the most consistent improvement across twelve independent trials, yielding approximately 40% more cells than TCP (*Figure 37c*). Consequently, S75H25 was selected for subsequent characterization.

Stereoscopic microscopy



Box-and-whisker plot



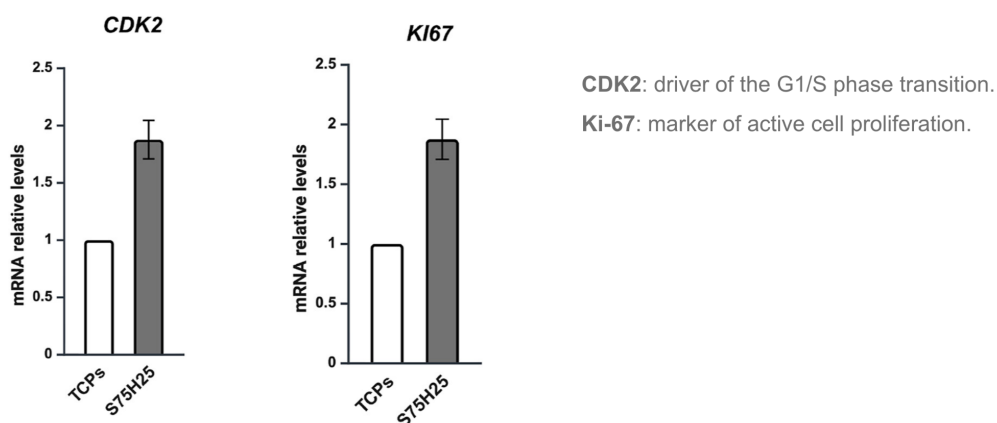


Figure 37. GAG-inspired polymer brushes promote hMSC proliferation.

a) Representative micrographs of hMSCs cultured in sulfonated polymer brushes, showing a typical spindle-like morphology characteristic of hMSCs. **b)** Relative mRNA expression of cell proliferation markers CDK2 and Ki67 between hMSCs cultured on TCPs and S75H25. **c)** Box-and-whisker plot comparing the hMSC proliferative capacity of different polymer brushes and TCPs controls. Scale bars are 100 μ M.

6.2.6 Sulfonated Brushes Promote Expression of bFGF Receptor and Integrin α 6

hMSCs expanded on S75H25 exhibited elevated expression of signaling and adhesion receptors that are associated with self-renewal. FGFR1 mRNA levels were quantified because bFGF–FGFR1 signaling is a primary driver of hMSC proliferation (**Figure 38a**). ITGA6 mRNA levels were also assessed, as integrin α 6 is an adhesion receptor associated with stem-like MSC subpopulations and cell–matrix interactions that facilitate growth (**Figure 38b**). Collectively, these findings suggest that sulfonated brush substrates promote a receptor expression profile that supports enhanced proliferative signaling and adhesion-mediated maintenance during hMSC expansion.

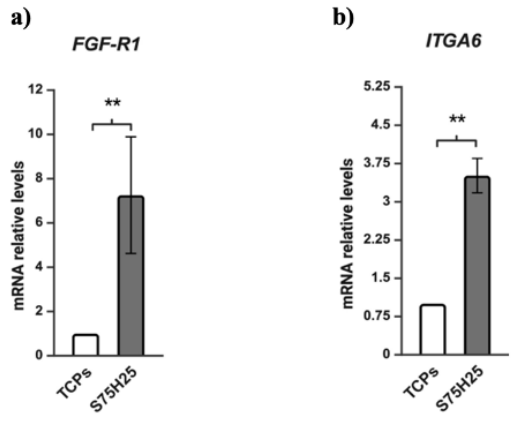


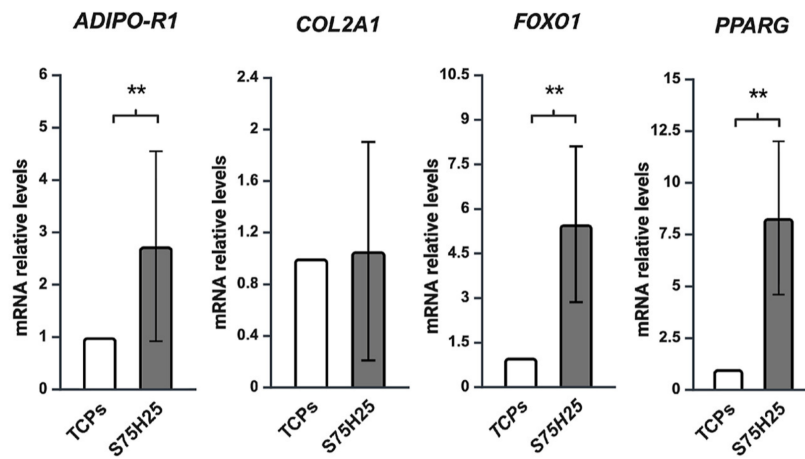
Figure 38. S75H25 promotes FGFR-1 and integrin signaling in hMSCs.

We observed a significant increment in the relative mRNA levels of **a) FGFR1** and **b) ITGA6** in hMSCs cultured on S75H25 substrates compared to TCPs. ** indicates p-values less than 0.01.

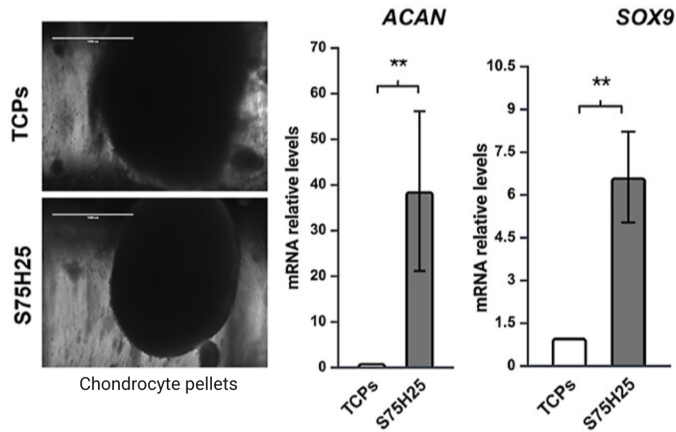
6.2.7 Retention of Trilineage Differentiation Capacity in hMSCs Cultured on S75H25

hMSCs expanded on S75H25 retained the capacity to differentiate into adipogenic, chondrogenic, and osteogenic lineages, comparable to cells expanded on TCP (**Figure 39**). Differentiation was induced using standard lineage-specific media. Following adipogenic induction, S75H25-expanded cells exhibited higher expression of the adipogenic genes FOXO1 and PPARG, whereas ADIPOR1 and COL2A1 expression levels were similar to those in TCP-expanded cells (**Figure 39a**). After chondrogenic induction, both groups formed distinct cartilage-like pellets; however, S75H25-expanded cells expressed higher levels of ACAN and SOX9 (**Figure 39b**). Upon osteogenic induction, both groups formed mineralized cultures and exhibited similar ALP and COL1A1 expression levels (**Figure 39c**). In summary, expansion on S75H25 did not diminish multipotency. Osteogenic differentiation remained unchanged, while adipogenic and chondrogenic outcomes were similar to or slightly higher than those observed with TCP.

