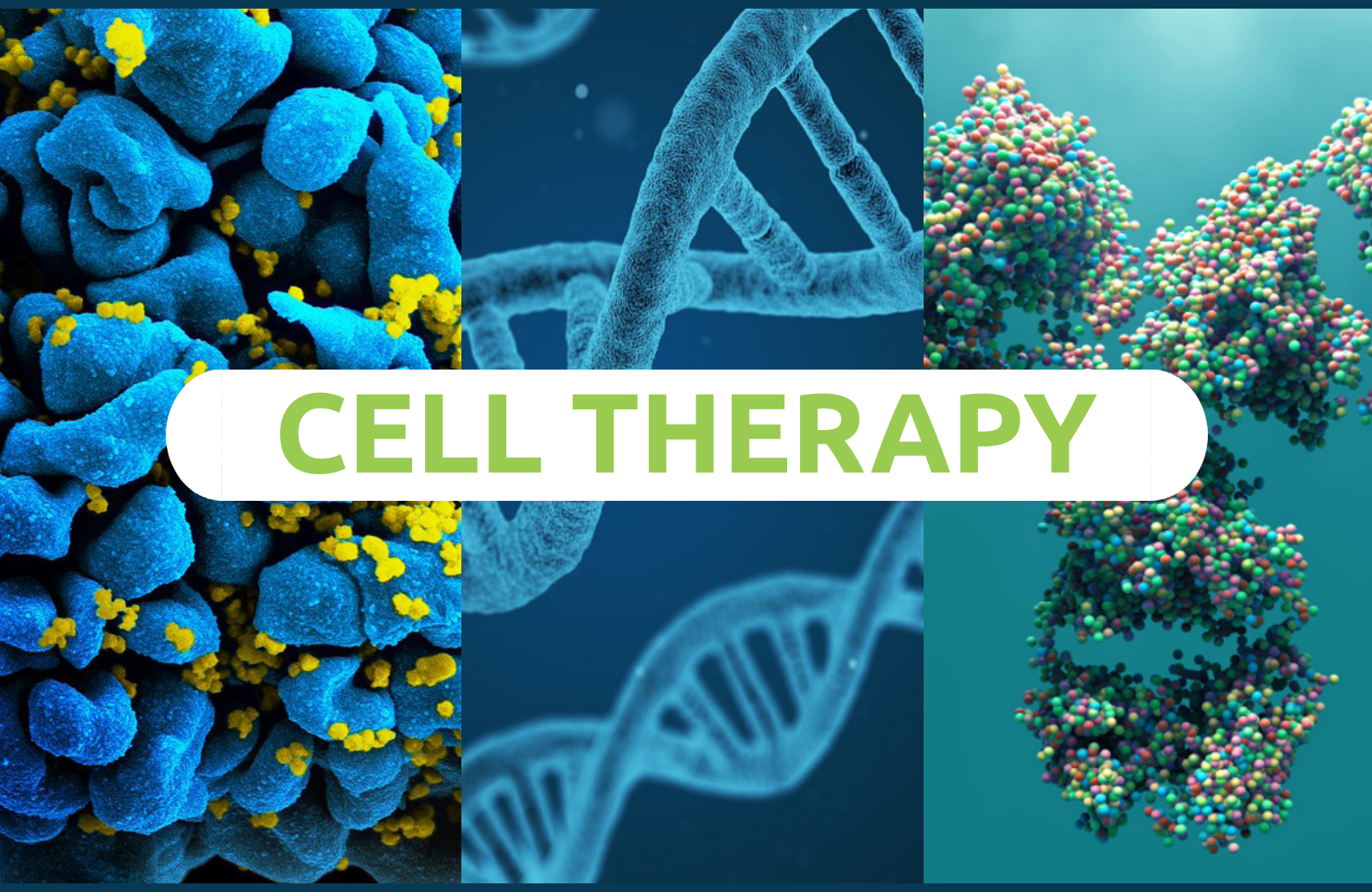
IMMUNOWATCH

EDITION N°5 - JULY 2022



CELL THERAPY

mabdesign
THE BIOTHERAPY EXPERT

INTRODUCTION

MabDesign's Immunowatch is a one-of-a-kind information monitoring newsletter in the field of biologics. Its aim is to provide members of our association with the most recent and pertinent data gathered or generated through the key expertise of MabDesign and its collaborators in scientific research, business intelligence, market analysis and intellectual property.

Each edition will focus on trending type of biologics. Its general format includes market study research, financial and economic data, invited contributions from scientific teams working in the industry or in academia and a section dedicated to intellectual property. The content of each edition is decided by an editorial composed of two field experts. Decision concerning the theme and conception of each newsletter is done in-house by the permanent members of our editorial team.

Finally, we would like to acknowledge the support of the Ambition Recherche & Développement (ARD) Biomédicaments 2020 Phase II programme, funded by the Centre Val de Loire region during the initial phases of launching this newsletter.

BIOPHARMACEUTICALS
Innovation Dynamics in Health
IN REGION CENTRE-VAL DE LOIRE

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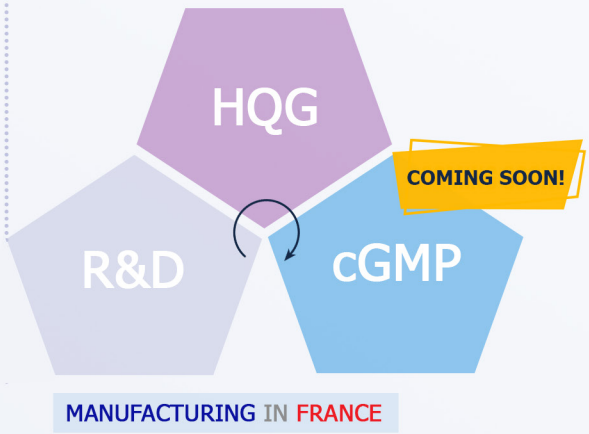

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Editorial

Sophie Derenne

Établissement français du sang

Frédéric Desdouits

Treefrog Therapeutics

Over the past 3 years, global investments in regenerative medicine doubled, reaching nearly $23 billion in 2021 (Alliance for Regenerative Medicine, 2021). Fuelled by the clinical success of CAR-T products against haematological malignancies, the cell therapy industry is now growing fast, and netted over $12 billion in global investment last year. However, manufacturing remains a critical bottleneck in the cell therapy field. According to the American Society of Clinical Oncology, the median drug price for CAR-T products is $411,278 (Brett Sahli et al. 2021), and it is estimated that manufacturing costs account for at least 50% of this price (BioProcess International, 2022). As of today, because of the manufacturing complexity and intrinsic limitations of autologous therapies, only a few thousands of patients can benefit from CAR-T cell products per year.

To address larger patient populations with readily available cell therapy products, the industry is now bringing allogenic strategies to the clinic, relying on stem cell- and donor-based cell sources. Such strategies further increase the need for scalable, reproducible and standardized manufacturing technologies. Automated systems have been developed for autologous approaches, but they only allow small and medium scale production to date: for example, the Cocoon™ platform (Lonza) integrating in a single automaton a complete GMP cell culture system incorporating all the constraints of containment, traceability and process management for the culture of adherent or suspension cells. In parallel, Miltenyi Biotec developed a production machine based on its CliniMACS system, the Prodigy®. This machine is ideally suited to the production of autologous CAR-T cell therapy products (Fraser, et al. 2017). The research grade CompacT SelecT™ automated machine marketed by the company Sartorius has proven its efficiency and reliability for the amplification and differentiation of several cell types derived from pluripotent stem cells in cell culture flasks. However, these automata still do not address the need for highly scalable manufacturing, especially with regards to strategies based on human pluripotent stem cells (hPSC).

The development of hPSC-based cell therapy products requires i) the large-scale expansion of hPSCs, and ii) their homogeneous differentiation and maturation into functional somatic cells. While 2D cell culture enabled to launch early clinical trials through scale-out, the industry is now moving to bioreactors use with the aim of scaling-up hPSC-derived cell therapies. However, the culture of hPSCs as aggregates or onto microcarriers within bioreactors has poorly met the needs of the industry so far. (Manstein F. et al. 2019) (Woon, Pandey et Ben-Nun 2022). Recently, the hybridization of biophysics and developmental biology led to the emergence of novel biomimetic approaches. For example, the C-Stem® platform developed by Treefrog Therapeutics, which relies on cell encapsulation to recapitulate the stem cell niche and protect hPSC and their progenies throughout bioreactor culture, recently demonstrated unprecedented yields in 10L bioreactors (>250-fold expansion within a week, 15 billion cells per batch).

Besides scale, cell bioproduction must be monitored to ensure the proper development and the safety of therapeutic cell-based products. Physico-chemical parameters (pH, O2, CO2,…) are easily monitored within bioreactors throughout bioproduction. However the continuous monitoring of biological parameters is more challenging. Cells are regularly sampled during culture to assess cell identity or viability. But this off-line approach remains insufficient to anticipate accurately production trends. New sensors are emerging, leading to the evaluation of cell metabolism or the composition of the culture medium (impurities for example). They can be directly connected to the bioreactor to monitor the culture parameters thanks to a feedback loop.

The combination of innovative sensors with artificial intelligence, mathematical modeling and finally, the development of digital twins, opens new possibilities for the in-line quality control of cellular products. They can redefine critical quality attributes and introduce the Quality-By-Design principle to cell therapy bioproduction.

Another challenge consists in the maintenance and control of genomic integrity during cell amplification and differentiation. Although karyotyping is required for the use of clinical-grade hPSC-based products, this readout poorly characterize product safety. New technologies, using NGS (Next-Generation Sequencing), are now capable of spotting copy number variations of specific interest within 2 or 3 days for fast batch release. These technologies, linked to those described above, allows for early interruption of improper batches, thus reducing costs.

Altogether, these innovations provide a better understanding of cell bioproduction, increase productivity, cell quality, and contribute to cost containment. This last point is critical if we want to be able to provide cell therapy to a large number of patients.

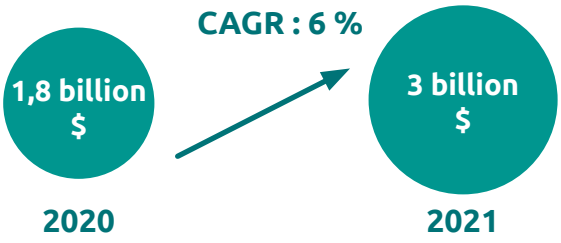
In the European Union, the cell therapy industry benefits from world-class scientific and clinical infrastructures, well-trained talents in stem cell biology and bioproduction, as well as a strong innovation ecosystem for the emergence of cell therapy biotech companies. There is therefore opportunity for the EU to keep up with the international competition, led by the USA and Japan, in the field of cell therapy, especially if European efforts are focused on the manufacturing front to address the main bottleneck of this industry.

GLOBAL CELL THERAPY market

Discover the marketed products, pipeline drug candidates, major deals and biopharmaceutical companies

Cell therapy market

Global market



35 marketed products

Marketed products

Drug Name	Drug Descriptor
Apceden	Autologous Therapy; Dendritic Cell Immunotherapy (DC Vaccine)
AstroStem	Autologous Therapy; Regenerative Medicine; Stem Cell Therapy
autologous cultured cartilage	Regenerative Medicine
CardioRel	Autologous Therapy; Stem Cell Therapy
Carticel	Autologous Therapy; Regenerative Medicine
Cartistem	Allogeneic Therapy; Regenerative Medicine; Stem Cell Therapy
Cellgram-AKI	Allogeneic Therapy; Regenerative Medicine; Stem Cell Therapy
Cellgram-AMI	Autologous Therapy; Regenerative Medicine; Stem Cell Therapy
Chondrosphere	Autologous Therapy; Regenerative Medicine
CreaVax-RCC	Autologous Therapy; Dendritic Cell Immunotherapy (DC Vaccine)
Cupistem	Autologous Therapy; Regenerative Medicine; Stem Cell Therapy
darvadstrocel	Allogeneic Therapy; Stem Cell Therapy
DCVax-L	Autologous Therapy; Dendritic Cell Immunotherapy (DC Vaccine)
Gintuit	Allogeneic Therapy; Regenerative Medicine
HeartSheet	Autologous Therapy; Regenerative Medicine; Stem Cell Therapy
Holoclar	Autologous Therapy; Regenerative Medicine; Stem Cell Therapy
Immuncell-LC	Adoptive Cell Therapy; Autologous Therapy
Jace	Autologous Therapy; Regenerative Medicine; Stem Cell Therapy
Kaloderm	Allogeneic Therapy
KeraHeal	Autologous Therapy
KeraHeal-Allo	Autologous Therapy
Maci	Autologous Therapy; Regenerative Medicine
Nepic	Regenerative Medicine; Stem Cell Therapy
Neuronata-R	Autologous Therapy; Regenerative Medicine; Stem Cell Therapy
Ocural	Regenerative Medicine; Stem Cell Therapy
Queencell	Autologous Therapy; Stem Cell Therapy
ReliNethra	Autologous Therapy; Regenerative Medicine; Stem Cell Therapy
ReliNethra C	Autologous Therapy; Regenerative Medicine; Stem Cell Therapy
remestemcel-L	Allogeneic Therapy; Regenerative Medicine; Stem Cell Therapy
Rethymic	Allogeneic Therapy; Regenerative Medicine
sakurashi	Regenerative Medicine
sipuleucel-T	Adoptive Cell Therapy; Autologous Therapy; Dendritic Cell Immunotherapy (DC Vaccine)
Stemirac	Autologous Therapy; Regenerative Medicine; Stem Cell Therapy
Stempeucel	Allogeneic Therapy; Regenerative Medicine; Stem Cell Therapy
StrataGraft	Allogeneic Therapy; Regenerative Medicine

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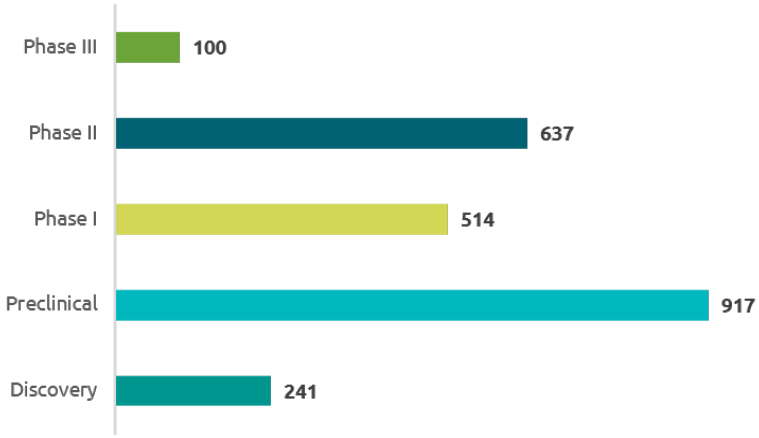
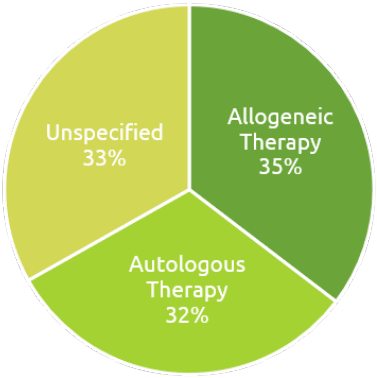
Cell therapy in development

Pipeline (from Discovery to Phase 3)

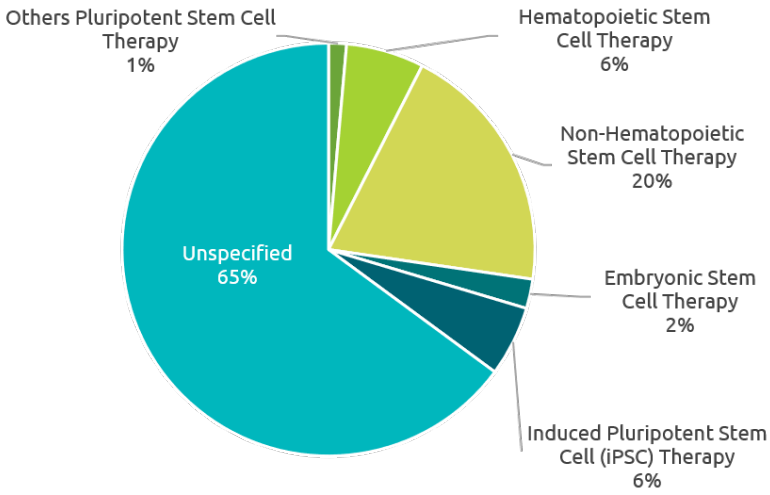
Phase distribution



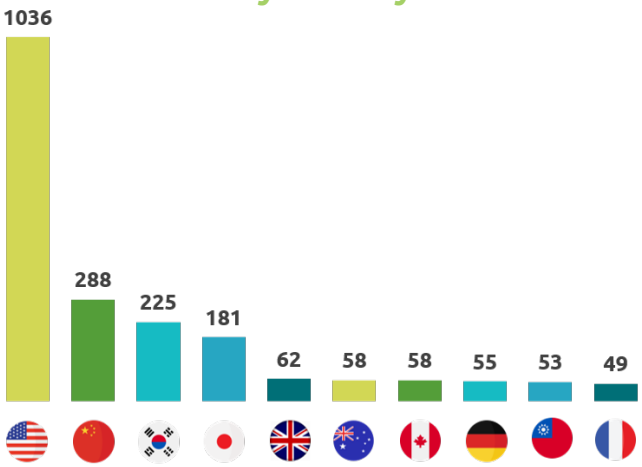
Cell therapy strategies (Allogenic Vs Autologous)



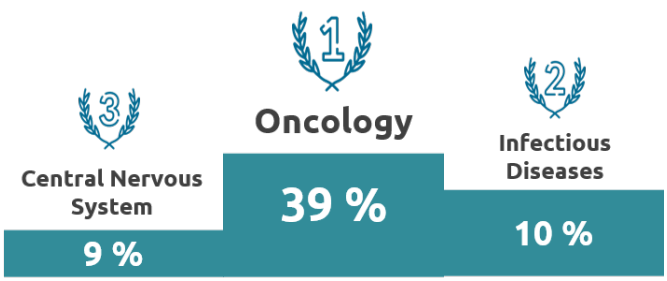
Cell therapy types



Number of developmental project by country



Top 3 therapeutic areas



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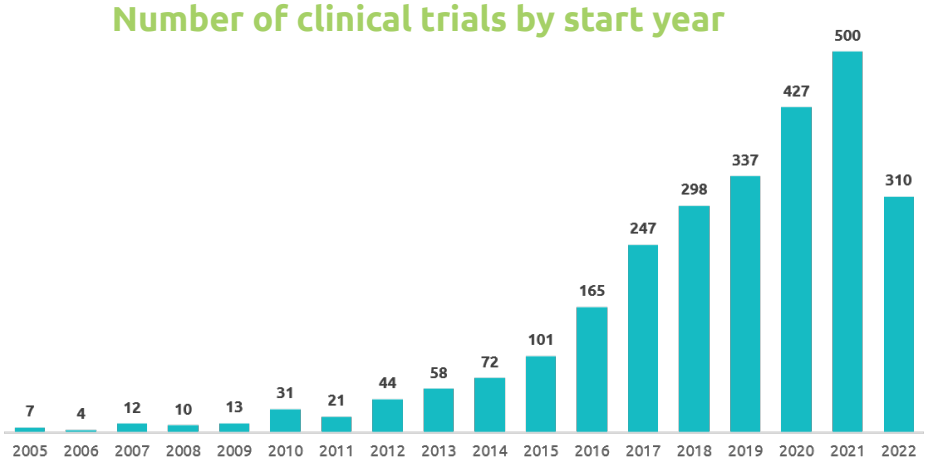
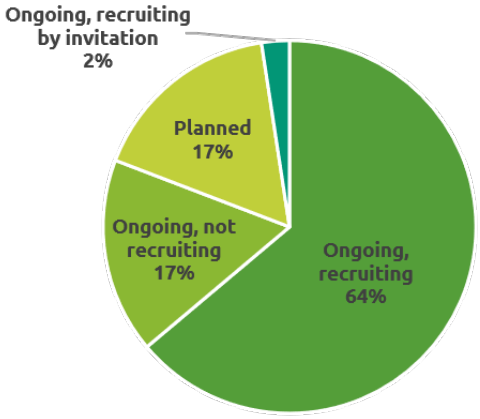
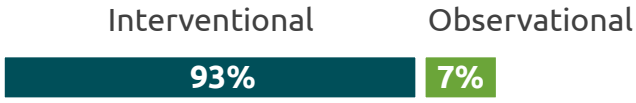
Cell Therapy Clinical Trials

