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The Significance of Cell-related Challenges in the Clinical Application of Tissue Engineering

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Abstract

Tissue engineering is increasingly being recognized as a new approach that could alleviate the burden of tissue damage currently managed with transplants or synthetic devices. Making this novel approach available in the future for patients who would potentially benefit is largely dependent on understanding and addressing all those factors that impede the translation of this technology to the clinic. Cell-associated factors in particular raise many challenges, including those related to cell sources, up- and downstream techniques, preservation, and the creation of *in vitro* microenvironments that enable cells to grow and function as far as possible as they would *in vivo*. This paper highlights the main confounding issues associated with cells in tissue engineering and how these issues may hinder the advancement of therapeutic tissue engineering.

Keywords: tissue engineering, cell manufacturing, tissue culture, clinical applications, stem cells.

1. Introduction

Tissue engineering (TE) is based on the premise that by exploiting cellular biology, it should be possible to create a functional substitute analogous to the natural tissue or organ that can be used for replacement therapy. Thus, TE may hold the potential to address the transplantation crisis caused by the increasing demand for tissues that far outweighs the available supply (Fig. 1).[1]

Since the advent of TE, remarkable progress has been achieved. However, many serious challenges still need to be addressed before this field can be exploited for widespread clinical applications. At the forefront of all challenges are those related to cells. As cells constitute the main pillar of TE strategies, whether the strategy is cell injection, cell induction, or cell-seeded scaffolds,[2] tackling cell-associated problems is inescapable.

To be effective for clinical purposes, cells should be easily procurable, scalable *in vitro*, and robust in culture and implantation. They should be obtained ethically and morally, be able to integrate functionally with the recipient tissue, and be non-immunogenic and safe; i.e., neither tumorigenic nor contaminated by any pathogen.[3] In addition, cells should be capable of being processed for availability off the shelf, and stem cells should have the capability to differentiate to the desired lineage.[4]

From the clinical perspective, obtaining cells that meet all these requirements for a particular therapeutic purpose presents a challenge. Consequently, only a handful of cytotherapeutic products are commercially available, in contrast to the considerable number of companies that are pursuing cell-based therapies.[5] This paper will discuss the cell-linked problems that may arise at different stages of cell processing, from sourcing to implantation into the living body, and how these problems might deter the utilization of TE in clinical therapy.

2. Cell Source

Cell acquisition represents a major hurdle that is linked with TE of every part of the body regardless of the tissue type or the strategy applied.[6] For clinical applications, cell origins could be autogenic or allogenic – from the same patient or a different human donor, respectively. Each type can be further described in term of maturity: differentiated or stem cells.[7] Every delineated type has potential drawbacks that could compromise its reliability (Fig. 2).

Allogenic cells are donor-dependent and are associated with genetic mismatch and possible immune rejection, which would necessitate immunosuppressive therapy. Conversely, autogenic cells eliminate the need for potential donor matching and remove the immunological risk.[4] However, many hurdles offset the therapeutic benefits. Firstly, the harvesting biopsy may yield limited quantities of viable cells; in particular, this may be the case where the source is a diseased organ, such as when harvesting cells for TE of the liver from a patient with liver failure.[8] Secondly, the target tissue may be inaccessible for direct biopsy, as in the case of the heart valve, or it may not be practical to biopsy at all, as with the spinal cord.[9] Thirdly, timing limitations that involve weeks to months of *in vitro* culture to obtain an adequate number of cells may not always be practical, particularly in urgent or life-threatening conditions.[10] Finally, the high production cost associated with patient-specific TE therapy compared with other treatments (even if they are less efficacious) can be a confounding factor when healthcare resources are limited.[11]

In terms of maturity, all mature differentiated cells have a finite life span. Cell viability gradually declines until the growth is irreversibly arrested as the cells progress to senescence. This can provide a limited number of population doublings that may be insufficient to create a clinically relevant tissue.[12] In contrast, stem cells – including adult stem cells (ASCs), embryonic stem

cells (ESCs) and induced pluripotent stem cells (iPSCs) – have a unique capacity for self-renewal, potency and differentiation into various specialized cell types. However, this usefulness brings with it other problems.[13]