a) Adipogenic lineages gene expression



b) Chondrogenic lineages gene expression



c) Osteogenic lineages gene expression

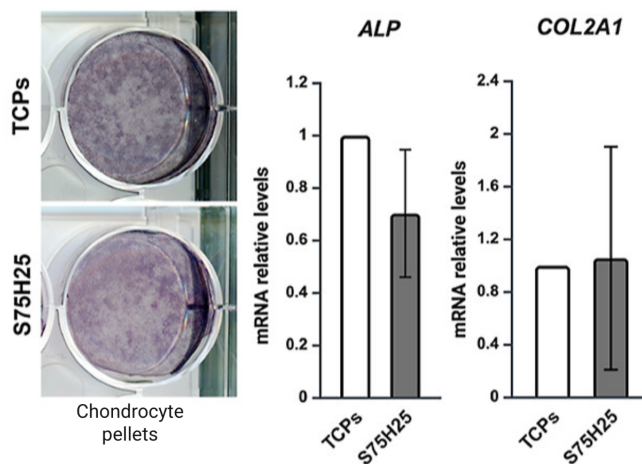


Figure 39. S75H25 substrates maintain the adipogenic, chondrogenic, and osteogenic differentiation capacity of hMSCs.

a) Relative mRNA expression of *ADIPOR-1*, *COL2A1*, *FOXO1*, and *PPARG* in hMSCs cultured on TCPs and S75H25 substrates and then induced to undergo adipogenic differentiation. **b)** Representative micrographs of chondrogenic pellets after differentiation of hMSCs cultured on TCPs and S75H25 substrates. Relative mRNA expression of *ACAN* and *SOX9* in chondrocyte pellets. **c)** Representative micrographs of mineralized osteocytes stained with alkaline phosphatase after osteogenic differentiation of hMSCs cultured on TCPs and S75H25 substrates. Relative mRNA expression of *ALP* and *COL1A1* in osteocytes. ** indicates *p*-values less than 0.01.

6.3 Discussion

Eight glycosaminoglycan-inspired polymer brush chemistries were systematically evaluated by varying sulfonation, hydroxylation, and hydrophobicity. This approach enabled the development of a structure–function map linking interfacial chemistry to hMSC expansion outcomes. Within this design space, the data reveal a narrow interfacial operating window where sulfonation is sufficient to recruit growth factors and surface hydration remains compatible with robust cell adhesion. These combined properties support rapid hMSC expansion while maintaining cellular potency. A central mechanistic focus was the interaction with growth factors. S75H25 and S75M25 adsorbed substantial amounts of bFGF, whereas tissue culture polystyrene exhibited negligible adsorption. These results support a model in which sulfonate-rich brushes provide heparin-like binding, concentrating and multivalently presenting bFGF at the cell–material interface to extend fibroblast growth factor receptor (FGFR) signaling. This interpretation aligns with the study's design logic: sulfonation enables electrostatic capture of heparin-binding proteins, while controlled hydrophilicity preserves cell attachment, which is necessary for productive signaling ([Ayala et al. 2011](#); [Qian et al. 2014](#); [Zehe et al. 2006](#)) In a fully defined, serum-free medium, several brush formulations improved hMSC expansion compared with tissue culture polystyrene. The lead compositions produced approximately 30–40% greater net expansion over the culture interval. Mechanistic profiling demonstrated that the lead brush (S75H25) increased expression of proliferation and

cell-cycle markers (CDK2, KI67) as well as the stemness and adhesion-associated marker ITGA6. Importantly, adipogenic, chondrogenic, and osteogenic differentiation potential was preserved after expansion. The optimal design window excluded super hydrophilic brushes, which impaired cell adhesion upon contact. These findings indicate that excessive hydration can negate the benefits of growth-factor capture by limiting productive cell–material engagement. These findings address a practical barrier to clinical translation. Therapeutic protocols often require large doses of hMSCs, yet proliferative capacity and therapeutic consistency typically decline with passaging and across manufacturing processes (Okolicsanyi et al. 2015). The present work supports a materials-based strategy for scalable biomanufacturing that aligns with the medicinal signaling cell concept and prioritizes potency preservation over differentiation bias (Caplan 2017). More broadly, combining chemically defined media with chemically defined GAG-mimetic brush interfaces offers a route to reduce batch-to-batch variability associated with ill-defined supplements and may enable more reproducible control of functional interactions relevant to therapeutic mechanisms (Olsen et al. 2018; Andrzejewska et al. 2019; Aijaz et al. 2018b).

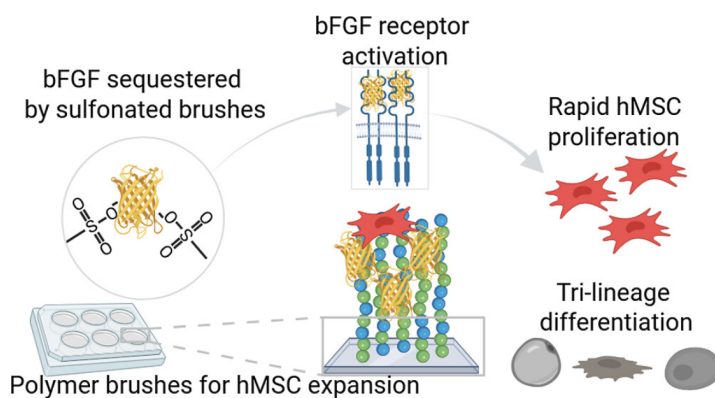


Figure 40. Recombinant stem cell substrate model

Sulfonated polymer brushes sequester and present bFGF to sustain FGFR signaling, boosting hMSC expansion while preserving trilineage differentiation potential.

