The bioanalytical tools that support cell and gene therapy development

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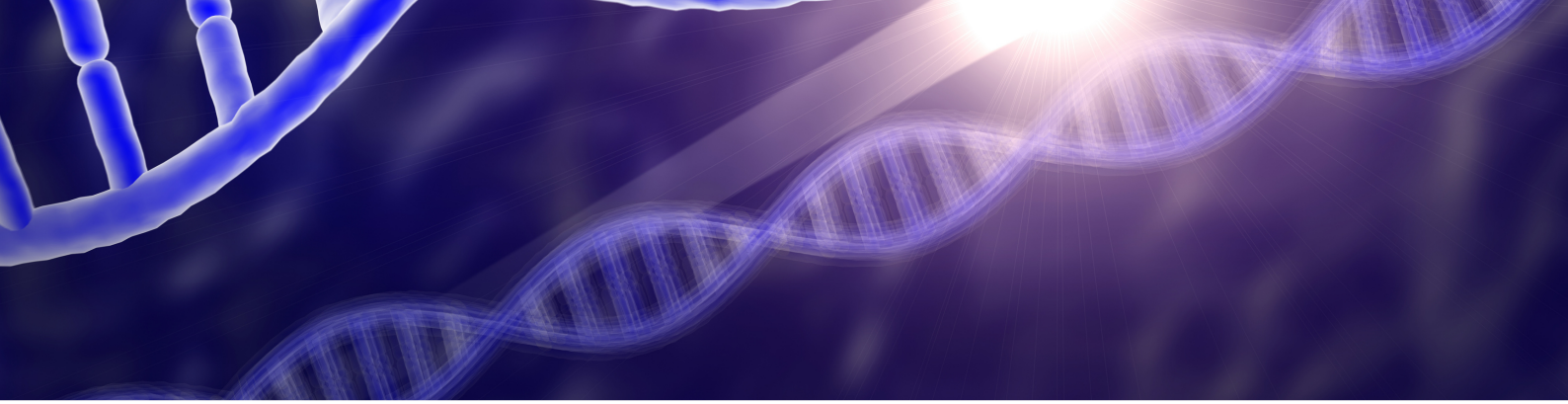

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Technology Digest: the bioanalytical tools that support cell and gene therapy development

by Naamah Maundrell (Editor-in-Chief, Bioanalysis Zone)

The promise of cell and gene therapies

Cell and gene therapies are promising new therapeutic modalities used in the treatment of inherited and acquired diseases [1]. These therapies have experienced a dynamic renaissance, particularly since the first market approvals of gene therapy products in 2017, and have become areas of great accelerated growth and promise in drug development to address unmet medical needs [2–4].

While both cell and gene therapies try to introduce biological function to the patient, the introduction mechanisms are very different. In gene therapy, the faulty gene is modified, and normal function restored by introducing genetic material (exogenous DNA or RNA) into targeted cells via a viral or nonviral delivery, whereas cell therapy involves the engineering and transfer of entire cells [1]. The vectors used in these therapies are designed to target specific cells or tissues and viral systems have demonstrated high transfection efficiencies. However, concerns have been raised over potential mutations, potential oncogenic effects and high costs. Nonviral vectors (such as lipid nanoparticles) have raised less safety concerns due to their relative simplicity [4].

These new therapeutic modalities are unique as they target previously 'undruggable' targets and have formed the third major drug platform after small- and large- molecule therapeutics [4]. Stephanie Pasas-Farmer, President and Founder of Ariadne Software, LLC (KS, USA), commented on the departure from traditional medical treatments:

"Cell therapy is the treatment of a disease through the injection, grafting or implanting of bioengineered cells that elicit a specific medicinal effect. This represents a significant departure from traditional medical treatment, primarily because the drug is no longer a single or collection of molecules but is an entire cell. This shift in treatment design demands a shift in the required bioanalytical approach where the scientist is required to determine the concentration of whole cells in biological matrices which, in turn, requires different analytical instrumentation."

