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# Toward a scalable and consistent manufacturing process for the production of human mesenchymal stem cells

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The development of novel, affordable and efficacious therapeutics will be necessary to ensure the continued progression in the standard of global healthcare. With the potential to address previously unmet patient needs as well as tackling the social and economic effects of chronic and age-related conditions, cell therapies will lead the new generation of healthcare products set to improve health and wealth across the globe. However, if many of the small-to-medium enterprises (SMEs) engaged in much of the commercialization efforts are to successfully traverse the 'Valley of Death' as they progress through clinical trials, there are a number of challenges that must be overcome. No longer do the challenges remain biological but rather a series of engineering and manufacturing issues must also be considered and addressed.

Revenues for the cell therapy industry recently exceeded US$1 billion [1], and with market approvals of stem cell therapy products, including Prochymal's (Osiris Therapeutics, Maryland, USA) human mesenchymal stem cell (hMSC) therapy, there is growing momentum and optimism that the cell therapy industry, learning from the failings of the tissue engineering field, will come to fruition [2]. This is buoyed by government investment and industry commitment, as evidenced in the UK by the creation of the Cell and Gene Therapy Catapult and Manufacturing Centre [3], in Canada by the emergence of the Centre for Commercialization of Regenerative Medicine and centre for advanced therapeutic cell technologies (with investment from FedDev Ontario and GE Healthcare) and a newly-introduced regulatory and reimbursement environment in Japan conducive for cell therapy manufacture [4]. Cell therapies are no longer solely a pursuit of scientific endeavor but a commercially viable industry in its own right – and a burgeoning one at that [1].

Human MSCs are a promising cell candidate for cell therapies due to their therapeutic efficacy, as determined by pre-clinical and clinical studies [5–8], their relative ease and multiple sources of isolation (Table 1), multi-lineage differentiation capacity and the ability to expand these cells *in vitro* [9]. With over 450 clinical trials involving the use of hMSCs by January 2015 [10], the interest in commercializing hMSC therapies is clear. However, in July 2011, the UK's Office for Life Science published a report which identified that "without the ability to manufacture, store, transport and distribute regenerative medicine products, the therapies would never become mainstream clinical practice" [11]. Bioprocess development and consistent manufacture is a key challenge that SMEs have faced, or will soon encounter, as they navigate through clinical trials; the ability to fulfil the increasing demand for cells at a quality and quantity required for therapeutic application.

**TABLE 1**

**Sources of mesenchymal stem cells.**

| Source | Refs |
|---|---|
| Bone marrow | [7], [9], [12], [13] |
| Adipose tissue | [15] |
| Synovium | [16] |
| Trabecular bone | [17], [18], [19], [20] |
| Skin Tissue | [21], [22] |
| Adult peripheral blood | [23], [24] |
| Umbilical cord (Wharton's Jelly) | [25] |
| Cord blood | [26], [27] |
| Deciduous teeth | [28] |
| Fetal blood, bone marrow, liver & lung | [29], [30] |
| Muscle | [31], [32], [33] |
| Pericyte | [34] |
| Periosteum | [35] |

## BIOPROCESSING CONSIDERATIONS FOR HMSC THERAPIES

There are a number of bioprocess challenges and considerations for the development of a hMSC therapy [36,37]; however this article will focus on those solely related to the expansion of hMSCs. Essentially, these challenges can be identified as follows:

- Cell quality – the cell forms the basis of the product
- Cell quantity – the number of cells required for therapeutic applications
- Cell nature – the anchorage-dependency of many cell therapy candidates
- Unprecedented and an undefined methodology

The large-scale *in vitro* expansion of cells, where the cell forms the basis of the product, is a paradigm shift for the biotechnology industry. Where the cell is the product, i.e., what is to be injected into the patient, there must be efficient harvest and the cell must retain its key quality attributes with respect to identity, potency, purity and safety [38] regardless of the intended application. In addition to cell quality, obtaining the numbers of cells required for therapeutic applications is another significant challenge. For the majority of applications, the expansion of hMSCs *in vitro* will be required to increase the number of functional cells to elicit a therapeutic benefit.

As illustrated in Table 2, the numbers of MSCs delivered to patients in clinical trials varies greatly but for a patient of 70 kg, 0.3 to 5 x $10^8$ cells per treatment may be required. For allogeneic treatments this will therefore mean generating lot sizes of potentially trillions of cells [39].


