Applying the Cytocentric Principles to Regenerative Medicine for Reproducibility

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Abstract

Purpose of Review Cell and tissue products do not just reflect their present conditions; they are the culmination of all they have encountered over time. Currently, routine cell culture practices subject cell and tissue products to highly variable and non-physiologic conditions. This article defines five cytocentric principles that place the conditions for cells at the core of what we do for better reproducibility in Regenerative Medicine.

Recent Findings There is a rising awareness of the cell environment as a neglected, but critical variable. Recent publications have called for controlling culture conditions for better, more reproducible cell products.

Summary Every industry has basic quality principles for reproducibility. Cytocentric principles focus on the fundamental needs of cells: protection from contamination, physiologic simulation, and full-time conditions for cultures that are optimal, individualized, and dynamic. Here, we outline the physiologic needs, the technologies, the education, and the regulatory support for the cytocentric principles in regenerative medicine.

Keywords Regenerative medicine · Tissue engineering · Cell culture · Organoids · Stem cells · Cytocentric

Introduction

As it is not one swallow or a fine day that makes a spring, so it is not one day or a short time that makes a man blessed and happy. — Aristotle.

The Cytocentric Principles are Basic Quality Principles for Cells

The quote from Aristotle is often interpreted as, "We are what we repeatedly do. Therefore, excellence is not a single act, but a habit." Likewise, cells and tissues, grown in our care, are not the product of a single set of conditions, but of the sum of those conditions over time.

Within the greater awareness of problems with biomedical research reproducibility [1], variability in regenerative medicine (RM) research has been identified as a major hindrance to advancement of the field [2]. While we are all taught that cell culture conditions are critical to reproducibility and translatability, we put those teachings aside when it is time to do experiments. We subject cells to environments that vary between non-physiologic conditions in

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the incubator to different non-physiologic conditions in the BSC. Swings in conditions are a preventable source of variability in cell and tissue culture. A recent set of landmark articles by Klein et al. [3, 4••] showcases critical cell parameters (CCPs) like pericellular O_2, CO_2, and pH levels that have been traditionally disregarded in cell culture. The authors call for improved control, monitoring, and reporting of cell culture conditions as neglected variables.

While traditional cell culture methods have produced successful cell therapies, such as CAR-T, it is not a secret that control of CCPs can improve cell yields [5, 6], improve predictability of in vitro models [7–10], and improve the efficacy of cell and tissue products [11•, 12]. It can also reduce the variability of cell and cell-derived products [13–16], yet most cell culturists do not monitor or control cell conditions.

We establish here a set of cytocentric principles, defined in Fig. 1, to improve the culture conditions for cells in RM. Just like quality principles have improved product quality in almost every other industry, from automobiles to computer chips, the cytocentric principles have been designed to improve cell and tissue product quality. Meeting the biological needs of yeast produces the most reproducible batch of bread. Meeting the needs of human cells will produce the best, most reproducible products for RM. We believe that the sensing and control of optimal environmental parameters will be a cornerstone of every manufacturing process for RM products and will pave the way for these technologies to become the next standard of care.

Where are we Now?

Current cell culture practices are best described as "people-centric." What equipment in the laboratory is designed for the needs of cells and tissues? The benches? The chairs? These are for the needs of people in the lab, not cells. Even a room air CO_2 incubator is designed for the needs of people in the lab, not the cells. It is also prone to contamination. It has supraphysiologic O_2. The CO_2 and temperature fluctuate every time the door opens. Yet, the standard room air CO_2 incubator persists because it is familiar, cheap, and easy to use for people. It is inherently peoplecentric, not cytocentric.

As Klein et al. [3] so elegantly pointed out, poorly controlled incubation conditions for cells are the norm, and the authors did not address all of the conditions cells face outside of the incubator. Cells are routinely taken out of incubators and transported to cell handling spaces or machinery, where they encounter conditions that vary significantly from the incubator and deviate from their physiologic needs [17]. Every day and everywhere, traditional peoplecentric cell culture practices ignore the needs of cells for the convenience of people.

So if we could erase what has become rote and start over, what do cells need?

Principle 1: Cells Need Protection from Microbial Contamination

A sterile environment for cell culture, in which proper aseptic techniques are rigorously followed, is a must to ensure reproducibility. The ease with which microbial contamination can devastate a lab should not be underestimated. At one point, it was estimated that up to one third of all cultures globally were infected with mycoplasma [18]. The consequences of this contamination include changes to cell physiology and metabolism, DNA fragmentation, mutation, and chromosomal defects. Wasted resources, irreproducible findings, and safety concerns for cell products are the results.