Number of clinical trials by start year

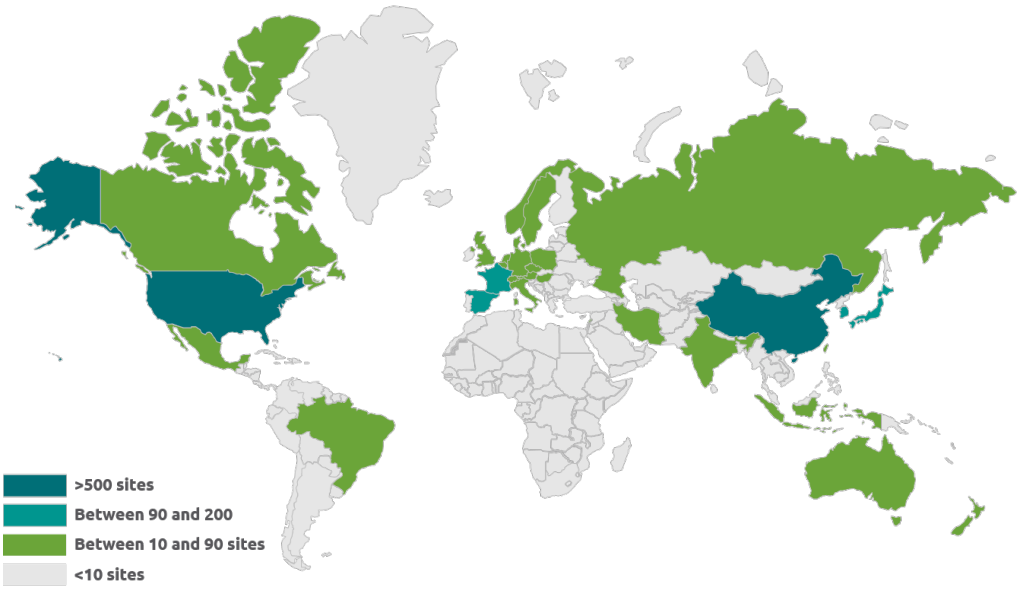


Clinical Trials By Status





Distribution of trial site by location



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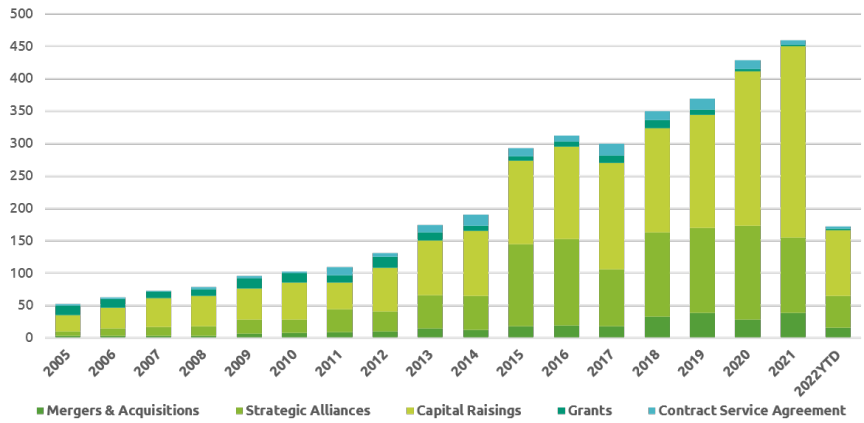
deals & companies

Cell therapy deal values

Number of deal by type and year



Top of deals

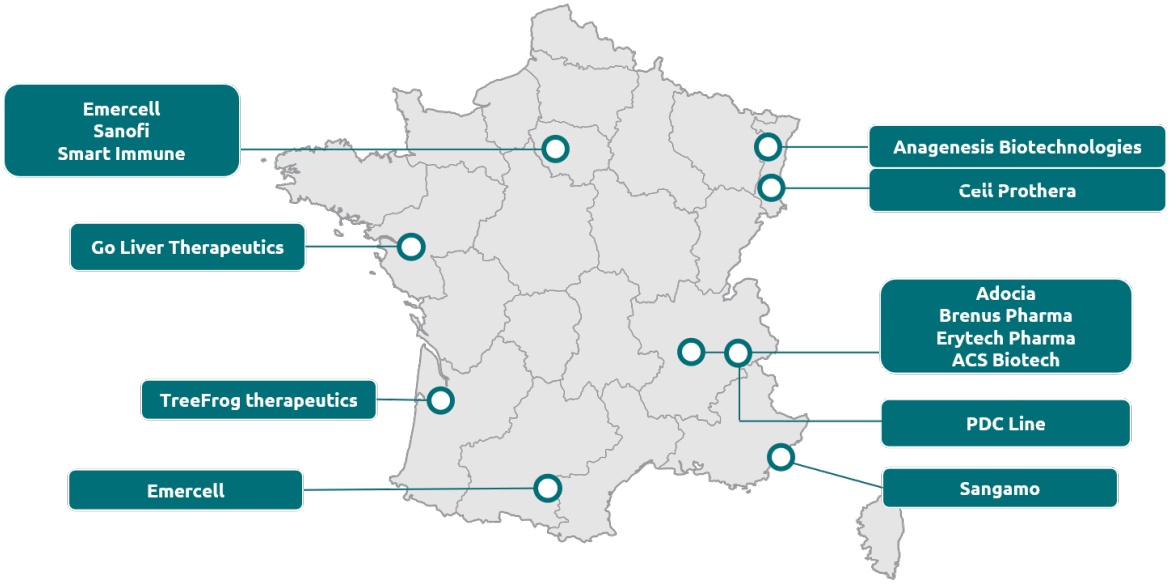
Capital Raising

Acquirers	Deal value
BioMarin Pharma	911 (millions US$)
Beam Therapeutics	800 (millions US$)
Intellia Therapeutics	690 (millions US$)

Strategic Alliances

Partners		Deal value	Deal description
Affimed	Genentech	5,1 (billions US$)	Affimed to Enter into Co-Development Agreement with Genentech
Genentech	Adaptimmune	3,25 (billions US$)	Genentech to Enter into Licensing Agreement with Adaptimmune
Kite Pharma	Sangamo Therapeutics	3,2 (billions US$)	Kite Pharma Enters into Licensing Agreement with Sangamo Therapeutics

List of cell therapy developers



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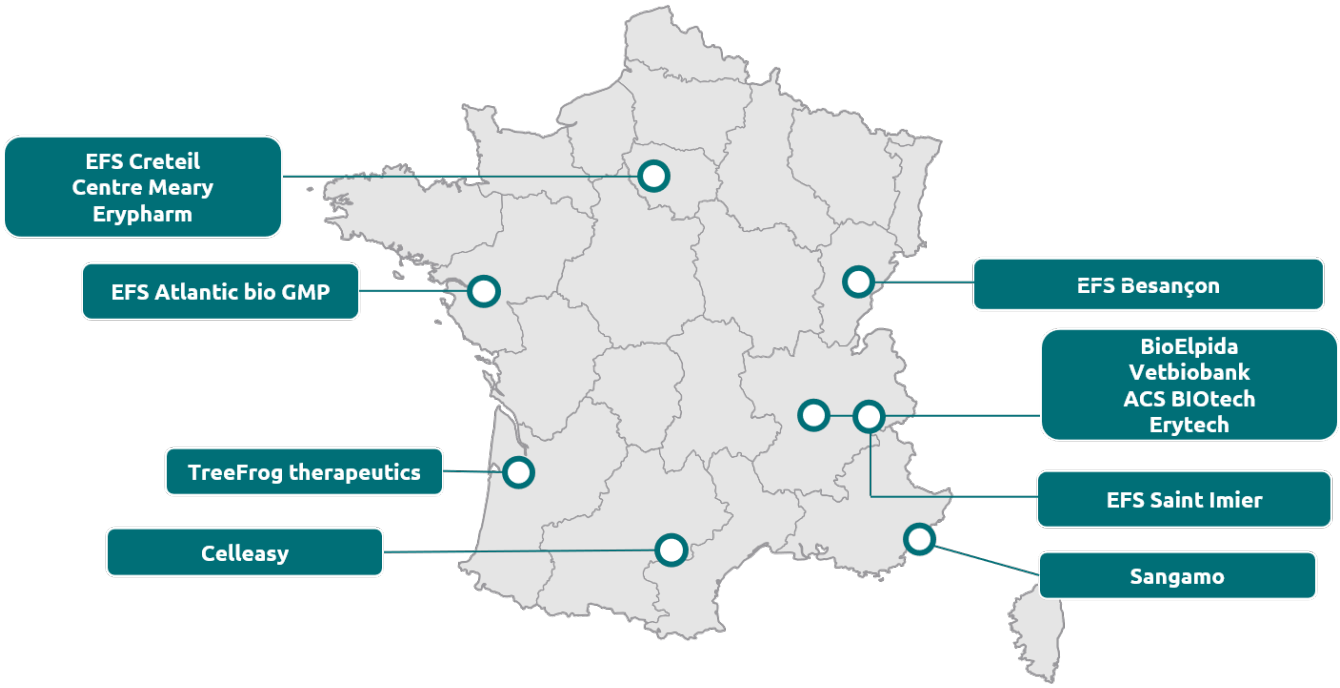
Cell therapy bioprocessing

Production sites of cell therapy in France



The SWOT is a strategic analysis tool use to evaluate the potential devlopment of cell therapy field.

S strenghts

- Improving regulatory framework
- High private as well as public investments in countries
- Technological advances in cell therapy manufacturing
- Clinical sucesses and cell therapy product on the market

W weaknesses

- Concerns associated with service/product quality
- High cost of infrastructure for development and manufacturing
- High chances of failure in cell therapy development
- Skilled personnel are required for developing new therapies

SWOT

O opportunities

- Government support for development through funds
- Presence of academic institutes and research centers : trend of spin-off of companies from research organizations
- Advancements in cell therapy technology provide ready-to-use fundamental support for development

T threats

- Ethical controversies related to use Cell Therapy
- Lengthy clinical trials that take years to complete
- The market is attributive to be highly competitive
- Need for high seed capital

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scientific articles

Read the different inputs from the scientific community on various aspects of cell therapy

INDUCED PLURIPOTENT STEM CELLS (IPSCS) VERSUS CD34⁺ /VERY SMALL EMBRYONIC-LIKE STEM CELLS (VSELS) FOR REGENERATIVE MEDICINE IN ISCHEMIC CARDIAC DISEASES

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INTRODUCTION

Current research in regenerative medicine is focused on finding pluripotent or multipotent cells with lower associated risks and fewer ethical problems when used as a treatment for patients. For a long time, embryonic stem cells (ESCs), which are immortalized pluripotent stem cells derived from an early embryo at the blastula stage, have been considered the ideal stem cells for applications in regenerative medicine, as they can give rise to cells from the three germ layers [1]. Their pluripotent differentiation potential theoretically enables them to generate any multipotent cell type, at least *in vitro* [2]. But there are still unresolved ethical and technical issues that have severily hampered their therapeutic use, which can up to now be considered to have clearly failed, despite the hype created by the media. The development of induced pluripotent stem cell (iPSC) technology by Yamanaka's research group in 2006 [3], has been considered as an exciting alternative source of pluripotent stem cells, avoiding ethical issues. However, their unexpected genomic instability and variability, resulting in a risk of tumorigenesis and of the development of immunogenicity, limits here again their clinical use [4]. In parallel, evidence has accumulated that adult bone marrow harbors different primitive cells which possess the ability to repair organs outside the hematopoietic system. It has been observed that in myocardial infarctions (MI), the injection of CD34⁺ cells close to the injured myocardial area can achieve a significant restoration of cardiac function, suggesting their direct and/or indirect involvement in the regeneration of heart tissue. This thought was sustained when pluripotent cells known as very small embryonic-like stem cells (VSELs), which are able to differentiate and regenerate different organic tissues, were identified and isolated among CD34⁺ stem cells [5]. They are able, in *in vivo* experimental models, of contributing to angiogenesis and myocardial repair representing a real alternative to ESCs and iPSC for cardiac regenerative medicine

iPSCs

iPSCs were developed based on the transduction of somatic mouse fibroblasts with a cocktail of four genes (Oct4, Sox2, c-Myc and Klf4) that encode transcription factors governing pluripotency via integrat¬ing vectors (virus, lentivirus), resulting in the acquisition of the morphology and growth properties of ESCs [3]. Yamanaka's team and others have further demonstrated that human adult cells can also be reprogrammed to restore ESC characteristics by introducing the same or additional set of transcription factors [6].

Despite their ability to multiply indefinitely and to differentiate again in all cell types, iPSCs remain distinct from ESCs. Indeed, it has been demonstrated that low-passage of mouse iPSCs-derived by factor-based reprogramming harbors residual DNA methylation signatures, and maintains characteristics of their somatic tissue of origin as epigenetic memory (methylation) and genetic signature (that extends to miRNA), which may affect their lineage-specific differentiation capacity [7]. In addition, iPSCs generated with reprogramming factors via either integrating retroviruses or lentiviruses might cause insertional mutagenesis [8]. Furthermore, major issues regarding iPSCs have presently limited their clinical use, for example, their teratoma formation ability [9] resulting from their somatic cells of origin or occurring during the stepwise reprogramming process to iPSCs [10], and the risk of associated cancer formation

linked to c-Myc and Klf4 known as potent oncogenes [11].

In order to increase the safety of iPSCs for clinical applications, human iPSCs technology has evolved through various non-integrative approaches [reviewed in 12]. All of these methods have made it possible to theoretically avoid the risk of insertional mutagenesis and genetic alterations.

iPSCs have aroused significant interest over the past decade in relation to cardiac repair, particularly in the treatment of MI through regeneration. Reprogramming of fibroblasts into cardiomyocyte-like cells has been first experimented in mice, but only a small proportion of these converted cells displayed spontaneous contractions and express cardiomyocyte global gene expression profiles. In humans, forced expression of cardiac transcription factors GATA-4, Hand2, TBX5, and myocardin combined with muscle-specific microRNAs have shown that fibroblasts can be reprogrammed to cardiac-like cells and displayed sarcomere-like structures and calcium transients. However, here again, only a small subset of such cells exhibited spontaneous contractility [13] Addition of Hand2 and a constitutively active form of AKT to the identified cardiac transcription factors synergistically ac-tivated genome-wide cardiogenic stage-specific enhancers [14]. In addition, human fibroblasts could be converted into functional cardiomyocytes by a combination of nine small molecules, resulting in induced cardiomyocyte-like cells that uniformly contracted and resembled human cardiomyocytes in their transcriptome, epigenetic, and electrophysiological properties [15].

Combining three human iPSCs-derived cardiac cell types in a three-dimensional micro-tissue could improve sarcomeric structures with T-tubules, enhance contractility and mitochondrial respiration, and promote electrical maturation associated to connexin 43 (CX43 gap junction) and intracellular cyclic AMP (cAMP) pathway activation [16]. Sustained activation of AMP-activated protein kinase in human iPSC-derived cardiomyocytes via Sirtuin activation improved differentiation, leading to decreased acetylation of histones H3 (at Lys9 and 56) and H4 (at Lys16), and increased mRNA and protein expression of both TNNI3 and TNNT2 [17].

Given the problems with rejection that can arise during the use of iPSCs due to the need for MHC matching and immunosuppressive treatment [18], another debate has emerged regarding the use of autologous versus « on the shelf » allogeneic iPSCs. The autologous setting implies a selection of cells specific to the patient, making their use lengthy, expensive, laborious and regulated. It could take several months of preparation, which may be unrealistic for the treatment of diseases such as MI. Transplanted allogenic iPSCs-derived cardiomyocytes, in association with immunosuppressors, can survive for up to 12 weeks in a macaque heart, but these animals present¬ed ventricular tachycardia, which could be due to the immature state of the transplanted cells [19].

Although some reports have demonstrated that cardio¬myocytes derived from iPSCs can be grafted into myocardium of animals and improve left ventricular function [20), in most cases iPSCs differentiate into immature cardiomyocytes - which are unable to restore massive loss of cardiac tissue responsible of fibrosis and scar formation and induce a po¬tential risk of arrhythmic complications - rather than to adult functional cardiomyocytes harboring their structures and gene expression profiles, particularly in larger animals [19].

A clinical pilot study using allogeneic iPSCs-derived cardiomyocytes directly injected in the myocardium during a CABG operation has been recently launched in China but is still in the recruitment process of five patients with ischemic heart disease.

Other groups have reported that iPSCs do not directly contribute to the regeneration of cardiac tissues but are sources of growth factors that provide only a paracrine effect. Recent studies using a mouse model of MI show that the transplantation of iPSCs can improve heart function via paracrine action [21]. In addition, iPSC-derived extracellular vesicles induce superior cardiac repair *in vivo* than cell transplantation,

representing a safer alternative [22]. In 2018, the Japanese Government approved a world-first study in which the investigators prefer to surgically implant degradable sheets of heart muscle tissues made from allogeneic iPSCs onto the external surface of the infarcted area as a source of growth factors, micro-vesicles, or exosomes, rather than integrating iPSCs-derived cardiomyocytes into the host cardiac tissue. The first of the ten patients scheduled was treated at the beginning of 2020, but no data are available yet.

Further investigations are firmly required to develop safer and more efficient iPSCs-derived cardiac cell therapy .

CD34⁺ cells and Very Small Embryonic-Like stem cells (VSELs).

To date, so-called "adult stem cells" have mainly been used for regenerative medicine in heart diseases. CD34⁺ stem cells (SCs) appear to emerge as the most convincing cell type among those that have been experimentally and clinically evaluated [23]. They are identified via the CD34 antigen, a membrane glycophosphoprotein that was discovered in 1984 by Civin et al. as a result of a strategy to develop antibodies that specifically recognize small subsets of human marrow cells, but not mature blood or lymphoid cells. CD34 antibodies specifically detect approximately 1% of low density mononuclear cells from BM aspirates of normal donors, when there is less than 0.1% CD34 labeling of PB cells [24]. CD34⁺ cells have long been considered as solely hematopoietic stem cells (HSCs), giving rise to all hematopoietic lineages. However, the "true" HSCs population, inducing and sustaining post-aplasia hematopoietic recovery in the long-term, and characterized by a lack of the CD38 differentiation marker [25], only represents a small portion (≈1%) of the total CD34⁺ cells, thus raising the question of the identity of remaining CD34⁺ cells. On 1997, Asahara et al., demonstrating that endothelial progenitor cells capable of inducing neo-angiogenesis also bore the membrane CD34 antigen, opened the door to new investigations into the existence of potential non-hematopoietic CD34⁺ SC subpopulations [26]. In the following years, researchers progressively established that the CD34 antigen was also a marker of liver, osteoblastic, cartilage, and cardiac progenitor cells which could be used for cell therapy, each of these CD34⁺ subpopulations representing approximately 1% of the total CD34⁺ cells [reviewed in 23]. In fact the intensi¬ty of CD34 expression on the cell's membrane is heterogeneous and correlates with the stage of cell immaturity/maturity, subdi¬viding cells into $CD34^{bright}$ or $CD34^{dim}$ subgroups. The $CD34^{bright}$ are smaller and less dense than the $CD34^{dim}$ and correspond to the earliest progenitors, i.e., stem cells, while the $CD34^{dim}$ are larger and denser and correspond to more committed progenitor cells, having lost their clonal growth properties [27].

Data from experimental MI studies performed in SCID-nude mice showed that human CD34⁺ SC engrafted in the ischemic area and that their progeny differentiated into cardiac and endothelial lineages, which correlated with sustained cardiac-function improvement [28, 29].

On the end of 2002, we launched a pilot study using autologous peripheral blood (PB)-CD34⁺ SCs, collected by leukapheresis (LKP) after granulocyte-colony-stimulating-factor (G-CSF) mobilization and then immuno-selected, in patients with very poor-prognosis MI scheduled for compassionate coronary artery bypass graft (CABG) operations not reperfusing the ischemic area [30].The patients'short- and long-term outcomes were very consistent, with an average progressive increase in left ventricle ejection fraction (LVEF) of 21 points from the baseline values 48 months after the procedure, and sustained structural and functional scar repair demonstrated by PET-scan imaging and 3D echography. 5/7 patients are still alive and well with an average follow up (FU) of 17 years (range 19-15 years). One, aged 84 years, died of a stroke at 12 years FU. Three of these patients had been initially scheduled for urgent heart transplant but have avoided it so far. In the same study, we had demonstrated that G-CSF-mobilized CD34⁺ cells included cells featuring immunophenotypic and gene characteristics of both endothelial and cardiac-muscle progenitor cells. Additionally, *in vitro* culture of still undifferentiated $CD34b^{right}$ cells in a specific and proprietary MV06™ medium induced the further development of adherent cells co-expressing characteristics of

endothelial (VEGFR-2/CD133) or cardiac-muscle (c-Troponin-T and sarcomeric-α-actin) lineages, suggesting the initiation of endothelial- and cardiac muscle-cell differentiation pathways [30]. By contrast, $CD34^{dim}$ mainly co-express CD133/CD45, characterizing them as granulocytic progenitor cells [27].