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## CONSIDERATIONS FOR THE CULTURE OF hMSCS

### Dissolved oxygen

In conventional mammalian cell culture (for the production of heterologous recombinant proteins), the level of dissolved oxygen in the growth medium is important and as such is always measured and often carefully controlled. Measurements in monolayer culture of hMSCs in T-flasks are usually limited to cell viability, confluency and those related to post culture functionality. However in order to inform the basis of the development of the larger scale production of hMSCs, the factors controlling the process need to be fully understood.

The general belief for hMSC expansion under controlled oxygen concentrations is that the concentration in the growth medium should mimic the *in vivo* physiological conditions from which the hMSCs have been derived, in this case, bone marrow. However, there are conflicting results. Work has shown [52–54] that under "normoxic" conditions (i.e. 20 % $O_2$ / 75% $N_2$, 5 % $CO_2$ v/v in the incubator, nominally ≡100 % $dO_2$), the expansion of hMSCs is inferior compared to that obtained under "hypoxic" conditions (~ 2–5% $O_2$ v/v in the incubator, nominally ≡ 10–25 % $dO_2$). Other studies, however, have demonstrated that, based on the concentration in the incubator, 10–25 % $dO_2$ ('hypoxia') can have an impact on either cell quality by attenuating cell differentiation [55] or cell quantity by reducing cell proliferation [56–58] in comparison to 100% $dO_2$ ('normoxia'). Clearly the problem is not yet fully resolved and there are a number of possible

**TABLE 2**

**Examples of treatments and the number of cells transplanted or injected into patients during each trial.**

| Condition | | Number of cells delivered per treatment | Refs |
|---|---|---|---|
| **AUTOLOGOUS** | | | |
| BM-MSCs | Ischemic heart failure | $20x10^6$, $100x10^6$ or $200x10^6$ cells/patient | [40] |
| BM-MSCs | Amyotrophic lateral sclerosis | $11–120x10^6$ cells/patient | [41] |
| BM-MSCs | Stroke | $60–160x10^6$ cells/patient | [42] |
| BM-MNCs | Stroke | $1 x 10^8$ cells/patient | [43] |
| BM-MSCs | Graft versus host disease | $1–2x10^6$ cells/kg | [44] |
| BM-MSCs | Cartilage repair (osteoarthritic knee) | $13x10^6$ cells/patient | [45] |
| BM-MSCs | Multiple sclerosis | $32–52x10^6$ cells/patient | [46] |
| BM-MSCs | Multiple sclerosis | $1–2x10^6$ cells/kg body weight | [47] |
| **ALLOGENEIC** | | | |
| Prochymal® | Graft versus host disease | 2 or $8x10^6$ cells/kg body weight | [48] |
| BM-MSCs | Graft versus host disease | $1.7–2.3x10^6$ cells/kg body weight | [49] |
| Prochymal® | Myocardial infarction | $0.5x10^6$, $1.6x10^6$ or $5x10^6$ cells/kg body weight | [50] |
| PD-MSCs | Diabetes | $1.2–1.5x10^6$ cells/kg body weight | [51] |

Prochymal® is an allogeneic bone marrow-derived MSC product from Osiris Therapeutics Inc (USA). Abbreviations: BM-MSCs: bone marrow-derived MSCs; BM-MNCs: bone marrow mononuclear cells; PD-MSCs: placenta-derived MSCs.

explanations for this difference, not least of which may be cell line specificity or culture conditions.

## Serum-free media

The culture of mammalian cells, be it for the production of proteins, vaccines or cell therapies, requires complex nutrients which have traditionally been provided in the form of growth-factor-rich media supplemented with Fetal Bovine Serum (FBS) [59]. It is widely acknowledged however that the addition of FBS is undesirable for a variety of reasons and efforts are being made to develop defined, serum-free processes [60]. The oft-cited reason for avoiding the use of serum is the risk of contamination through the introduction of adventitious, xenogeneic agents [60]. This is often the biggest driver for the switch from serum-based to serum-free processes given the perceived FDA aversion towards serum [61]; however this risk is mitigated in part by the rigorous screening and selection process required for GMP-grade serum.