Antibiotics and antimycotics are often used in cell culture media to prevent microbial growth. Not only can they mask mycoplasma contamination, but they also alter gene expression while impairing cell growth and differentiation [19–24]. Antibiotic/antimycotic residues from cell and tissue processing can also compromise the health of some patients.

How can we protect cells from microbial risks without antibiotics and antimycotics? With good laboratory/manufacturing aseptic practices, antibiotics can be avoided.

The Cytocentric Principles

1. **Cells Need Full-Time Protection from Contamination**
2. **Cells Need Physiologic Simulation**
3. **Cells Need Proper Conditions Full-Time, Not Part-Time**
4. **Cells Need Individualized Conditions**
5. **Cells Need Dynamic Conditions as Populations Evolve**

Fig. 1 The cytocentric principles. The cytocentric principles are general quality principles that outline the needs of cells and tissues in culture. Putting the needs of cells and tissues first is essential for a high quality and reproducible cell or tissue product in regenerative medicine

However, complete closure of the cell environment is the most effective strategy. Available technologies for controlling the physical attributes of a closed cell handling space, particularly relative humidity (RH) and temperature, can also actively reduce microbial risk to cells without the need for antibiotics [25]. Improved manual cell culture technique and closing the cell handling space fulfills the needs for cells to have a microbe-free environment.

Principle 2: Cells Need Physiologic Simulation

People have been culturing cells in room air for over 80 years [26], even though this is an alien environment for cells. Uncontrolled peoplecentric laboratory conditions are not physiologic or reproducible from site to site. We can provide more cytocentric conditions for cells in culture and improve biomedical reproducibility at the same time.

Physiologic Media

The culture media utilized in growing cells ex vivo present critical elements for maintaining healthy, proliferating cells. Natural media include highly variable biological fluids such as plasma, serum or tissue extracts, while synthetic media are either undefined or fully defined based on the formulation. Free of animal derivatives, fully defined media are more reproducible.

Glucose levels in traditional cell culture media are far in excess of in vivo levels and limiting glucose to physiologic levels benefits cells [27] and tissues [28] in culture. The combination of more physiologic medium with more physiologic O_2 levels makes a difference in the metabolic mechanisms that underlie all cell functions [29].

Physiologic Gas Levels

People experience full-time room air, but cells inside the body do not. Room air mixes in the upper airways with the gasses being exhaled such that even the lungs are at about half of room air O_2 [30]. Likewise, live skin cells get their O_2 from capillaries under the dead cell layer [31]. Unless there is a major injury to the body, living cells in vivo do not experience room air O_2 levels. This means that the term "normoxia" for room air cell culture describes a peoplecentric concept, not a cytocentric one. It is the wrong frame of reference for talking about cellular O_2.

Likewise, calling normal physiologic O_2 levels "hypoxia" can lead to confusion with pathological hypoxia. Terms like "physioxia," the oxygenation state found in the normal physiology of a tissue [32], are replacing older peoplecentric concepts of O_2 status with more cytocentric ones.

It is the pericellular gas levels that needs to be controlled, not just the cell culture headspace [31, 33]. Pericellular O_2, as a CCP, is different from incubator O_2 [34]. Protocol-specific factors that impact what the cell experiences in a cell culture vessel including cell density and distance of the cells from the fluid/gas interface.

Exposing cells to room air O_2 changes their function [5, 10, 11•, 35], introducing artifact into traditional cell culture assays [9]. It not only affects cell proliferation and viability, but also cell integrity at the gene level [35]. Extraphysiologic oxygen shock, which happens during initial isolation of cells from tissues, causes cell stress, irreparable cell damage, and cell loss [5, 10].

Klein et al. [3] detail the shifts in pH that cells can experience in culture over time. Incubator CO_2 levels and bicarbonate-based buffers are employed in tandem to control pH in cell culture media. However, CO_2 diffuses quite readily out of aqueous fluids. When cells are removed from a traditional incubator into low-CO_2 room air, media can shift in pH.

Researchers often resist adopting new technologies for controlling gases around cell cultures because of additional costs; however, the most expensive research is work that does not reproduce and does not translate to patients.

Controlling gas levels around cell cultures as CCPs is critical for controlling sources of variability in cell and tissue production. Controlling them to constant physiologic levels is cytocentric.