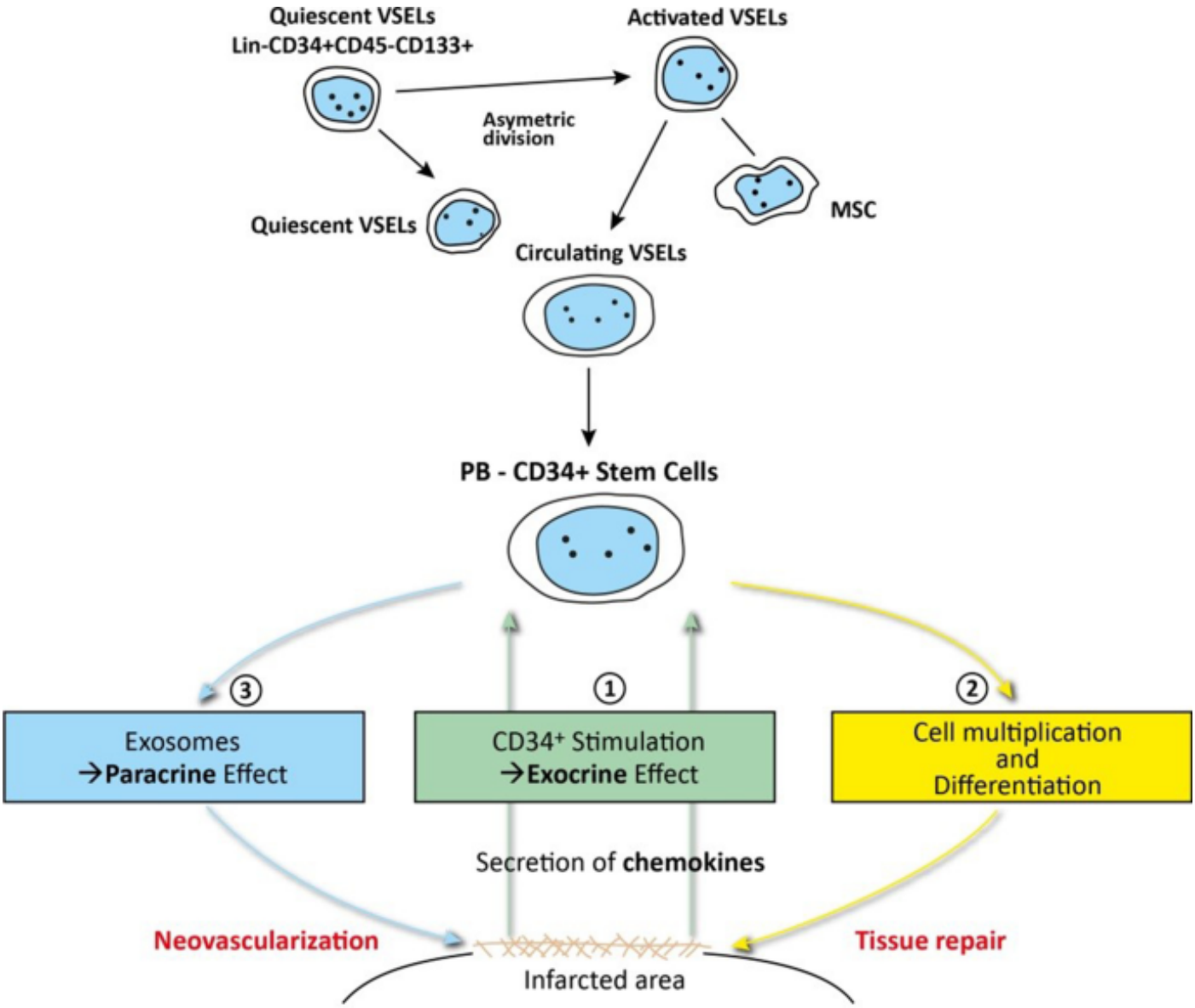
Thus, considering their large capacity to differentiate in various cell tissues, it now seems that some, if not all, $CD34^+$ cells might be pluripotent rather than multipotent stem cells, capable of giving rise to a large panel of organic tissues under specific stimulations. This hypothesis appears now to be realistic since Ratajczak's team described in 2006 a type of cells which have kept their embryonic capacities in the bone marrow of adult mice [5]. Indeed, these cells he called « VSELs» have similar char-acteristics to ESCs and are believed to originate from primordial germ cells in the yolk sac, after which they migrate, escape specifi¬cation into tissue-committed stem cells, retain their pluripotency, settle in different organs and persist throughout life [31]. Their existence was further demonstrated in humans by the same group and several others [reviewed in 4]. Interestingly, VSELs are phenotypically characterized as being $CD34^+$, $CD133^+$ and/or $CXCR4^+$, but do not express lineages (Lin^-) and hematopoietic ($CD45^-$) markers [5, 32]. They can be sorted and isolated on the basis of their phenotypic features and their small size (5 to 6 µm). They express pluripotent ESCs specific mark¬ers, such as SSEA-4 and TRA-1-81, on their surface, and Oct-4, Nanog and Sox-2 transcription factors at the protein level. VSELs constitute a tiny, homogeneous and quiescent pluripotent fraction of $CD34^+$ stem cells. Their quiescence under the steady state prevents them from over-prolifera¬tion and potential risk of teratoma formation and is related to the expression of low levels of genes involved in proliferation and cell signaling, which become upregulated during cell activation. In addition to the bone marrow and peripheral blood, VSELs have also been identified in umbilical cord blood and other organs [32], diversifying their source of collection and sampling. They can support vessel formation *in vivo*, and be specified to cardiomyocytes, neurons, epithelial lung, and HSCs, both *in vitro* and in animal models under appropriate physio-pathological stimuli. [reviewed in 33]

The affiliation between VSELs and $CD34^+$ stem cells thus now appears clearer. Purified $CD34^+$ cells contain only a tiny fraction of $CD45^-$ cells containing VSELs, which explains the need for a large number of injected cells to have a significant effect on organs repair. However, VSELs can be mobilized from the BM into the PB in larger numbers by G-CSF, and expanded ex vivo [33, 34]. It has also recently been shown that expanded VSELs can efficiently give rise to endothelial colony-forming cells when stimulated by nicotinamide acid [35].

We have developed a GMP automated manufacturing process allowing the expansion of autologous $CD34^+$ cells for their production on a large clinical scale to be used for cardiac repair after MI. The final product, which has been labelled as « ProtheraCytes® », is considered advanced therapy medicinal product (ATMP) by the authorities [36]. We have recently demonstrated that this industrialized process also expands accurately the $CD34^+$ VSELs among ProtheraCytes® by around a ten-fold from baseline. Furthemore, beside pluripotent VSELs , ProtheraCytes® also contain multipotent $CD34^+$ cells expressing early markers of the cardiac and endothelial pathways, the combination of both making them very good candidates for sustained heart repair after MI [37]. A Phase I/IIb clinical trial using autologous ProtheraCytes® endocardially reinjected in the heart of patients suffering of severe MI is ongoing (EXCELLENT trial, EUDRACT 2014-001476-63).

The underlying cardiac-repair mechanisms of VSEL-derived $CD34^+$ subpopulations are probably multi-faceted (reviewed in 37, and Fig. 1). First, a complex blend of cardioactive chemokines secreted by the inflammatory scar chemoattracts the injected $CD34^+$ SCs to home in on the ischemic zone and induces their commitment along the endothelial and cardiac pathways. Then, once activated by the scar chemokines, $CD34^+$ SCs may release soluble paracrine factors and exosomes that can enhance the proliferation of resident cardiomyocytes or support neoangiogenesis, respectively, reducing fibrosis and attenuating remodeling effects. These cellular and molecular events are strongly dependent on changes in myocardial stiffness that occur after MI. Such commitment is crucial for the induction of cardiac tissue repair after

ischemic heart disease. Furthermore, the hypoxic environment of the infarct zone increases vascular endothelial growth factor (VEGF) expression by transplanted cells, which may accelerate the proliferation of endothelial cells and α-SM actin-positive cells.



1) Secretion of chemokines by damaged tissue will stimulate injected CD34+ stem cells

2) CD34+ are chemoattracted by the lesion, multiply and differentiate in situ to repair the damaged tissue

3) CD34+ then secrete exosomes containing mRNA capable of inducing revascularization of the damaged tissue

Figure 1: *Proposal for the different biological mechanisms involved in CD34+ stem cell cardiac regenerative medicine (MSC : mesenchymal stem cells ; PB : peripheral blood)*

CONCLUSIONS

Besides ESCs, other cell types have been retained by different groups of researchers (i.e., CD34⁺ SC, iPSCs and VSELs) to determine the benefits and drawbacks related to their future development with a view to applications in cardiac regeneration (Figure 2). Despite the emergence of iPSCs and the hope they have aroused as an alternative to overcome the frustrating problems raised by ESCs, no clinical trial in heart disease has been successfully achieved to date. The main hurdles to overcome are the risk of tumorigenesis, the development of life-threatening arrhythmias and immunogenicity. Moreover, engineering iPSCs is a lengthy and costly process. The iPSCs should not be realistically employed in the clinic in the foreseeable future until significant progress in their clinical safety has been achieved.

CD34⁺ stem cells taken from blood or bone marrow have displayed effectiveness in the treatment of blood diseases, but other unexpected therapeutic dimensions related to these cells have been identified in recent years. It is worth considering whether isolated and expanded CD34⁺ VSELs could be safer and more efficient to improve heart function and cardiac remodeling when used in regenerative medicine. They represent a credible alternative to ESCs and iPSCs, as an easier-to-access source of pluripotent stem cells, with no ethical issues, no epigenetic modifications and no undesirable side effects as risk of teratoma or cancer formation. However as purified CD34⁺ cells, that have already been demonstrated to be effective as a treatment for MI, contain both already committed cardiac and endothelial progenitor cell subpopulations and VSELs, it would be better to use the total CD34⁺ product rather than purified/expanded VSELs alone. It is likely that already committed cells would begin straight on the process of cardiac repair, followed in a second phase by the stimulation of quiescent VSELs, that will induce their long term muliplication and differentiation. These complementary mechanisms would both together allow a rapid and long-term sustained cardiac repair. It is in that way we have developed our industrialized platform for production of ProtheraCytes® and launched the EXCELLENT trial for their clinical use in ischemic cardiac diseases.

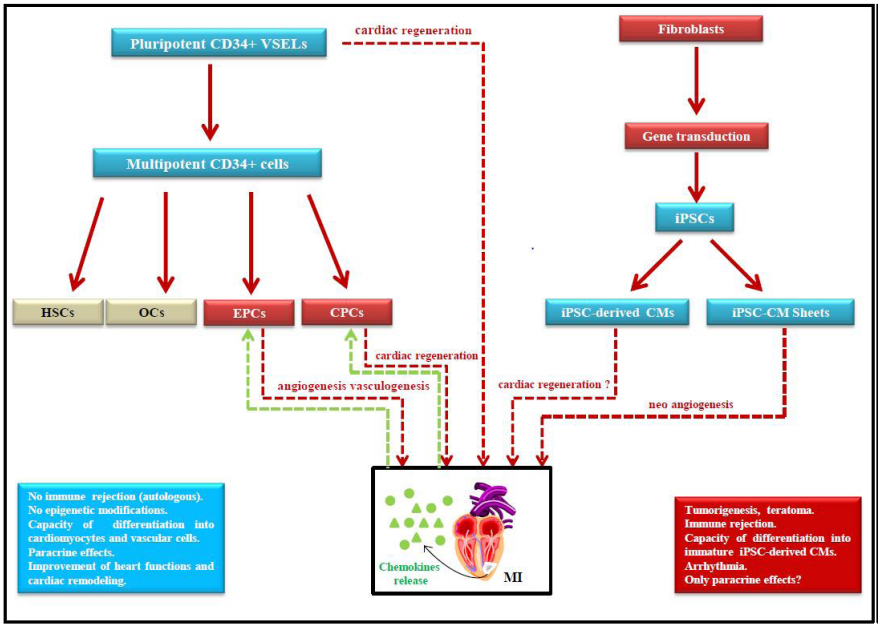


Figure 2: *Regenerative medicine in ischemic heart disease. Advantages and limitations of CD34⁺ and iPSCs cell-based therapies in the treatments of myocardial infarctions. HSCs indicates hematopoietic stem cells; OCs, osteogenic cells; EPCS, endothelial progenitor cells; CPCs, cardiac progenitor cells; iPSCs, induced pluripotent stem cells; CMs, cardiomyocytes; MI, myocardial infarction.*

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IMPACT OF GENETIC INSTABILITY OF IPSC CLONES FOR THE GENERATION OF ABNORMAL TISSUE AFTER TRANSPLANTATION

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Abstract

Cell therapy using induced pluripotent stem cell (iPSC) derivatives may result in abnormal tissue generation because the cells undergo numerous cycles of mitosis before clinical application, potentially increasing the accumulation of genetic abnormalities. Genetic tests may predict abnormal tissue formation after transplantation if both are correlated. Here, we administered iPSC derivatives with or without single nucleotide variants (SNVs) and deletions in cancer-related genes with various genomic copy number variant (CNV) profiles into immunodeficient mice and examined the relationships between mutations and abnormal tissue formation after transplantation. No positive correlations were found between the presence of SNVs/deletions and the formation of abnormal tissues; the overall predictivity was 29%. However, a copy number higher than 3 was correlated, with an overall predictivity of 86%. Furthermore, we found CNV hot spots at 14q32.33 and 17q12 loci. Thus, CNV analysis may predict abnormal tissue formation after transplantation of iPSC derivatives and reduce the number of tumorigenicity tests.

Results

Genetic profiles of iPSC clones changed constantly during culture

De novo mutation may occur at any time during long-term culture. The variant allele frequency (VAF) percentage is determined by the number of sequence reads harboring mutations in genes relative to the total reads, and the VAF (%) profile can be used as a genetic marker of cells in culture, providing information regarding the degree of genetic heterogeneity among the cells in culture. Furthermore, changes in the cell population during culture can alter the VAF profiles, and the genetic stability of cells in culture can be assessed by measuring epithelium organized around a central cavity: the epiblast rosette. This 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, chronological changes in VAF profiles. To explore the relationships between genetic abnormalities and tumorigenic events after transplantation, we prepared five iPSC test clones with genetic mutations in the COSMIC (COSMIC - Catalogue of Somatic Mutations in Cancer, May 2022) Cancer Gene Census oncogene database (16E84, 16E85, 16H12, 15M38, 1210B2), which were selected under the hypothesis that abnormal tissues would be generated after transplantation of the iPSC derivatives, and two subclones derived from iPSC clones (Ff-WJs1401, Ff-I01) without mutations in the listed oncogenes. H9 embryonic stem cell (ECS) derivatives without mutations in the Census oncogene database and Shibata's List were used as normal transplantation controls. Notably, sequence errors in whole-genome sequencing (WGS) and whole-exome sequencing (WES) may occur, and the validity of VAF values, the limit of detection (LOD), and the decision limit of VAF are directly related to interpretation and judgment of the presence or absence

of single-nucleotide variants (SNVs) or deletions. We calculated the LOD and decision limit based on the relative standard deviations (RSDs) of VAFs obtained by WGS and WES for common SNVs/deletions (SNVs/del) in 16E84, 16E85, 16H12, and 15M38 and their derivatives, as previously reported (20, 21). Additionally, we set a VAF of 24% as the LOD in WGS, yielding an RSD of 0.3 (i.e., 1 / 3.3) and a VAF of 12% as the decision limit, yielding an RSD of 0.61 (i.e., 1 / 1.65). The VAF values of the genes in 1210B2 (Table 1A), Ff-WJ14s01 (Table 1B), Ff-I01 (Table 1C), 16E84 (Table 1D), 16E85 (Table 1E), 16H12 (Table 1F), and 15M38 (Table 1G) reached nearly 50% in autosomal chromosomes and nearly 100% in the X chromosome if a male sample was used, and most of these VAF values were maintained throughout the culture period, even after differentiation. These findings suggested that all of the iPSC clones examined in this study originated from single cells and that the majority of cells in culture consisted of clonal expansion from the single cell and its derivatives acquiring additional genetic mutations during culture, owing to their superior growth advantage over other cells under the given culture conditions.

Changes in the VAF profiles of iPSC clones maintained in the undifferentiated state can be used to assess genetic heterogeneity in iPSC clones. The VAF profiles of undifferentiated iPSC clones 16E84, 16E85, and 15M38 having mutations in the Census database varied; for example, the VAF for MYH9 increased from 7.0% (below the decision limit) to 29.6% (above the LOD) in 16E84-iPSCs (Table 1D), that of BCOR increased from 7.9% (below the decision limit) to 18.9% (above the decision limit) in 16E85-iPSCs (Table 1E), and that of MYH9 dropped from 17.4% (above the decision limit) to 0% in 15M38-iPSCs (Table 1G) during culture. Notably, the mutation in BCOR found in clones 16E84 and 16E85 was a deletion (Table 1D, 1E), and the other mutations in all tested cell lines were SNVs. Change in the VAF profiles of cells cultured with the same medium suggested that cells in culture may undergo constant mutation and that the proportions of cell populations may change if such mutations affected the proliferation potential. In some cases, the VAF profiles changed definitively before and after differentiation. For example, the VAF for BCOR dropped from 91.2% to 0%, that for BRD3 dropped from 49.4% to 0%, and that for MYH9 dropped from 29.6% to 1.1% when 16E84-iPSCs differentiated into retinal pigment epithelial cells (RPEs; Table 1D). Similarly, the VAF for BCOR dropped from 18.9% to 0% when 16E85-iPSCs differentiated into RPEs (Table 1E), suggesting that cells in culture included a variety of cells owing to constant acquisition of mutations, despite being derived from a single cell. Additionally, these findings suggested that specific cell populations benefitting from the RPE differentiation protocol expanded and became dominant during the long culture period (3 months).

Relationship between mutations in genes from the oncogene lists and histological outcomes after transplantation

Next, we explored whether mutations in the Census oncogene database and Shibata's List were related to abnormal tissue or tumor formation after transplantation. For this, we prepare two types of animal transplantation models. One was a subcutaneous transplantation model using severely immunodeficient NOG mice. This model is not a tumorigenicity test that is used in the clinical setting, but serves as a long-term QC test for *in vivo* culture when using mice as the ultimate incubator. Such results cannot be obtained using *in vitro* culture assays. The model has the advantage of permitting the histological examination of a large numbers of transplants. The other model uses orthotopic transplantation of cells via clinical route.

In this study histology of transplants of iPSC-RPEs (tissue differentiated into RPEs), iPSC-non RPEs (tissue not differentiated into RPEs), iPSC-derived cardiomyocytes (iPSC-CMs; tissue differentiated into CMs, and iPSC-non CMs (tissue not differentiated into CMs) derived from several clones (Table 1C–G). Notably, 16H12-iPSCs and 15M38-iPSCs could differentiate into RPEs, but not into CMs, suggesting a tendency to differentiate into a specific lineage and demonstrating that a cardiomyocyte differentiation protocol could be employed as a test to identify and eliminate cells with differentiation bias. H9-RPEs with no mutations in the Census database and Shibata's List showed normal RPE morphology. These

cells were used as positive controls of RPE transplantation derived from the other iPSC lines.

In a series of transplantation experiments, we did not find correlations between mutations in the Census database or Shibata's List with the formation of abnormal tissues or tumors in NOG mice. Then, predictivity of the detection of SNVs/del in cancer-related genes for abnormal tissue formation, including tumorigenesis, was analyzed among cell clones differentiated into target cells (RPEs, CMs, or NSCs). Thus, these findings may be useful for "go/no-go" clinical decision making by Institutional Review Boards. Hayashi's quantification method type II was used for statistical analyses (Table 2). In final target cells (RPEs, CMs, or NSCs) for transplantation, no deletions were detected; however, some cells had cancer-related SNVs/del. Notably, abnormal tissues were generated more frequently from iPSC-derived final products without mutations than from those with mutations. Among five abnormal transplants, including tumors, no tissues were generated from iPSC-derived products with cancer-related SNVs/del, and all five cases of abnormal tissue formation were generated from iPSC-derived products with no detectable cancer-related SNVs/del. By contrast, among nine normal transplants, five tissues were generated from iPSC-derived products with cancer-related SNVs/del and four tissues were generated from iPSC-derived products with no detectable cancer-related SNVs/del (discriminative ratio [= test specificity] was 44%). The overall predictability of the occurrence of abnormal tissue after transplantation based on the detection of SNVs/del in iPSC derivatives was 29% (less than 50%), with a correlation ratio of $\eta = 0.56$.