Batch-to-batch variability of serum is another reason for the shift away from serum-based processes [62]. Serum is poorly defined and there can be significant variation between batches, resulting in a lack of reproducibility. For an industry where the "process is the product", standardization is crucial and variation in culture conditions outside of pre-defined limits is unacceptable. Moving towards a well-defined medium will allow for the development of standardized, reproducible manufacturing methods and would avoid costly serum batch testing.

Moreover, Brindley *et al* posit that the biggest concern with employing serum-based processes is not necessarily due to the perceived regulatory issue, as GMP-grade serum can be sourced, but rather a supply and availability issue [61].

In light of the concerns regarding the use of serum, there is now a growing body of literature investigating the use of serum-free media for monolayer culture [60,62–67] and microcarrier-based culture of hMSCs [68–70] with varying degrees of success. Table 3 provides an overview of various commercially available serum/xeno-free hMSC media.

# EXPANSION TECHNOLOGIES

To be able to obtain a sufficient number of cells for a cell therapy, be it autologous or allogeneic, *ex vivo* cell expansion is an essential step in the development process. There are numerous techniques which are currently employed for the scale-up or scale-out of adherent cells with their own respective advantages and disadvantages. This article will focus specifically on microcarriers. A more detailed comparison of the various expansion systems is provided by the author [71,72].

## Microcarriers

Microcarrier technology provides a significantly larger surface area per unit volume of bioreactor [73] compared to monolayer culture, and combines the potential ease of scalability, process monitoring and control capability associated with bioreactor cultures that makes bioreactor culture common place in the biopharmaceutical arena. Numerous types of microcarrier particles are commercially available with varying surfaces, charge, structures and other properties (Table 4).


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**TABLE 3**

**Comparative summary of different hMSC serum-free media and FBS-based medium.**

| | Manufacturer | Glutamine supplement required? | Attachment substrate required? | Xeno-free available? | Dissociation reagent | Medium Exchange | Days until passage | Shelf-life of prepared medium | Notes |
|---|---|---|---|---|---|---|---|---|---|
| Mosaic | BD | ✗ | ✓ | ✗ | Accutase | Not required | 3 | 28 days | Attachment substrate can be applied to as many flasks as necessary and stored for 3 weeks. No longer available for purchase. |
| DMEM + FBS | Multiple | ✓ | ✗ | ✗ | Trypsin-EDTA | After 3 days | 6 | 28 days | Simplest and most user-friendly protocol with no attachment substrate. |
| TheraPeak | Lonza | ✗ | ✗ | ✓ | Any non-animal derived dissociation reagent | After 3 days | 6 | 28 days | Identical to DMEM passage protocol therefore most user-friendly. No attachment substrate needed |
| MesenCult | Stem Cell Technologies | ✓ | ✓ | ✓ | Manufacturer's own dissociation and inhibition reagents | After 5 days and if medium appears "yellow" | 5 or 6 days | 5 days | Most involved protocol with prepared medium only available for 5 days (therefore needing many working aliquots). Attachment substrate needed 24 h before culture |
| StemPro | Life Technologies | ✓ | ✓ | ✓ | TrypLE Select | Every 2 days | 6 | 14 days | Requires 2 medium exchanges during a routine passage. Attachment substrate required 1 h prior to culture |
| Prime-XV | Irvine Scientific | ✗ | ✓ | ✓ | TrypLE Express | Every 2 days | 6 | 28 days | Requires 2 medium exchanges during a routine passage. Attachment substrate required a minimum of 1 h prior to culture |
| Xuri | GE Healthcare | ✗ | ✓ | ✓ | TrypLE Select | Every 2-3 days | 6 days | 30 days | Attachment substrate required. Coated vessels can be stored for 1 week when covered with Parafilm. |