Chapter 7. Integrated Discussion and Unified Working Model

7.1 Overview

This dissertation presents a microenvironmental control framework in which stem cell fate is determined by the integration of (i) extracellular context, including ECM availability and remodeling, (ii) $\beta 1$ -integrin activation state, (iii) FAK routing and nuclear NT-FAK signaling, and (iv) nuclear fate-control circuitry centered on the FAK–MDM2–p53 axis. In hPSC studies, undifferentiated cells maintain adhesion within a restrained “baseline” signaling configuration. Forced $\beta 1$ -integrin activation disrupts this baseline, engaging nuclear stress–fate programs that destabilize OCT4 and promote differentiation. Two additional factors influence interpretability and translational relevance: feeder-derived mitochondrial transfer, which introduces a bioenergetic confounder that can bias fate-associated readouts, and defined biomaterials, which serve as engineering tools to standardize ligand and growth-factor presentation for reproducible, scalable culture. These findings integrate mechanistic control (integrin/FAK/nuclear signaling) with process control (confounder mitigation and biomaterial design) to enable interpretable stem cell biology under defined conditions.

7.2 Integrated summary of key findings

7.2.1 $\beta 1$ -integrin activation switches hPSCs from a pluripotency-permissive baseline to a fate-destabilizing nuclear FAK state

The principal conclusion from Chapter 4 is that integrins $\alpha 6$ and $\beta 1$ do not function solely as phenotypic markers of undifferentiated hPSCs; rather, they define an adhesion system whose *activation state* governs downstream fate control. Under self-renewal conditions, hPSCs maintain robust adhesion while exhibiting restrained canonical focal-adhesion signaling, consistent with a pluripotency-permissive “low-FAK baseline”. In this baseline, OCT4, SOX2, and NANOG remain stable, and FAK is present but does not operate primarily through classical focal-adhesion output as the dominant fate driver. Increased $\beta 1$ -integrin

activation induces a transition to a distinct signaling configuration. Two activation strategies were employed: Mn^{2+} , which broadly enhances integrin affinity by stabilizing β -subunit conformations, and an ITGB1-activating antibody, which selectively activates and clusters $\beta 1$. Although these perturbations differ in specificity, both elevate integrin outside-in signaling and promote a “FAK state switch” characterized by FAK relocalization and engagement of nuclear fate-control circuitry. While pFAK^{Y397} serves as a supportive activation readout, the primary mechanistic feature is the routing of FAK toward a nuclear NT-FAK–linked program that destabilizes OCT4-centered pluripotency stability and increases lineage priming.

7.2.2 Nuclear NT-FAK operates as a gene-regulatory scaffold coupled to MDM2–p53, linking adhesion state to OCT4 stability

A second major conclusion from Chapter 4 is that hPSCs contain a functionally relevant nuclear FAK module. Confocal localization, biochemical fractionation, and co-immunoprecipitation support a model in which NT-FAK is present in the nucleus of undifferentiated hPSCs and associates with pluripotency transcriptional regulators, positioning FAK as a compartment-switching integrator of adhesion state and gene-regulatory control. Upon $\beta 1$ -integrin activation, this module is reconfigured: FAK routing and NT-FAK nuclear signaling are coupled to the MDM2–p53 axis, resulting in increased p53 pathway activity (including p21 induction) and a concomitant reduction in OCT4 signaling and loss of pluripotency stability. These findings support a mechanistic sequence in which $\beta 1$ -integrin activation not only intensifies adhesion signaling but also reprograms nuclear proteostasis and transcriptional control through a coordinated FAK–MDM2–p53 module. This mechanism enables extracellular activation cues to be directly translated into fate-relevant changes in pluripotency circuitry.

7.2.3 $\beta 1$ -integrin activation drives context- and time-dependent transcriptional remodeling consistent with lineage priming

A third integrated conclusion is that early fate destabilization following integrin activation does not represent a binary transition between pluripotency and differentiation. Instead, integrin activation induces graded, time-dependent transcriptional changes in pluripotency factors, lineage-associated transcripts, and stress network outputs. Targeted RT-qPCR kinetics and pathway-level profiling reveal that Mn^{2+} treatment and ITGB1 activation generate distinct temporal programs: Mn^{2+} induces an earlier, more acute activation, whereas ITGB1 activation results in more sustained remodeling over extended exposure. Under these conditions, the transcriptional signature indicates reduced stability of the pluripotency network and increased lineage priming, consistent with imaging-based readouts, such as reduced OCT4 and increased p53/p21. These data support a model in which $\beta 1$ -integrin activation imposes a context-dependent fate bias, mediated by nuclear FAK routing and engagement of the p53 network, rather than causing an immediate loss of all pluripotency markers.

7.2.4 Feeder-derived mitochondrial transfer is a high-frequency, EV-enriched confounder that perturbs bioenergetics and biases fate-associated interpretation

Chapter 5 identifies a culture variable with direct implications for mechanistic inference: mitotically inactivated feeder cells can transfer mitochondrial material to hPSCs at high frequency, and this transfer occurs both by direct contact and via feeder-derived conditioned medium. Orthogonal validation by species-specific PCR demonstrates mouse COX2 mtDNA detection in exposed hPSCs, with persistence across multiple passages, indicating that xenogeneic mitochondrial cargo can remain detectable for weeks after exposure.

Mechanistically, the data indicate that transfer is predominantly extracellular-vesicle enriched, while tunneling nanotube-like structures may form but are not required for transfer under the tested conditions. Acquisition of xenogeneic mitochondrial material is associated with disrupted mitochondrial membrane potential and altered ATP production profiles, indicating that feeder-derived mitochondrial exchange constitutes a bioenergetic perturbation

rather than a neutral labeling artifact. Given the close relationship between metabolism, chromatin state, and fate responsiveness in hPSCs, this transfer acts as a confounder node. It can alter stress signaling and lineage responsiveness independently of intended experimental perturbations, thereby biasing interpretation of fate-associated endpoints following feeder exposure.

7.2.5 Defined bioengineered substrates enable translational microenvironment control for scalable expansion without potency loss

Chapter 6 extends the dissertation's microenvironmental control logic to a translational manufacturing problem: expansion of hMSCs at scale while preserving multipotency and function. Clinically aligned workflows require large doses and consistent product quality, yet expansion can reduce potency and increase variability. The key finding is that GAG-mimetic sulfonated polymer brushes create a chemically defined, growth factor–interactive interface that enhances hMSC proliferation while maintaining the capacity for tri-lineage differentiation. Mechanistically, sulfonated brushes are designed to sequester and stabilize bFGF, thereby promoting its multivalent presentation to cells and supporting FGFR signaling. Because extreme hydrophilicity can impair adhesion, hydrophilicity is treated as a tunable design parameter. Within the material screen, a specific design window that combines sulfonation with optimal wettability supports approximately 30–40% greater expansion relative to tissue culture polystyrene (TCP), while preserving adipogenic, chondrogenic, and osteogenic differentiation and elevating proliferation-associated markers and ITGA6. In the integrated framework, these results establish defined biomaterials as programmable microenvironments that reduce batch variability and enhance scalability while maintaining potency. This engineering solution complements the mechanistic control principles described in the hPSC chapters.