Hundreds of these new drug candidates are currently under evaluation in clinical trials [1]. However, as well as understanding the biological nature of the therapy, it is important to recognise that the efficacy of the bioanalytical methods used plays a vital role in therapy success [4].

The bioanalytical tools used to support cell and gene therapies

Due to the inherent differences, cell and gene therapies can require different bioanalytical tools to support the preclinical and clinical studies beyond the commonly used chromatographic and ligand-binding assays (LBA) of most traditional small- and large-molecule drug products. A host of different techniques are required to assess biodistribution, persistence, efficacy and immunogenicity [1]. Evaluating the PK of the therapy is essential in discovery research as well as preclinical and clinical development. The bioanalytical tools used to support these programs are varied and can include quantitative polymerase chain reaction (qPCR), droplet digital PCR (ddPCR), flow cytometry and next generation sequencing. Each platform presents benefits and challenges. The qPCR assays are known for their high sensitivity, accuracy and practical ease, but face challenges of assay contamination and cross reaction with other sources [4]. As an alternate ddPCR assays are considered more precise, providing an absolute quantification of copies/ml without the use of a standard curve. While ddPCR can reduce the amount of background DNA allowing for a greater detection of low copy amplicons, it has a smaller dynamic range and is more expensive [5].

Owing to the diverse range of analytical platforms that support these modalities, access to laboratories with a broad variety of bioanalytical capabilities is essential to ensure success [2]. As this field has grown so has the demand for viral vector manufacturing and companies who offer access to experts and assets for product development. It has become common for innovators to outsource the manufacturing and downstream processing stages due to product complexity and challenges with dosing, undefined analytics and long-term traceability [3]. Pasas-Farmer emphasised the key questions being asked of those developing these products:

"Some key questions being asked are 'how do we validate a pharmacokinetic approach for cell and gene therapies?' and 'how do we measure an entire cell to examine the impact of a cell therapy?' Although not fully validated and regulated for bioanalysis, flow cytometry is a good platform to measure whole cells."

The regulatory landscape of cell and gene therapies

Bioanalytical labs have inconsistent approaches during preclinical and clinical phases. These inconsistencies are due to the use of various platforms and reagents as well as the lack of detailed regulatory guidance of method validation.

Although guidance documents exist for cell and gene therapy products, there are currently no specific regulatory guidance documents for bioanalytical validation of these assays [4].

"At this time, there are no clearly defined method validation parameters with their associated acceptance criteria for cell and gene therapy platforms. The data output is also very different when compared to more traditional drug development bioanalytical platforms such as LBA and LC–MS/MS data sets, thereby leading to confusion for quite a few of us. We need to come together as a community to address these questions with best practices that can then be suggested to regulatory agencies to be formalized into guidance. Although this process has begun through a few consortiums, we need to be more open with what has been working for us and what doesn't," – remarked Pasas-Farmer on the state of current regulations.

The regulatory landscape is therefore incomplete, and gaps include a lack of guidelines that describe expectations for bioanalysis of transgene protein expression by LC–MS or other methods and on the methodologies to conduct cellular immune response assessment. The data output is also different when compared to more traditional drug development bioanalytical platforms such as LBA and LC–MS/MS data sets leading to confusion when less experienced auditors or regulatory reviewers are presented these types of data in support of preclinical and clinical studies [2]. The guidelines published by the US and European agencies reflect on the general principles of cell and gene therapy development but have a limited description of bioanalysis specific questions, with some guidance's stating that gene therapies are excluded. Therefore, with companies having to navigate validations without a specified set of regulatory guidance documents it is paramount that both regulators and industry leaders work together to educate each other on what is and isn't appropriate to follow. It is hoped that a more comprehensive guidance will be published in the future [2,5].