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## TABLE 4

### Properties of commercially available microcarriers

| Microcarrier | Manufacturer | Diameter (µm) | Matrix | Avg. density | Surface coating | Surface charge | Carrier porosity |
|---|---|---|---|---|---|---|---|
| MAMMALIAN PROTEIN-COATED MICROCARRIERS | | | | | | | |
| Collagen | Pall SoloHill® | 125–212 | Polystyrene | 1.02 | Type I porcine collagen | None | Non-porous |
| Cultispher-G® | Percell-Biolytica | 130–380 | Type I porcine gelatin | 1.04 | None | None | Macroporous (porosity: 50 % pore size: 10 – 30 µm) |
| Cytodex 3™ | GE Healthcare | 141–211 | Dextran | 1.04 | Type I porcine collagen | None | Non-porous |
| FACT III | Pall SoloHill® | 125–212 | Polystyrene | 1.02 | Cationic Type I porcine collagen | + | Non-porous |
| Global Euraryotic Microcarrier (GEM™) | Global Cell Solutions | 75–150 | Polysaccharide alginate | 1.02 | Porcine gelatin | Magnetically charged | Non-porous |
| SphereCol® | Advanced BioMatrix | 125–212 | Polystyrene | 1.03 | Type I human collagen (VitroCol®) | None | Non-porous |
| RECOMBINANT PROTEIN-COATED MICROCARRIERS | | | | | | | |
| Pro-Nectin® F | Pall SoloHill® | 125–212 | Polystyrene | 1.02 | Recombinant fibronectin | None | Non-porous |
| XENO-FREE MICROCARRIERS | | | | | | | |
| Cytodex 1™ | GE Healthcare | 147–248 | Dextran | 1.03 | DEAE | + | Non-porous |
| Cytopore 1 and 2™ | GE Healthcare | 200–280 | Cotton cellulose | 1.03 | DEAE | + | Micro/Macroporous (porosity: > 90 % pore size: 30 µm) |
| Enhanced Attachment | Corning | 125–212 | Polystyrene | 1.02 | CellBIND® | None | Non-porous |
| Glass | Pall SoloHill® | 125–212 | Polystyrene | 1.02 | High silica glass | None | Non-porous |
| Hillex® CT | Pall SoloHill® | 90–212 | Polystyrene | 1.12 | Cationic trimethyl ammonium | + | Non-porous |
| Hillex® | Pall SoloHill® | 160–180 | Dextran | 1.11 | Cationic trimethyl ammonium | + | Non-porous |

**TABLE 4 CONT.**

| Microcarrier | Manufacturer | Diameter (µm) | Matrix | Avg. density | Surface coating | Surface charge | Carrier porosity |
|---|---|---|---|---|---|---|---|
| MicroHex™ | Nunc | Side-length: 125 µm Thickness: 25 µm | Polystyrene | 1.05 | Nunclon™ surface | Not specified | Non-porous |
| Plastic | Pall SoloHill® | 125–212 | Polystyrene | 1.02 | None | None | Non-porous |
| Plastic Plus | Pall SoloHill® | 125–212 | Polystyrene | 1.02 | None | + | Non-porous |
| PVA | Loughborough University | 100–220 | PVA | 1.03 | None | None | Non-porous |
| Star-Plus | Pall SoloHill® | 125–212 | Polystyrene | 1.02 | None | Charged | Non-porous |
| Synthemax II® | Corning | 125–212 | Polystyrene | 1.02 | Synthemax II® | None | Non-porous |

## MSC MICROCARRIER STUDIES

### Microcarrier culture conditions

It has become increasingly clear that to develop an optimal microcarrier-based hMSC culture process, parameters for the culture must be identified and optimized. Table 5 provides a list of these various parameters for different stages of the process.

#### Microcarrier selection

At present, there is no unified set of culture conditions for the expansion of hMSCs on microcarriers given the infancy of the research. Some groups have demonstrated successful growth on one particular microcarrier over another, for example Schop and colleagues found that when comparing nine different microcarriers, Cytodex-1 was selected after it demonstrated the highest seeding efficiency [74]. In contrast, Dos Santos and colleagues found that having previously used Cultispher-S [75] (a gelatin-based microcarrier), a xeno-free approach was required for the adoption of such a process for clinical-grade expansion, and therefore selected Plastic P102-L. Our group developed a systematic microcarrier screening process for hMSCs including 13 commercially available microcarriers and found that Collagen and Plastic P102-L microcarriers were optimal for hMSC growth for three different donor cell lines [76].