7.3 Unified working model

The findings from this dissertation support a unified model that can be expressed as:

Microenvironment input → β 1-integrin activation state → FAK routing (nuclear NT-FAK) → FAK–MDM2–p53 regulatory output → OCT4 stability and lineage priming, with an additional bioenergetic confounder node introduced by feeder-derived mitochondrial transfer.

Node 1. Microenvironment boundary conditions (substrate, ECM, and remodeling)

Initial conditions are defined by ligand identity/density and the cell's capacity to remodel its pericellular matrix. Defined substrates constrain variability by controlling the initial presentation of the starting ligand and by influencing endogenous ECM reinforcement over time.

Node 2. β 1-integrin activation state (not expression alone)

Undifferentiated hPSCs can maintain adhesion with restrained downstream signaling. Forced β 1-integrin activation (global or β 1-selective) pushes the system past an activation threshold, triggering integrin clustering and outside-in signaling.

Node 3. FAK routing and nuclear NT-FAK signaling

The core fate-control feature is FAK relocalization and routing toward a nuclear NT-FAK–linked program. pFAK^{Y397} functions as a readout of activation, but the key mechanistic transition is the redistribution of FAK between adhesion-proximal and nuclear-associated pools.

Node 4. FAK–MDM2–p53 output and OCT4 stability

Upon β 1-integrin activation, the nuclear module engages MDM2–p53 signaling and increases p53 functional output (including p21), correlating with reduced OCT4/pluripotency stability and increased differentiation priming.

Node 5. Phenotype-level endpoints

Observable outcomes include OCT4 destabilization, increased heterogeneity, lineage priming, and altered stress responsiveness.

Node 6. Confounder node: feeder-derived mitochondrial transfer

Feeder EV-enriched mitochondrial cargo perturbs $\Delta\Psi_m$ and ATP allocation, biasing fate-associated readouts by shifting bioenergetics and stress signaling independently of the intended perturbation.

Node 7. Engineering node: defined biomaterials to enforce reproducibility and scalability

GAG-mimetic brushes and other defined surfaces reduce variability by standardizing growth-factor presentation and cell–material interactions, enabling scalable expansion while maintaining potency and functional competence.

7.4 Impact on reproducibility and stem cell engineering

Collectively, these findings support both a practical and conceptual shift in stem cell workflows. First, $\beta 1$ -integrin activation state and FAK routing should be regarded as controllable determinants of fate stability, rather than as downstream correlates of adhesion. Second, feeder-based systems must be interpreted as active co-culture environments, since EV-enriched mitochondrial transfer can perturb bioenergetics and bias fate-associated endpoints even after brief exposure. Third, defined biomaterials offer an engineering approach to standardize microenvironmental inputs, reducing biological variability and enabling scalable, potency-preserving expansion in both pluripotent and mesenchymal systems. Thus, microenvironmental definition emerges as both a translational requirement and a mechanistic necessity for reproducible inference and robust manufacturing design.

7.5 Study Limitations

Several limitations should be considered when interpreting these findings. While Mn^{2+} and ITG β 1-activating antibodies are effective experimental tools for inducing integrin activation, these methods do not fully recapitulate the spatial, temporal, and ligand-dependent features of physiological extracellular matrix-mediated signaling. As a result, the observed responses primarily reflect experimentally induced activation states rather than a comprehensive model of native integrin regulation. Evidence for mitochondrial transfer from feeder cells to hPSCs was based on fluorescent tracing and species-specific mtDNA detection; however, these approaches do not confirm long-term functionality, persistence, or stable integration of transferred mitochondria. Similarly, extracellular vesicle (EV) involvement was inferred mainly from particle size and isolation profile, which is insufficient for definitive subtype identification without marker-based validation. The use of pharmacological inhibitors may also affect multiple aspects of vesicle biology, complicating the interpretation of the precise mechanism of transfer. In polymer brush studies, donor-to-donor variability remains a significant limitation to translational relevance. Although proliferation and trilineage differentiation were assessed, additional long-term functional parameters, such as immune-regulatory potency and broader therapeutic fitness, were not comprehensively evaluated. These limitations underscore the need for further research to enhance mechanistic resolution and translational applicability across all three aims.

7.6 Future Research Directions

Future research should directly assess whether nuclear NT-FAK is essential for fate control by perturbing FAK nuclear routing via NLS/NES mutations or targeted degradation, followed by measuring OCT4 stability and p53/p21 outputs. NT-FAK-dependent gene programs should be defined using CUT&RUN or CUT&Tag (targeting p53, OCT4, and candidate NT-FAK partners) combined with time-resolved transcriptomics following β 1-integrin activation.

Surface integrin activation states should be quantified using conformation-specific $\beta 1$ antibodies or ligand-mimetic probes across defined substrates to establish links between extracellular matrix identity, activation thresholds, and FAK-state switching. In mitochondrial transfer studies, future work should determine the composition and function of EV mitochondrial cargo by measuring mtDNA copy number across EV fractions, performing EV proteomics, and employing high-resolution imaging. Routine quality control, including mouse COX2 screening across passages and standardized EV depletion and characterization, should be implemented to minimize confounding in metabolic and differentiation assays. GAG-mimetic polymer brushes should be adapted for scalable formats, such as microcarriers, suspensions, or closed systems. Potency metrics should be expanded beyond trilineage differentiation to include immunomodulatory function, senescence, and therapeutic secretome profiling.

7.7 Concluding synthesis

In summary, the results described in this dissertation demonstrate that precise control of the microenvironment is fundamental to directing the fate and function of human stem cells. Engagement of integrin $\beta 1$, along with the spatial distribution and activation of FAK, establishes a regulatory axis linked to the nucleus that can either maintain or disrupt pluripotency via NT-FAK–MDM2–p53 signaling. Mitochondrial transfer from feeder cells is a significant confounder that can alter cellular energetics and influence the interpretation of fate. The implementation of defined bioengineered substrates, such as GAG-mimetic polymer brushes, offers a practical strategy to enhance reproducibility and scalability by standardizing the cellular microenvironment.

Chapter 8. Conclusions and Future Perspective

8.1 Conclusions

This dissertation establishes that stem cell fate decisions are regulated by the cellular microenvironment, which operates through three interconnected levels: extracellular matrix (ECM)–cell interactions, cell–cell interactions, and ECM–soluble factor–cell interactions.