Abnormal histological images could be partly evaluated by tracing changes in copy number variants (CNVs)

Our series of *in vivo* transplantation studies support the hypothesis that not all cancer-related mutations may cause abnormal tissue or tumor formation after transplantation. Therefore, we next performed additional genetic tests to analyze if CNV profile could be a predictor of the formation of abnormal tissues after transplantation and evaluated whether it could be a relevant QC test predictive of IPS cell line safety. Accordingly, we used an SNP array (Karyostat HD; Thermo Fisher Scientific, MA) that could detect copy numbers at loci of 50 kb or more with 2.5 million probes to quantitatively evaluate CNVs. Notably, the sites of CNVs changed during the differentiation process (Figs. S3, S4); that is, some sites became hard to detect, whereas others were newly detected, suggesting the profiles of CNVs could serve as a genomic marker of the dominant cell population in the given culture. Additionally, this dominant population may change if the acquired de novo mutations affected the proliferation potential of the cell during culture in the undifferentiated state or if the new culture conditions during differentiation selectively supported the growth of a specific cell subpopulation. Although cells in culture had CNVs at various loci, their copy numbers seldom exceeded 3 (Figs. S3, S4). Moreover, two common hot spot loci with a copy number of 4 were detected in iPSC clones and their derivatives, regardless of culture medium or differentiation status. These loci were 14q32.33 (16E84-CMs, 16E84-non-RPEs, 16E85-non-RPEs, 15M38-iPSCs, and all derivatives, i.e., Ff-WJ14s01, Ff-I01-RPEs, and Ff-I01-NSCs) and 17q12 (16E84-iPSCs, 16E85-non-CMs, 16E85-RPEs, 16E85-non-RPEs, 16H12-non-RPEs, and Ff-I01-RPEs; Table 1 and Figs. S3, S4). Digital droplet DNA polymerase chain reaction (PCR) for the amplicons covering the locus at Chr.14:106260714 in Chr.14q32.33 or Chr.17:36120285 in Chr.17q12 (GRCh38) was performed (Fig. S5), and the results supported the findings of the CNV array. The predictivity of CNVs for abnormal tissue formation was analyzed among cell clones differentiated into the target cells (RPEs, CMs, and NSCs), and the results are shown in Table 2C. Out of five abnormal transplants, five tissues were generated from iPSC-derived products with a copy number of 4 (discriminative ratio [= test sensitivity] was 100%). Additionally, out of nine normal transplants, seven tissues were generated from iPSC-derived products with a copy number of 3 or less (discriminative ratio [= test specificity] was 78%). The overall predictability of abnormal tissue formation by detection of a copy number greater than 3 was 86% with as correlation ratio η of 0.75.

Discussion

Notably, the results of WGS may also vary depending on the sequencing method, reagents, analysis software, and coverage depth as well as the version of the reference genome assembly (GRCh37 or GRCh38). The LOD or decision limit of VAFs scored needs to be discussed to make an appropriate interpretation of WGS/WES results. Indeed, several reports have shown that WGS/WES sensitivity for detection of SNVs can vary by the sequence error rate, coverage rate, and detected variant reads. The standard read depth of 30–50× with WGS has been reported to be insufficient to detect SNVs with rates of less than 15% for analysis of tumor complexity. Moreover, one report showed that calculation of the LOD of VAF should be performed with coverage depth and variant reads above a minimum threshold to minimize the probability of false-positive and false-negative results. Based on their calculations (http://app.olgen.cz/clc/), an LOD of VAF of 24% could be obtained with a recommended coverage of 42 and a minimum variant reads number of 3, consistant with our proposed LOD of VAF of 24%, calculated with an RSD of 0.3, as determined by VAF values from an approximately 60 reads average. Therefore, judgment of the detection of SNVs/del may vary dramatically based on these sequencing parameters. Furthermore, the cancer-related oncogene Census database is revised frequently as new reports are published. These findings supported the necessity of understanding the limitation of sequence results and the sensitivity of detection to genomic changes (variant types and their allele frequencies) when we interpret genomic/epigenomic data obtained from state-of-the-art technologies, such as NGS. Mutations in the Cosmic Census and Shibata's List have been suggested to play some roles in cancer development on the basis of correlation and/or causality.

In this study, we found a positive correlation between the presence of copy number greater than 3 and the formation of abnormal tissues after transplantation; the overall predictability was 86%. CNVs have been used to diagnose genetic abnormalities in congenital diseases and characterization of cancer. Although commercially available SNP-based CNVs arrays are not designed for the detection of genetic structural abnormalities or aberrations generated during long culture periods, several hot spot loci for CNVs have been reported previously. In the current study, all cells in the culture showed genomic loci with CNVs. Although their copy numbers seldom exceeded 3, we found two hot spots with copy numbers exceeding 3 at 14q32.33 (n = 6), 17q12 (n = 6), or both (n = 1) among the 24 cases examined, consistent with previous reports showing a copy number of 4 at 14q32.33. In particular, four of 18 iPSC clones in the Korea National Stem Cell Bank showed a copy number of 4 at either 14q32.33 or 17q12, and no other locus was found to have a copy number greater than 3. The mechanism for the selective DNA amplification at 14q32.33 and/or 17q12 and the biological impact of this mutation on abnormal tissue formation from iPSC derivatives are currently unclear. One plausible explanation is that the 14q32.33 locus, located at the tip of chromatin, and genes nearby the telomere have a higher chance of amplification to maintain the chromatin structure. Moreover, amplification of JAG2 encoded in the 14q32.33 locus could confer tumorigenic proliferation to the cells, as has been reported in cancer cells. The HER2 gene is encoded in the 17q12 locus, and amplification of HER2 triggers multiple proliferation signals and drives genomic instability along chromosome 17q, leading to different patterns of gene amplification. Therefore, amplification of the 17q12 locus confers cells with uncontrolled proliferation, and if cells have 4 copies of HER2, the cells would become dominant in the culture.

Contributions

Cell preparation: TY; WGS: NT ; animal transplantation study and histology: TY; RPE differentiation: MT; CM differentiation: TY; NS/PC differentiation: YK and HK; PCR study: TY; statistical analysis: YS; manuscript preparation: TY, SK, and YS; manuscript review: SY, SK.

A

Cell line	1210B2	SNV (VAF (%) in WGS)										CNV		Outcome	
		SCNN1D	SH2D5	RYR2	FOXO3	HMCN2	ARID5B	PTPRCAP	SLFN12	MEGEB1		Max. CN	Locus	Graft	Cell/Tissue
Cell typing	iPSC-C p12	45,0	51,1	54,7	37,9	47,5	49,4	45,2	19,8	44,0		ND	NA	NA	NA
	iPSC-K p18	47,4	58,3	49,4	44,7	60,6	53,1	44,2	27,3	50,7		3	Multiple	NA	NA
	NSC	51,3	55,2	44,4	43,5	56,1	52,8	49,3	17,5	43,2		3	Multiple	Normal	Neural cells

B

Cell line	Ff-WJ14s01	SNV (VAF (%) in WGS)										CNV		Outcome	
		ADAM33	SYNE2	PITPNM3	ZFN677	SLC12A4	EIF4A1	TMEM178B	TMEM63A	GML		Max. CN	Locus	Graft	Cell/Tissue
Cell typing	iPSC-C p15	43,5	36,6	57,4	53,4	31,3	21,3	18,6	0,0	0,0		4	14q32.33	NA	NA
	NSC p4	43,5	46,5	55,9	46,9	17,5	13,6	21,7	3,8	11,8		3	Multiple	Normal	Neural cells
	NSC p8	54,9	50,0	65,1	57,5	4,1	2,8	6,9	24,1	29,5		ND	ND	ND	ND

C

Cell line	Ff-I01	SNV (VAF (%) in WGS)							CNV		Outcome	
		PTPN5	NPHS1	EXOC2	GDPD2	C7orf72	POU2F1		Max. CN	Locus	Graft	Cell/Tissue
Cell typing	iPSC-C p12	46,9	49,4	48,4	100	50	3,5		NA	NA	NA	NA
	RPE p3	52,9	49,2	46,1	100	58,6	4,7		4	14q32.33, 17q12	Abnormal	Connective tissue
	NSC p8	48,1	42,9	51,4	94,7	44,3	0		4	17q12	Abnormal	Tumor

D

Cell line	16E84	SNV (VAF (%) in WGS)										CNV		Outcome	
		FASTKD1	NPHS1	GDPD2	PTPN5	BRD3	BCOR	ZFYVE27	MYH9	FAM171A2		Max. CN	Locus	Graft	Cell/Tissue
Cell typing	iPSC-C p23	42,5	41,4	100,0	53,3	51,9	97,4	16,7	7,0	0,0		ND	NA	NA	NA
	iPSC-F p28	50,0	45,0	100,0	47,1	49,4	91,2	33,8	29,6	0,0		4	14q32.33	NA	NA
	RPE	53,6	48,8	100,0	41,2	0,0	0,0	0,0	1,1	48,1		4	14q32.33	Abnormal	RPE + Cartilage
	non-RPE	45,5	48,1	100,0	54,6	5,8	13,8	11,9	7,0	41,9		4	17q12	Abnormal	including Cartilage
	CM	51,3	62,7	100,0	66,7	59,3	95,7	64,6	48,2	0,0		4	17q12	Normal	CM
	non-CM	49,4	48,7	100,0	50,8	56,3	91,5	39,2	50,8	0,0		3	Multiple	Normal	Connective tissue

E

Cell line	16E85	SNV (VAF (%) in WGS)									CNV		Outcome	
		ESRRG	FASTKD1	EXOC2	SLFN12	GDPD2	PTPN5	UBR4	BCOR	ZFN23b		Max. CN	Locus	Graft	Cell/Tissue
Cell typing	iPSC-C p20	23,2	51,5	56,0	30,9	96,8	39,0	35,3	7,9	0,0		ND	NA	NA	NA
	iPSC-F p25	28,6	43,3	53,0	33,3	100,0	43,5	40,0	18,9	0,0		3	Multiple	NA	NA
	RPE	36,4	49,3	59,6	37,5	100,0	40,4	47,7	0,0	22,2		4	17q12	Normal	RPE
	non-RPE	42,0	49,5	50,0	34,9	100,0	58,9	37,3	0,0	0,0		4	17q12	Abnormal	including Cartilage
	CM	20,8	43,3	54,7	31,9	100,0	60,3	45,2	25,0	1,4		3	Multiple	Normal	CM
	non-CM	32,6	37,9	43,2	30,3	100,0	57,9	32,1	26,8	1,5		4	14q32.33, 17q12	Normal	Connective tissue

F

Cell line	16H12	SNV (VAF (%) in WGS)									CNV		Outcome	
		NEB	HIPK2	CYP2A7	EP300	REPS2	IGSF1	ADGRG4	VWC2	USP54		Max. CN	Locus	Graft	Cell/Tissue
Cell typing	iPSC-C p5	49,4	42,4	50,0	42,2	73,3	100,0	97,6	39,2	23,2		ND	NA	NA	NA
	iPSC-F p10	48,1	47,4	41,3	56,0	90,9	96,7	96,3	50,0	3,7		3	Multiple	NA	NA
	RPE	51,2	56,1	51,6	51,2	90,9	93,3	100,0	40,0	2,7		3	Multiple	Normal	RPE
	non-RPE	44,8	50,7	60,2	50,0	88,0	100,0	100,0	42,4	4,8		4	17q12	Normal	RPE
	non-CM*	49,4	48,4	43,4	45,2	80,5	100,0	100,0	49,1	5,4		3	Multiple	Normal	Connective tissue

* non-cardiomyocytes obtaind by cardiomyocyte induction treatment on iPSCs

G

Cell line	15M38	SNVs (VAF (%) in WGS)							CNV		Outcome	
		MYH9	ZXDA	CELA3B	POU5F1B	OR6C76	MMP17		Max. CN	Locus	Graft	Cell/Tissue
Cell typing	iPSC-C p7	17,4	18,2	9,8	7,0	23,6	6,6		ND	NA	NA	NA
	iPSC-F p13	0,0	77,4	47,5	49,7	44,4	5,5		4	14q32.33	NA	NA
	RPE	0,0	60,5	47,1	52,9	18,0	5,3		4	14q32.33	Abnormal	Teratoma
	non-RPE	0,0	75,6	45,2	57,6	26,6	11,9		3	Multiple	Abnormal	Teratoma
	non-CM*	0,0	55,0	51,4	45,5	27,9	7,1		4	5q35.3	Abnormal	Teratoma

* non-cardiomyocytes obtaind by cardiomyocyte induction treatment on iPSCs

H

Cell line	H9	SNV (VAF (%) in WGS)										CNV		Outcome	
		ZNF717	FOXD1	MUC3A	NUDT18	B3GNT6	SARM1	CDC27	PPP1R9B	ZCCHC3	GAS2L1	max. CN	locus	Graft	Cell/Tissue
Cell typing	Undiff.	81,3	0,0	0,0	0,0	100,0	0,0	90,1	0,0	0,0	0,0	3	Multiple	NA	NA
	RPE	0,0	0,0	0,0	0,0	0,0	0,0	88,3	0,0	0,0	0,0	3	Multiple	Normal	RPE
	non-RPE	59,3	0,0	98,9	0,0	0,0	0,0	91,1	0,0	0,0	0,0	3	Multiple	Normal	RPE connective tissue
	CM	62,1	0,0	0,0	0,0	0,0	0,0	92,5	0,0	0,0	0,0	3	Multiple	Normal	CM
	non-CM	66,4	100,0	97,0	100,0	100,0	98,3	94,7	100,0	100,0	100,0	3	Multiple	Normal	Connective tissue

Table 1. Genetic profiles of iPSC clones and their outcomes after transplantation into NOG mice

VAF (%) of genes listed in the COSMIC ver.88 by WGS and copy number variants of iPSC clones 1210B2 (A), Ff-WJ14s01 (B), Ff-I01 (C), 16E84 (D), 16E85 (E), 16H12 (F), or 15M38 (G) and their derivatives are shown in the table. Genetic mutations detected by WGS are SNVs, except deletions in BCOR in 16E84 (D) and 16E85 (E). VAFs less than 24% (below the detection limit [LOD]) are shown in blue cells, and VAFs less than 12% (below the decision limit) are shown in gray cells. VAFs of genes in the Census database and Shibata's List showing values above the LOD are highlighted with a pinkish color. Gene whose VAFs reached around 50% or 100% related to the clonality of cells are shown in blue, suggesting that all of the iPSC clones tested in this study consisted of clonal expansion from a single cell and its derivatives. VAFs of POU5F1B in 15M38 suggesting the integration of extrinsic POU5F1 (thick border). H9 ESCs and their derivatives (H) were used as controls. Maximum copy number (max.CN) of relevant cell clones and the loci showing CN = 4, if detected, are added to the table. Notable or abnormal findings of the transplants are described in blue or red in the table where applicable. p: passage number, NA: not applicable, ND: not determined, NSC: neural stem cell, iPSC-C: iPSC cultured at CiRA, iPSC-K: iPSC transferred to and cultured at Keio Uni. for differentiation, iPSC-F: iPSC transferred to and cultured at FBRI used for differentiation and engraftment, RPE: retinal pigment epithelial cell, CM: cardiomyocyte.

A

Explanatory variables in PSC-derivatives				Outcome variable
Cell line	Cell typing	SNV	CNV	Histological finding
16E84	RPEs	SNV(-)	CNV(+)	Abnormal
16E84	CMs	SNV(+)	CNV(+)	Normal
16E85	RPEs	SNV(-)	CNV(+)	Normal
16E85	CMs	SNV(+)	CNV(-)	Normal
16H12	RPEs	SNV(+)	CNV(-)	Normal
16H12	non-CMs	SNV(+)	CNV(-)	Normal
15M38	RPEs	SNV(-)	CNV(+)	Abnormal
15M38	non-CMs	SNV(-)	CNV(+)	Abnormal
1210B2	NSCs	SNV(+)	CNV(-)	Normal
Ff-WJ	NSCs	SNV(-)	CNV(-)	Normal
Ff-101	RPEs	SNV(-)	CNV(+)	Abnormal
Ff-101	NSCs	SNV(-)	CNV(+)	Abnormal
H9	RPEs	SNV(-)	CNV(-)	Normal
H9	CMs	SNV(-)	CNV(-)	Normal

B. Explanatory variable: SNV

Explanatory variable		SNV(-)	SNV(+)	Discriminative ratio		Overall predictabi
expectancy		Normal	Abnormal			
Outcome variable	Normal	4	5	44%	(Specificity	29%
	Abnormal	5	0	0%	(Sensitivity	
Predictivity		44%	0%			
overall predictivity		29%				
Likelihood ratio for abnormal outcome		2,3	0,0			

Correlation ratio η: 0,56

C. Explanatory variable: CNV

Explanatory variable		CNV(-)	CNV(+)	Discriminative ratio		Overall predictabi
expectancy		Normal	Abnormal			
Outcome variable	Normal	7	2	78%	(Specificity	86%
	Abnormal	0	5	100%	(Sensitivity	
Predictivity		100%	71%			
overall predictivity		86%				
Likelihood ratio for abnormal outcome		0,0	4,5			

Correlation ratio η: 0,00

Table 2. Outcomes of transplantation of iPSC-derived RPEs, CMs, or NSCs with SNVs/del in cancer-related genes or with a copy number exceeding 3

A. Table of iPSC-derivatives and their histology after transplantation. Cell typing: identities of differentiated cells transplanted, RPEs: retinal pigment epithelial cells, CMs: cardiomyocytes, NSCs: neural stem cells. All of the mutations in cancer-related genes listed in the table are SNVs, SNV (+): cells with SNVs/del in cancer-related genes, SNV (-): cells with no detectable SNVs/del in cancer-related genes, CNV (+): cells with a maximum copy number exceeding 3, CNV (-): cells with copy numbers of 3 or below. B: Predictivity of abnormal tissue formation after transplantation by detecting cancer-related SNVs/del in the final product. C: Predictivity of abnormal tissue formation after transplantation by detecting a maximum copy number exceeding 3 in the final product. Categorical variables were analyzed with Hayashi's quantification method type II.

Cell Therapy Process Development with the Breez™

With the recent investments in and approval of CAR-T cell immunotherapy products, there is a growing need for manufacturing technologies to improve efficacy, decrease variability, and reduce cost. Major challenges that exist include experiment reproducibility and robustness during process development. For autologous processes, donor variability and differences between healthy donor-derived versus patient-derived material can confound experiment results. In addition, there is typically not enough donor or patient material to run a full DOE experiment campaign. Materials are also costly, including peripheral blood mononuclear cells (PBMCs), growth factors, viral vectors, serum and chemically defined media.

Therefore, process development experiments in cell therapy are typically done in milliliter sized, often static culture with minimal environmental control. While these systems can generate data quickly, the lack of control and monitoring can result in variabilities that may prove difficult to translate to larger culture systems.

To address this gap, Erbi Biosystems has developed the Breez™ bioreactor with True Perfusion™. The data presented in this article shows that the Breez can be used in process development to carry out activation, transduction, and expansion of T-cells in a controlled and closed system.

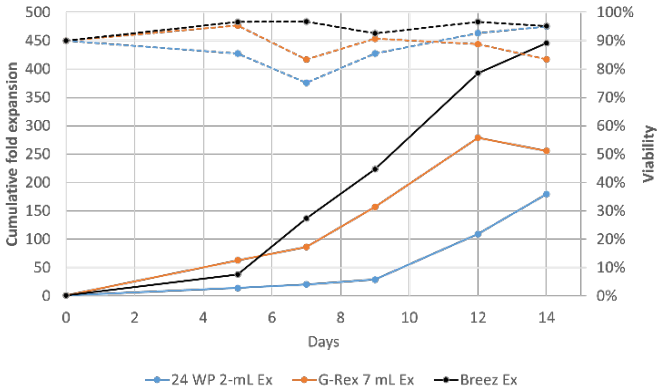
Materials and Methods

Activation, transduction, and expansion were performed in three different culture systems, a 24 well plate (24-WP) at 2 mL, a G-Rex®24 at 7 mL, and an Erbi Breez at 2 mL working volume. Healthy Donor cells sourced from Stemcell were grown in AIM-V media + 2% Serum + 100 U/mL IL-2, activated with Dynabeads in a 1:1 ratio, and inoculated into the 24-WP and the Breez. After 24 hours, lentivirus (MOI 5) was added to the 24-WP with retronectin and spinoculated then the cells were split into a 24-WP and a G-Rex®24. Lentivirus was also added to the Breez with no enhancers. Post-transduction starting cell densities were 4e5,3e5, and 5e5 cells/mL for the 24-WP, G-Rex®24, and Breez respectively.

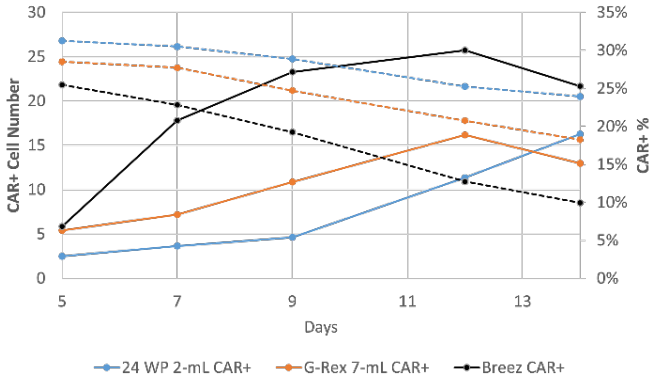
Activation, Transduction, and Expansion

Results

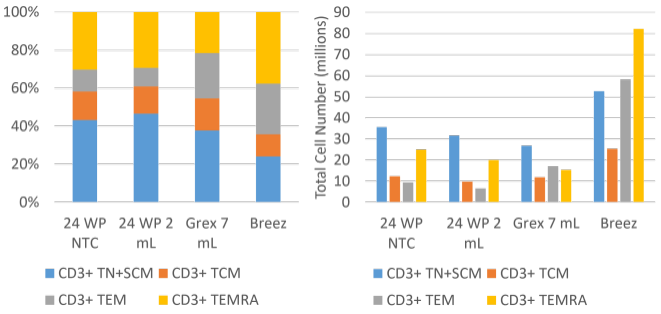
- High density T cell culture in the Breez – maximum VCD of > 100e6 cells/mL, compared with 11e6 cells/mL in the G-Rex 24
- Initial transduction efficiency without enhancers in the Breez was similar to that with retronectin in the 24-WP
- Improved expansion rates of all cell types, including CAR+ cells in the Breez vs controls



The Breez enables high cell expansion rates (solid) and viable cell number (numbers in millions) with high cell viability (dotted).