Physiologically Relevant Structures for Cytocentric Conditions

The move from flat plastic to more physiologic 2D and 3D structures for cells in culture is inherently cytocentric. Conventional cell culture methods use flasks or bioreactors with media that promote cell growth and proliferation in a controlled environment. However, it is the interaction of the cell with other cells and the environment in a tissue that determines cell phenotype.

Organoids are 3D tissue structures starting to be used in research, toxicology, and drug development settings. Derived from primary tissue, embryonic stem cells, or induced pluripotent stem cells (iPSCs), organoids are made from cells capable of self-renewal and differentiation. An organoid is a miniaturized version of an organ produced in vitro, demonstrating more realistic micro-anatomy, and also capable of self-renewal and self-organization. Organoids have been used as in vitro models of a vast array of human organs [36] and may exhibit similar functionality as the tissue of origin [37]. The interaction of the starting materials (iPSCs or adult stem cells/tissue) with the extracellular matrix is critical for the physiological development of the organoids in culture [38].

The utility of organoids as accurate models of human tissue for RM depends on the reproducibility of their performance [39, 40]. Culture in peptide hydrogels has been demonstrated to stimulate in vivo-like secretion of extracellular vesicles from organoid-like spheroids [41]. There is still a need to develop criteria for comparisons of organoids to the respective organ. Matching in vitro organoid conditions to in vivo organ conditions should be a goal for organoid culture.

Typically, organisms with low plasticity better correlate their phenotype to their genotype. In RM, genotypes and phenotypes are more dissociated, adding a layer of complexity. For example, human umbilical vein endothelial cells may have their native genotype but portray a drastically different phenotype when seeded into porous scaffolds, a structure not encountered in the human body [42]. These cells will differentiate even with no changes in their genome, an event defined by cellular interactions with the environment, changes in gene expression and epigenetics. Changes in the genome may also occur via mutations [42, 43].

Providing an environment that is more physiologically relevant to cells in culture through fully defined media, physiologic gas levels, and tissue-like structures can help reduce cellular stress and more faithfully replicate in vivo tissues in vitro for RM.

Regardless of methods used, we emphasize the importance of both appropriate phenotype and genotype in RM, and believe a significant factor for maintaining both arise from cytocentric approaches. To improve RM quality through reproducibility, we must shift from a user-centered approach to a cell-centered dogma: a cytocentric approach.

Principle 3: Cells Need Full-time Optimal Conditions

Going back to the quote at the beginning of this article, providing more physiologic conditions for cell culture for only part of the time is suboptimal when the final product is the sum of all the conditions. Even transient temperature shifts introduce variability into cell-derived biologic products [13].

In 3D structures like cultured cartilage [12] and islets of Langerhans [44], maintenance of proper O_2levels in the cellular environment is critical for tissue viability. Cyclical changes in O_2 levels can induce organ injury and mimic the pathology of obstructive sleep apnea [45]. Cells may experience unstable pH and O_2 levels caused by cell handling or imaging outside the incubator. Extracellular scaffolding may serve as an O_2 buffer, minimizing the fluctuation levels. For example, iPSCs cultured in 3D showed more stable cell growth performance and better maintenance compared to 2D culture [38]. In addition, iPSCs cultured on 2D extracellular matrix had higher expression of REX1. This gene is critical to protecting iPSCs from the higher O_2 levels in 2D culture that can trigger mitochondrial oxidative phosphorylation. In a 3D hydrogel system, the O_2 level was buffered by hydrogel scaffolding, and the expression level of REX1 was lower. Human embryonic stem cells (ESCs) and mesenchymal stem cells are influenced by fluctuating O_2 levels due to cell handling and analysis [35].

Separating the cell incubation and handling environment from room air can also provide controlled O_2 conditions for cells and tissues [17]. Full-time control of physiologic O_2 and temperature throughout all cell culture incubation and handling operations has been shown to enhance the engraftment and efficacy of cardiac progenitor cells [11•].

By controlling cell handling spaces and extracellular structures, we can reduce the stresses that cells experience in our care and tissue product variability can be reduced for better reproducibility.

Principle 4: Cells Need Individualized Conditions

In the body, CCPs differ between cell types depending on the location of tissue, its function, and its proximity to blood vessels. Putting different cell types together in a single room air incubator is the lowest common environment for all involved. Different cell types may perform adequately under the same culture conditions and in mixed cultures, individualized conditions may not be possible. However, the use of individualized culture conditions for different cell types may improve culture performance.