ASCs are multipotent and have a finite potential for expansion. They are capable of generating the cell types of the pertinent tissue, but not all cell lineages.[14] For instance, the proliferation and differentiation of mesenchymal stem cells (MSCs) to an osteogenic lineage are passage-dependent. After the fourth passage, the alkaline phosphatase expression and calcium deposition are abrogated, the cell growth is impaired, and the doubling time is increased.[15] In addition, the availability of MSCs is extremely limited; for example, the proportion of MSCs in the bone marrow is 1:100,000 nucleated cells.[16] Furthermore, increased donor age has a negative impact on cell viability, proliferation and differentiation, with concomitant display of senescent features, when compared with cells isolated from a young donor.[17,18] Moreover, spontaneous transformation of ASCs has been reported with 4–5 months' cultivation, which raises a biosafety concern regarding cancer formation.[19,20]

In contrast to ASCs, ESCs are indefinitely proliferative and pluripotent, i.e., they are able to differentiate to all tissue types.[21] Therefore ESCs offer an unparalleled opportunity to circumvent the cell paucity that impedes tissue fabrication. However, the benefit of ESCs in TE could be seriously undermined by several complications. Firstly, the ethical and moral controversy surrounding the sacrifice of viable embryos to isolate ESCs.[22,23] Secondly, the immunogenicity and potential rejection that can be triggered by immune-mismatched ESC-derived tissue. Grinnemo *et al*.[24] state that ESCs are unlikely to be immune-compatible and postulate that this problem might be counteracted by altering the isolation method from immune to mechanical, and elimination of any 'xeno' products, including the feeder layers. However, it can be argued that

this immune incompatibility may not result merely from prolonged cell propagation in culture containing animal or synthetic constituents but, rather, from immune immaturity. The accelerated *in vitro* differentiation mechanism that recapitulates the long period of normal development within a short time span will cause a lack of immune inhibition ligands and retention of residual antigens within the differentiated cells that are eventually recognized as foreign antigens.[25] The third hurdle relates to the identification of a cocktail of factors that drive the differentiation pathway to the desired lineage and optimize the differentiation efficiency, which with current isolation protocols does not yield a 100%-pure population. This incomplete differentiation poses a problem regarding culture heterogeneity, requiring an effective technique to purify the differentiated cells and prevent them from being contaminated by residual undifferentiated cells.[4] Failure in this regard will give rise to a further serious issue – i.e., the potential risk of tumorigenicity, namely benign teratoma or malignant teratocarcinoma.[26,27] The reason for such propensity appears to be that stem and cancer cells share many cellular and signaling pathways.[28] In addition, aberrancy in the cultural environment adds another risk factor for triggering oncogenesis.[29] According to Vacanti,[3] cancer cells are originally derived from natural stem cells that, under severe disruption of their environmental cues, deviate from the normal repair process and shift to malignancy, resulting in uncontrolled multiplication without maturation.

In an attempt to overcome problems associated with ESCs, iPSCs have been derived through cell reprogramming by delivering four transcription factors – Oct4, Sox2, Klf4 and c-Myc – that dedifferentiate the somatic cells and turn them back to the ESC-like state.[30,31] iPSCs have been assumed to be as pluripotent as ESCs and are readily accessible and patient-specific. The ethical and immune issues associated with allogenic ESCs should therefore be eliminated.[32] However,

the accumulative evidence has revealed that iPSCs may share many of the ESCs' problems and, indeed, these may be greater.

