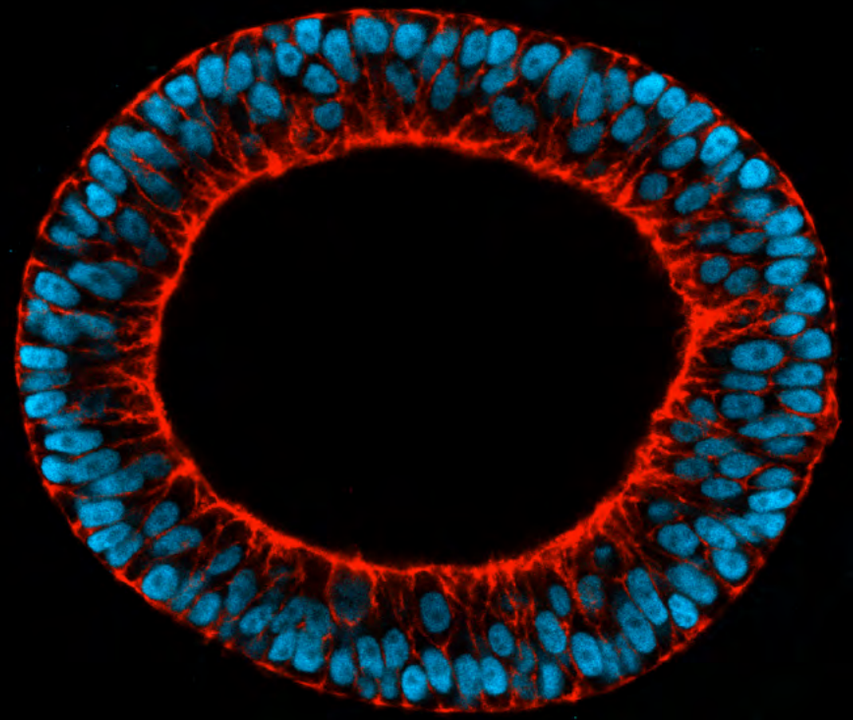
White Paper

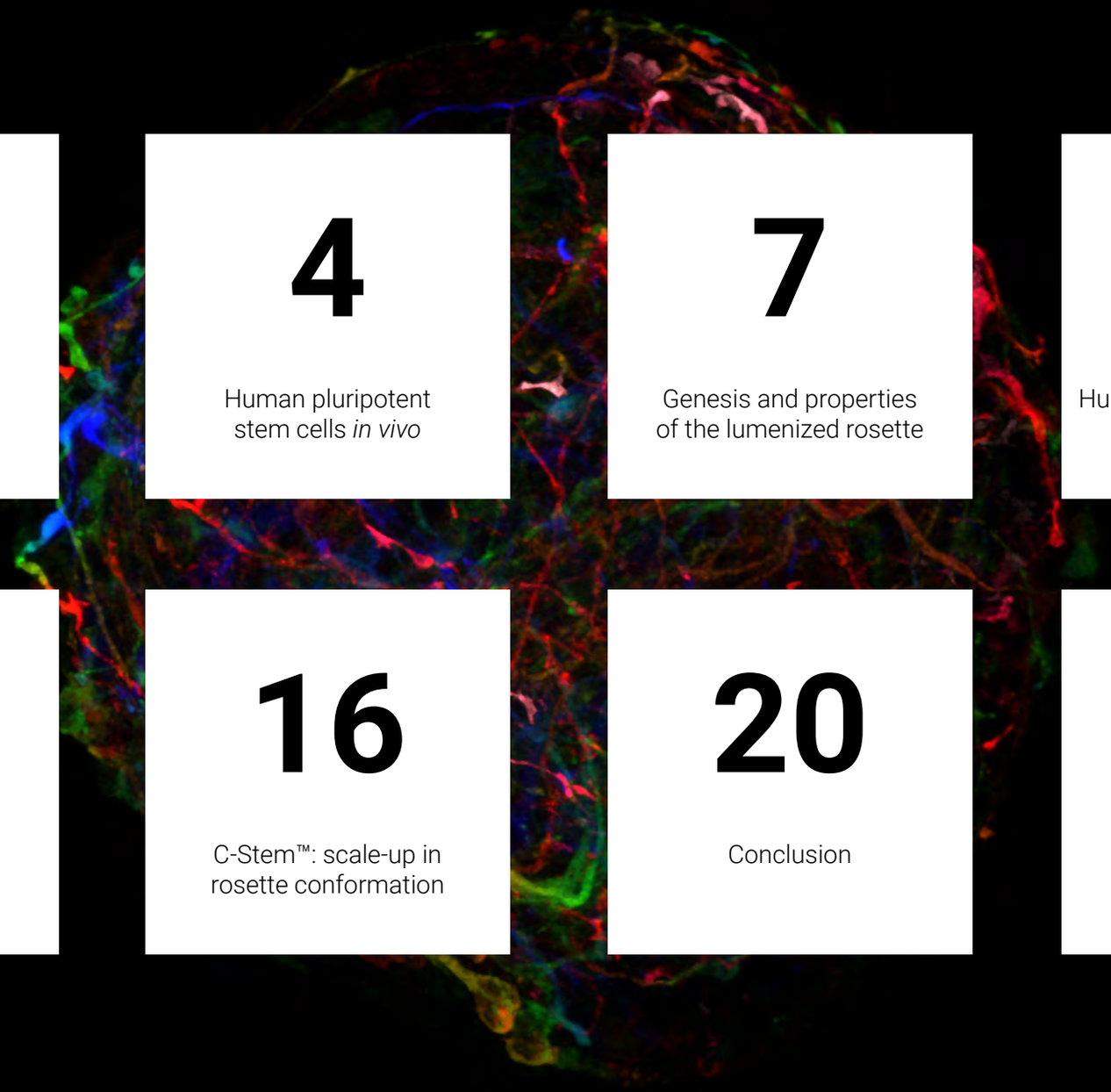
TreeFrog therapeutics



THE LUMENIZED ROSETTE

Mimicking the *in vivo* micro-environment of human pluripotent stem cells

Contents



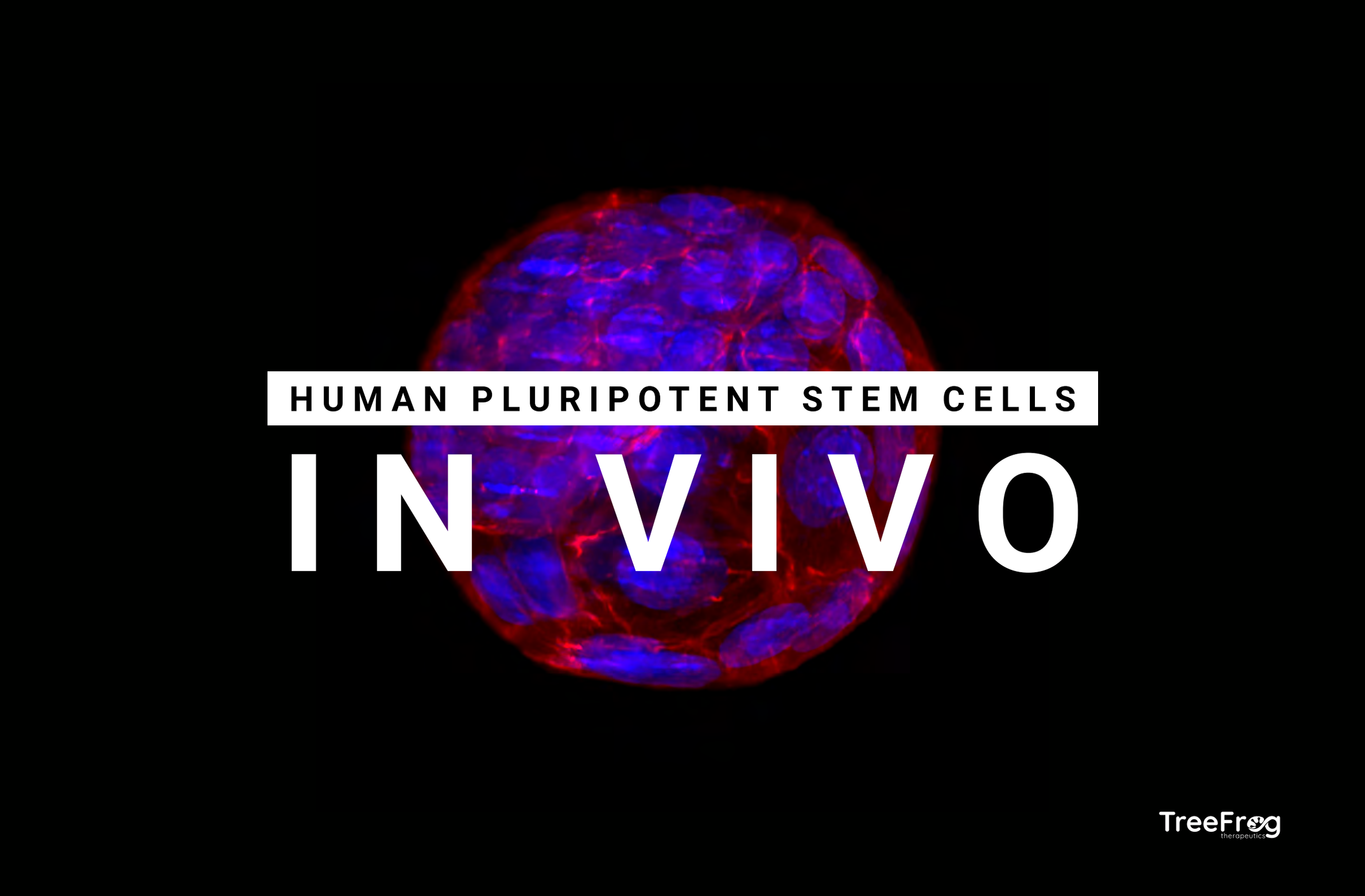
Cover: Confocal image of a colony of human induced pluripotent stem cells (hiPSCs) forming a lumenized rosette. hiPSCs were cultured with the C-Stem™ technology. Cytoskeletal actin in red and OCT4 in blue.

Introduction

The discovery by Shinya Yamanaka in 2006[1] of key transcription factors enabling the reprogrammation of somatic cells to the pluripotent state triggered massive access to human pluripotent stem cells (hPSCs). Induced hPSCs are often touted as an infinite source of cells for research, disease modelling, and therapeutic applications. Being adherent cells, hPSCs have originally been cultured as 2D colonies in Petri dishes or T-flasks. However, despite their apparent unlimited proliferation capacity, hPSCs only exist for a few days *in vivo* and do not grow as 2D colonies. *In vivo*, pluripotency (here restricted to human primed pluripotency) is only observed during the second week of human development, and exclusively within 3D epithelialized arrangements of cells. To overcome the intrinsic limitations of 2D cell culture systems with respect to hPSC expansion – especially high mortality and genomic alterations[2,3] –, novel cell culture technologies that mimic the hPSC niche have recently been developed, bringing a new breath to academic research and industrial applications.

This white paper aims at:

- → Describing the *in vivo* environment of hPSCs, with a focus on the lumenized rosette conformation
- → Comparing the *in vivo* organization of hPSCs to standard *in vitro* cell culture systems