Study 1 demonstrated that interactions between the extracellular matrix and cells are central to regulating hPSC fate. Integrin $\alpha 6\beta 1$ -mediated adhesion was found to control FAK signaling state and subcellular localization, thereby linking matrix engagement to nuclear gene-regulatory outcomes. The findings support a model in which self-renewing hPSCs maintain a low basal adhesion-signaling state associated with nuclear FAK. In contrast, integrin $\beta 1$ activation disrupts this equilibrium, activates the FAK–MDM2–p53 axis, increases p53/p21 signaling, reduces pluripotency-associated gene expression, and promotes early differentiation responses.

Study 2 revealed that cell–cell interactions within feeder-based culture systems are biologically active and can influence stem cell behavior. Feeder cells were observed to transfer mitochondrial material to hPSCs via TNTs and extracellular vesicles, leading to altered mitochondrial membrane potential and ATP-linked bioenergetics. These results indicate that feeder exposure can bias metabolic and fate-associated measurements and should be treated as an experimental variable requiring explicit control, rather than as a neutral support condition.

Study 3 demonstrated that ECM–soluble factor–cell interactions can be engineered to enhance stem cell expansion. GAG-mimetic sulfonated polymer brush substrates increased hMSC proliferation while maintaining trilineage differentiation capacity. These findings indicate that biomaterials can regulate cell behavior by controlling the extracellular growth factor

environment. This establishes a translational principle: matrix chemistry can be leveraged to support scalable expansion without compromising functional potency.

Collectively, these studies demonstrate that stem cell fate is regulated by multiple aspects of the microenvironment, including matrix engagement, intercellular exchange, and growth factor–matrix presentation. This dissertation provides both a mechanistic framework for understanding stem cell regulation and an engineering framework for developing more reproducible, defined, and scalable stem cell culture systems.

8.2 Future Perspective

The findings presented here offer a broader perspective on stem cell culture, in which microenvironmental cues function not only as explanatory variables but also as programmable tools for regulating cell state. Across the three project areas, a consistent theme is evident: extracellular adhesion, metabolic exchange, and biomaterial chemistry can be modulated to preserve stemness, induce lineage priming, or enhance translational culture performance.

A significant future direction involves the controlled application of integrin $\beta 1$ activation as a strategy for early fate specification.

In this research, both Mn^{2+} -mediated global integrin activation and ITG $\beta 1$ -selective activation did not merely disrupt pluripotency; instead, they elicited distinct early differentiation-associated responses. These findings suggest that precisely timed $\beta 1$ activation could be integrated into directed differentiation protocols to enhance the efficiency or synchrony of lineage entry. Given that Mn^{2+} and ITG $\beta 1$ activation produce distinct signaling and transcriptional outcomes, subsequent studies should investigate whether this activation modes can bias cells toward specific developmental trajectories or yield more uniform progenitor states for disease modeling, organoid initiation, and regenerative applications.

The mitochondrial transfer results indicate two primary future directions.

Within feeder-based hPSC culture, xenogeneic mitochondrial exchange should be recognized as a potential source of biological confounding and monitored accordingly. Conversely, controlled mitochondrial transfer may possess therapeutic potential. In other biological systems, mitochondrial donation has been associated with metabolic rescue and enhanced cellular recovery following stress or injury. Future research should therefore differentiate between unintended feeder-derived contamination and intentional mitochondrial delivery and assess whether mitochondrial cargo or extracellular vesicle (EV)-associated mitochondrial components can be utilized to improve bioenergetic function in models of mitochondrial dysfunction or cellular injury.

The sulfonated polymer brush platform also demonstrates utility beyond feeder-free expansion.

These materials likely regulate the local presentation of growth factors, particularly within the fibroblast growth factor (FGF)–FGF receptor (FGFR) axis. They may serve as reductionist systems for investigating how extracellular chemistry influences the strength, duration, and receptor activation of growth factor signaling. Expanding this platform to scalable culture formats, while assessing not only growth and differentiation but also immune potency, senescence, and secretory function, will enhance its translational relevance.

Collectively, these studies support a future in which stem cell microenvironments are engineered with increased precision: integrin activation serves as a mechanism for fate control, mitochondrial exchange is addressed as both a quality-control consideration and a potential therapeutic strategy, and polymer-based substrates are employed as defined tools for growth factor biology and scalable cell manufacturing.

8.3 Closing statement

This dissertation demonstrates that the microenvironment governs stem cell fate through three principal regulatory axes: ECM–cell interactions, cell–cell interactions, and ECM–soluble factor–cell interactions. By elucidating the role of integrin–FAK signaling in regulating hPSC fate, identifying the introduction of mitochondrial confounders by feeder cells, and showing that sulfonated biomaterials enhance hMSC expansion, this work establishes microenvironmental control as both a fundamental biological principle and a translational strategy for stem cell research, manufacturing, and therapeutic applications.