Compared with ESCs, iPSCs are more prone to tumorigenicity.[27] This can be attributed to the multiple mutations with subsequent genetic and epigenetic instability,[33] the imperfect induction process,[34] and the reprogramming genes themselves that clearly demonstrate oncogenicity and overexpression in progressive and poorly prognostic tumours.[35] Another challenging issue, contrary to what was expected, is that the autogenic iPSCs may provoke an immune response due to epigenetic modifications that can cause inappropriate gene and antigen expression.[36] Although the immunogenicity of iPSCs is not as intense as that of ESCs, this immune evasion may be problematic rather than advantageous because it might be exploited by engrafted aberrant cells and eventually result in tumor development.[34]

Putting aside the obstacles stated above, the critical question that is still under debate is whether the iPSCs are truly equivalent to ESCs at a molecular and functional level.[32] To date, there is no conclusive evidence proving that iPSCs are identical to ESCs. In fact, many studies have pointed out substantial differences between the two cell types and have shown that iPSCs retain the epigenetic memory of their source, an issue that may restrict the differentiation only into cell types of their original lineage.[37,38]

3. Cell Manufacturing

Once the cell source is secured, the next challenge to be considered relates to the processing stages of current Good Manufacturing Practice (cGMP), namely upstream processing (production of the desired cell quantity), downstream processing (harvesting and purification), and preservation.[39]

3.1. Upstream processing

Whether the target application is for patient-specific (autologous) or for off-the-shelf (allogenic) therapy, scaling up of cells to approximately 10^7–10^{10} is necessary.[5] Expansion of limited harvested cells to a clinically relevant *quantity* while preserving the fundamental *quality* parameters such as identity, genetic stability, purity, potency, and functionality is imperative for clinical therapy.[39] Achieving this requires manufacturing technologies that are robust, scalable, predictable, economical, and compliant with cGMP.[40]

A 2D static culture employing T-flasks or well plates is inconvenient when scaling up large quantities of monolayer cells due to the limited surface area/volume ratio. The use of a large number of culture vessels leads to real issues concerning high incubator occupancy, time, labor and media costs, as well as the contamination risk associated with the open culture system. In addition, the static environment permits gas exchange only at the liquid/gas interface (diffusion) and lacks the tight control of oxygen and nutrients.[41] Furthermore, the production of large cell numbers requires frequent enzymatic passages that may lead to dedifferentiation of cells and elimination of the supportive extracellular matrix (ECM).[42]

Roller bottles provide a greater surface area, and mechanical agitation prevents sedimentation of ingredients, however there is a lack of online monitoring and difficulty in handling large bottles manually.[43] Automation and robotics may alleviate some of these issues by housing up to 90 x T-175 culture flasks that produce approximately 2.5 x 10^9 cells, eliminating the manual effort and variability as well as minimizing the contamination risk. However, the high cost, static condition, and use of animal-derived products remain.[44]

Bioreactor-based culture systems, by contrast, have evolved not only to mitigate the biological constraints in terms of providing a dynamic, sealed, automated, reproducible, tightly controlled

and strictly monitored environment, but also to apply mechanical and electrical stimulation.[45] 3D cell culture strategies such as self-aggregated spheroids, microcarriers and microencapsulated cells suspended in stirred or spinner vessels provide a potential strategy for stem cells expansion, although this can be at the expense of mass transfer and downstream processing. Central necrosis or unpredictable differentiation may arise due to a progressive decrease in oxygen and nutrients as the cell aggregates or microcarrier clumps increase in size. In addition, cell damage may be caused by the detrimental effects of hydrodynamic physical forces e.g. shear and perfusion. Moreover, it is difficult to maintain cell viability during cell cluster dissociation or cell-bead separation of aggregates and microcarriers, respectively. Microencapsulation may counteract agglomeration and excessive shear stress, however it shares similar mass transfer and decapsulation step limitations, as well as difficulties in visualization and issues of material/equipment cost.[40,46]

3.2. Downstream processing

The ability to undertake cell detachment from the culture surface without compromising the cell quality is as equally important as cell attachment and proliferation, particularly if the cells are intended for a clinical purpose. Cell harvesting requires development of large-scale enzymatic or mechanical dissociation technologies that gently decontaminate cells from any unwanted materials (e.g. carrier) with minimum hydrodynamic shear force.[39]