- → Reviewing biomimetic systems that scientists can implement in their lab to culture hPSCs in lumenized rosette conformation
- → Introducing C-Stem™ – a biomimetic technology allowing for the mass-production and differentiation of hPSCs in large-scale bioreactors


HUMAN PLURIPOTENT STEM CELLS
IN VIVO

Human pluripotent stem cells in vivo

Human primed pluripotency: a 7-day window

Pluripotency is a transient cell property *in vivo*. At the end of the first week of development, naïve hPSCs transition to primed pluripotency. Within the following 5 to 7 days, the hPSC colony, benefiting from a very fast 15h to 16h[4] cell cycle, grows exponentially to reach over 1,000 cells. Following this exponential amplification, hPSCs exit pluripotency as they commit to one of the three germ layers that undergo gastrulation. Overall, among the 40 cell divisions required to constitute an adult body (~37 trillion cells)[5], 10 are estimated to occur within the second week of development[6], highlighting the importance of the pluripotent stage.

A protected microenvironment

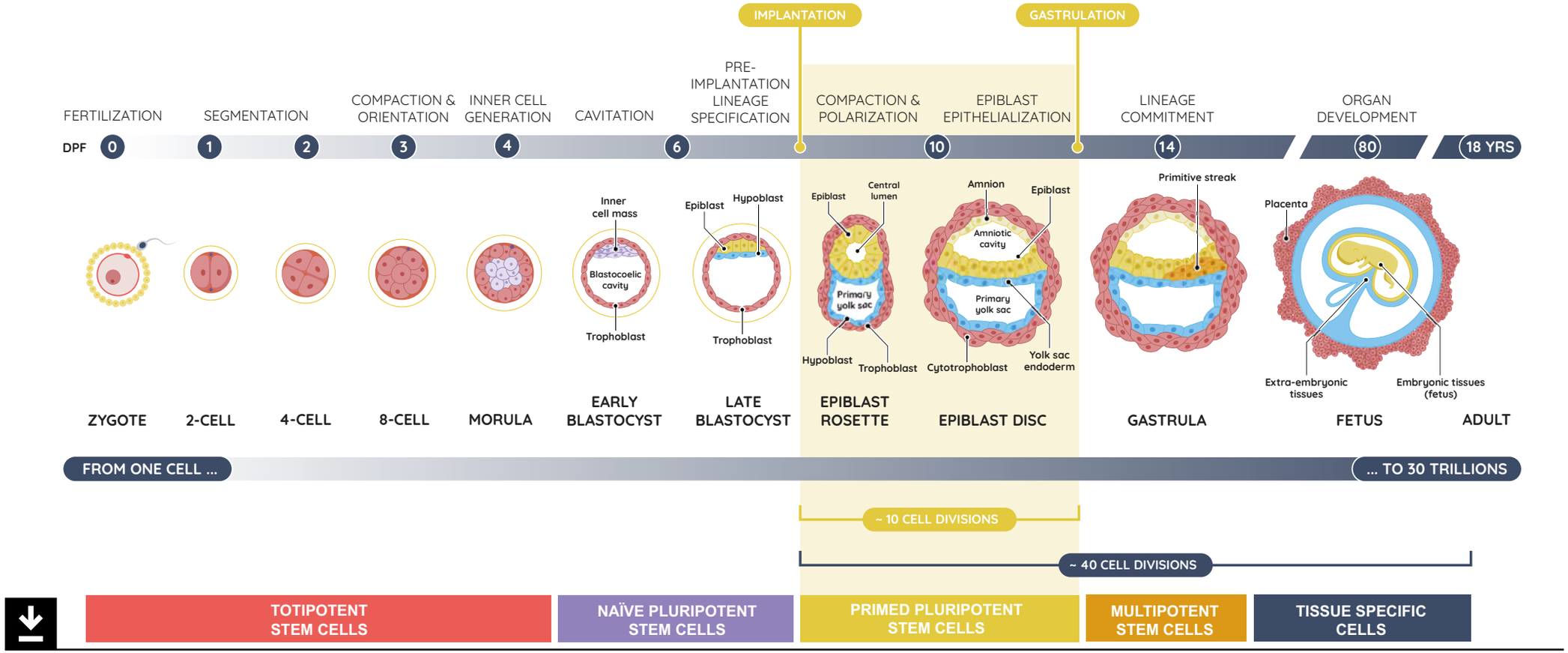
Before implantation, the fertilized egg and its subsequent totipotent stem cell progeny grow for a few days within the zona pellucida - a 5-10 µm thick spherical shell composed of glycoproteins. The zona pellucida is a mechanically protective capsule that allows for metabolic exchanges with the environment of the uterus. This structure is hatched around day 7 post-fertilization to enable implantation into the uterine wall. This capsule is then functionally replaced by an extra-embryonic outer layer of trophoblast cells, which provides mechanical protection, enables metabolic exchanges, and creates a controlled 3D niche in which hPSCs can grow exponentially.