#### Medium selection

As mentioned previously, there is a growing body of literature focusing on hMSC monolayer culture with serum-free medium. Given

**TABLE 5**

**Parameters for each hMSC microcarrier culture process step with a list of studies which have investigated this particular parameter.**

| Stage of process | Parameter | Studies |
|---|---|---|
| Vessel configuration | Impeller selection | [79] |
| | Baffles | - |
| | Vessel geometry | - |
| Inoculation | Impeller delay | [82], [83] |
| | Intermittent/constant agitation | [80] |
| | Cell/microcarrier seeding density | [81], [80], [79] |
| Agitation | Agitation speed | - |
| | Intermittent/constant agitation | [68], [74] |
| Culture | Medium selection | [69], [70], [84] |
| | Microcarrier selection | [74], [85], [68], [86] |
| | pH | [87], [76] |
| | $dO_2$ / $dCO_2$ | [87], [85] |
| | Sparging | - |
| | Addition of extra microcarriers | [85] |
| | Mode of operation | - |
| Medium exchange | Level of medium exchange | [75] |
| | Frequency of medium exchanges | [75] |
| Harvest | Dissociation reagent | - |
| | Agitation speed | [88, 89] |

A (-) indicates no studies were found to have investigated this parameter as of yet.

that work is still ongoing to determine the optimal monolayer hMSC culture conditions, it is likely that microcarrier culture conditions will lag behind, as is evident by fewer studies investigating serum/xeno-free hMSC microcarrier cultures. Dos Santos and colleagues have employed a completely xeno- and serum-free microcarrier culture process, where they used the StemPro® MSC xeno-free medium [68]. They found that they were able to effectively culture bone-marrow- and adipose-derived hMSCs under such conditions, reaching a maximum cell density of 2.0 x $10^5$ cells/mL for the bone marrow derived hMSCs in a working volume of 80 mL. Using Prime-XV, we achieved a maximum cell density of > 3.0 x $10^5$ cells/mL in a working volume of 100 mL on Plastic P102-L microcarriers [69].

## Seeding density

It has been suggested in the literature that seeding density has an effect on the proliferation of hMSCs grown as a monolayer, with lower seeding densities (100 cells/$cm^2$) demonstrating increased proliferation compared to higher seeding densities (5000 cells/$cm^2$) [77,78]. In animal and human MSC microcarrier culture, this is an area which has also received attention, with studies investigating the effect of different cell-to-bead ratios [79–81]. Frauenschuh and colleagues described the cell attachment process as following a Poisson distribution [81], and found that initial cell seeding densities ranging from 1–3 x $10^6$ cells/100 $cm^2$ surface area had little effect on attachment. With respect to the cell-to-bead ratio, there appears to be consistency in the data presented with studies by Hewitt and colleagues [79] and Yuan and colleagues [80] suggesting that a ratio of 5 cells-to-bead may be optimal.

## Operating conditions

The combination of microcarrier culture with a bioreactor system provides all of the benefits associated with bioreactors such as a greater level of culture homogeneity achieved via agitation as well as process monitoring and control. This however means there is the need to consider and optimize the operating parameters of the bioreactor also.

Much of the research carried out thus far attempts to demonstrate the effect of some of the aforementioned parameters on hMSC yield and quality. Dos Santos and colleagues opted to employ an intermittent agitation


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**TABLE 6**

**Studies of MSC culture on microcarriers.**

| Ref. | MSC source | Microcarrier | Working volume (mL) | Max. cell density (cells/mL) | Volume harvested (mL) | Notes |
|---|---|---|---|---|---|---|
| [81] | Porcine bone marrow MSCs | Cytodex-1 | 40 | ~1.0 x $10^6$ | 40 | |
| [74] | Human bone marrow MSCs | Cytodex-1 | 50 | ~1.5 x $10^5$ | 2 | Volume harvested was based on sample size |
| [83] | Porcine bone marrow MSCs | Cytodex-1 | 200 | ~4.0 x $10^5$ | - | No sample or harvest volume specified |
| [79] | Human placental MSCs | Cytodex-3 | 80–100 | ~3.8 x $10^5$ | - | Volume harvested was based on sample size |
| [90] | Human placental MSCs | Cytodex-3 | - | ~1.05 x $10^6$ | 2 | It is unclear what the working volume of the microcarrier culture was |
| [86] | Human bone marrow MSCs | Modified Cytodex-3 | 30 | ~5.0 x $10^5$ | 30 | |
| [75] | Human bone marrow MSCs | Cultispher-S | | ~4.2 x $10^5$ | 50 | |
| [91] | Rat ear MSCs | Cultispher-S | 1000 | ~9.0 x $10^5$ | 0.5 | Harvest volume was based on sample size |
| [80] | Human bone marrow MSCs | Cultispher-S | 125 | - | 125 | |
| [92] | Human bone marrow MSCs | Cultispher-G | 200 | ~3.4 x $10^5$ | 1 | Harvest volume was based on sample size |
| [68] | Human bone marrow and adipose tissue MSCs | Plastic P102-L | 80 | 2.0 x $10^5$ (bone marrow MSCs) 1.4 x $10^5$ (adipose tissue MSCs) | 0.5 | Carried out in xeno-free medium. Harvest volume was based on sample size |
| [93] | Human bone marrow MSCs | Collagen | 2000 | 9.0 x $10^4$ | - | Volume harvested was based on sample size |
| [69] | Human bone marrow MSCs | Plastic P102-L | 100 | 3.2 x $10^5$ | 100 | Serum-free medium culture. Full volume harvested |