8.4 Summary (Graphical Abstract)

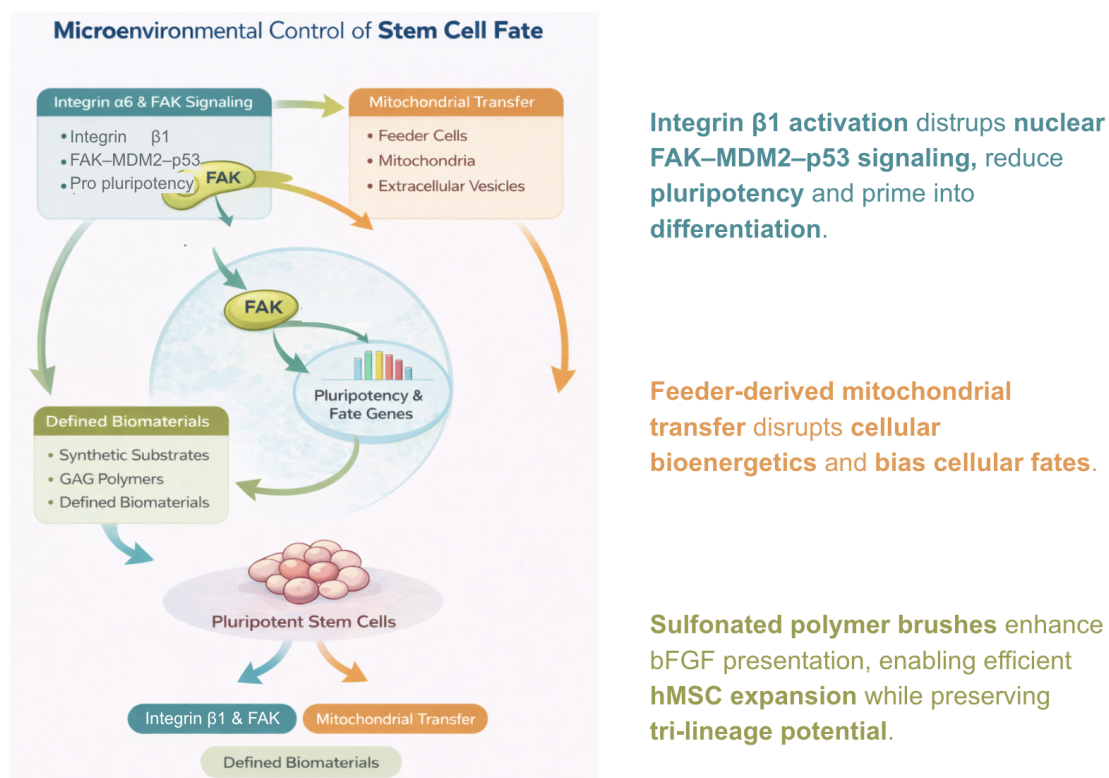


Figure 41: Graphical Abstract of Ph.D. Dissertation

Controlling the stem cell microenvironment—both biological and engineered is essential for reproducible stem cell biology and reliable translational applications.

Chapter 9. Materials and Methods

9.1 Overview of Experimental Strategy

The microenvironmental regulation of human stem cell fate was investigated across three primary axes: (i) integrin $\beta 1$ activation and FAK nuclear signaling in hPSCs; (ii) feeder-derived mitochondrial transfer as a culture confounder influencing hPSC bioenergetics; and (iii) defined bioengineered materials, including synthetic substrates for hPSC stabilization and GAG-mimetic sulfonated polymer brushes for the potency-preserving expansion of hMSCs. All assays incorporated matched controls, standardized acquisition parameters, and consistent normalization to facilitate cross-condition comparisons.

9.2 Cell Lines, Culture, and Quality Control

9.2.1 Cell Lines

Human foreskin fibroblasts (hFF) were obtained from ATCC (Manassas, VA, USA). hiPSC GFP reporter lines (SOX2-GFP and TOM20-GFP) were obtained from the Coriell Institute (Camden, NJ, USA). Irradiated C57BL/6 mouse embryonic fibroblasts (iMEFs) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). hESC lines H1 (WA01) and H9 (WA09) were obtained from WiCell Research Institute (Madison, WI, USA). All cell stocks were confirmed mycoplasma-free before use.

9.2.2 Matrigel Coating for Feeder-Free hPSC Culture

hESC-qualified Matrigel (Corning, Glendale, CA, USA) was thawed on ice and diluted to 0.1 mg/mL in cold DMEM/F-12 (Gibco, Thermo Fisher Scientific). All handling was performed on ice. Matrigel solution was applied at ~ 0.1 mL/cm² to tissue culture-treated plates and incubated at room temperature for 2 h. Excess solution was removed and the surface rinsed once with warm sterile PBS (1 $\times$, without Ca²⁺/Mg²⁺; Cytiva, Marlborough, MA, USA). Cells were seeded immediately in pre-warmed medium.

9.2.3 hPSC Maintenance and Passaging

hESCs and hiPSCs were cultured on Matrigel-coated plates at 37 °C and 5% CO₂ in StemFlex medium (Thermo Fisher Scientific) supplemented with 1% penicillin–streptomycin (Gibco). Colony morphology was evaluated every 48 hours using a stereomicroscope (Leica MC170 HD camera on Leica stand; Leica Microsystems Inc., Buffalo Grove, IL, USA). Differentiated foci were removed with a sterile pulled-glass pipette. Cells were passaged weekly using L7 hPSC passaging solution (Lonza, Walkersville, MD, USA), dissociated into single cells, and counted with a Countess II FL (Invitrogen, Thermo Fisher Scientific). For routine expansion, 200,000 cells (2.0×10^4 cells/cm²) were seeded on Matrigel-coated dishes and cultured for 7 days. Medium was exchanged every other day. Brightfield images were captured using an EVOS FL Cell Imaging System (Thermo Fisher Scientific).

9.3 Reprogramming of Human Somatic Cells

9.3.1 Sendai Virus–Mediated Reprogramming

hFFs (1×10^5 cells/dish) were plated on 0.01% gelatin-coated dishes in MEM supplemented with 10% FBS (GeminiBio, West Sacramento, CA, USA), 1% NEAA (Gibco), 1% GlutaMAX (Gibco), and 1% penicillin–streptomycin. Upon reaching approximately 50% confluence, cells were transduced with CytoTune-iPS 2.0 Sendai vectors encoding KLF4, SOX2, OCT3/4, and c-MYC (Thermo Fisher Scientific) in Opti-MEM (serum-free, glucose-containing, antibiotic-free) following the manufacturer's protocol. After 8 hours, the medium was replaced with supplemented MEM. At 72 hours post-transduction, cells were subcultured into six dishes (three on iMEFs and three on Matrigel). After 24 hours, cultures were switched to StemFlex medium supplemented with 10 μM Y-27632 (STEMCELL Technologies, Vancouver, Canada). Medium containing Y-27632 was refreshed daily until weeks 3–4, when hiPSC colonies appeared. Colonies were identified by alkaline phosphatase activity and morphology, then subcloned to establish isogenic hiPSC lines derived with or without iMEFs.

9.4 Mitochondrial Transfer Assays (Direct and Indirect)

8.4.1 Direct Mitochondrial Transfer by Co-Culture

iMEFs (2.0×10^5 cells) were seeded onto 35 mm imaging dishes (Falcon, Corning) coated with 0.01% gelatin and maintained in MEF medium [MEM (Gibco) supplemented with 1% GlutaMAX, 1% NEAA, 1% penicillin–streptomycin, and 20% FBS (Benchmark, Sayreville, NJ, USA)]. After 24 hours, iMEFs were stained with MitoTracker Red CMXRos (Invitrogen) at 200 nm. for 45 minutes at 37 °C, then washed 8 times with PBS. hPSCs (1.0×10^5 cells) were then added and co-cultured for 24 hours in StemFlex, with 10 μ M Y-27632 included during initial attachment. Cultures were washed twice with PBS and imaged using an EVOS FL system with both brightfield and fluorescence channels (MitoTracker excitation/emission 581/644 nm; GFP excitation/emission ~470/510 nm when applicable). Mitochondrial transfer was quantified from confocal images in ImageJ/Fiji using fixed ROIs and consistent thresholding.