CAR+ cell number (solid) (> 20e6 cells) potentially satisfies dosage for pediatric infusion. All systems have similar initial transduction efficiency (dotted).



Phenotypes assayed on day 14 show improved expansion of all subtypes compared to other systems.

Conclusions

- The Breez 2 mL perfusion system successfully demonstrated an **integrated workflow for CAR-T processing** including activation, transduction, and expansion, greatly simplifying experiments and reducing contamination risk.
- The Breez system was able to **match or exceed performance of larger volume processes** such as G-Rex or rocking bags with phenotypes and growth characteristics similar to other systems.
- **Viable Cell Densities in excess of 100e6 cells/mL** suggests future potential for dose level production and use as both a **development tool as well as a manufacturing platform**.
- **Online sensors and continuous monitoring** enable enhanced process knowledge and new control capabilities to explore environmental influence on T-cell growth and differentiation.

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C-STEM™: BUILDING ON THE *IN VIVO* BIOLOGY OF HUMAN PLURIPOTENT STEM CELLS TO SCALE-UP HIPSC MANUFACTURING

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Introduction

The discovery of the induced pluripotent stem cells (iPSC) technology by Shinya Yamanaka in 2006[1] triggered massive access to human pluripotent stem cells (hPSC). hPSC are often touted as an infinite source of cells for research, disease modeling, and therapeutic applications. Being adherent cells, hPSC have originally been cultured as 2D colonies in Petri dishes or T-flasks. However, despite their apparent unlimited proliferation capacity, hPSC 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. Given the limitations of 2D cell culture systems for hPSC expansion – especially high mortality and susceptibility to 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.

In this article, we aim at i) describing the *in vivo* environment of hPSC and highlighting core features of the epiblast rosette conformation, ii) comparing the *in vivo* organization of hPSC with current *in vitro* cell culture systems, and iii) introducing C-Stem™ – a biomimetic technology that allows for the mass production and differentiation of hPSC in large-scale bioreactors.

Human pluripotent stem cells *in vivo*

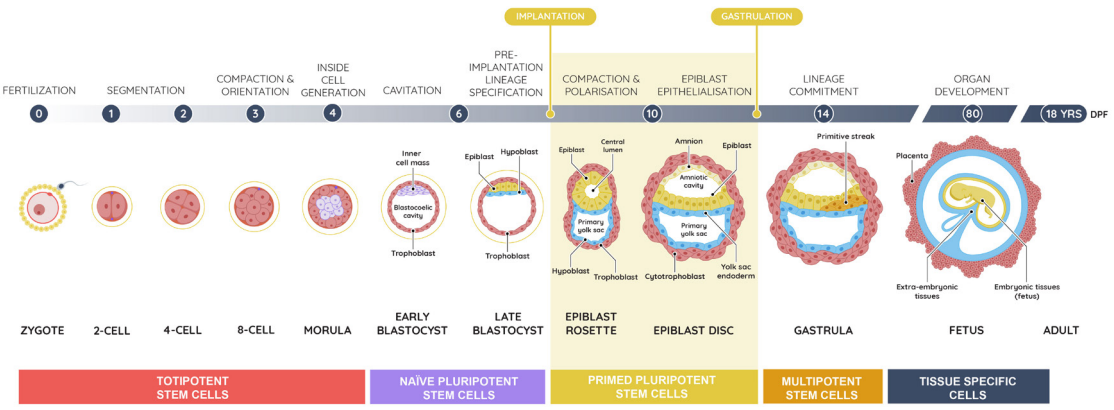

Fig. 1. Human primed pluripotency in vivo: a 7 day-window, a protected microenvironment, and a 3D epithelial organization.

Adapted from Wamaitha & Niakan, 2018 Human pre-gastrulation development[4] ; Shahbazi & Zernicka-Goetz, 2018a Deconstructing and reconstructing the mouse and human early embryo[5]; Shahbazi et al., 2020. Mechanisms of human embryo development: From cell fate to tissue shape and back[6] & Roelen, 2018 Epiblast Development[7]

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 hPSC transition to a state of primed pluripotency. Within the following 5 to 7 days, the PSC colony, benefiting from a very fast 15h to 16h[8] cell cycle, grows exponentially to reach over 1,000 cells. Following this exponential amplification, hPSC exit their pluripotent state and commit to one of the three germ layers during the process of gastrulation. Overall, among the 40 cell divisions required to constitute an adult body (~37 trillion cells)[9, 10] are estimated to occur within the second week of development, 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 then hatched to enable implantation into the uterine wall, which functionally replaces the zona pellucida in conjunction with the underlying layer of trophoblasts, the trophectoderm. This extra-embryonic outer layer provides mechanical protection, enables metabolic exchanges, and creates a controlled 3D niche in which hPSC can grow exponentially.

A distinctive rosette architecture

Following implantation, hPSC acquire primed pluripotency and arrange as a spherical single cell-layered epithelium organized around a central cavity: the epiblast rosette. This 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 hPSC have their apical domains clustered toward the lumen, which acts as central signaling hub, enabling synchronous and homogeneous growth.

Finally, the epiblast 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 hPSC divide, and locally decrease the tangential mechanical resistance to accommodate new cells. Facilitating cell division[10], this conformation also prompts the depletion of abnormal cells,[11] thus safeguarding the genomic fitness of the hPSC colony.[11]

As it proliferates, the pluripotent epiblast rosette gradually transitions to a disc conformation before a symmetry break occurs on day 14. Recent studies support that the radial symmetry of the epiblast and concentration of several factors within the lumen, like the developmental signaling molecule BMP, or fibroblast growth factor (FGF) molecules, promote robust symmetry breaking and differentiation of hPSC during gastrulation.[6,12,13,14]

Genesis & properties of the epiblast rosette

The human epiblast is a lumenized and polarized PSC colony

The epiblast rosette architecture emerges during the transition from naïve to primed pluripotency. In response to extracellular matrix cues, naïve PSC start to polarize. As transcriptional factors trigger primed pluripotency, hPSC form de novo intracellular actin-rich compartments called apicosomes, which accumulate Ca2+ and express apical proteins[15]. As the apicosomes regroup, concomitant mechanisms promoting water influx towards the center of the epiblast participate in the establishment of a fluid-filled lumen.[16] The resulting rosette architecture displays apical-basal polarity: hPSC apical domains face the lumen, while basal domains face the external environment of the epiblast, lined with a basal

face the lumen, while basal domains face the external environment of the epiblast, lined with a basal membrane.

Lumen formation is required for optimal proliferation

hPSC in epiblast rosette conformation are characterized by prolonged undifferentiated proliferation without undergoing senescence or quiescence. Kim et al. showed in 2021[16] 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.[17] 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[17] 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*, PSC that escape the 3D rosette architecture are differentiating, thus losing their pluripotent phenotype.[18]

The epiblast architecture maintains hPSC fitness

In 2018, Knouse et al. demonstrated that polarized epithelial tissue architecture is required for high-fidelity chromosome segregation[10]. 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[10,19] in epithelial cells. Genomic abnormalities are quite common in early human development. 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.[20] Recent research has shown that the pluripotent epiblast undergoes natural quality control through cell competition mechanisms, leading to the elimination of abnormal cells through apoptosis and autophagy.[21,22] In the mouse epiblast, 35% of PSC were found to be eliminated by the onset of gastrulation.[23] Several groups demonstrated that defective cells, harboring mis-patterning, karyotypic[11], or mitochondrial abnormalities[21] are efficiently depleted in epiblast conformation. Normal diploid PSC were also found to increase their proliferation to compensate for the elimination of abnormal cells in the epiblast.[11] Overall, the competition of PSC within the epiblast, which involves the signaling pathways YAP/TAZ[24], MYC[22], p53[23], and mTOR[23], is considered as a critical mechanism, safeguarding pluripotency and genomic integrity before germline commitment.

Pluripotent stem cells: *in vitro* cultures vs *in vivo*

2D hPSC culture vs *in vivo*

Standard 2D hPSC culture consists of seeding hPSC 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, hPSC 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 hPSC is the intermittent control of cell culture parameters, including temperature, pH, dissolved oxygen (DO), glucose, and growth factor concentrations,[25] caused by medium changes. Second, while it has been reported that hPSC cultured in 2D spontaneously form lumens, such rosette-like structures were found to rapidly collapse (in ~5 days or less)[26]. hPSC 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 epiblast rosette versus

cell culture medium in standard 2D culture.[18]

Furthermore, in contrast with the epiblast 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[27]. This phenomenon is described as edge/center heterogeneity[28,27]. 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[29]. This lateral competition is independent of cellular fitness markers and promotes the selection of fast-growing apoptotic-resistant clones, likely to harbor oncogenic mutations.[29]

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] highlighting the limitations of monolayer cultures for scaling up the production of clinical-grade hPSC-derived products.

hPSC aggregates in bioreactors vs *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 hPSC. 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[30]. Several factors may affect performance in bioreactors. *In vivo*, an extra-embryonic tissue layer protects hPSC from external stress. In bioreactors, hPSC aggregates are directly exposed to impeller-induced hydrodynamic stress, which can trigger either cell death or spontaneous differentiation[31]. Agitation also leads to spontaneous fusions of aggregates[32], resulting in large hPSC spheroids harboring necrotic cores instead of lumens[31,33]. 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.

C-Stem™: hPSC mass production in epiblast-like conformation

The epiblast rosette conformation is critical to the behavior of hPSC *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 epiblast conformation.[34,35,36] 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)[33]. The inner wall of the capsule is decorated with extracellular matrix, thus mimicking the basement membrane of the hPSC niche.[37] In this biomimetic microenvironment, hPSC spontaneously self-organize in 3D and form epiblast-like 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 PSC 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[38]

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 PSC were limited by impeller-induced shear stress, causing significant cell death[2,3] and spontaneous differentiation[2,3]. By shielding hPSC within alginate capsules, the C-Stem™ technology removes the hydrodynamic stress constraints, thus permitting biomimetic PSC 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 hiPSC 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 hiPS cells 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 PSC amplified with C-Stem™ demonstrated the benefits of the epiblast format for the containment of pre-oncogenic mutations over prolonged culture (15-fold fewer pre-oncogenic mutations than in standard PSC culture in 2D, and 25-fold less than in agitated PSC 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 the hPSC populations expanded with the technology.

Conclusion

In vivo, the epiblast rosette architecture allows for robust and rapid expansion of hPSC with high 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 conformations 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 epiblast 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 that contain 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 two times faster than progenitor-based strategies, while mitigating proliferation risks.[39] This proprietary program for Parkinson's disease is scheduled to enter the clinic in 2024. 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.

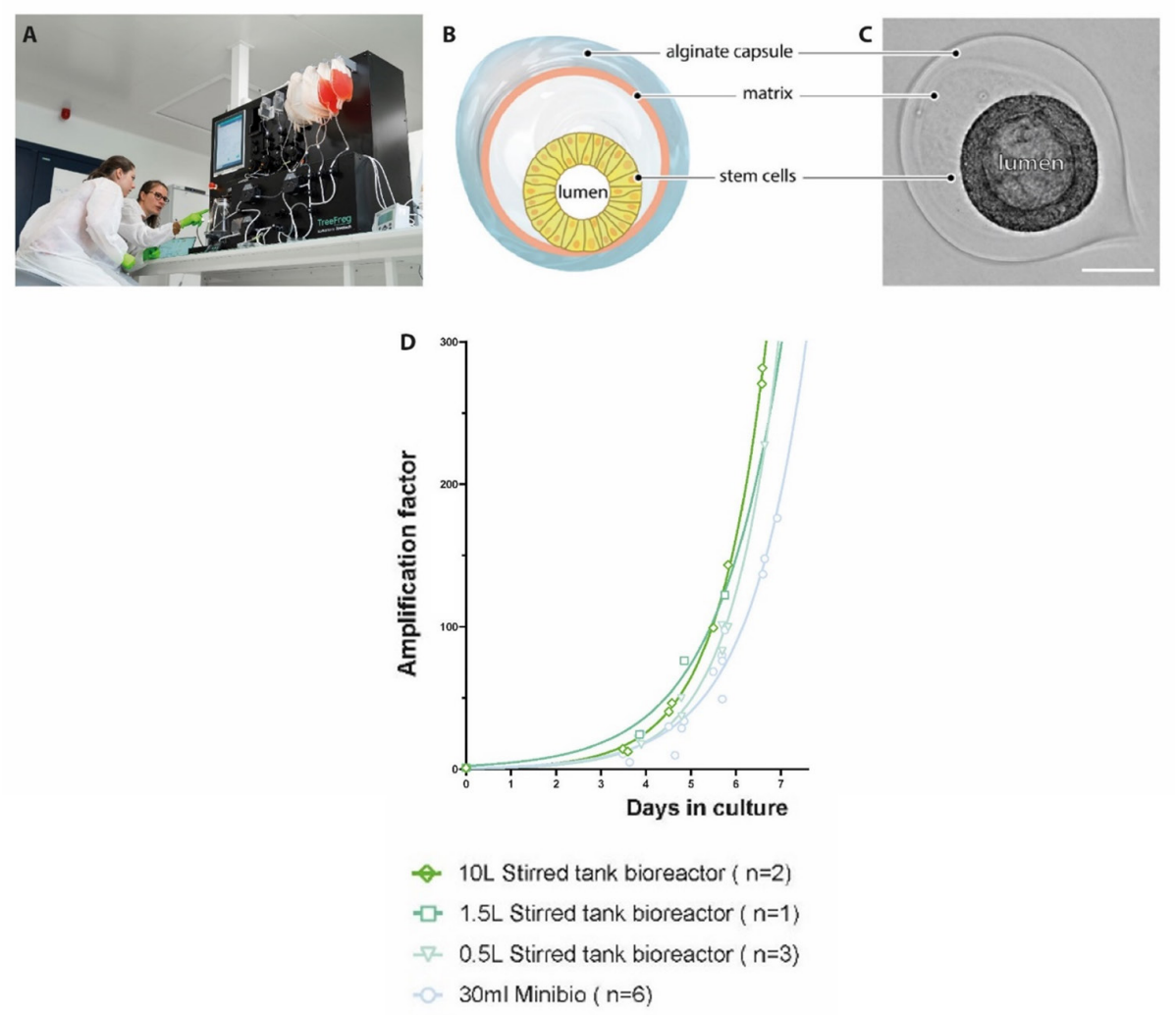

Fig. 2. C-Stem™-enabled encapsulation preserves the exponential expansion profile of hPSC grown in rosette conformation regardless of the bioreactor volume.

(A) Proprietary high-throughput cell encapsulation device. (B, C) hiPSC 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. (D) C-Stem™ enabled fast hiPSC scale-up, from 30mL to 10L bioreactors, while preserving expansion curve profiles and cell culture parameters across scales.

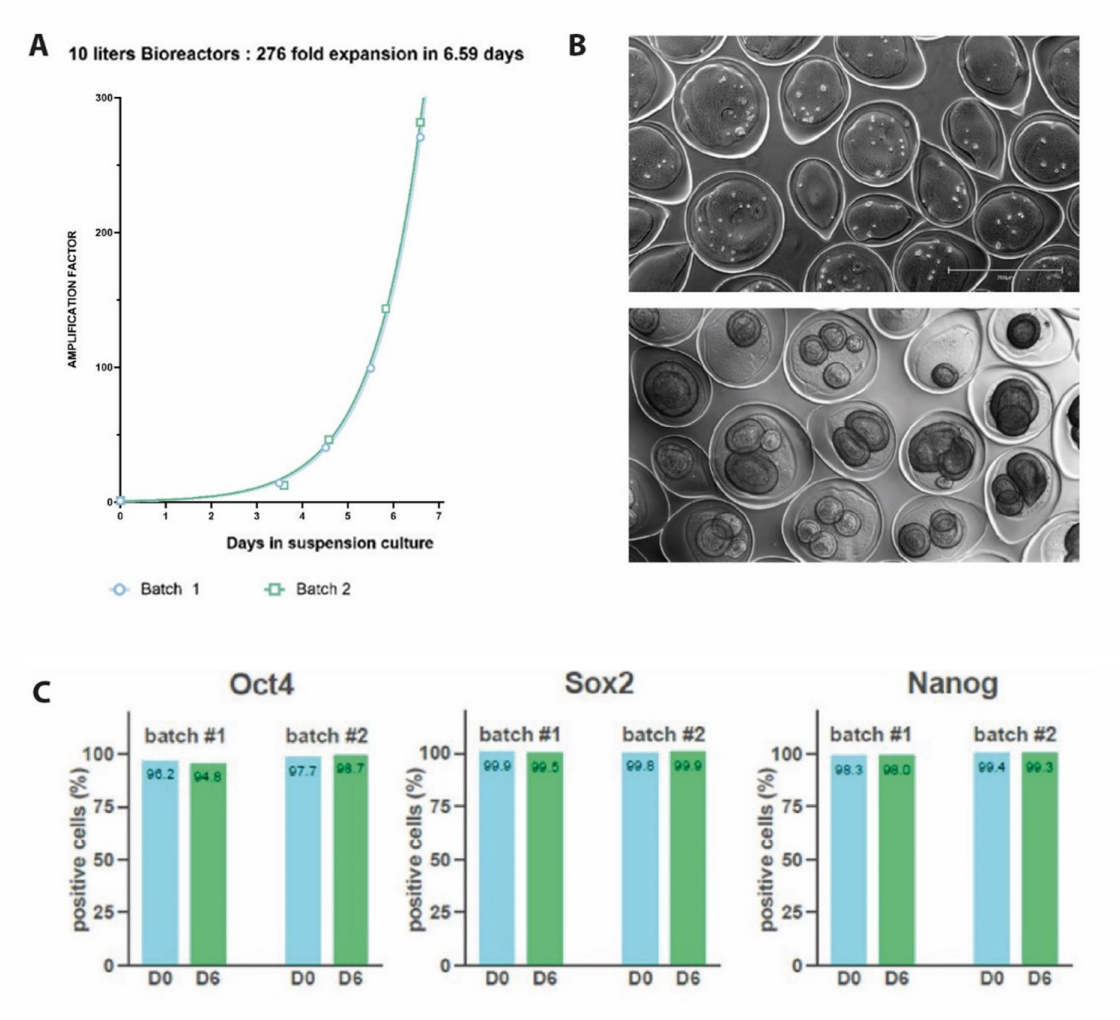

Fig. 3. Highly reproducible production of 2 single batches of 15Bn hiPSC in 10L bioreactors, with unprecedented 276-fold amplification factor per week, and conservation of cell quality

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

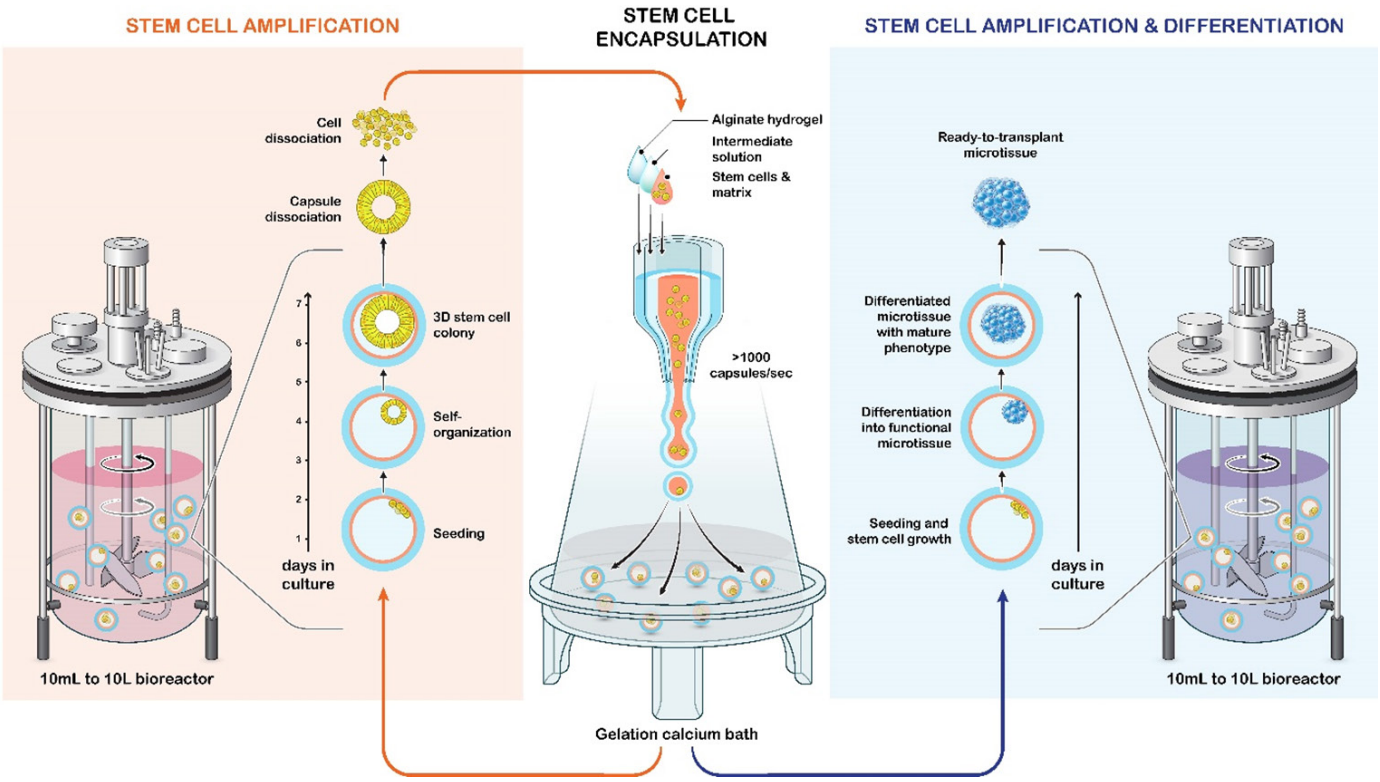

Fig. 4. C-Stem™, a single platform for hiPSC amplification and differentiation in large-scale bioreactors

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.