Enzymatic digestion with trypsin is one of the most commonly used methods for cell recovery in 2D culture and 3D microcarrier-based expansion systems.[47,48] However, trypsin causes downregulation of growth- and metabolism-related proteins and upregulation of apoptosis-related proteins.[49] In addition, higher trypsin concentrations and longer incubation times are associated with damage to the structural integrity of cell surface integrins (adhesion proteins),

which subsequently reduces the cell's adhesive ability.[50] Furthermore, the vitality of cells cultured in scaffolds is also negatively affected by prolonged incubation in trypsin, which is necessary for ECM degradation. An increased trypsin incubation time not only releases more cells from the matrix, but also causes progressive cell destruction, which may be as high as 35%.[51]

Non-enzymatic harvesting has been suggested as a preferred alternative to avoid cell damage. Yang *et al*.[52] demonstrated the possibility of using microcarriers coated with a thermo-responsive polymer to harvest MSCs and eliminate the need for enzymes. Following cell expansion on the microcarriers, the culture temperature was decreased to 32 °C and the cells were able to detach. However, this method involved the additional step of carrier coating with a polymer, which in turn may affect the original cell surface properties. In addition, it is particularly difficult to obtain an even polymer coating on scale-up.[48] Cell conditioning with enzyme-free buffer is another method that has been utilized in place of trypsinization. However, it results in a significant reduction in cell viability and reattachment of dissociated cells.[53] Mechanical dissociation of cell colonies by manual scraping has also been investigated and shown to be detrimental to cell viability and freeze-thaw survival rate when compared with trypsinization with gentle pipetting.[54]

Recently, a novel microfluidic device has been designed by Qiu *et al.*[55] to improve mechanical detachment and recovery of cells. This device works by shortening exposure of cells to enzymes and enables the use of non-enzymatic treatment such as the calcium chelator ethylenediaminetetraacetic acid (EDTA). Microfluidic dissociation augments cell separation with trypsin, resulting in a higher yield cell number (up to 93%) when compared with vortexing and pipetting. In addition, it can eliminate the enzymatic treatment when used with small cell clusters

or monolayer sheets. For more complex models, however, the combination of brief EDTA exposure followed by device processing is particularly useful as a non-enzymatic method. Nevertheless, optimization of this device to improve channel dimensions, flow rate, processing speed, prevent sample clogging, and facilitate milder enzymes remains to be reported.

cGMP guidelines, particularly in terms of safety, make the above problems even more challenging to solve. The regulations imply that the bioprocessing should be automated, chemically-defined, economic, xeno-free, and pathogen-free. Any exposure to animal products such as a serum or feeder cell layer renders the cells irrelevant to clinical therapy.[42] A further problem in the downstream stage is that the raw material itself is the final product i.e. cells, and thus cannot be terminally sterilized.[56]

3.3. Cell preservation

The difficulties in cell sourcing and expansion impose challenges in generating a robust preservation method to ensure a steady supply of high quality and well-characterized cell lines of various types. Bio-banking systems aim to cope with such demand, yet certain critical issues should be addressed, particularly cryopreservation and storage, in addition to the distribution, recording, tracking and database management. There are also issues in using a standardized protocol for cells that are different in source and require tailored culture conditions, passage number, and cryopreservation.[57]

Cryopreservation normally utilizes controlled slow freezing (1–2 °C/min) and then rapid thawing with a cryoprotective agent, often 10% dimethyl sulfoxide (DMSO), to minimize the detrimental effects of ice recrystallization and cell shrinkage. However, it is still questionable whether this technique is convenient for banking and therapeutic applications. DMSO has a toxic effect that varies from cell type to cell type and is time-, temperature- and concentration-dependent.[58] Its

clinical adverse effects, particularly with regard to the cardiovascular and respiratory systems, have been well documented.[59] In addition, a systematic review of toxicity events linked to DMSO over the last two decades identified 779 adverse reactions, ranging from transient mild nausea or vomiting to more serious effects such as cardiac arrest, encephalopathy, respiratory stress, anaphylaxis or even death.[60] Moreover, current washing protocols may not ensure complete elimination of DMSO, which can then result in different cell responses under similar conditions.[61] Furthermore, the slow freezing protocols are not reliable for all cell types; e.g., the viability, functionality and potency of cryopreserved ESCs are significantly reduced in slow freezing, pushing the cells towards senescence and apoptosis.[62-64]