9.4.2 Indirect Transfer Using iMEF-Conditioned Medium (iMEF-CM)

iMEFs (2.0×10^5 cells/35 mm dish) were plated on 0.01% gelatin, stained with MitoTracker Red CMXRos (200 nm., 45 min, 37 °C), washed eight times with PBS, and incubated in StemFlex for 24 h to generate iMEF-CM. CM was collected, centrifuged at $300 \times g$ for 5 min, filtered through a 0.22 μ m filter, and applied to hiPSCs grown on Matrigel for 24 h at 37 °C and 5% CO₂. Cultures were washed twice with PBS and imaged using identical acquisition settings. Transfer was quantified in ImageJ/Fiji using fixed ROIs and constant thresholding.

9.5 Nucleic Acid Workflows and PCR-Based Detection

9.5.1 DNA and RNA Extraction, Quantification, and cDNA Synthesis

Cells were washed with PBS. hPSCs were dissociated using L7 hPSC passaging solution, and iMEFs were collected with 0.25% trypsin (Gibco). Cells were counted, pelleted ($300 \times g$, 5

minutes, 4 °C), and processed for nucleic acid extraction. RNA was lysed in TRIzol (Invitrogen) and purified using Direct-zol RNA Miniprep Plus (Zymo Research, Irvine, CA, USA) with on-column DNase I treatment. RNA quantification was performed using Qubit 1X RNA BR. For reverse transcription, 1,000 ng RNA was converted to cDNA using SuperScript II VILO and diluted to 10 ng/μL. DNA and cDNA were stored at -20 °C, and RNA at -80 °C. Quality control included A260/280 assessment (1.8–2.1), no-RT controls, and matched input amounts across samples.

9.5.2 Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was performed in triplicate using TaqMan Gene Expression Assays on a Bio-Rad CFX Opus 96 system. Relative gene expression was calculated using the $\Delta\Delta C_t$ method. Statistical analysis of qRT-PCR data was conducted in Microsoft Excel (Data Analysis ToolPak) using an unpaired two-tailed Student's t-test, with $p < 0.05$ considered statistically significant. In addition to statistical significance, a fold-change threshold of ≥ 2 (upregulation) or ≤ 0.5 (downregulation) relative to control was used to define biologically meaningful changes.

9.5.3 PCR Amplification and Agarose Gel Electrophoresis

Genomic DNA was extracted using Quick-DNA Miniprep Plus (Zymo Research), quantified by Qubit 1X dsDNA BR, and normalized to 50 ng/μL. PCR reactions (25 μL) contained 100 ng DNA, DreamTaq PCR Master Mix (2×), and gene-specific primers (IDT). Amplicons were resolved on 2% agarose gels (EZ Pack tablets in 1× TAE) pre-stained with SmartGlow pre-stain. Electrophoresis was performed at ~100 V for 45–60 min using a Mini-Sub Cell GT system with a 1000 bp ladder. Gels were imaged on a ChemiDoc Touch (Bio-Rad) and analyzed using identical exposure settings.

9.6 Microscopy and Imaging Pipelines

9.6.1 Confocal Microscopy

Cells were imaged using a Nikon Eclipse Ti2 inverted microscope with a C2 Plus confocal system. Excitation was provided at 405, 488, 561, and 640 nm, with emission collected across 400–720 nm. Acquisition parameters (laser power, gain, pinhole, step size) were kept constant across conditions. Additional fluorescence imaging was performed on an EVOS FL system. Image processing and quantification were performed in ImageJ/Fiji using fixed ROIs and identical thresholds.

9.7 Flow Cytometry (FACS) for Mitochondrial Transfer

9.7.1 Acquisition and Analysis

hPSCs were washed, dissociated with L7 hPSC passaging solution, and suspended in PBS. FSC/SSC gated cells, and $\geq 10,000$ events were acquired per sample on a BD FACS Canto II (488 nm and 405 nm lasers). APC fluorescence histograms and MFI were computed in FlowJo (v10) using consistent gates across conditions.

9.8 Mitochondrial Function and Bioenergetic Profiling

9.8.1 JC-1 Mitochondrial Membrane Potential Assay

hPSCs were analyzed under untreated control, CCCP-depolarized positive control (50 μ M, 5 min), and iMEF-CM exposure (24 h). Cells were stained with JC-1 (2 μ M) for 20–30 min at 37 °C, washed with PBS, and analyzed by microscopy or flow cytometry using 488 nm excitation. JC-1 monomers were detected in the FITC channel (530/30 nm) and aggregates in the PE channel (585/42 nm). Membrane potential ($\Delta\psi$ m) was reported as the red/green fluorescence ratio with identical gating.

9.8.2 Seahorse XF Real-Time ATP Rate Assay

Bioenergetic profiling was conducted using the Seahorse XF Real-Time ATP Rate Assay (Agilent XF96 analyzer). Cells were seeded in poly-D-lysine-coated XF plates ($2-3 \times 10^4$ cells/well) 24 hours before testing. On the day of the assay, cells were washed and incubated for approximately 60 minutes in XF base medium (pH 7.4; 37 °C; non-CO₂) supplemented with 10 mM glucose, 1 mM pyruvate, and 2 mM glutamine. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured at baseline and after sequential injections of oligomycin (1.5 μM) and rotenone/antimycin A (0.5 μM each). Mitochondrial ATP production was calculated from oligomycin-sensitive OCR, and glycolytic ATP production from glycolytic proton efflux. Rates were normalized to post-assay nuclei counts. Technical triplicates and at least three biological replicates were included, with exclusion of failed wells based on quality control criteria.

9.9 Extracellular Vesicle-Enriched Fraction Preparation and Nanoparticle Tracking

9.9.1 Micro vesicle-Enriched EV Isolation and NTA

Conditioned medium was processed by sequential centrifugation: $300 \times g$ for 10 min (remove cells), $2,000 \times g$ for 30 min (remove debris/apoptotic bodies), and $10,000 \times g$ for 30 min (pellet micro vesicle-enriched EVs), all at 4 °C. Pellets were resuspended in 50 μL PBS and diluted 1:40 for sizing. NTA was performed on a Zeta View system (Particle Metrix) with a 488 nm laser. The flow cell was flushed with PBS, then with water. Instrument performance was verified using polystyrene beads (1:250,000 dilution) to set focus/threshold. Samples were loaded without bubbles, allowed to settle for 1–3 min, and measured with consistent positions, cycles, and analysis parameters across runs.

9.10 Pathway Arrays and Gene Expression Arrays

9.10.1 Phospho-Kinase Array

Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems; ARY003C) was used to profile phosphorylation-state changes under the indicated experimental conditions, using standardized exposure and densitometry procedures.