A distinctive lumenized rosette architecture

After implantation, hPSCs acquire primed pluripotency and arrange as a spherical single cell-layered epithelium organized around a central cavity. This lumenized rosette architecture is instrumental for the *in vivo* function of the hPSC compartment.

First, this radial organization around a central lumen ensures that each hPSC within the colony has similar environmental conditions, including topology, mechanical characteristics and access to oxygen, nutrients, extracellular matrix, and growth factors. Its radial symmetry *per se* avoids local variations, which could compromise the establishment of ontogenic axes organizing the body plan.
Second, in the epiblast rosette, all hPSCs have their apical domains clustered toward the lumen, which acts as central signaling hub, enabling synchronous and homogeneous growth.
Finally, the lumenized rosette is a plastic architecture suited for growth. Its roundness suggests a positive intraluminal osmotic pressure. Such a pressure gradient may help expand the surface area of the epithelium as hPSCs divide, and locally decrease the tangential mechanical resistance to accommodate new cells. Facilitating cell division[7], this conformation also prompts the depletion of abnormal cells, thus safeguarding the genomic integrity of the hPSC colony.[8]

As it expands, the pluripotent epiblast rosette gradually transitions to a disc conformation before a symmetry break occurs around day 14. Recent studies support that the radial symmetry of the epiblast, as well as the concentration of several factors within the lumen, such as BMP or fibroblast growth factor (FGF), promote robust symmetry breaking and differentiation of hPSC during gastrulation.[9,10,11,12]



Adapted from Wamaitha & Niakan, 2018 Human pre-gastrulation development[13] ; Shahbazi & Zernicka-Goetz, 2018 Deconstructing and reconstructing the mouse and human early embryo[14] ; Shahbazi et al., 2020. Mechanisms of human embryo development: From cell fate to tissue shape and back[9] ; Roelen, 2018 Epiblast Development[15]
DPF = Day post-fertilization, YRS = Years

Fig. 1. Human primed pluripotency *in vivo*: a 7-day window, a protected microenvironment, a 3D epithelial organization

At day 0, the fusion of the oocyte with the sperm creates a single diploid zygote. Then, the human embryo undergoes a series of cleavage divisions. At the four/eight-cell stage transition, the embryonic genome becomes activated. Around day 4, a morula is formed and inside cells are generated. The cavitation begins with early blastocyst formation comprising a fluid-filled cavity - the blastocoel - and an inner cell mass (ICM), surrounded by an outer layer of trophectoderm cells. The ICM is further segregated into a naïve pluripotent epiblast that will give rise to the embryo proper, and a primitive endoderm layer (hypoblast) that gives rise to extraembryonic endoderm cells. Thus, the late blastocyst is composed of three main tissues: epiblast, hypoblast and trophoblast. Around day 7, at the peri-implantation stage, the embryo undergoes a global morphological transformation, and the embryonic epiblast loses its naïve pluripotent character. At this stage, primed pluripotent stem cells self-organize in rosette conformation around a central lumen. Subsequently, a subset of epiblast cells differentiates to form the amnion, while the remaining epiblast acquires a disc shape, resulting in the formation of the amniotic cavity. Around day 12, gastrulation is initiated in the posterior epiblast. Cells undergo an epithelial-to-mesenchymal transition, lose their pluripotent character and commit to one of the germ layers: ectoderm, mesoderm or endoderm.

GENESIS & PROPERTIES

OF THE LUMENIZED ROSETTE

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Genesis & properties of the lumenized rosette

The human epiblast is a lumenized and polarized PSC colony

The lumenized rosette architecture emerges during the transition from naïve to primed pluripotency. In response to extracellular matrix cues, naïve PSCs start to polarize. As transcriptional factors trigger primed pluripotency, hPSC form *de novo* intracellular actin-rich compartments called apicosomes, which accumulate Ca^{2+} and express apical proteins.[16] As apicosomes regroup, concomitant mechanisms promoting water influx[17] towards the center of the epiblast participate in the establishment of a fluid-filled lumen. The resulting rosette architecture displays apical-basal polarity: all apical domains are facing the lumen, while basal domains are facing the external environment of the epiblast.

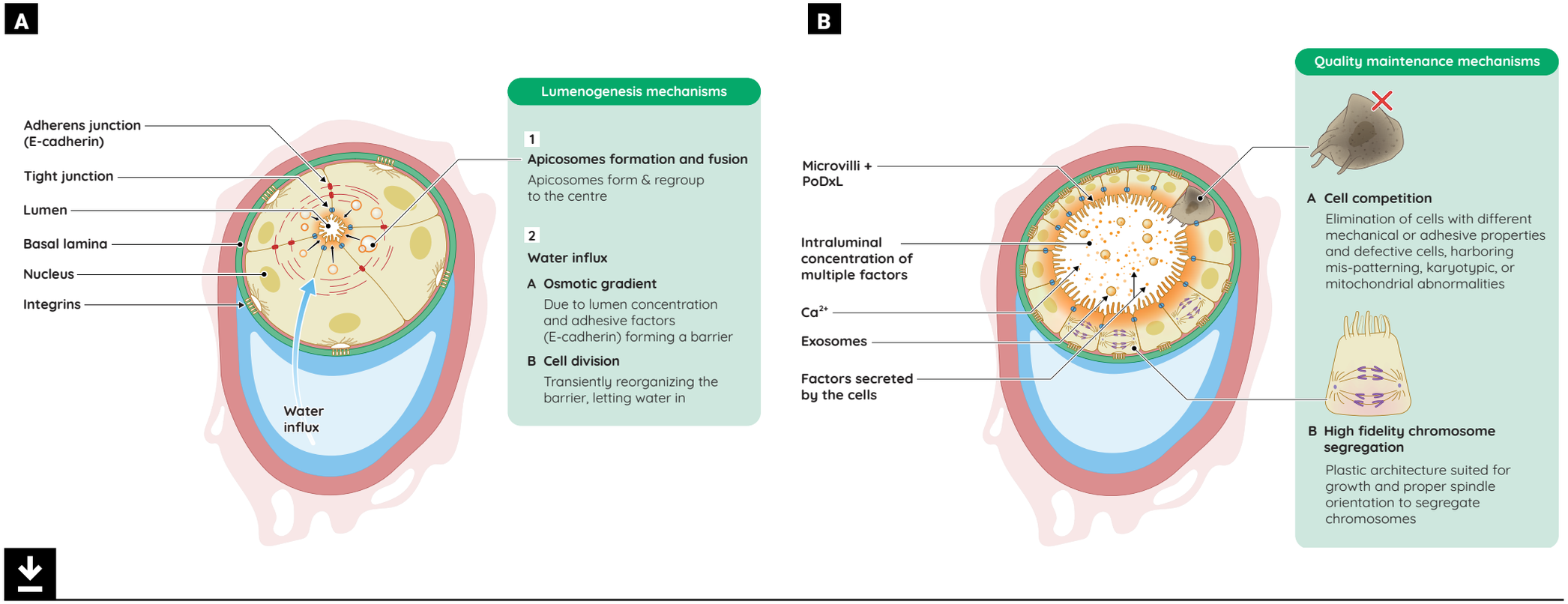
Lumen formation is required for optimal proliferation and homogenous pluripotency