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IN VITRO EXPANDED MESENCHYMAL STROMAL CELL FOR BONE TISSUE ENGINEERING

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Member of the MAXIBONE consortium supported by EC (GA n° 779322)

Clinical background

Every year 4 to 6 million dental implants are placed in Europe. However, in 5 % of the cases, the height of the jawbone is insufficient, which makes it impossible to treat the patients. They have lost teeth secondary to traumatic shock or chronic infection. These pathologies are also responsible of bone loss, which usually results in alveolar ridges with deficient bone volume for adequate dental implant placement. In these patients, predictable rehabilitation by implant supported prosthesis can only be reached by partially regenerating the jaw bone either in conjunction or prior to implant placement. The European project MAXIBONE (GA n° 779322) aims to create personalized jaw bone reconstruction using a combination of autologous Mesenchymal stromal cells (MSCs) and biomaterial prior to dental implant placement. This study coordinated by Dr Pierre Layrolle (INSERM Toulouse, France) and Pr Kamal Mustafa, (Bergen University, Norway), financed by Europe, brings together a partnership of clinical services, industrialists, research laboratories and European public establishments accredited in the production of stem cells (Etablissement Français du Sang, Creteil- France and Institute for Transfusion Medicine, University Hospital Ulm, Germany).

Mesenchymal stromal cell for bone tissue engineering

Mesenchymal stromal cells (MSCs) are defined as non-hematopoietic progenitors characterized by their ability to adhere to plastic, the expression of non-specific phenotypic markers and their potential to differentiate into cells of the mesodermal pathway: bone, cartilage and adipose tissue. MSCs associated with biomaterials emerge as an innovative strategy for bone repair. The diversity of scaffolds is a source of heterogeneity for bone formation efficiency. We demonstrated previously that a biomaterial architecture similar to cancellous bone is important to promote MSCs adhesion and ensure cell survival *in vivo*. The alternative to cancellous bone is to use synthetic biomaterials whose composition is similar to natural bone. Thus the combination of hydroxyapatite (HA) and beta tricalcium phosphate(TCP) offers a promising scaffold for bone repair (Brennan MÁ et al Stem Cell Res Ther. 2014). The ability of MSCs to form bone tissue can be studied in a model of ectopic bone formation in the immunodeficient mouse. Early survival of MSCs during the first week post-implantation is associated with the most rapid bone formation (detectable at two weeks), the most quantitatively important with elements of vascularized marrow niche reformation (Mebarki et al Acta Biomater. 2017). The surviving MSCs are also able to differentiate into osteoblastic cells and can thus contribute directly to bone formation. MSCs with low osteogenic potential participate in the osteoblastic differentiation of host cells, probably by a paracrine mechanism. We demonstrated that the grafted MSCs play a direct role coupled to a paracrine effect to enhance bone formation and that both of those roles are governed by the used scaffold.

MSC manufacturing in Good Manufacturing Practice (GMP)

The MAXIBONE consortium has established a protocol for expansion over 15 days of culture from a 30 ml sample of autologous bone marrow (BM). MSCs are isolated *in vitro* by their ability to adhere to plastic in 5-stage cellstacks chamber (Corning). BM was directly seeded without any further manipulation

in Minimal Essential Medium Eagle, alpha formulation (alpha-MEM medium) supplemented with 5% platelet lysate (PL) and 1 IU heparin/mL at day 0 and incubated at 5% CO2 atmosphere, 95% relative humidity at 37°C. The platelet lysate allows to obtain a higher level of amplification compared to the use of foetal calf serum in culture and to limit the risks of transmission of prion-like viruses. Moreover, even in the absence of a differentiating agent, the culture of MSCs in the presence of LP initiates the expression of some osteoblastic genes such as Bone Sialoprotein, Alkaline Phosphatase, Osteopontin and BMP2 (Chevallier et al; Biomaterials. 2010). MSCs are produced in accordance with Good Manufacturing Practice (GMP) in a clean A/B areas; environmental and non-viable particulate monitoring is performed throughout the production process. Tubing sets (Macopharma) allow changing the culture medium every 3-4 days without opening the containers in a closed system. At day 10, cells are rinsed with Phosphate Buffered Saline (PBS) and detached with TrypZean (Lonza). Harvested cells (passage 0) are re-seeded in alpha-MEM medium supplemented with 8% PL and 1UI heparin/ml. at day 15, cells were rinsed with PBS and harvested using TrypZean. Cells are resuspended in 5% human albumin solution (Octapharma) to obtain the final MAXIBONE product. Fresh MSCs are packaged in closed syringe and labeled before the shipment toward clinical centers. The cells are maintained at 4-10°C during shipment and must be used within 24 hours (Rojewski et al; cytotherapy 2019).

Release parameters for the medicinal product include microbial, endotoxin and mycoplasma testing, tests for viability, clonogenicity, identity, purity and functional tests. MSCs fulfilled quality criteria requested by the competent authorities.

Clinical trial

The MAXIBONE clinical study is an interventional study designed as a phase IIb randomized multi-center clinical trial using advanced therapy medicinal product (ATMP) aiming to evaluate the efficacy, safety, and tolerability of the clinical single local application of autologous bone marrow MSCs combined with biphasic calcium phosphate (BCP) granules as bone replacement graft in patients with jaw atrophy, before dental implant placement. The trial was approved by the National Competent Authorities (NCAs) and the Ethical Committees (ECs) in each participating country (Spain, France, Germany, Norway, Denmark). This approval by NCAs was based in a Voluntary Harmonization Procedure (VHP1528) in October 2019. The clinical trial MAXIBONE followed the European guidelines for advanced therapeutic medicinal products. The EudraCT number of the trial was 2018-001227-39 and the trial was incorporated in the database ClinicalTrials.gov with the identifier NCT04297813. The Principal Investigator is the Pr Cecilie Gudveig Gjerde in UiB (Norway).

The adult patients are recruited based on the criterion of having vertical and lateral loss of alveolar bone volume (width less than 4 mm) in edentulous ridges of both mandible and maxilla, where the placement of a dental implant could not provide adequate primary stability. These patients were selected based on a three dimensional radiographic examination (CBCT imaging) confirming the loss of bone volume posterior to the canine teeth of the maxilla and/or the mandible. This is a randomized study with 50 control patients (autologous bone graft) and 100 patients treated with cell therapy / biomaterial. Expanded MSCs are associated with biphasic calcium phosphate bioceramic granules (biomatlante) associated during 1 hour and then implanted into the patient. The surgical site is covered with a membrane that allows for the maintenance and integration of the biomaterial/stem cell mixture. Five months after the operation, the implants are placed which allows the prosthetic rehabilitation.

The primary outcome is to measure bone volumes and quality by CBCT 5 months after reconstructive surgery. The secondary objective is to assess whether it is possible to insert an implant in the reconstructed area 5 months after the grafting procedure. The safety of the tested interventions were assessed by collecting adverse effects (AEs) and soft tissue healing outcomes at 2 and 4 weeks and 5 months post-surgery. Inclusions are still ongoing on the protocol. The quality of the regenerated bone 6 months

following bone augmentation procedures was considered by the operating surgeons as satisfactory and allowed the surgical placement of implants with primary stability.

Perspectives

BM autologous harvesting is complicated to organize: a team experienced in the exercise is required, as well as an operating room. Moreover, patients requiring a jaw enhancement are most often older than 60 years. The number of MSCs in the bone marrow decreases with age. Also the frequency of osteoprogenitors may not be sufficient to expect enough MSCs expansion *in vitro*. This exposes to a risk of batch failure in autologous situation. Therefore, a new study design is being considered to introduce a treatment arm with allogeneic MSCs. A pre-clinical animal model in pigs treated with allogeneic MSCs from another pig is under analysis. The potential use of allogeneic MSCs makes it possible to consider a scale-up of the production process on a large scale, with virological qualification of a batch of more than 8x109 MSCs allowing the preparation of at least 80 therapeutic doses with 100x109 MSCs. In addition, this strategy would allow a significant reduction in production costs and easier access to a larger number of patients.

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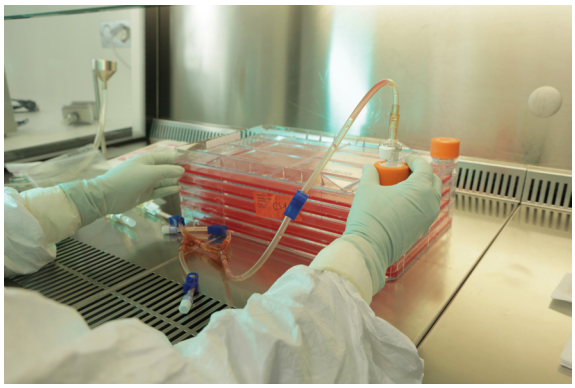

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ISSUE OF CELL SOURCING IN THE BIOENGINEERING OF SKIN ORGANOIDS FOR CLINICAL PURPOSES

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Biomedical scope of skin organoid bioengineering

Skin was the first organ that could be reconstructed ex vivo by tissue bioengineering, which opened an avenue for the development of epidermal sheets or more complex dermo-epidermal skin composites suitable for therapeutic skin replacement strategies. A pioneer clinical achievement of skin substitute grafting, obtained more than three decades ago, is the permanent coverage of large-surface burn wounds (Gallico et al., 1984). Since then, this base tissue bioengineering technology has increased its clinical perimeter, as it was combined with vector-driven genetic correction, leading to a successful gene therapy protocol (Hirsch et al., 2017). Therapeutic uses of bioengineered skin substitutes are presently considered for other medical indications that concern large populations of patients, such as chronic wounds and ulcers (Kyriakidis et al., 2021).

Considering the diversity of clinical needs and pathophysiological contexts, not one single cell- and tissue-therapy product will cover all specifications. The parallel development of complementary strategies will contribute to enrich the catalog of graftable products available to meet the specific requirements of different medical indications. As exemplified here, research and development efforts concern different components, stages, and methodologies involved in skin bioengineering architectures. Focus of interest comprise the different options of skin cell sourcing and parameters of bioproduction.

Adult skin cells as biological raw material for graft bioengineering

Resident epithelial stem cells from adult epidermis, also called epidermal or keratinocyte stem cells, have been extensively characterized both at the functional level and for their clinical relevance. Major therapeutic applications that could emerge thanks to their efficient regenerative capacity are the permanent skin replacement in the treatment of massive full-thickness skin burns (Gallico et al., 1984), and as target cells for long-term gene therapy (Kueckelhaus et al., 2021). The terminology of 'holoclones' has been attributed to these epithelial stem cells (Barrandon & Green, 1987), based on their potentialities that comprise an extensive proliferative capacity exceeding 100 population doublings, associated with efficient potential for three-dimensional (3D) epidermis organoid reconstruction (Fortunel et al., 2010). They represent a highly potent cellular raw material for the generation of cutaneous graft clinical products. To date, bioengineered skin grafts produced for permanent skin restoration are prepared using epidermal keratinocytes extracted from biopsies of patient's own skin (i.e. autologous keratinocytes). Of note, the process used for the production of epidermal sheets still very similar to that initially set-up in the 80's. This tissue therapy is patient-specific, and its present clinical perimeter is compational indications. A current need is implementation of technological breakthroughs that will enable large-scale skin graft production, together with cost reduction and standardization.

A first major challenge concerns the problem of immune rejection that currently limits the use of allogeneic cell banks for the bioengineering of skin substitutes compatible with long-term grafting. Strategies aiming at disrupting HLA-class I and class II molecules expression are proposed as an issue to prevent the rejection of allogeneic skin cells. An alternative option proposes combining β2-Microglobulin knock-out together with overexpression of non-classical HLA class I molecules (HLA-E) (Gornalusse et al., 2017). A

third explored option is based on the immuno-modulatory properties of the HLA-G immune checkpoint molecules, which were initially described in the context of maternal-fetal tolerance (Carosella et al., 2003). Recent works have shown that vector-induced expression of HLA-G in adult human keratinocytes may open a new avenue for the bioengineering of tolerogenic skin cells (Mestrallet et al., 2021).

A second major challenge concerns the culture condition that are currently required for the preservation of keratinocyte stem cells (holoclones) throughout the bioengineering process, and consequently for conferring a long-term grafting capacity to skin substitutes. Indeed, the maintenance of holoclones in an ex vivo context still requires undefined biological constituents of animal origin (serum) and of feeder accessory cells, which is not in line with the guidelines of good manufacturing practices (GMP) in the perspective of large-scale industrialization. Strategies aiming at replacing serum and feeder cells by defined molecular effectors driving pro-'stemness' signals. Targeting the Krüppel-like factor 4 (KLF4) transcription factor using interfering RNAs or active molecules was identified as an option to presence stemness and improve the ex vivo expansion of immature keratinocytes in a serum- and feeder-layer-free culture system (Fortunel et al., 2019). Pharmacological inhibition of the mTOR and ROCK signaling pathways may also contribute to the design of chemically defined culture conditions supporting epithelial stem cell ex vivo expansion (Centonze et al., 2022).

Of note, these researches conducted on adult skin native epithelial stem cells may bring innovation applicable to the production of keratinocytes derived from pluripotent stem cells.

Pluripotent stem cells for skin cell therapies

Beside adult stem cells, human pluripotent stem cells (hPSCs) have been considered a promising cell source for regenerative medicine. hPSCs are self-renewable and give rise to any cell type of the human body. They can be obtained from supernumerary *in vitro* fertilized embryos (human embryonic stem cells or hESCs) or after the conversion of adult primary cells to pluripotency by the overexpression of a cocktail of transcription factors (human induced pluripotent stem cells or hiPSCs). Their proliferative and differentiation capacities are highly convenient for cell substitution therapy because they enable the propagation of cells to obtain the required amounts and the possibility of creating any cell type from the human body. Obtaining all needed cells from a single donor facilitates production processes and quality controls required for clinical productions, and ensure no variability between batch productions, potentially observed with multiple donors. Research grade protocols were developed allowing the differentiation of hPSCs into various type of cells, including major populations present in skin: keratinocytes (Guenou et al., 2009), fibroblasts (Shamis et al., 2011) and melanocytes (Nissan et al., 2011).

GMP keratinocytes derived from hPSCs presenting basal and juvenile keratinocyte markers such as keratin 5, keratin 14 and keratin 19 were recently obtained (Domingues et al., 2022). In addition, fibroblasts derived from hPSCs expressed also fibroblast markers such as, fibronectin, vimentin, and podoplanin, which are characteristics of papillary dermal fibroblast cells. These type of "juvenile-like" cells could be interesting in a clinical perspective since aged skin failed to heal after grafting due to a decrease in proliferative potential. The functionality of these clinical grade cells was also confirmed by their capability to form a pluristratified epidermis on a dermal equivalent scaffold *in vitro* (Domingues et al., 2022)

As mentioned above, autologous transplantation does not need an immunosuppressive regimen and favors an optimal cell survival. However, the cost of autologous cell sample generation is high. In addition, the delay inherent to derivate hPSCs from a patient then differentiate cells to a particular cell type and finally quality controls to ensure safety is time consuming. This strategy is not optimal when the need for a treatment concerns millions of patients, as foot ulcers associated to diabetes. In contrast, allogenic cell banks allow an off-the-shelf product that could be distributed and used on demand. However, although epidermal cells derived from hPSCs seems have some immune privilege (Guenou et al., 2009) due to

their juvenile stage, cells could be acquired immunogenic stage with time. To prevent graft rejection, an immunosuppressive regimen is required but associated side effects can be important and deleterious. The hPSCs cell line could be developed from specific donors to obtained haplobanks (Sullivan et al, 2020) or could be genetically manipulated by inactivating the Beta-2-Microglobulin gene using CRISPR/Cas9 to evade the immune system (Bogomiakova et al., 2021) in order to obtain "universal graftable" engineered composite skin avoiding tissue rejection. Altogether, this strategy could lead to a reduction in the cost in the engineered skin production in order to offer a treatment to the greatest number.

Of note, raw cellular materials extracted from adult skin samples or derived from hPSCs constitute the bases of parallel complementary strategies that will both drive engineering breakthroughs.

Questions related to bioproduction

The development of robust and precisely adjusted bioproduction processes is a critical issue in the design of cell-based regenerative therapies, as they have to meet both qualitative and quantitative requirements. Firstly, processes have to consider the physiological requirements of the desired cells in order to avoid alteration of their characteristics and potentialities, while respecting the GMP guidelines. Secondly, the objective of large-scale production that will be needed to ensure an increased availability of skin substitutes, and thus enlarge their therapeutic uses. Some groups approach these bottlenecks by developing xeno-free and feeder-layer cell free protocols (Fortunel et al., 2019; Frese et al., 2021; Domingues et al., 2022). The development of bioproduction protocols has promoted usage of automated systems to upscale the cell expansion and increase production standardization. The use of modular platforms was reported for the long-term maintenance and passaging of different cell types in a closed automated cell-culture system (for example the CompacT SelecT® system developed by Sartorius, or the Miltenyi Prodigy® system). An achievement of fully automated process was the large-scale production of retinal pigmented epithelial cells from hPSCs, raising a yield of production capacity reaching several billion of mature and functional cells, within 12 weeks (Regent et al., 2019). Different bioreactors technologies are also presently available, using different micro-carriers that have to be selected according to cell types (Bellani et al., 2020).

In addition to efforts concerning the epithelial component of skin substitutes (i.e. pluristratified epidermis), bioengineering architectures comprise the design of dermis equivalents, which are usually composed of a matrix biomaterial in which fibroblasts can be embedded. The choice of the matrix raw biomaterial is a critical issue, as its impacts the biophysical and biological properties of resulting bioengineered tissues. Approaches based on the use of synthetic biomaterials can provide structural scaffolds, but are limited by the lack of biological functionalities. Researches on extra-cellular matrix (ECM) composition and properties have driven the development of new biomaterials that can reproduce some of the biomechanical and biological aspects of native tissue matrixes. Biomaterials potentially suitable for the reconstruction of dermis equivalents can be synthetic, natural biological components, or 3D decellularized tissue structures. They have to be biocompatible, biodegradable, and bioresorbable. Biomaterials used for the manufacturing of dermal equivalents comprise for example collagen, chitosan, elastin, hyaluronic acid, or fibrin (Sierra-Sanchez et al., 2021). Of note, the choice of using chemical raw materials versus animal- or human-derived products greatly influences the design of bioengineering processes. The specific structure of biomaterials (porous, fibrous, hydrogel etc…) can bring advantages, but can also generate drawbacks, depending on desired tissue properties.

Skin bioengineering prospects

To date, the offer of cell- and tissue-therapy products required for the treatment of severe skin injuries and chronic wounds in general is still not sufficient to meet the wide needs of this clinical domain. Billions of euro-equivalents have been invested worldwide to develop treatment options, including clinically

reliable models of bioactive skin substitutes. Bioengineering strategies using patient's autologous cells have the great advantage of immune compatibility, but they are by definition not dedicated to large-scale bioproduction and uses. In contrast, strategies using large banks of characterized cells as raw cellular material for the bioengineering of skin substitutes dedicated to allogeneic uses are more compatible with the development of large-scale bioproduction architectures. They however have to integrate the question of immune rejection, which presently restricts their therapeutic applications to transient wound dressing. In a broader view, ruptures in the skin-bioengineering domain will depend on interdisciplinary inputs, integrating new designs and innovations concerning cellular and biomaterials, combined with developments on bioproduction processes including automation. In addition to the classical currently used cell-culture manual methods, machine-driven technologies (3D bioprinting) are explored as an alternative option for skin composite assembly. To conclude, the domain of skin bioengineering has a great potential for driving technological innovation in cell and tissue therapies. The development of rupture concepts and products will be based on efficient translational architectures bridging basic research and the ultimate objective of bringing engineered skin products to patients. Two proposed bioengineering architectures that respectively use native adult skin cells and iPSC-derived skin cells are schematized in **Figure 1** and **Figure 2**.