strategy whereby during the first 24 h, the culture was agitated for 15 min at 25 rpm after which followed a period of non-agitation for 2 h [68]. After this, the culture was agitated constantly at 40 rpm for the duration of the culture. Schop and colleagues instead employed an agitation strategy of constant agitation at 30 rpm for 18 h, after which the culture was constantly agitated at 40 rpm [74].

Medium exchange regime is another key consideration; the regularity and amount of medium exchange has to be controlled carefully. With respect to hMSC microcarrier culture, the effect of medium exchange regime was demonstrated by Eibes and colleagues who compared two medium exchange regimes; (i) a 25% medium exchange every 48 h and (ii) a 25% medium exchange every 24 h starting after day 3 [75]. They found that the first medium exchange regime resulted in a significant depletion of glucose during the exponential phase of cell growth, with an associated increase in ammonium concentration which reached inhibitory values. The second medium exchange regime did not result in such adverse metabolite concentrations, with glucose present throughout the exponential phase and the level of ammonium not reaching inhibitory values [75].

### Microcarrier harvest

A key factor in the choice of microcarrier is the ability to efficiently harvest the cells after hMSC expansion, a decision which will impact downstream processing. In the majority of the studies listed in Table 6, the working volume is below 200 mL and there is little focus on the harvesting procedure, or the ability to effectively harvest should the process increase in scale. This would appear to suggest that most, if not all of the work involving the expansion of hMSCs on microcarriers, has focussed solely on the attachment, expansion and culture conditions of hMSCs. Detachment of cells from the microcarrier surface and subsequent retention of cell quality is equally as important as cell attachment and proliferation given that the product of interest for cell therapies is the cell itself. This problem will only be exacerbated as expansion scale increases and therefore it is crucial to consider cell harvesting strategies from the outset so as to ensure a viable, holistic bioprocess.

## TRANSLATIONAL INSIGHT

The successful development of clinically-relevant, reimbursable cell therapy products and other advanced therapeutics will require a shift in mindset toward one that values, understands and applies translational research – the type of research that takes science from the bench and addresses technological, manufacturing, commercial, regulatory and clinical challenges, thereby enabling the delivery of healthcare and economic benefits. It is my contention that early-career researchers (ECRs), from both industry and academia, with a strong grounding in translation research, are therefore critical to accelerate the development process [82]. Although the translational pathway can be resource intensive, application-specific and fraught with obstacles, there are fundamental principles which can provide guidance along the prickly path [82]. Further understanding of how a product's CQAs correlate with clinical efficacy and better characterisation techniques will result in more consistent, standardized manufacturing processes, and as with biologics


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production, there will be a continued increase in the cell densities that are obtained. But perhaps most importantly, with ever-increasing numbers of highly-trained, multidisciplinary ECRs emerging from world class training centres (the Catapult, CCRM and UK EPSRC Centres for Doctoral Training to name but a few), there is great reason to be optimistic and I fully believe that this generation of ECRs will harvest (pun intended) the crops planted and nurtured by the previous generation of translational scientists, engineers and clinicians who have pioneered the field.

## FINANCIAL & COMPETING INTERESTS DISCLOSURE

*The author has no relevant financial involvement with an organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock options or ownership, expert testimony, grants or patents received or pending, or royalties.*
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