hPSCs in lumenized rosette conformation are characterized by prolonged undifferentiated proliferation without undergoing senescence or quiescence. Kim et al. showed in 2021[17] that inhibiting lumen formation in PSC colonies results in reduced proliferation, and that the lumen is required for the establishment of critical signaling pathways, such as the Nodal cascade.[18] Although the composition of the luminal fluid and the signaling molecules found within this central cavity are not fully characterized, the lumen is known to enhance intercellular communication[18] within the rosette, and this architecture is considered to contribute to the maintenance of stemness during hPSC exponential growth. In a 2021 interview, developmental biologist Marta Shahbazi reported that *in vitro*, PSCs that escape the 3D rosette architecture are differentiating, thus losing their pluripotent phenotype.[19]

The rosette architecture participates in the fitness of pluripotent stem cells

In 2018, Knouse et al. demonstrated that polarized epithelial tissue architecture is required for high-fidelity chromosome segregation.[7] Loss of polarity and disruption of tissue architecture around a central lumen are in fact correlated with mitotic failures, aneuploidy and accumulation of DNA damage[20,7] in epithelial cells. This observation gains a particular relevance in the context of early human development, where genomic abnormalities are extremely common. In 2020, a single-cell RNA sequencing analysis reported that more than 70% of human embryos contain karyotypically abnormal cells at the pre-implantation stage, prior to epiblast formation.[21]

Recent research has shown that the pluripotent rosette undergoes natural quality control through cell competition mechanisms, leading to the elimination of abnormal cells through apoptosis and autophagy.[22,23] In the mouse epiblast, 35% of PSCs were found to be eliminated by the onset of gastrulation.[24] Several groups demonstrated that defective cells, harboring mis-patterning, karyotypic[8], or mitochondrial abnormalities[22] are efficiently depleted in epiblast conformation. Normal diploid PSCs were also found to increase their proliferation to compensate for the elimination of abnormal cells in the epiblast.[8] Overall, the competition between PSCs in the epiblast, which involves YAP/TAZ[25], MYC[23], p53[24], and mTOR[24] signaling pathways, is considered as a critical mechanism, safeguarding pluripotency and genomic integrity before germline commitment.



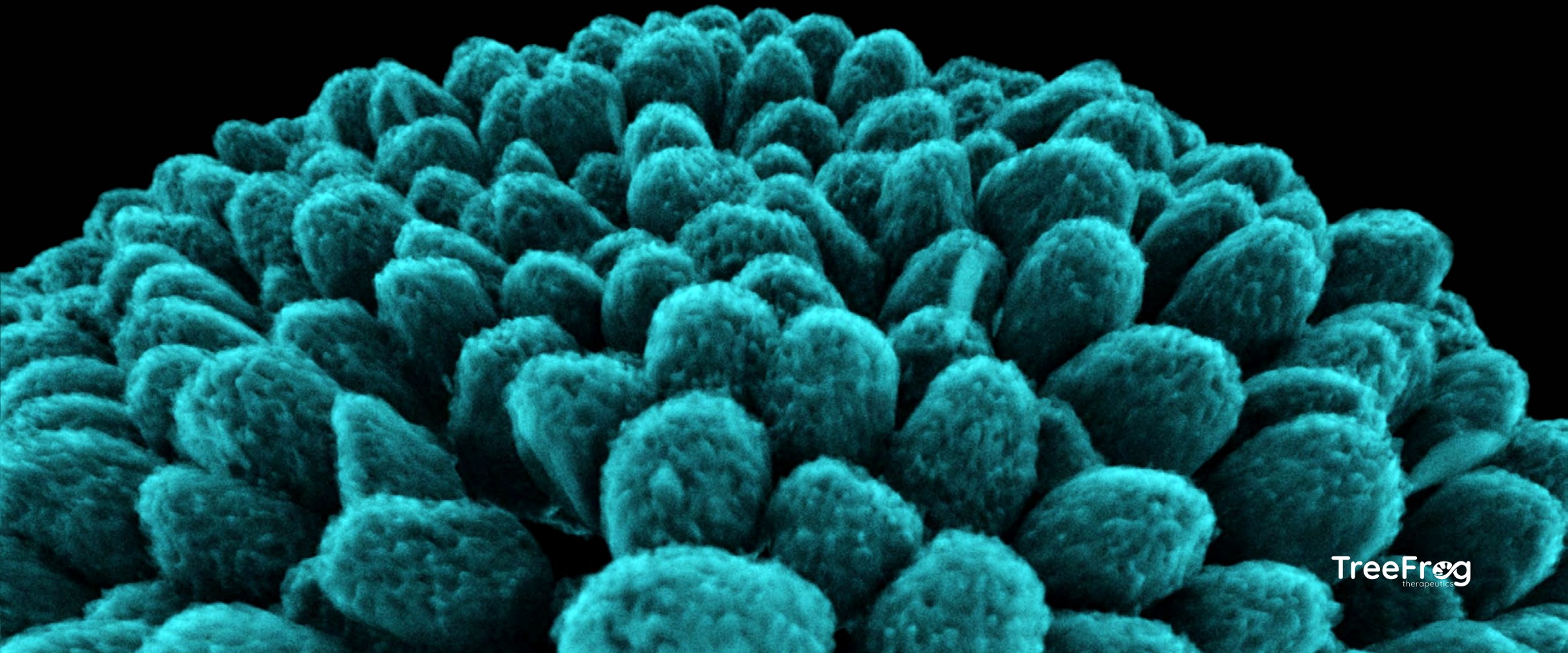
Adapted from Knouse et al., 2018[7] ; Kim et al., 2021[17] ; Taniguchi et al., 2015[26] ; Taniguchi et al., 2017[27] ; Shahbazi et al., 2017[28]

Fig. 2. Lumenogenesis & rosette expansion

(A) Lumenogenesis is an intrinsic property of isolated pluripotent stem cells.[26] This phenomenon relies on the secretion of vesicles called "apicosomes"[27]. The apicosomes regroup and merge together at the center of the epiblast. Concomitantly, several factors, including cell division, osmotic gradients and reorganization of adherens junction (E-cadherin), promote water influx toward the central cavity. (B) The 3D stem cell colony organizes around a central lumen, promoting the conservation of stemness and pluripotency even beyond 10 consecutive passages (Freedman et al., 2015). Stem cell viability has also been reported to be improved in lumenized rosette compared to the aggregate configuration (Y. Zhu et al., 2020). In the lumenized rosette configuration, chromosome segregation is facilitated and cell competition mechanisms lead to the elimination of abnormal cells.