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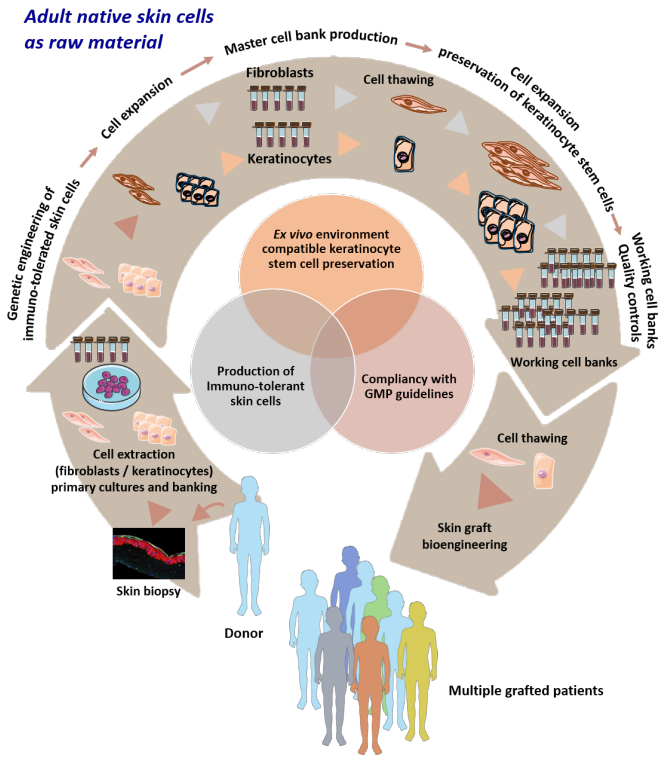

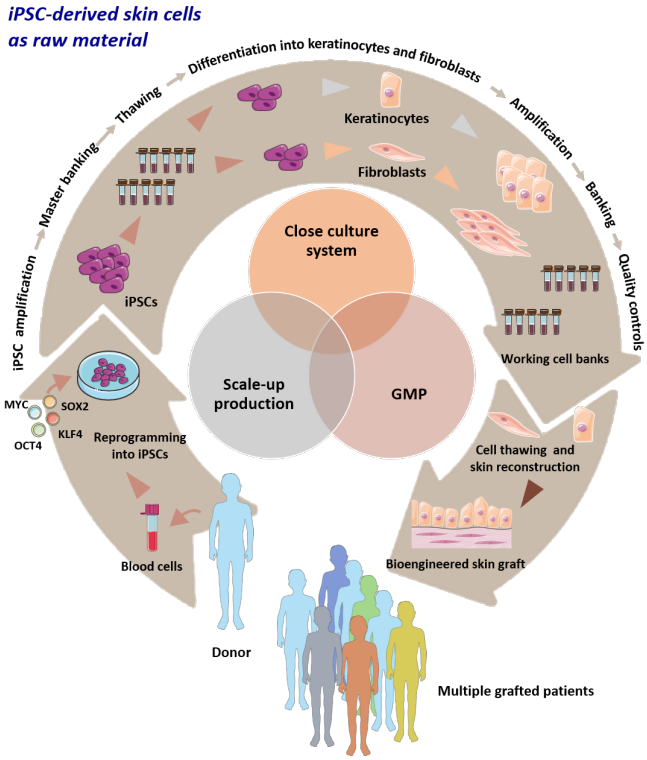

Figure 1: Proposed scheme for the bioproduction of immunotolerated skin substitutes using native adult skin cells.

Fibroblasts and keratinocytes are extracted from one skin biopsy from a selected donor, and amplified in culture and genetically-engineered to acquire an immuno-tolerated status, and then stored as master frozen banks. This frozen cellular material is available for secondary amplification and generation of secondary frozen working banks. Finally, this raw cellular material is used for the bioengineering of graftable three-dimensional skin composites. Of note the preservation of keratinocyte stem cells status during the amplification steps is a critical issue to ensure skin graft long-term regenerative potential.

Figure 2: Proposed scheme for the bioproduction of immunotolerated skin substitutes using iPSC-derived skin cells.

Blood cells are obtained from a selected donor and reprogrammed into iPSCs, which are amplified and stored as master banks. This frozen cellular material is available for amplification and lineage-oriented differentiation into skin cells (keratinocytes and fibroblasts), which are then stored as master cell banks. Finally, this raw cellular material is used for the bioengineering of three-dimensional skin composites.

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PROSPECTIVE FOR ADOPTIVE IMMUNOTHERAPY BASED ON B-CELL EDITING

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Abstract

While biotherapies based on mAbs still continue to rapidly develop and benefit from the constant development of new last-generation mAbs, another revolution is ongoing with the growing success of adoptive immunotherapy protocols based on autologous T-cells carrying chimeric antigen receptors (CAR-T-cells). In several instances, such protocols endowing patients with cells engineered on-purpose for binding cancer-specific antigens have indeed demonstrated long-term efficacy after a single cure. The optimization of adequate adoptive immunotherapy strategies thus becomes a grail for multiple chronic diseases and beyond T-cells designed for direct contact and cell cytotoxicity, other lymphoid lineages could be of interest for adoptive immunotherapy. In the context of long-term treatments, for which long-lived cells are thus suited, it notably makes a lot of sense to consider using B-lineage cells which could be edited on-purpose for prolonged *in vivo* secretion of biotherapies.

1. Introduction

More than one century ago, *passive immunotherapy* first began, notably with Behring and the serotherapy of diphtheria, based on the administration of sera from convalescent patients. These methods were then developed in multiple applications for treating or preventing infectious diseases and beyond. During the last 40 years, development of passive immunotherapy has been boosted by the availability of recombinant monoclonal antibodies (mAbs), hereby revolutionizing the treatment of multiple human disorders. In parallel, cell therapy also followed major changes since the early applications of bone marrow transplantation for treating patients with leukemia. In addition to replacing the patient's hematopoietic cells, these methods were used for their "graft versus tumor" effects, notably helping to cure leukemia. More specifically, "tumor-infiltrating lymphocytes" (TILs) extracted from tumors and expanded *ex vivo* proved efficient to treat some cancers[1]. TILs in fact mostly paved the way for developing *adoptive immunotherapy*, through the use of *ex vivo* engineered autologous T-cells modified in order to express chimeric antigen receptors (CAR-T-cells)[2]. While specific cytotoxic CAR-T-cells now constitute an attractive therapeutic opportunity for some severe cancer cases, most notably leukemia and lymphoma, there is a growing interest for also using other immune cell lineages accessible to genetic reprogramming, including TREG, NK, NKT or dendritic cells. B-cells now appear in this growing list, due to their dual ability not only to efficiently secrete Ig but also to potentially support immune memory.

2. Current aspects of passive immunotherapy

Passive immunotherapy currently relies on a broad spectrum of products. Besides the wide usage of polyclonal immunoglobulins (Igs) obtained from human sera or less frequently from animals immunized against a specific antigen (Ag), the range of monoclonal antibodies (mAbs) available for immunotherapy is constantly extending. New products now mostly consist into recombinant humanized mAbs mimicking endogenous human Igs and thus efficiently targeting the Ag while being well tolerated. The modular organization of Ig molecules into independent bricks, either variable (V) domains binding the Ag or constant (C) domains) providing effector functions, has promoted the design of multiple molecular

variants. Chimeric molecules can then mix and associate Ig domains of various origins or eventually combine them with non-Ig soluble receptors. Single-chain variable fragments (scFv) are notably often used in new-generation biotherapies. scFvs are composed of linked VH and VL domains and can eventually be associated with other protein domains or be combined as dimers (*diabody*), trimers (*tribody*) or even tetramers (*tetrabody*), in order to increase their avidity for the target. Associating different scFvs can then cumulate their specificities. Building next-generation Abs smaller than regular mAbs, such as nanobodies, can also provide them a broader biodistribution[3].

Such strategies are notably used for the design of therapeutic mAbs with a specific ability to recruit effector cells, as for bispecific T-cell engagers (BiTEs) aimed at bridging a target cell with effector T-cells. Blinatumomab, for example, combines an anti-CD19 with an anti-CD3 scFv, bridging CD19+ malignant target cells with cytotoxic T-cells, while Wu L *and al* reported a trispecific Ab targeting CD3 (T-cells), CD38 (myeloma cells) and CD28 (a co-stimulatory signal for T-cells)[4]. Natural Killer Cell Engagers (NKCEs) are similar multivalent Abs, associating one anti-CD16 scFv binding NK cell with one (BiKE) or two (TriKE) other scFv specific for cancer cells. Some TriKE additionally bind a cytokine enhancing NK activity[5]. Instead of three Fabs, another format of NKCEs includes an Fc domain, naturally binding CD16, together with an anti NKG2A "checkpoint inhibitor" Fab further increasing NK activity (by blocking the NKG2A/MHC class I inhibitory signal)[6].

3. Adoptive immunotherapy as established with CAR-T-cells

ScFvs from therapeutic mAbs have also provided bricks for building artificial chimeric Ag receptors (CARs) anchored to the cell membrane and coupled with strong signaling cascades for transforming autologous T-cells into therapeutic agents for adoptive immunotherapy.

CAR-T-cell therapy in fact obviously constitutes the latest success story in cell therapy. It is based on the forced expression of a new Ag-binding receptor, able to activate on purpose transduced or transfected primary T-cells against a given Ag (usually a tumor Ag)[7]. Additional modifications of engineered T-cells were also proposed in order to ensure local secretion by T-cell of a soluble molecule acting against the cancer, thus using CAR-T cells as "micro-pharmacy"[8].

CAR-T-cells transduction currently mostly relies on retroviruses and lentiviruses, *i.e.* vectors with efficient genomic integration and a potential risk of oncogenic insertion. This calls for the development of safer strategies, either based on naked DNA, RNA included into nanoparticles, or transposase-based systems [9,10,11,12,13, 14].

Since random insertion is afflicted with safety issues, precise genomic edition using the CRISPR/Cas9 system is currently the most attractive strategy for future cell therapy projects based on *ex vivo* genetic re-programming and it begins to enter into therapeutic applications [15,16]. As for immune cells, either T or B cells, precise genome editing of the TCR or Ig locus also carries the advantage to really edit antigen-specificity, *i.e.* to simultaneously disrupt endogenous TCR/BCR expression and trigger expression of a knocked-in cassette encoding a new receptor with an on-purpose specificity.

Such a strategy notably allowed to insert in the TCR locus a CD19-specific CAR and to yield persistent and functional CAR-T-cells[17]. Several reports have now documented TCR gene replacement after bringing DNA templates either with AAV or with naked DNA[18]. This has also supported projects of multi-engineered universal "off-the-shelf" CAR T-cells, after simultaneous CRISPR-inactivation of TCR, B2M and PDCD1 genes, without HLA class I expression and not susceptible to PD-1 checkpoint inhibition[19].

4. Stategies for *in vivo* mAb production

Continuous refinement and optimization of genomic edition with CRISPR-Cas9 tools obviously open

avenues for future precise edition of Ag receptor genes, while progresses in regenerative medicine and *ex vivo* expansion of human cells now make it reasonable to consider re-tailored B-cells as future tools available for adoptive immunotherapy.

Indeed, B-lineage cells undoubtedly constitute the best suited Ig factory. As terminally differentiated long-lived plasma cells (LLPCs), they can secrete Ig at high level on the long term. As resting B cells, they can also produce a membrane-bound BCR and patrol in blood and lymphoid organs while waiting for activation upon antigen encounter. While pre-immune (naïve) B-cells normally express an unmutated IgM/IgD-class BCR, the affinity of the BCR for the Ag can increase along somatic hypermutation (SHM) of V(D)J sequences in germinal centers (GCs). Effector functions of the BCR (and the secreted Ab produced hereafter) can also be reshaped by class switch recombination (CSR) in activated cells. The abovementioned modular organization of Ig into structurally independent domains is also perfectly suited for on purpose engineering.

Given that molecular genetics now makes it doable to express recombinant Ig in primary B-cells, *in vivo* usage of such re-programmed B-cells might become a therapeutic strategy for addressing several needs. B-cell based therapy might notably help provide patients therapeutic mAbs in situations needing prolonged (lifelong) treatments. Among the specific and numerous advantages of such a strategy stand the permanent infusion of a biotherapy (avoiding pharmacodynamic variations for mAbs with short half-lives), the local delivery of immunoregulatory mAbs by LLPCs in some lymphoid tissues, the administration of mAbs for which *in vitro* production is not mastered due to their structure or their glycosylation, and the potential ability of edited primary B cells to deliver not only a single mAb but a collection of its various class-switched variants.

Beside the direct re-programming of primary B-cells, it would also be doable for the same goals to modify B-cell precursors, *i.e.* either hematopoietic stem cells or even induced pluripotent stem (iPS) cells since strategies for differentiating iPS into immune cells are emerging[20,21].

a. Immunotherapy with virus-encoded, DNA-encoded or RNA-encoded mAbs

Gene delivery based on adeno-associated viruses (AAVs) or naked DNA has initially been applied to mAb delivery, in so-called "vectored immunoprophylaxis" (VIP), notably for experimentally protecting laboratory animals by making them produce mAbs against a variety of pathogens such as *P. Falciparum* [22], Ebola virus [23], SIV[24], Zika Virus…[25]. Similar non-specific DNA delivery procedures have also been experimented in mice against HER2+ tumors[26]. If prolonged therapy is required, such procedures are however likely to need repeated gene administrations, each of them with the risk of oncogenic random genomic insertion, *i.e.* with safety issues.

More recently, *in vivo* production of an anti-HIV neutralizing mAb has also been obtained in mice through safer RNA-based approaches, using lipid nanoparticles as carriers[27]. The same was obtained with RNA encoding a Chikungunya-neutralizing mAb, yielding protection in mice challenged with the virus. However, as for proteins, an RNA-based strategy requires repeated injections in order to yield long-term protection.

b. B-cell editing for *in vivo* mAb production

Several reports have pioneered *in vivo* mAb production by engineered B-cells. B-cell modification was first obtained by targeting their lymphopoietic precursors using lentiviral transduction for finally producing anti-HIV broadly neutralizing Abs in mice, hereby yielding some functional anti-viral effects (bnAbs)[28–30]. Since non-specific lentiviral insertions could potentially endanger cell viability or function, such methods are however unlikely to find clinical applications[31].

CRISPR-Cas9 technology now expands the possibilities of manipulating the human genome and could clearly be developed in order to edit antigen-specificity in B lineage cells, notably since circulating B-cells are readily available from blood and can thus easily be manipulated.

A major safety advantage of *ex vivo* manipulation lies in a tighter control of which cell type is specifically targeted, and which gene modification indeed occurs within targeted cells before their reintroduction back into the host[32]. A strategy following which B-cells are isolated from blood, edited *ex vivo* for mAb secretion and injected back into the patient (**Figure 1**) then stands as highly attractive and could find applications in multiple instances, from infections, to genetic disorders, chronic inflammatory diseases and cancers[31,33].

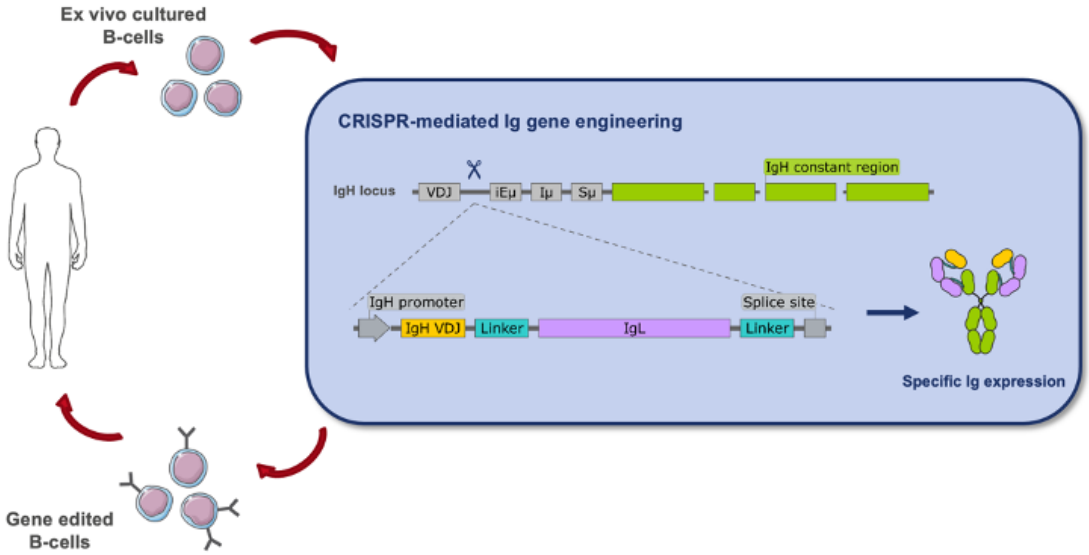

Figure 1: *Potential protocol of B-cell-based adoptive immunotherapy including ex vivo genome edition*

By contrast to lentiviral insertion, CRISPR/Cas9 cleavage of genomic DNA can result in precise site-specific insertion by homology-directed repair (HDR)[32,31]. A mix of the Cas9 endonuclease, with the specific guide RNA (gRNA) and the DNA template to be inserted, can be brought into target cells in various forms. A convenient system shown to work in primary human B-cells brings the DNA template together with a ribonucleoprotein (RNP), *i.e.* a pre-mix of Cas9 protein and gRNA [34,31]. In addition to gene edition parameters, the conditions in which primary B-cells are grown prior to their transduction has a strong influence on the rate of successful edition, notably since HDR optimally occurs in cells at the S/G2 phases of the cell cycle [35,36,37]. Several studies have thus tried to define optimal B-cell expansion parameters for *ex vivo* B-cell editing[34,31,38]. Successful expression of re-tailored Ig loci has thus been obtained by several teams. This was notably done for producing mAbs against HIV after simultaneously disrupting the κ L chain locus in primary B-cells using RNPs, and inserting a transgenic "H and L chains" cassette, in the IgH intron following JH[39]. Anti- TNF-α mAbs or nanobodies were also obtained by inserting ssDNA templates into the H or L chain loci[34].

Since immunoglobulins are H2L2 polymers, engineering their expression for therapeutic goals needs to face several challenges. In B cells previously producing an endogenous Ig, this notably requires to obtain high and stoichiometric expression of both the new H and L chains while preventing or minimizing the expression of the endogenous Ig chains. Failure to do so would result into assembly of chimeric Ab molecules including Ig chains of diverse origins, and most probably of undesired specificity. Any attempt to express transgenic Ig thus requires parallel termination of endogenous Ig chain production. In this regard, engineering the IgH locus stands as a very attractive strategy and has notably been

accomplished efficiently in murine B-cells by using RNP while bringing the DNA template included into an AAV vector[31,38,40].

By targeting a site upstream of the Eμ enhancer for inserting a single gene cassette, which encoded both the Ig L and H chain under a single-chain format, Moffett *et al* notably obtained the disruption of the endogenous IgH chain production together with the production of a neutralizing mAb against respiratory syncytial virus (RSV)[40]. By similarly disrupting the endogenous IgH locus expression through insertion in-between JH and Eμ, we recently expressed a singular form of single-chain complete Ig molecule (scFull-Ig), the sequence of which starts with an scFv fragment[41]. In this configuration, both the VH and VL domains were included in the IgH SHM domain, and could eventually benefit from affinity maturation *in vivo*, a feature of potential interest for dealing with viral or cancers antigens prone to antigenic variations.

4. Concluding remarks

Altogether and despite remaining challenges, editing primary B-cell is highly promising and currently motivates the efforts of several academic teams in various countries, while having also provided a framework for research and development in two young U.S. Biotech companies, Immusoft and Bebiopharma.…

Multiple applications are indeed possible, even by just exploiting the remarkable ability of LLPCs to secrete proteins (either Ig or other therapeutic proteins) in high amounts and for long periods. If applied to therapeutic mAbs, this would eliminate the need for periodic injections of some short-lived mAbs[31,30]. It could also develop immune protection on purpose in patients with some immune deficiencies and a defective responses to vaccines[31]. By contrast to the homogeneity of therapeutic mAbs administered as proteins, mAbs produced by edited B cells as a collection of class-switched variants sharing the same V-regions would be a strong asset, then cumulating the effoctor functions of the various Ig classes. Such a goal can clearly be reached by engineering the IgH locus, provided the expression of the recombinant is obtained by engineering the JH region and preserves the CSR process[39,42]. In edited B-cells, an adoptive BCR could also potentially be further "improved" *in vivo* in terms of affinity maturation if the edited cells are able to enter into germinal centers and participate to immune reactions after challenge with their cognate antigen[40,42]. Ability to undergo SHM would be especially attractive for immunity against pathogens which undergo frequent genetic changes, such as for HIV, or SARS-CoV2, and probably also against mutating cancer cells.

Besides methodological challenges concerning the efficiency of B-cell genetic edition and the long-term survival of edited B cells, the other obvious challenges are at the level of safety. These safety issues are crucial in a lineage spontaneously exposed to genetic anomalies related to aberrant recombination mediated by RAG enzymes or to off-target mutations driven by the activation-induced deaminase activity[43,40,39,42,44]. Cas9 off-target mutations might come with an additional layer of genetic anomalies. These aspects can however be mastered and new CRISPR/Cas9-related tools, such as nickase enzymes or mutated Cas9 with lowered off-target rate, are now increasingly safe and strongly limit the risk of off targets genomic alterations[44]. Various strategies can also now be proposed in order to eradicate edited cells if needed, notably by using suicide genes able to trigger inducible apoptosis[45,46].