9.10.2 TaqMan Array: Pluripotency and p53 Signaling

TaqMan Array formats were used for pathway-scale profiling: Human Stem Cell Pluripotency (Fast 96-well; Thermo Fisher Scientific; 4418722) and Human p53 Signaling (Thermo Fisher Scientific; 4414168), following the manufacturer's workflows and consistent normalization across groups.

9.11 Immunoprecipitation for Mass Spectrometry (IP–MS)

9.11.1 Lysate Processing and Clarification

Tissue was homogenized in 5 volumes of Buffer A (or detergent-free buffer), transferred to low-retention tubes, and clarified by centrifugation at 14,000 rpm and 4 °C for 10 min. Supernatants were transferred to clean low-retention tubes.

9.11.2 Bead Equilibration and Pre-Clearing

Magnetic beads were equilibrated in 1 mL of Buffer A (or detergent-free buffer) by vortexing, spinning, and removing the buffer, repeated 2–3 times. Pre-clearing was performed by adding an equilibrated bead slurry (typically 20–25 µL) to each lysate and rotating at 4 °C for 3–4 hours. The lysate was transferred to clean tubes after pre-clearing.

9.11.3 Immunoprecipitation, Capture, and Flow-Through

Protein concentration was determined by BCA assay, and total protein for IP was calculated (standard input: 2 mg). Samples were divided into an IP condition (pre-cleared lysate plus antibody) and a beads-only control (pre-cleared lysate without antibody). Final volumes were equalized with Buffer A. Antibody was added to IP tubes (e.g., Tau13: 2 µL undiluted; antibody-dependent). Samples were rotated overnight at 4 °C (16–18 h). Equilibrated beads

were then added, and the samples were rotated for 3–4 hours at 4 °C. The flow-through was removed and saved.

9.11.4 Washes, On-Bead Digestion, and Sample Cleanup

Beads were washed 3–4 times with 1 mL 25 mM AMBIC (pH 8) using magnetic separation. Beads were resuspended in 50 μ L 25 mM AMBIC / 50% ACN, and trypsin (1 μ g) plus rLys-C (500 ng) were added for digestion at 37 °C for 16–18 h. Supernatants were transferred to clean tubes, dried under a speed vacuum at 30 °C (~20 min), resuspended in 50 μ L of 25 mM AMBIC/5% ACN, and transferred to mass spectrometry vials.

9.11.5 Buffer A Composition

Buffer A consisted of 20 mM Tris base (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 5 mM sodium pyrophosphate, supplemented with 1 $\times$ protease inhibitor cocktail.

9.12 Polymer Brush Fabrication, Characterization, and hMSC Functional Testing

9.12.1 Materials for Polymer Brush Fabrication

SPMA, HEMA, MEMA, CuBr₂, dopamine hydrochloride, Et₃N, BiBB, EDTA, TRIS base, anhydrous DMF, PMDETA, heparin sodium salt, and toluene were purchased from Sigma-Aldrich. Ascorbic acid was purchased from Ambeed. Sodium periodate was purchased from Oakwood Chemicals. Silicon wafers were purchased from University Wafers. eBMPUS was purchased from Gelest, and eBMPMD from Prochimia Surfaces. HEMA and MEMA were passed through basic alumina and stored at –18 °C before ATRP.

9.12.2 Substrate Functionalization (Silicon, Gold, and TCP)

Silicon wafers were cleaned and functionalized with eBMPUS via toluene soaking (0.005 wt%) at –18 °C for 24 h, followed by sonication in ethanol. Gold wafers were prepared by titanium/gold deposition and functionalized by eBMPMD thiol adsorption (2 mM, 24 h). TCP

substrates were functionalized with pDOPA-Br by reacting dopamine with BiBB in DMF under nitrogen, transferring to pH 9 TRIS, and oxidizing with sodium periodate during deposition onto the substrates. Substrates were rinsed thoroughly and stored until grafting.

9.12.3 ARGET ATRP Brush Grafting

CuBr₂, PMDETA, and ascorbic acid stock solutions were prepared in 1:1 MeOH/DI water, and SPMA stock was prepared in water. Reaction mixtures were degassed by bubbling nitrogen for 30 min, activated by adding ascorbic acid, and transferred to initiator-functionalized substrates in a nitrogen glove bag at 23 °C. Polymerization proceeded for 1 h. Post-reaction, substrates were rinsed thoroughly; TCP substrates were treated with EDTA and CaCl₂ to remove copper and washed repeatedly before drying and storage.

9.12.4 Characterization of Polymer Brushes

Brush thickness was quantified by spectroscopic ellipsometry. The composition was analyzed by XPS (survey and high-resolution C 1s, S 2p, and O 1s). FTIR spectra were collected using a grazing-angle accessory on gold substrates. Water contact angles were measured by goniometry. Brush swelling in water, and PBS was measured by in situ ellipsometry using effective medium approximation modeling.

9.12.5 bFGF Adsorption by ELISA

Brush-coated wells and controls (HS-coated positive control; TCP negative control) were blocked with Super Block. bFGF was incubated at 50 ng/mL for 2.5 h with gentle shaking, followed by extensive washing. Bound bFGF was detected using a biotinylated FGF antibody, HRP-streptavidin, and TMB development, with absorbance read at 450 nm (background 720 nm). Statistical testing used one-way ANOVA with Tukey's HSD.

9.12.6 hMSC Culture, Proliferation, Differentiation, and Gene Expression

hMSCs derived from H1 were expanded and validated using surface marker profiling and assessment of trilineage differentiation potential. Cells at passages 3–4 were seeded onto polymer brushes and TCP controls in chemically defined medium and cultured for 10 days. Cell counts were acquired using an automated cell counter and normalized to TCP. Adipogenic, chondrogenic, and osteogenic differentiation were performed using standardized commercial differentiation media with lineage verification by Oil Red O staining (adipogenesis), pellet culture (chondrogenesis), and alkaline phosphatase staining (osteogenesis). Gene expression was quantified using $\Delta\Delta C_t$ normalization with GAPDH and 18S internal controls.

9.13 Statistical Analysis

Data are presented as mean $\pm$ SEM unless otherwise indicated. Unless specified otherwise, analyses were performed using three independent biological replicates. Statistical analyses and graph generation were conducted using Microsoft Excel (Microsoft Corp.) with the Data Analysis Tool Pak add-in. For comparisons between two groups, an unpaired two-tailed Student's t-test was applied. For comparisons involving more than two groups, one-way ANOVA followed by Tukey's HSD post hoc test was used. Statistical significance was defined as $p < 0.05$, with significance levels indicated as follows: $p < 0.05$ (*), $p < 0.005$ (), and $p < 0.0005$ (***)).

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