HUMAN PLURIPOTENT STEM CELLS

IN VIVO VERSUS IN VITRO



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Human pluripotent stem cells: *in vitro* versus *in vivo*

2D culture versus *in vivo*

Standard 2D hPSC culture consists of seeding hPSCs as single cells or, most frequently, cell clusters in culture flasks or Petri dishes coated with extracellular matrix. Cells are then incubated at 37°C under normoxic or hypoxic conditions. Every 24-48 hours, hPSCs are taken out of the incubator for media change. When the colonies approach or reach confluence, the cell monolayer is detached from the substrate, often resulting in a combination of cell clusters and single cells, which are then replated for further expansion.

The first obvious limitation of 2D culture for the recapitulation of the *in vivo* environment of hPSCs is the intermittent control of cell culture parameters, including temperature, pH, dissolved oxygen (DO), glucose, and growth factor concentrations,[29] caused by medium changes.
Second, while it has been reported that hPSCs cultured in 2D spontaneously form lumens, such rosette-like structures were found to rapidly collapse (in ~5 days or less)[26]. hPSCs end up establishing an inverted topology in 2D. As opposed to the rosette architecture, in Petri dishes the apical domain is facing the cell culture media, while the basal pole is established at the bottom of the dish, with limited access to external cues and nutrients. As a result, in 2D culture, signaling molecules secreted at the apical domain are diluted in a large volume of media, which is rinsed at every media change. Developmental biologist Marta Shahbazi estimated a 2,500-fold difference in the concentration of autocrine factors in the lumen of hPSC colonies in rosette conformation versus cell culture medium in standard 2D culture.[19]
Furthermore, in contrast with the lumenized rosette conformation, in 2D, as the colony expands and reaches confluence, cells at the center of the colony experience a very different environment from the ones at the edge, translating into spatially heterogeneous expression of pluripotency markers.[30] This phenomenon is described as edge/center heterogeneity.[31,30] The cellular crowding and compaction observed in 2D hPSC colonies also induce a competition for space, known to inhibit epithelial cell proliferation and trigger apoptosis through caspase-dependent mechanisms.[32] This lateral competition is independent of cellular fitness markers and promotes the selection of fast-growing apoptotic-resistant clones, likely to harbor oncogenic mutations.[32]

Together, intermittent control of cell culture conditions, unphysiological apico-basal polarity, edge-center heterogeneity, and competition for space may explain the high mortality rates, spontaneous differentiation, and genetic drift reported in 2D hPSC colonies[2,7], highlighting the limitations of monolayer cultures for scaling up the production of clinical-grade hPSC-derived products.

3D aggregates in bioreactors versus *in vivo*

With the aim of meeting industrial needs for scale-up, hPSC are also cultured in agitated bioreactors, which provide continuous control over key cell culture parameters. In standard bioreactor culture, agitation induces the formation of 3D aggregates of hPSCs. However, productivity and cell quality are consistently found to be lower in this configuration than in 2D. In a benchmark presented in 2021 at the American Society of Gene & Cell Therapy (ASGCT), prolonged agitated hiPSC culture over 28 days resulted in a 6,000-fold lower expansion than in 2D, and a higher mutational load was reported in hPSC aggregates.[33] Several factors may affect performance in bioreactors. *In vivo*, an extra-embryonic tissue layer protects hPSCs from external stress. In bioreactors, hPSCs aggregates are directly exposed to impeller-induced hydrodynamic stress, which can trigger either cell death or spontaneous differentiation.[34] Agitation also leads to spontaneous fusions of aggregates[35], resulting in large hPSC spheroids harboring necrotic cores instead of lumens.[34,36] Cellular overcrowding, heterogeneous access to gas, nutrients, and signaling molecules in large hPSC aggregates, together with failure to establish apical-basal polarity, and epiblast-like cell competition, may altogether explain the reduced proliferation and increased rate of mitotic errors observed in bioreactor culture.

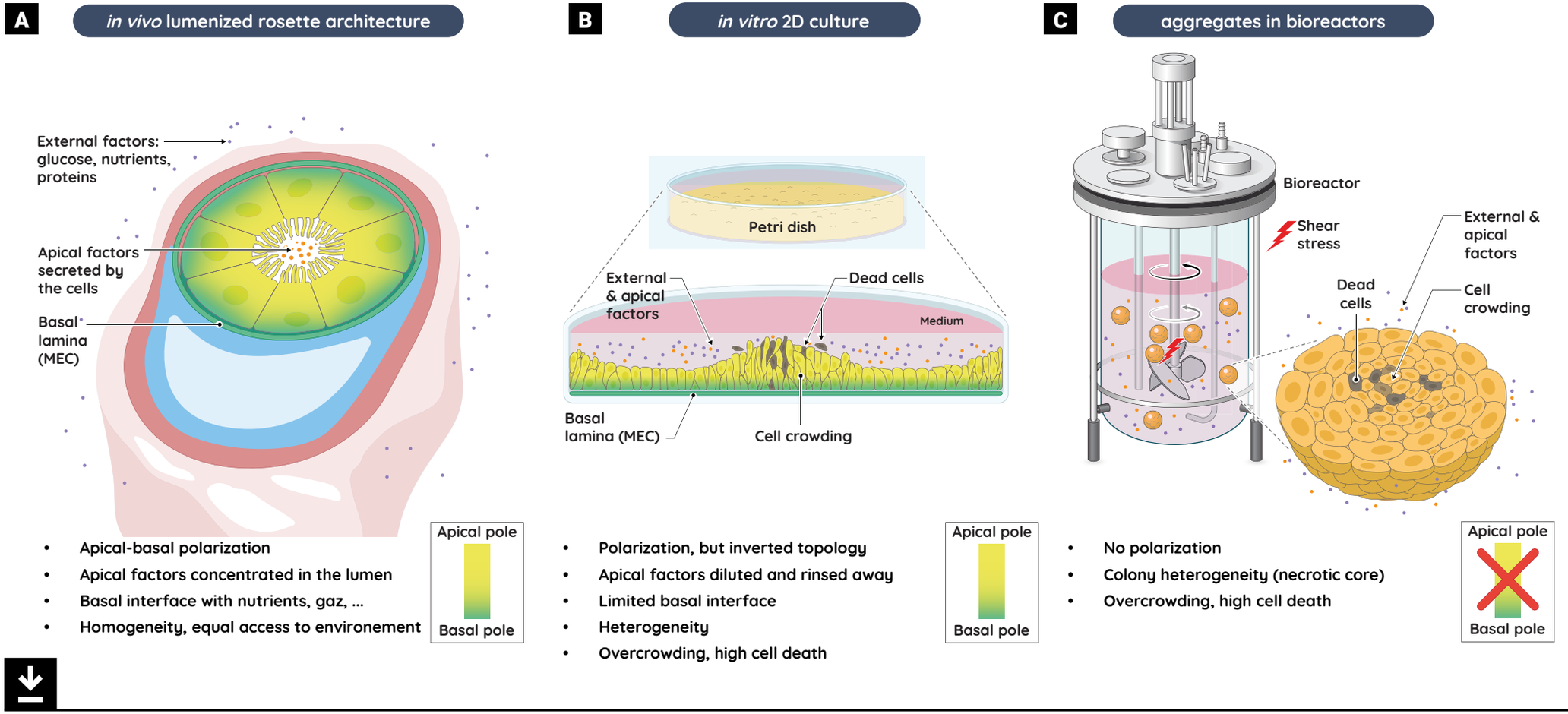

Fig. 3. Comparison of standard 2D culture and 3D aggregate culture to the *in vivo* lumenized rosette


GROWING LUMENIZED ROSETTES
IN THE LAB
TreeFrog
therapeutics

Growing lumenized rosettes in the lab

Over the past decade, developmental biology entered a golden era,[37,26] fueled by novel biomimetic approaches to culture hPSCs. *In vitro* recapitulation of the lumenized rosette conformation was described by the group of Magdalena Zernicka-Goetz in 2014 using mice PSCs cultured in micro-wells coated with Matrigel.[37] This approach was then successfully applied to human ES and iPS cells in 2016 by the same team.[38]

With the view of generating hPSC rosettes at scale while automating their maintenance, several groups have explored droplet microfluidics, confining hPSCs in agarose, alginate or PEG,[39,40] while other teams engineered microfluidic circuits to entrap and grow hPSCs in 3D.[26,41,42,43] While successfully generating lumenized rosettes, such microfluidic systems were primarily designed for academic research, and thus feature limitations either in throughput or total loading capacity.