Altogether and as shown by several recent reports, the progresses of genomic edition and of B-cell culture conditions clearly indicate that adoptive B-cell immunotherapy is feasible and likely to soon become a new strategy for addressing a number of unmet needs in treating human diseases.

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Intellectual property

Learn about the challenges of patenting cell therapy

IP CONSIDERATIONS FOR CELL-THERAPIES

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Introduction

Cell therapy refers to the transfer of autologous or allogeneic cellular material into a patient for medical purposes. It is a promising, rapidly advancing field with the potential to transform medicine across disease areas with significant therapeutic need. For example, since the discovery of stem cells over half a century ago, more than 5000 US clinical trials have utilised stem cells. Currently, most cell therapies are in early stages of development (phase 1/2), with several exceptions being either a current best practice in specific settings (e.g., bone marrow/stem cell transplants, hepatocyte transplantation, skin equivalents), or approved for specific indications, such as PROVENGE® (sipuleucel-T), LAVIV® (azficel-T), MACI® (autologous cultured chondrocytes on porcine collagen), and KYMRIAH™ (tisagenlecleucel) among others.

Cell therapy combines stem cell- and non–stem cell-based unicellular or multicellular therapies. It typically employs autologous or allogeneic cells; might involve genetic engineering or manipulations in formulation; and can be administered topically or as injectables, infusions, bioscaffolds, or scaffold-free systems. Cell therapy spans multiple therapeutic areas, such as regenerative medicine, immunotherapy, and cancer therapy.

Treatment using cell-based therapies relates to a global market size estimated to expand from USD 9.5 billion in 2021 to USD 23.0 billion in 2028. It is also one that involves significant investment and, therefore, can be expected to have concomitantly significant costs to patients and their insurers. Accordingly, patent exclusivity will be important to defray high development and regulatory compliance costs. The nature of such therapies, and recent patent law trends regarding natural products and methods relating to the practice of medicine, suggest these therapies may not be given the type of robust patent protection conventionally available for small-molecule drugs.

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Different types of cell therapies

Cell therapies come in all sizes and shapes and can be classified in various overlapping categories.

A first, commonly-used classification is based on the origin of the cells used. For example, these therapies may use cells taken from and administered to the same individual (autologous). In this case, the cells are isolated and/or derived from a patient, concentrated, modified, or otherwise manipulated and then returned to the patient. Examples of current technologies using autologous cells include haematopoietic stem cells (HSC) isolated from bone marrow or umbilical cord blood. Other, more nascent sources of autologous cells include adult stem cells or progenitor cells isolated from tissues; these include e.g., skin stem cells and mesenchymal stem cells (MSC) derived from adult tissue or stimulated to differentiate from embryonic stem cells. A variety of immune cells, such as tumour infiltrating lymphocytes (TILs), viral reconstitution T cells, dendritic cells, γδT cells, regulatory T cells (Treg) and macrophages are also somatic cells that are being developed as cell therapies.

Another broad area corresponds to cells derived from a donor who is different from the ultimate patient benefiting from the treatment (allogeneic). Such cells have the advantage of being capable of mass production and being available off-the-shelf and, to some extent, standardised with regard to biochemical, metabolic, and antigenic properties. Disadvantages include the possibility that for any particular patient, immunological rejection may be triggered. These cells include cells having the broadest applicability, such as human embryonic stem cells and induced pluripotent cells (iPS), which can be generated by introducing four well-defined genes (Oct3/4, Sox2, c-Myc, and Klf4) into cells from appropriate tissues. Human embryonic stem cells have the broadest potential applicability because they are the most pluripotent, but their use can raise ethical issues, whereas iPSCs although less robust but also less ethically challenging.

Cell therapies can also be classified by the therapeutic indication they aim to address, e.g., neurological, cardiovascular, ophthalmological. On a more general level, they can be classified as being directed to regenerative medicine, immune system disorders, cancer therapies, or others.

In particular, many stem cell-based therapies are currently used in regenerative settings, either as investigational or established treatments with the rationale of repopulating damaged cells or resetting tissue homeostasis. In regenerative medicine, adipose stem cells (ASCs) are considered the most promising among cell therapies. HSCs have been widely used for treating haematopoietic disorders such as those resulting from myeloablative treatment. On the other hand, the clinical application of embryonic stem cells (ESCs) or iPS seems to be more distant, due to preparation and standardisation obstacles and lack of therapeutic evidence.

Most commonly, cell-based therapies are classified by cell types and the modifications involved. For example, the EU regulatory classification of cell-based therapies discriminates between minimally manipulated cells for homologous use (transplants or transfusions) and those regulated as medicines which are required to demonstrate quality, safety and efficacy standards to obtain a marketing authorisation before becoming commercially available (referred to as Advanced Therapy Medicinal Products; ATMPs) which are further subdivided into somatic cell, gene therapy and tissue engineered products. In this regard, CAR-T cells therapy is particularly promising; questions regarding patent protection and freedom to operate of this specific technology were previously discussed[1] and will not be addressed herein.

Patent protection prospects

The patentability of pluripotent stem cells has given rise to a number of fundamental questions, both legal and ethical, to which answers have been given on a country-by-country basis. Specifically, attitudes towards the patentability of hESC research findings are undergoing dynamic adjustment based on benefit weighing and the general evolution of jurisprudence. This question is particularly pregnant in Europe and the US wherein more than 35 % of all patent applications with stem cell technology are filed.

[1] Gillet & Bouquin (2020) Disruptive CAR-T therapy is driving a new wave of patenting challenges. Immunowatch n° 2, p. 37.
[2] 447 U.S. 303 (1980)

Patentability of hESCs in the U.S.

US law poses no morality-based barrier to patenting human stem cells. In the US, patent eligible subject matter is "any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof." (35 USC §101).

Until recently, this section has been interpreted broadly by the courts, including in Diamond v. Chakrabarty[2], when the Supreme Court stated that "everything under the sun made by man" should be patent-eligible. The important companion case to Chakrabarty is In re Bergy, wherein the United States Court of Customs and Patent Appeals (CCPA) opined that the biologically pure culture was not a product of nature and that patentability was not affected by the microorganism being alive.

Stem cells were protected under this pretence for about thirty years. The patentability of hESC-related findings suffered not much resistance in the United States. For instance, the Wisconsin Alumni Research Foundation (WARF) held a series of fundamental patents, including patents covering hESCs. Both inventions were patented in the United States. In reexamination proceedings, USPTO concluded that the previously existing technologies were too unpredictable to allow other scientists to culture hESCs and accepted the non-obviousness of the WARF patents. This case shows the American attitude toward the patentability of hESCs-related findings.

This situation changed with the passage of "America Invents Act" ("AIA") into law. The AIA provides that "no patent may issue on a claim directed to or encompassing a human organism"[3]. The legislative history of AIA clarifies that under this act, stem cells are patent eligible but patent claims directed to or encompassing a human organism, including human embryos are prohibited. However, the AIA does not define "human organism". There is unfortunately no way of predicting how the courts will interpret these words. In particular, if, in the future, courts construes "human stem cells" as being a kind of "human organism", stem cells would be patent ineligible.

New court decisions in the last ten years have however seriously restricted the scope of patent eligibility in the U.S.

In Association for Molecular Pathology v. Myriad Genetics, Inc. (2013)[4], hereafter referred to as the Myriad decision, the Supreme Court ruled that DNA isolated from nature was patent ineligible, while at the same time deciding that cDNA was patent eligible. The court's rationale, though, was particularly worrisome with regard to patenting natural products. This rationale was that "mere" isolation of a portion of human DNA (a "gene") from a chromosome was not enough to render the isolated DNA patent eligible. There has to be "something more", in accordance with another Supreme Court decision, this time involving diagnostic method claims, Mayo Collaborative Services v. Prometheus Laboratories[5], which has been interpreted to mean a structural difference.

This strongly suggests that all products — nucleic acids, proteins, cells, etc. — which are not significantly different from a product of nature might be patent-ineligible. For example, it is because the claimed clone did not present any difference with the organism that they were derived from that the Federal Circuit found that claims directed to Dolly the sheep were not patent eligible[6]. Genetically engineered or otherwise modified cells likely remain patent eligible under Myriad. However, other types of cells used in cell-based therapies (e.g., hematopoietic cells, embryonic and adult stem cells, and products of such cells such as dermal sheets used to treat burn victims) are clearly at risk with regard to whether patent protection is or will be available.

On the other hand, process patents are less likely to be patent-ineligible laws of nature. In Rapid Litigation Management v. Cellzdirect, Inc., the claims were directed to a method of preparation of hepatocytes. The court reasoned that these claims differed from patent-ineligible law of nature claims because they did more than just observe the law of nature — they are directed to a new method of better preserving hepatocyte cells. In addition, the steps of that method were not "routine or conventional" in relation to the prior art. In its holding, the court repeatedly emphasized that these claims were eligible because they claimed a

[3]AIA, Pub. L. 112-29, sec. 33(a), 125 Stat. 284
[4]Association for Molecular Pathologists v. Myriad Genetics, 133 S. Ct. 2107 (2013)
[5]Mayo Collaborative Services v. Prometheus Laboratories Inc., 132 S. Ct. 1289(2012).
[6]In re Roslin Institute, 750 F.3d 1333 (Fed. Cir. 2014)

method or process, and the result may have been different if the claims only covered the frozen hepatocyte product.

Over the last five years, the United States has significantly narrowed the scope of patent-eligible subject matter in the field of biotechnology. Subject matter that was previously considered patentable may now be rejected as unpatentable. Even though the Supreme Court has not classified stem cells as patent-ineligible subject matter, the worry that stem cells may be so classified is on many investors' minds.

Patentability of hESCs in Europe

Nothing is ever simple in Europe. When considering patent law, three overlapping layers of jurisdiction coexist and must be taken into account. A first level is provided by the European Patent Convention ("EPC"), an international treaty signed in 1973. The EPC created the European Patent Office ("EPO") and also created the substantive law as to what is patentable in the member states. The provisions of the EPC are interpreted by the Boards of Appeal, the judicial instances of the EPO. It is important to note that the EPC is distinct from the EU. Indeed, a second level of jurisdiction relevant for patentability of stem cells is constituted by the European Union ("EU"). The EU is an important source of legislation in Europe, both direct (directives and regulations) and indirect, through the case law of the Court of Justice of the European Union ("CJEU"). Finally, national patent laws and their interpretation by the national patent organisations and national courts add another layer of complexity. Notably, national patent laws must be compatible with the EPC whilst respecting the EU legislation.

The discussion of the legal protection of biotech inventions, including stem cells, originated in the EU. The result was eventually the Directive 98/44/EC of 6 July 1198, widely known as the Biotech Directive. This directive defines the legal basis for protection of all biotechnological inventions, including stem cells. Firstly, the recitals state that a mere discovery cannot be patented. Secondly, however, articles 3 and 5 open the doors for patenting isolated biological matter or human body elements, even if their structure is identical to a structure occurring in nature. The principles of patenting stem cells and gene sequences are regulated in Article 6 of the Biotech Directive. This article prohibits patenting inventions that use the human embryo for industrial or commercial purposes, as their commercial exploitations would be contrary to ordre public or morality.

The EPO, which as previously noted is separate from the EU, was under no obligation to make any legislative adaptation of its own. Yet the EPO's Administrative Council nevertheless decided in 1999 to adapt the Implementing Regulations to the EPC (the EPC Rules), in view of the large overlap between EU member states and EPC contracting states. It thus incorporated the criteria and definitions set out in the Biotech Directive into the European Patent Convention (EPC) Implementing Regulations (Rules 26 to 29) and Guidelines for Examination (Part G, II.5.2). According to Rule 28(c) of the EPC Implementing Regulations (corresponding to Article 6(2)(c) of the Biotech Directive), European patents cannot be granted to inventions that use human embryos for industrial or commercial purposes. This provision must be read in conjunction with Article 53 (a) of the EPC which states that, if the commercial exploitation of the invention is contrary to ordre public or morality, it is excluded from patenting. In other words, the prohibition of inventions using human embryos for industrial or commercial purpose is based on morality which strongly suggested that it would be a blanket interdiction.

Decision G 2/06 (November 2008) of the Enlarged Board of Appeal of the EPO was based on an application relating to a cell culture comprising primate embryonic stem (ES) cells. The board held that subject matter relating to products (i.e., stem cells) which on the filing date can be exclusively prepared by methods necessarily involving the destruction of human embryos from which said products are derived is not patentable under the EPC. Technical developments after the filing date are irrelevant. Importantly, this applies even if the destructive method is not explicitly part of the claims. In other words, the application must be taken as a whole for this assessment

A few years after G 2/06, a similar case was heard by the CJUE. The patent at stake was directed

to methods maintaining hESCs in culture in an undifferentiated state, as well as a cell culture comprising hESCs. In this case (C-34/10; Oliver Brüstle v Greenpeace e.V.), the Luxembourg judges had to consider for the first time the term 'uses of human embryos for industrial or commercial purposes' within the meaning of Article 6(2)(c) of the Biotech Directive.

The CJUE decided that according to the Biotech Directive, inventions are excluded from patentability where the technical teaching that is the subject-matter of the application requires the prior destruction of human embryos or their use as base material, whatever the stage at which that takes place and even if the description of the technical teaching claimed does not refer to the use of human embryos. Therefore, it is not possible to isolate the subject-matter of the patent from the prior destruction of the embryo, even if the link between both is not immediate. The decision of the CJEU appears to be in line with G 2/06, as it confirms that an invention is excluded from patentability where the technical teaching which is the subject matter of the patent application requires the prior destruction of human embryos or their use as a base material.

In the same decision, the Court had to interpret the term 'embryo', since it is nowhere defined in the Biotech Directive. The CJUE opined that the term 'embryo' had to be widely interpreted. This term should encompass all stages of human development after fertilisation of a human egg, as well as cells "capable of commencing the process of development of a human being".

This immediately provoked an outcry. It was argued that such a broad definition would permanently stifle European stem cell research, since it is well known that the possibility of obtaining a patent in a technical area has a significant impact on whether and how research organisations move into an area. Moreover, the decision was criticised on scientific grounds as seemingly including pseudo-fertilised eggs, or parthenotes, in the definition of 'embryo, when, in reality, these cells do not develop in the same way as normal embryos; nor are they developmentally viable if made without nuclear transplant.

This last point was corrected in a later decision (C-364/13; International Stem Cell Corporation v. Comptroller General of Patents) wherein, contrary to the previous decision, the court held that an unfertilised human egg whose division and further development has been stimulated by parental genesis does not constitute a 'human embryo' within the meaning of the Biotech Directive. In light of current scientific knowledge, it does not, in itself, have the inherent capacity to develop into a human being. Consequently, the term 'embryo' covers only cells that have the capacity to develop into a human being and parthenotes-based inventions may be patentable in Europe.

A question immediately arising from the decisions relates to the "relevant" date of the invention. Both G 2/06 and Brüstle (in combination with ISCC) prohibit patenting inventions involving the destruction of an embryo. To establish whether cells are obtained by the destructive use of a human embryo, one has to take into consideration not only the teaching of the application, but also «the state of the art at the filing date». It is therefore necessary to determine the point in time when new technology for obtaining hESC lines without destroying embryos rendered such practices more flexible.

In T 1441/13 the Board of Appeal concluded that at the 2001 filing date of the patent in suit, the known and practised method for achieving cultures of human ES cells (i.e., the starting material of the claimed method) necessarily included preceding steps that involved the destruction of human embryos. Another source of pluripotent cells was not specified in the application. According to the evidence before the board, the first public disclosure of a method by which human embryonic stem cells could be obtained without destroying a human embryo was published in January 2008.

This date was further pushed back in time in T 385/14. The claimed method involved using an in vitro differentiated cardiomyocyte with the proviso that this cell was not derived from a human embryonic stem cell. The board, referring explicitly to Brüstle and ISCC, found that, at the priority date (May 2004), it was possible to derive hESCs from parthenotes since a protocol had already

been made available by the publication of a patent application[7] on 5 June 2003.

Another important question raised by G 2/06 and Brüstle (in combination with ISCC) relates to the identity of the person 'destroying the embryo'. In particular, one could ask whether the exemption would apply only when the embryo was destroyed (so to speak) by the person seeking or owning a patent.

However, in T 2221/10, the competent EPO board of appeal answered negatively to that question. This case was based on an application relating to methods for maintaining hESCs in culture in an undifferentiated state by the addition of certain human foreskin cells, as well as a cell culture comprising hESCs. The claim thus did not require the destruction of a human embryo. The board considered that if such hESCs were derived from human embryos that had been destroyed, it was irrelevant how early in the performance of the invention such destruction occurred. The board thus held that Article 53(a) and Rule 28(c) EPC do not merely exclude the patentability of biotechnological inventions that make use of human embryonic stem cells obtained by de novo destruction of human embryos, but also apply to inventions which employ publicly available cell lines which were initially derived by a process resulting in the destruction of human embryos.

The law that governs the patentability of stem cell technologies in Europe is thus complex, at least insofar as the stem cells are embryonic in origin. It has a convoluted genesis and is overlaid with case law from the CJEU, the EPO, and national courts, not all of which have historically taken the same approach, although the CJEU and EPO now appear to be aligning.

Patentability of hESCs in China

As in Europe, some inventions in China's patent law are exempted from patentability on ordre public and morality grounds. In China, ethical concerns about embryos mainly emerge in areas of human cloning and the destruction of human genetic consistency. Hence, ethical and moral restrictions placed on human embryos in China have restrained the regulation and practice of hESCs patentability.

Article 5(1) of China's Patent Law (CPL) provides that "[n]o patent shall be granted for an invention that contravenes any law or social morality or that is detrimental to public interests."

Whether an invention contravenes social morality is subject to preliminary examination under China's Patent Law. Article 5(1) also provides a basis for rejecting a Chinese patent application during substantive examination, and for invalidating a Chinese patent claim. Therefore, passing the preliminary examination or even obtaining an issued patent is no guarantee that China National Intellectual Property Administration (CNIPA) will not come back to reject a patent application or invalidate an issued patent for contravention of social morality.

This provision was originally interpreted very strictly, so that both "utilisation of human embryos for industrial or commercial purposes" and utilisation of human embryos for the purpose of treatment or diagnosis and are beneficial to human embryos were considered to be patent-ineligible. Over time these restrictions relaxed. In particular, in 2015, it was determined that human cells without developmental totipotency were not embryos and, as such, were not excluded from patentability.

In addition to the CPL and Implementing Regulations thereof, the Guidelines for Patent Examination (GPE) are crucial in the practice of patent review as a specific standard for patent applications and requests in accordance with the other two regulations. The GPE were reviewed in 2020, with in particular the introduction of a specific provision forbidding patentability of inventions directed to the use of embryos for commercial or industrial purposes. On the other hand, hESCs are now explicitly defined as not reflecting a stage of human development. This means that there exists no blanket prohibition of patenting hESCs and methods for preparing them.

Unlike in the United States, where patent eligibility or the lack thereof is frequently the subject of heated legal battles and often subject to judicially crafted tests, China's primary approach to this issue has been administrative. Even though the GPE supposedly only binds patent examiners, it is unlikely that Chinese courts will take a different position from that of the GPE.

[7] WO 2003/046141

Conclusion

Cell-based therapy is a rapidly-growing field of clinical research, sustained by substantial global investment. Strong patent protection is essential for investors. However, recent legislative and jurisprudential developments have converged towards limitations of patent eligibility of cell-based therapy-related inventions, notably hESCs, around the world.

In the US, the AIA and recent Supreme Court decisions have casted doubt on the patentability of hESCSs and other cell products identical to natural products. In Europe, the Biotech Directive and its application by courts of both the EU and the EPO has excluded patentability for hESCs on a morality basis. In China, strict interpretation of the morality clause led to severe restrictions on human cell patentability; however, CNIPA has since re-adjusted its construction of the provision, reaching a position more akin to Europe.

While many worry that unduly restrictive patents will hinder innovation, the long-term effect is not clear cut. However, the harmonisation of the concept of patentability-excluded subject-matter in relation to cell therapy in the major markets may bring a level of stability most welcome by investors.

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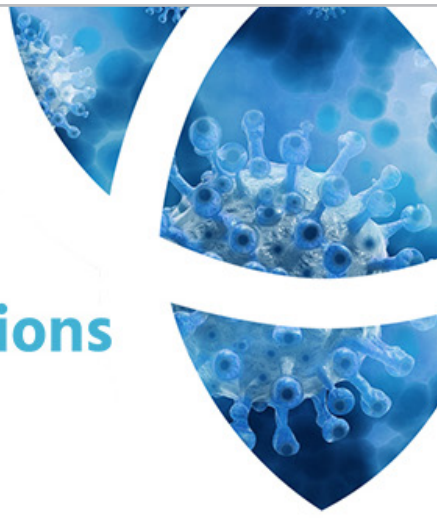

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