Compared with conventional freeze–thawing, vitrification (ultra-rapid cooling without crystallization) demonstrates a higher cell recovery rate. The main drawbacks of this technique are again increased toxicity due to the high DMSO concentration required, increased contamination risk due to direct exposure of cells to liquid nitrogen, labor intensiveness, and inefficiency in large-scale cell production.[63] One attempt to counter these problems is to use a 3D integrated expansion and cryopreservation strategy by microencapsulation of immobilized cells on microcarriers cultured in a stirred tank. In comparison with the non-encapsulated method, microencapsulation demonstrated cell recovery that was greater than 70% following cryopreservation.[65] High expansion ratios up to twenty-fold were evident and post-thawing cell survival improved up to three-fold; more importantly, cellular characteristics were not compromised. Although this strategy successfully preserved the ESCs and eliminated the need for feeder cells, it involved the extra steps of encapsulation, decapsulation, and cell separation from the microcarriers.

Cell storage requires stringent methodology and quality control testing to ensure that the cells remain viable, contamination-free, and maintain their original phenotype.[57] Storage problems related to misidentified and cross-contaminated cell lines have been reported in 18–36% of repositories.[66] Contamination with environmental and microbial organisms can happen despite the strict sterilization techniques. Mycoplasma infection, for instance, has a high incidence rate, affecting approximately 5–30% of cell lines worldwide: it is particularly problematic, being resistant to antibiotics and visually undetectable.[67]

Finally, cell shipping and delivery requires a strict time frame as cellular products have a limited shelf life, usually less than a few days. Therefore, it is essential to develop an efficient delivery system as well as simple qualitative assays to detect expiration of the cells.[68]

4. Functional characteristics of cells

In vivo, cells are integrated into a highly sophisticated matrix environment in which their function and response is not discrete from their surroundings.[4,69] Therefore, functional characterization of cells *in vitro* to achieve the desired behavior poses a significant challenge (Fig. 3).

The inter-relationship between structure and function means that cells will not function as efficiently *in vitro* as they function *in vivo*, unless a 3D architectural microenvironment analogous to that of the natural state is appropriately designed. *In vivo*, the local microenvironment is made up of an orchestrated and synergistic symphony of signals including cell–cell contact and cell–ECM interaction, as well as biomechanical and physical forces. These interrelated factors determine cell behavior interdependently rather than individually.[70] This

issue is particularly significant for stem cells because any interruption in cells' contact with their specific niche cues will not only affect the cells' function but also their commitment and fate.[71]

Another question to answer is to what extent the imitation of such intricate biological system is attainable *in vitro*. Cell–cell contact, for instance, is mediated *in vivo* by different extracellular molecules, namely **endocrine** signals (via the systemic vasculature), **autocrine** signals (generated and bound to the secretory cell), and **paracrine** signals (diffused from the adjacent cells). The latter is either homocrine (the signaling and responding cells are of the same type) or heterocrine (the signaling and responding cells are of different types). *In vitro* by contrast, only autocrine and homocrine signaling usually occur (Fig. 4).[12]

In the natural environment, the ECM composition and architecture affect the reciprocal cell–matrix interactions that elucidate a cascade of intracellular events influencing cell survival, proliferation, and protein synthesis. Failure to recapitulate the ECM function which exceeds the mechanical support will result in a passive cell–matrix interface fails to trigger such events and deprives cells of the substantial benefits of ECM that may alter cell phenotype.[4] Unlike the ECM, the synthetic scaffold (which represents the artificial analogue to the authentic ECM) may lack this informative role and be unable to transmit growth cues due to the lack of functional binding ligands (domains) that cells can recognize and with which they can interact.