Core features of biomimetic approaches

Despite the apparent diversity of *in vitro* approaches for the generation of hPSC lumenized rosettes, key parameters are generally preserved:

- Cells are biophysically contained and a critical aggregate size needs to be reached to form a polarized epithelium with a central lumen (~50-300 µm).[42]
- Matrigel® remains the standard extracellular matrix[42], as it mimics the basal membrane niche and supports PSC polarization and lumenogenesis.[37]
- E8 or mTESR feeder free media are used, minimizing or eliminating the use of poorly characterized biological components additives such as serum, thus avoiding inconsistencies and variations.[44,45]
- Hypoxic culture conditions (~ 5% O2)[46] are adopted when possible. Hypoxia was indeed reported to reduce mutation rate and promote polarization in hiPSC cultures.[46,37]

10 steps to obtain lumenized hPSC colonies[47]

1. Place filter tips and ibiTreat 8-well µ-plates in the refrigerator and thaw matrigel slowly on ice.
2. Detach hPSCs using StemPro Accutase by incubating at 37°C for 3 min.
3. Add E8 or mTESR1 and pipette the cell suspension in a 15 ml conical tube. Centrifuge for 3 min at 1000 rpm.
4. Wash once with PBS and centrifuge the cell suspension for 3 min at 1000 rpm. Re-suspend the cells in either E8 or mTESR1. NOTE: At this point, ROCK inhibitor may be added to prevent dissociation-induced cell death.
5. Count the cells using a hemacytometer. 2.5 $\times 10^4$ cells are needed per well.
6. Coat the entire surface of the pre-chilled ibiTreat 8-well µ-plate with 40 µL of ice-cold matrigel. CRITICAL STEP: Use pre-chilled tips and keep the matrigel on ice at all times. Increasing temperatures lead to premature polymerisation. Make sure the matrigel covers the entire surface of the well.
7. Place in a 37 °C incubator for 2 min or until the matrigel has solidified. Immediately after, add 2.5 $\times 10^4$ cells in a final volume of 250 µL per well (cell suspension prepared in 5).
8. Incubate the cell suspension for 5 min at 37°C or until most of the cells have attached to the matrigel.
9. Gently remove the supernatant. Add fresh E8 or mTESR1 medium, supplemented with 5% matrigel. NOTE: At this point, ROCK inhibitor may be added.
10. Analyze lumen formation 24 or 48 hours after plating.

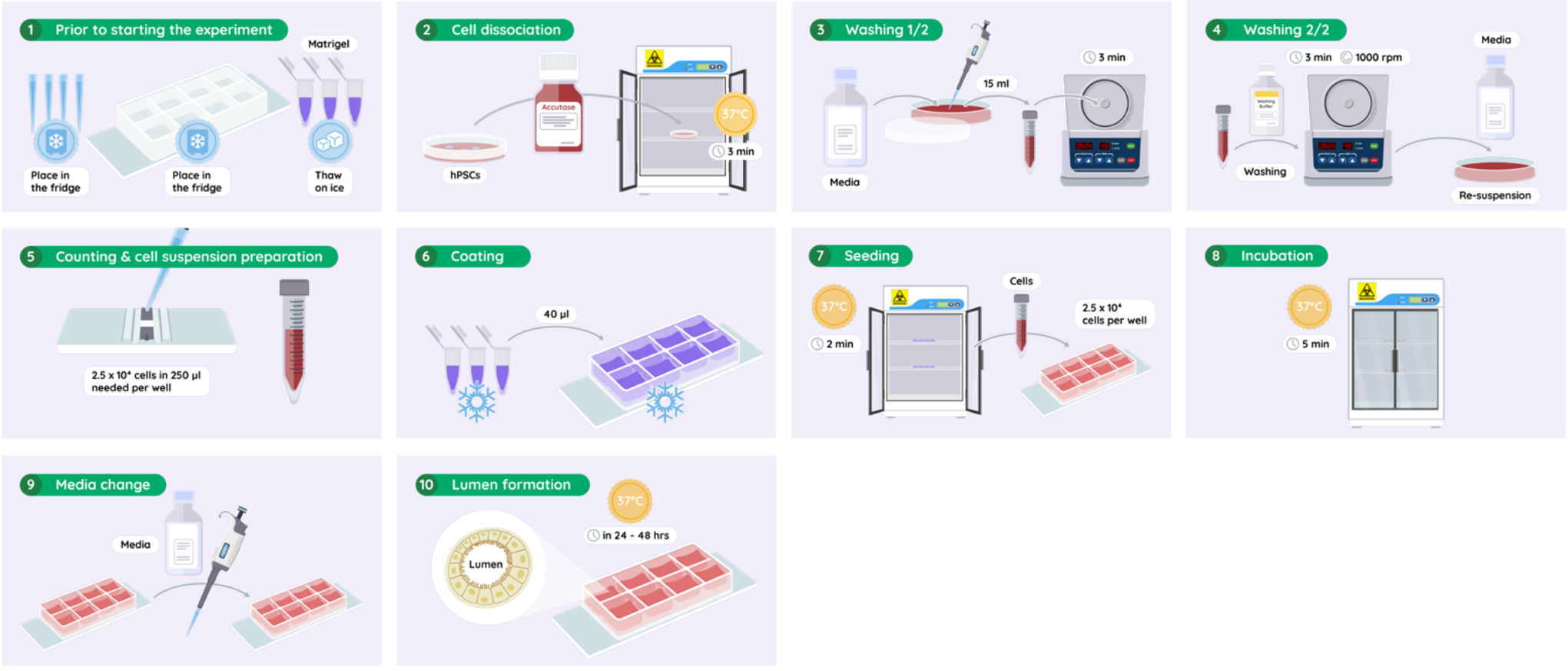

Fig. 4. A 10-step protocol to culture hPSC colonies in 3D in lumenized rosette conformation (Shahbazi et al. 2016)[47]


Cstem
by TreeFrog therapeutics
SCALE-UP IN ROSETTE CONFORMATION
TreeFrog
therapeutics

C-Stem™: scale-up in rosette conformation

The lumenized rosette conformation is critical to the behavior of hPSCs *in vivo*. Replicating this architecture seems a logical approach to achieve *in vivo*-like exponential growth while preserving cell quality. However, current protocols for hPSC culture in lumenized rosette conformation[47,28,39] were not designed for scale-up. The C-Stem™ platform was invented to fill this gap.