Now that our understanding of cell biology is more nuanced than it was when the cell incubators were developed decades ago, it is time for a more sophisticated cell production environment for RM [44]. Lung cells [30] and bone marrow [46] exist in vivo under very different conditions, and proper oxygenation is critical for the function of both [31]. Cartilage in vivo is normally avascular and almost anoxic. Islets of Langerhans in the pancreas enjoy high blood perfusion rates. 3D structures like cultured cartilage [12] and pancreatic islets [44] need very different environments in vitro. They need individualized environments. Tuning the cell in vitro environment to the cell type-specific needs can deliver the proper culture conditions for RM for each tissue type.

Principle 5: Cells Need Dynamic Environments as Populations Evolve

Cells do not pop into existence at the opening of the incubator door; they have their own histories. Subpopulations of cells can grow, compete, differentiate, senesce, and drift

phenotypically. In fact, the very definition of life requires change over time [47]. Although some cells may perform adequately in static conditions, changes to the environment may improve the results as populations double and their nutrient requirements increase. As cells differentiate, their environmental and nutritional requirements are also likely to evolve. Part of improving reproducibility in cell culture is not only responding to the changing needs of cell populations over time, but also recording the cells' history, so when unexpected things happen, the data may reveal the point at which things went awry. Frequent optical microscopic imaging can give insights into cell health that can raise an alarm when the cells are behaving abnormally. Metrics such as variations in growth rate or motility can be used to flag abnormal conditions and they have the advantage of being sensitive to both known and unknown environmental factors.

Using the Cytocentric Principles to Guide Advancements for RM

There are new technologies that can improve conditions for cells in culture. Incubator subchambers can control O_2 to physiologic levels. Next-generation isolators can provide unbroken controlled conditions for cell handling. New sensors for O_2 and metabolites can provide real-time read-outs of pericellular conditions. Imaging systems can monitor cell coverage changes without human eyes. With each of these advances, the control of cell conditions is getting more sophisticated. At the level closest to the scale of the cell, microphysiologic systems (MPS) are finding new applications.

Microphysiologic Systems (MPS)

In the body, cell nutrients are replenished and wastes are removed. As Klein et al. [3] discussed, the changing needs of cell populations over time are not well served by static cultures that are tended to twice a week. New technologies are addressing these needs for cells in culture. MPS can supply a continuous nutrient flow and waste removal, controlling CCPs like pH and O_2, as well as incorporating extracellular matrix, compartmentalization, and chemical gradients. This control of the environment is expected to reduce the variability in organoids that has limited their translatability [48].

The organ-on-a-chip device is an MPS for customized tissue/organ disease modeling, and predictive high-throughput drug screening for pre-clinical applications [49–54]. They have generally four key elements: a microfluidic chip, biofabricated micro-tissues that are cultured in the chip, components for stimulus loading, and sensors [54, 55] for monitoring the physiological behavior of micro-tissues. Organ-on-a-chip devices can provide not only individualized environments for different tissue and cell types, but also adjust to the changing needs of the cells over time [56]. Organ-on-a-chip technology may even enable perfusion of vascularized micro-tissues and organoids which is expected to improve their reproducibility and usability [57, 58].

Although current organ-on-a-chip systems are significantly more physiological than long-used flat, static plastic cultures, many of them remain as a local 2D environment for cells. Integrating 3D cellular aggregates like spheroids or organoids [59], or extracellular matrix structures built by various biofabrication strategies [49, 60], into MPS devices, may bring improvements. The microfluidic configuration allows dynamic stimuli to take place, while cell aggregates or scaffolding ensure spatial control of cellular arrangements.

No cell or tissue in our body is disconnected from others, nor are their interactions with pharmaceutical compounds, toxins, or infectious species. A more advanced form of the MPS is the multi-organ-on-a-chip platform or the body-on-a-chip [61, 62]. Connecting individual organ types into a single microfluidic circulation makes these systems more cytocentric [54, 63, 64].

MPS, when combined with physiologically relevant media, physiologically relevant structures, advanced sensors, and next-generation isolators for handling, meet the cells' needs for 1) protection from contamination, 2) physiologic simulation, 3) full-time optimal conditions, 4) individualized conditions, and 5) dynamic conditions. With the potential to be automated both in operations and in monitoring [54], MPS have a unique position to advance RM. Not only can pre-clinical testing on human tissues be done with unprecedented accuracy, but the same platforms may also lead to improved therapeutic potential by enabling cell expansion and tissue maturation in a cytocentric manner for RM.