Summary

From reviewing the current trends, it is reasonable to expect that the number of cell and gene therapy products will continue to increase [5]. With this expansion and considering the complexity and diversity of the bioanalytical platforms related to in vivo and ex vivo cell and gene therapy modalities, more established documentation and aligned guidance is required [2]. It is also likely that discussions will continue around the relevance of certain tests and the degree of analytical method fit-for-purpose validation [5].

In the future, it is expected that cell and gene therapy approaches will be treated as separate entities due to their significant differences and the different bioanalytical tools used to study them. However, as processes and analytical platforms are optimized and a better understanding of the required end points are obtained, the cell and gene therapies of the future will be more widely embraced. The ultimate end goal is that the complete capabilities of cell and gene therapies will be uncovered, enabling the treatment of individuals with previously fatal genetic disorders [2,5].

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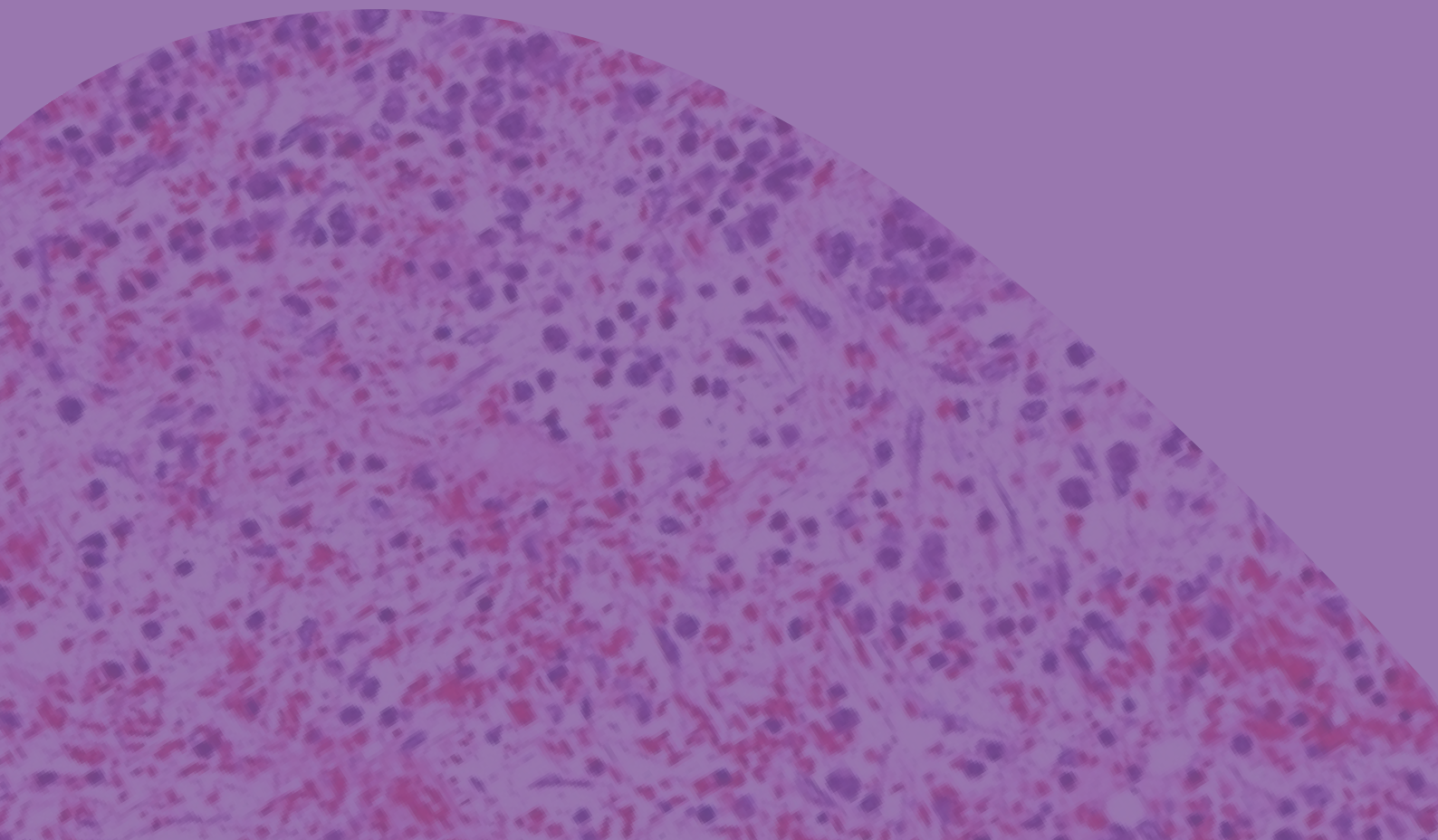