Biomimetic capsules

The C-Stem™ technology utilizes proprietary microfluidics to encapsulate hPSC in hollow alginate shells at very high throughput (> 1000 capsules per second).[36] The inner wall of the capsule is decorated with extracellular matrix, thus mimicking the basement membrane of the hPSC niche.[48] In this biomimetic microenvironment, hPSCs spontaneously self-organize in 3D and form *in vivo*-like lumenized rosette structures. The size of the capsule (tunable from 100 to 800 µm radius) and the porosity of the alginate allow for optimal diffusion of oxygen and nutrients, thus preventing the formation of a necrotic core. On the outside, the 30 µm thick wall of alginate constitutes a highly resistant shell, which protects PSCs from hydrodynamic stress. When the capsule reaches confluence, the PSC colony is easily harvested, simply by dissolving the alginate shell with a calcium chelator.

Bioreactor scale-up[49]

Widely used to scale-up bioproduction processes, stirred-tank bioreactors provide full control over key cell culture parameters, such as pH, temperature, oxygen level, and media change regimens. So far, the obvious benefits of bioreactors to finely replicate the *in vivo* conditions of hPSCs were limited by impeller-induced shear stress, causing significant cell death[2,3] and spontaneous differentiation.[2,3] By shielding hPSCs within alginate capsules, the C-Stem™ technology removes the hydrodynamic stress constraints, thus permitting biomimetic hPSC culture within large-scale industrial bioreactors.

In 2021, just a few months after the commissioning of its first industrial encapsulation device, TreeFrog Therapeutics announced the production of two single batches of 15 billion hiPSCs in 10L bioreactors, with an unprecedented amplification factor of 276-fold within a week. Data demonstrated remarkable reproducibility (95% confidence interval), cell viability (>98.8%) and pluripotency (95% OCT4, 99% SOX2, 98% NANOG). This result contrasts with the best performance described in the literature (Huang et al., 2020), consisting in the 37-fold expansion of 1 billion hiPSC over 6 days in a 10L bioreactor and reporting a significant drop in stemness.

Genomic integrity

As expected, preliminary results assessing the genomic integrity of hiPSCs amplified with C-Stem™ demonstrated the benefits of the lumenized rosette format for the containment of pre-oncogenic mutations over prolonged culture (15-fold fewer pre-oncogenic mutations than in standard hiPSC culture in 2D, and 25-fold less than in hiPSC aggregates). In particular, the high hPSC viability obtained with this technology has demonstrated the capacity to mitigate the pro-survival advantage conferred by 20q11.21 copy number variation. In a more general sense, the limited cell death observed in C-Stem™ may reduce the selective advantage of mutations that affects cell survival, thus positively impacting the safety profile of hPSC populations expanded with the technology.

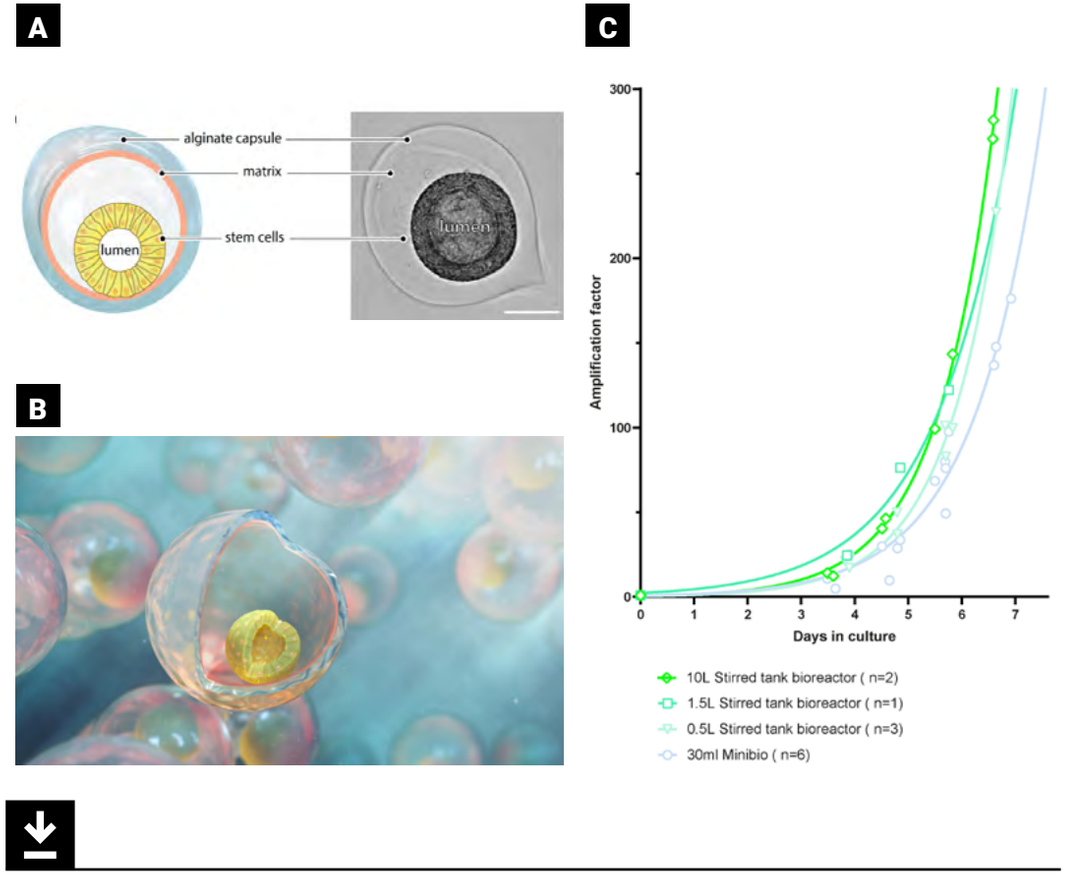

Fig. 5. C-Stem™-enabled encapsulation preserves the exponential expansion profile of hPSCs grown in rosette conformation regardless of the bioreactor volume

(A) hiPSCs encapsulated in core-shell alginate capsule decorated with extracellular matrix self-organize into an *in vivo*-like rosette conformation. On the right panel, magnified phase contrast image. Scale bar = 100 µm. (B) Artist view of a hPSC colony organized in lumenized rosette conformation with a C-Stem™-generated alginate capsule (C) C-Stem™-enabled fast hiPSC scale-up, from 30mL to 10L bioreactors, while preserving expansion curve profiles and cell culture parameters across scales.