In addition to the biological effect, the chemical, mechanical, electrical, topographical and mass transport properties need to be well adjusted to maintain a homeostatic balance. For example, increased porosity is essential for mass transport and elimination of central cell necrosis; however, this should not be at the expense of sacrificing mechanical integrity.[72]

Equally important is increasing the surface area and cell concentration without compromising cell viability by mass transfer limitations. Oxygen transport is linked to its concentration, which

is ~220 μM in fully-oxygenated culture medium, compared with 8699 μM in oxyhemoglobin. The low oxygen concentration in media limits diffusion to the outer cell rim and deprives the core cells of oxygen and nutritional supply.[73] As the cells dependent on oxygen diffusion cannot survive at a distance greater than 100–200 μm, considerable cell death in the scaffold's deep areas will occur.[74] In addition, once the cells start to secrete their ECM, another diffusion barrier is formed by the matrix components such as proteins and proteoglycans, which are relatively larger molecules with low diffusion coefficients; this may further hinder nutrient diffusion.[75,76] Cell death due to hypoxia will arise *in vivo* following implantation because the available oxygen is consumed by implanted cells within hours, while the angiogenesis process takes several days to form new capillary plexuses.[7]

5. Conclusion

Despite the significant advances witnessed by TE in the past few years, many challenges still lie ahead. At the heart of all obstacles are those related to the cells themselves, which constitute a major hurdle for the clinical application of engineered tissue equivalents. Cell sourcing, bioprocessing, shipping, marketing, delivery, and reconstruction of the cell microenvironment are sensitive procedures that have a significant impact on cells. Creating an optimal microenvironment for the cells appears to be the most challenging step in tissue engineering for clinical applications. Potential solutions rely partly on a better understanding of the cellular and molecular mechanisms that govern tissue regeneration *in vivo*, and partly on the ability to identify reliable strategies to enable us to mimic the *in vivo* environment and translate the *in vitro* system into clinical application.

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Figure Captions

Fig. 1 Increasing number of people on the organ transplant waiting list, compared with the stagnant donation and transplantation rates in the US.

Fig. 2 The different categories of cells used in TE and their limitations.

Fig. 3 The influencing factors on cells and their impact in determining the cell behavior. Cells perceive, read, interpret and respond to diverse external stimuli; in many cases the output determines the prospective inputs.

Fig. 4 The four different biomolecule signals affecting cell behavior *in vivo*.

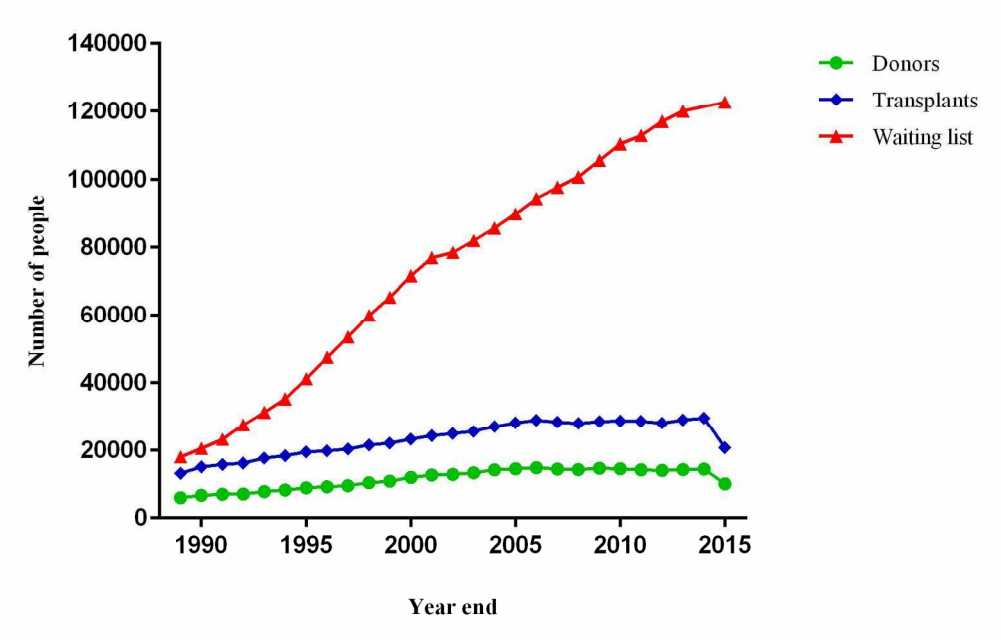

Fig.1 Increasing number of people on the organ transplant waiting list, compared with the stagnant donation and transplantation rates in the US.
158x101mm (300 x 300 DPI)