Standards to Support Cytocentric Principles

There are many standards for environmental control that can support cytocentric principles [65–85]. Of these, ISO 13408–6 [70] and ISO 14644–7 [77] are the most relevant with a focus on separative devices such as hoods, gloveboxes and isolator systems. A separative device is defined as "equipment utilizing constructional and dynamic means to create assured levels of separation between the inside and outside of a defined volume" [70]. These standards have a general focus on biotechnology applications and addressing particulates and aseptic technique. Live cells also require control of CCPs: temperature, humidity, CO_2, and O_2.

Standards on how to achieve control of these parameters in separative devices that are designed for cell culture and processing are required. They should address topics such as system design, sensor placement, parameter monitoring, and equipment requirements. It is often desirable to place equipment, such as centrifuges and microscopes, into the

separative device so that cells can be processed without leaving the controlled environment. Humidity is particularly challenging since it facilitates growth of microorganisms and can degrade mechanical and electrical parts (ISO 14644–3 Annex B) [74]. Guidance on which equipment should be placed within separative devices and how to achieve a stable environment during cell processing steps is needed. To assure better cell quality, the cytocentric principles could help bring the needs of cells into focus for future guidances.

Creating the Cytocentric Technical Workforce

Implementing the cytocentric principles and addressing the appropriate standards requires appropriate knowledge, skills, and abilities (KSA's) for a cytocentric workforce. A 2020 survey of skill gaps in RM found a significant need for KSA's consistent with cytocentric principles [86•]. Over 90% of survey respondents expressed a need for basic cell biology lab/production skills. Between 30 and 40% of respondents noted an unmet need for KSA's in documentation, validation, standards, regulation, bioprocessing, and biomechanics. Incorporation of cytocentric principles and their supporting technologies into RM-related biology, biotechnology, and bioprocessing curricula in higher education programs will help prepare the next generation of skilled RM technicians with a cytocentric focus.

Improving Reproducibility for RM Through the Cytocentric Principles

It has been over 15 years since a landmark article by John Ioannidis brought attention to reproducibility as a barrier to scientific progress [88]. A major contributor to this lack of reproducibility in cell culture is the failure to control CCPs like O_2 [7, 44, 89] and pH [90]. Journals are starting to take a stand in requiring publications to record necessary environmental parameters in their cell culture experimental results [91]. We believe this is a step in the right direction, but still not enough if we want more reproducible scientific publications.

While cGMP cell production does improve on process controls and documentation, cells in GMP facilities are still subjected to variable non-physiologic conditions in room air incubators and BSCs. Controlled critical cell parameters should be the new standard for culturing cells. Cell and tissue products produced under uncontrolled room conditions are simply not optimal, not robust, and not reliable. Automation has been predicted to improve the financial return on investment for iPSC manufacturing (92). There is also a business value to adopting cytocentric principles to improve the reproducibility and reliability of cell culture conditions for RM manufacturing.

Conclusions

Large-scale RM still has to prove that high quality tissue products for patients are viable as an industry. New technological advances, new guidances, and new educational efforts are needed to improve the reproducibility of tissue culture conditions and practices from the perspective of the needs of cells. The most "yeastcentric" conditions for yeast produce the best, most reproducible loaf of bread. With new cytocentric technologies, guidances, and a trained workforce, the best, most reproducible human cells and tissues will be produced for RM.

Abbreviations 2D: 2-Dimensional; 3D: 3-Dimensional; BSC: Biological safety cabinet; CCPs: Critical cell parameters; ESC: Embryonic stem cells; hiPSCs: Human-induced pluripotent stem cells; iPSCs: Induced pluripotent stem cells; KSA: Knowledge, skills, and abilities; RM: Regenerative medicine; RH: Relative humidity; MPS: Microphysiological system

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Author Contribution All authors discussed the contents and participated in writing the manuscript. AH handled final editing.

Compliance with Ethical Standards

Conflict of Interest Alicia Henn, Xiuzhi Susan Sun, Mark Nardone, Ramon Montero, Alan Blanchard, and Randy Yerden are employed by for-profit companies (eg, BioSpherix, Akron Biotech, Thrive Bioscience) working to advance regenerative medicine and so have a financial interest. Kunal Mitra, Joshua Hunsberger, Sita Somara, Gary Green, and Carl G. Simon, Jr. declare that they have no conflict of interest.

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- • Of importance
- •• Of major importance

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