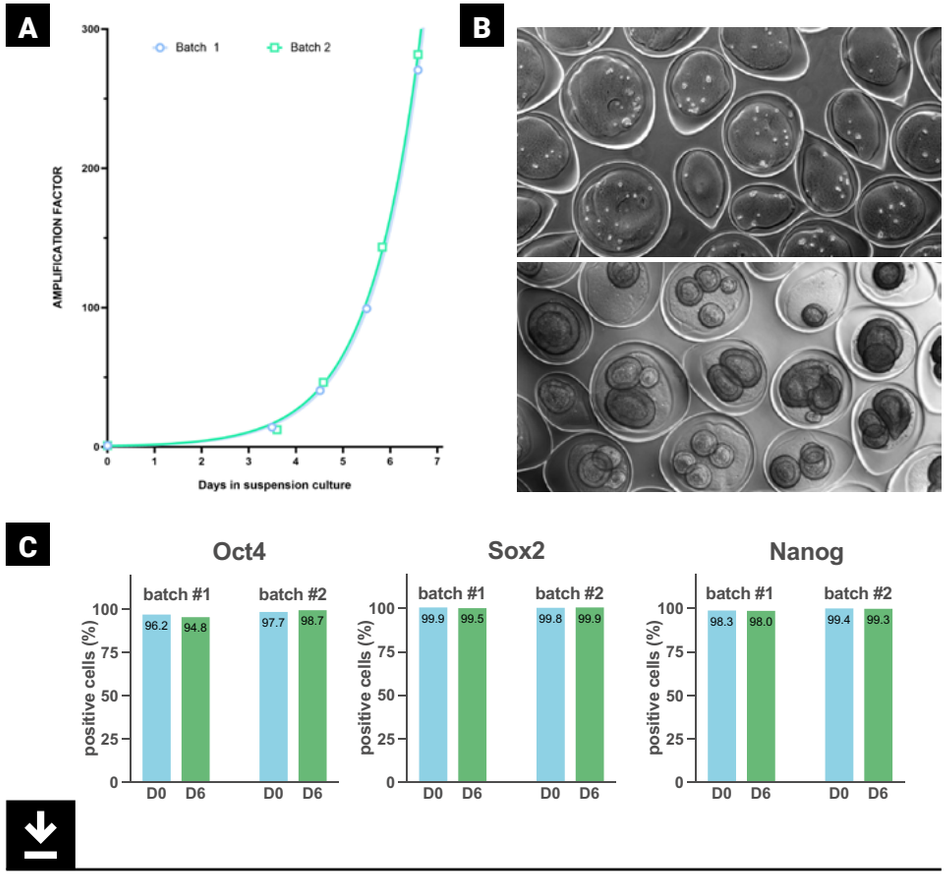

Fig. 6. Highly reproducible production of 2 single batches of 15Bn hiPSCs in 10L bioreactors, with unprecedented 276-fold amplification factor per week, and conservation of cell quality

(A) hiPSC amplification data in two independent 10L bioreactors. For each run, 50 million C-Stem™-encapsulated hiPSCs were cultivated under hypoxic conditions for 6 days. Each run resulted in a 15Bn hiPSC batch. Population doubling time is approximately 19h. Final hiPSC viability : 98.8%. (B) Phase contrast micrographs of encapsulated hPSCs at day 0 (top) and day 6 (bottom). (C) Assessment of key pluripotency markers by flow cytometry at day 0 and day 6 demonstrates robust maintenance of stemness across batches.

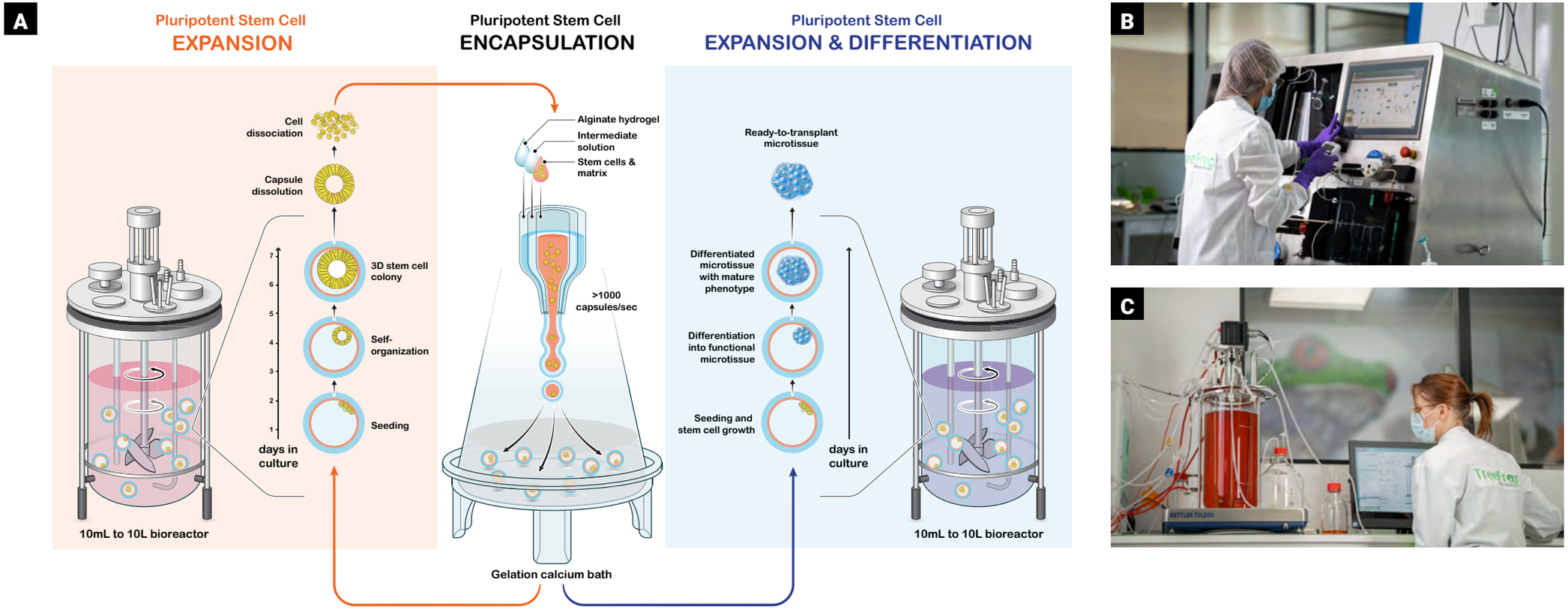

Fig. 7. C-Stem™, a single platform for hiPSC amplification and differentiation in large-scale bioreactors

(A.) Following encapsulation using high-throughput proprietary microfluidics (middle panel), hPSC in matrix-laden capsules can be either amplified serially to create master and working cell banks, or differentiated into functional microtissues. In each case, the cellular content is easily harvested by dissolving the capsule with a calcium chelator. (B) Proprietary GMP-grade encapsulation device delivered in October 2022. Capable of seeding bioreactors from 200mL to 10L, the GMP system is fully closed, automated, and 21 CFR part 11 compliant. (C) Expansion of encapsulated hiPSCs in a 10L bioreactor.

Conclusion

Recent research brought new light on the *in vivo* biology of hPSCs, mirroring the limitations of current *in vitro* systems for the amplification and maintenance of high-quality PSC.[50] *In vivo*, the lumenized rosette architecture allows for robust and rapid expansion of hPSCs while preserving genomic integrity. Therefore, engineering cell culture systems that closely recapitulate the *in vivo* environment of hPSC and favor their intrinsic capacity to grow in epiblast-like rosette conformation seems rational to unlock the potential of hPSC-based applications.

As of today, the C-Stem™ platform is the only biomimetic technology that demonstrated the capacity to harness the potential of the lumenized rosette conformation at scale, resulting in unprecedented expansion of hiPSC batches with state-of-the-art quality. More importantly, the same platform also enables the differentiation of hPSC rosettes into ready-to-graft functional microtissues within large-scale bioreactors, under closed, automated, GMP-compatible conditions. While creating a new manufacturing paradigm for hPSC-derived products in terms of productivity and quality, the C-Stem™ platform also opens opportunities to create entirely new cell therapy products. For instance, until very recently, transplanting mature neurons was considered as a non-viable option because of the complexity of harvesting neurons in 2D without severely damaging neurites. With C-Stem™, hiPSC rosettes can be differentiated into neural microtissues containing mature dopaminergic neurons.

In preclinical models of Parkinson's disease, such microtissues demonstrated rapid and efficient integration into the host tissue, triggering complete motor-function recovery within 4 months, while mitigating proliferation risks.[51] This proprietary program for Parkinson's disease is scheduled to enter the clinic in 2025.

In addition to neurodegenerative disorders, the C-Stem™ technology is currently being used for the development of novel cell therapy modalities in the field of cardio-metabolic disorders and immuno-oncology, through strategic alliances with biotechnology and pharmaceutical players.

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