	Autogenic	Allogeneic
Differentiated	Associated with donor morbidity. Requires Extra surgical step. Inaccessible areas for harvest. Culture is time consuming . Limited quantity. Cost ineffective. Have finite life span.	Donor- dependent. Immune- incompatible.
Stem	ASCs Limited differentiation lineages. Function is donor age-dependent. Limited availability. iPSCs Tumorigenicity. Immunogenicity. Being identical to ESCs is controversial.	ASCs Limited differentiation lineages. Function is donor age-dependent. Immune- incompatible. ESCs Ethical issue. Immunogenicity. Tumorigenicity. Difficulty in optimizing differentiation and culture purification.

Fig.2 The different categories of cells used in TE and their limitations.
338x190mm (300 x 300 DPI)

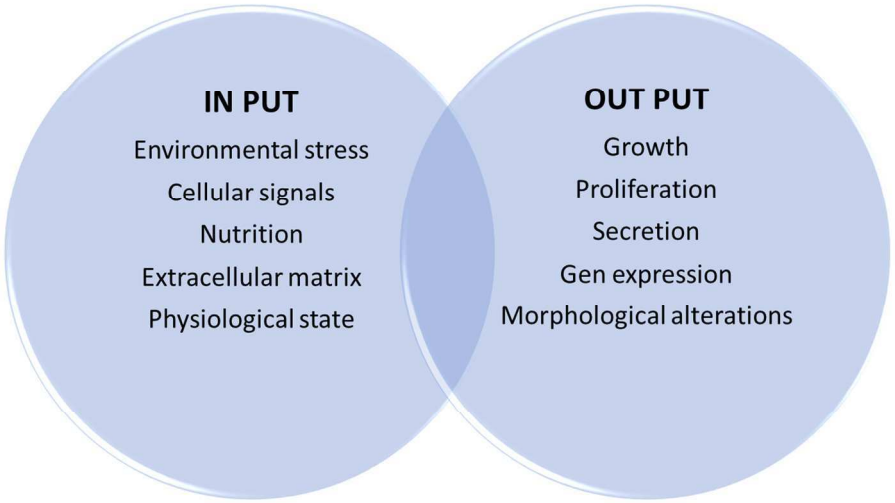

Fig.3 The influencing factors on cells and their impact in determining the cell behavior. Cells perceive, read, interpret and respond to diverse external stimuli; in many cases the output determines the prospective inputs.
254x190mm (300 x 300 DPI)

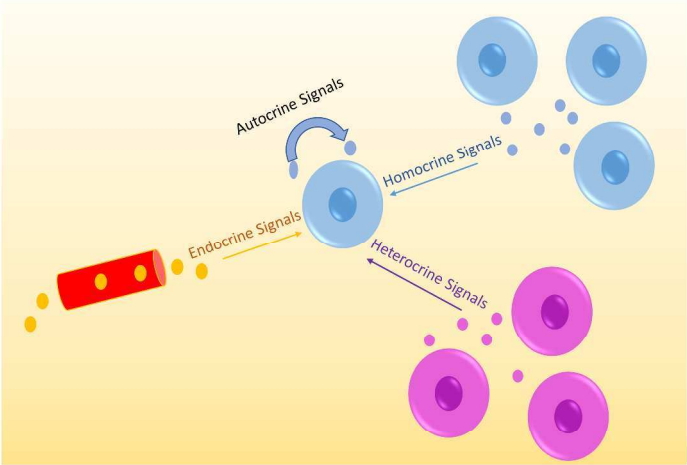

Fig.4 The four different biomolecule signals affecting cell behavior in vivo.
338x190mm (300 x 300 DPI)