Editorial

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A dozen years of clinical trials performing advanced cell therapy with perinatal cells

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"The number of clinical trials registered worldwide each year is flat for cells that are unique to cord blood, but rapidly growing for other perinatal cells"

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Keywords: cell therapy • clinical trial • cord blood • perinatal cells

Since we published our article [1], 'The first decade of advanced cell therapy clinical trials using perinatal cells (2005–2015)', we have continued to gather data on cell and gene therapy clinical trials, and we can now report on trends in perinatal cell therapy for the years 2005–2017.

Figure 1 illustrates the most striking trends in the field of cell and gene therapy with perinatal cells. The number of clinical trials registered worldwide each year is flat for cells that are unique to cord blood, but rapidly growing for other perinatal cells. Since 2010, the number of clinical trials per year that rely on the action of perinatal cells unique to cord blood has been between 10 and 20 trials per year, averaging 14 trials per year. Note that this does not include standard hematopoietic stem cell transplantation trials because they do not meet our criteria of only archiving advanced cell therapy [2]. Meanwhile, the number of clinical trials that rely on cells from other perinatal sources doubled over the 5 years from 2013 to 2017, from 28 to 56. The majority of this growth, averaging 84% of the other perinatal trials since 2010, is from clinical trials that rely solely on mesenchymal stem/stromal cells (MSCs) from any perinatal sources, including cord blood, cord tissue and the placenta.

The rapid rise in clinical trials with MSCs from perinatal sources is even more striking when compared with the overall growth of advanced cell therapy with MSCs from any source. Our data at CellTrials.org show [3] that, over the 5 years from 2013 to 2017, the number of clinical trials that relied on the action of isolated MSCs from any source increased by 50%, whereas the number of trials with perinatal MSCs doubled during this time. As a result, the fraction of the MSC trials from perinatal sources rose from about 20 to 30%. In 2016 and 2017, trials with perinatal MSCs outnumbered trials with MSCs from bone marrow or adipose tissue.

Another clear difference between the advanced cell therapy trials that rely on cord blood cells versus other perinatal cells, is that perinatal MSCs have been applied to a much wider variety of indications for use. Admittedly diagnosis categories can be somewhat subjective, so we will clarify below which indications for use were included in each category.

Cumulatively from 2005 through 2017 there are a total of 131 advanced cell therapy clinical trials with cells unique to cord blood. Among these trials, 75% of the indications for use are either hematology/oncology (39%), or neurology (36%). No other diagnosis category has exceeded a 9% share of the trials with these cells over a dozen years. Since traditional stem cell transplants do not qualify as 'advanced cell therapy', the hematology/oncology trials we are counting here are focused on isolating and expanding specific cell lines from cord blood in order to enhance engraftment during transplants for malignancies or blood disorders. By comparison, most of the neurology trials are using unmanipulated cord blood for nonhomologous applications. The neurology indications include Alzheimer's disease, amyotrophic lateral sclerosis, autism, cerebral palsy, hypoxic-ischemic encephalopathy, Parkinson's disease, spinal cord injury and stroke.

Cumulatively from 2005 through 2017 there are a total of 238 advanced cell therapy clinical trials with perinatal MSCs. Among these trials, 64% of the indications for use fall into the five categories of autoimmune disorders

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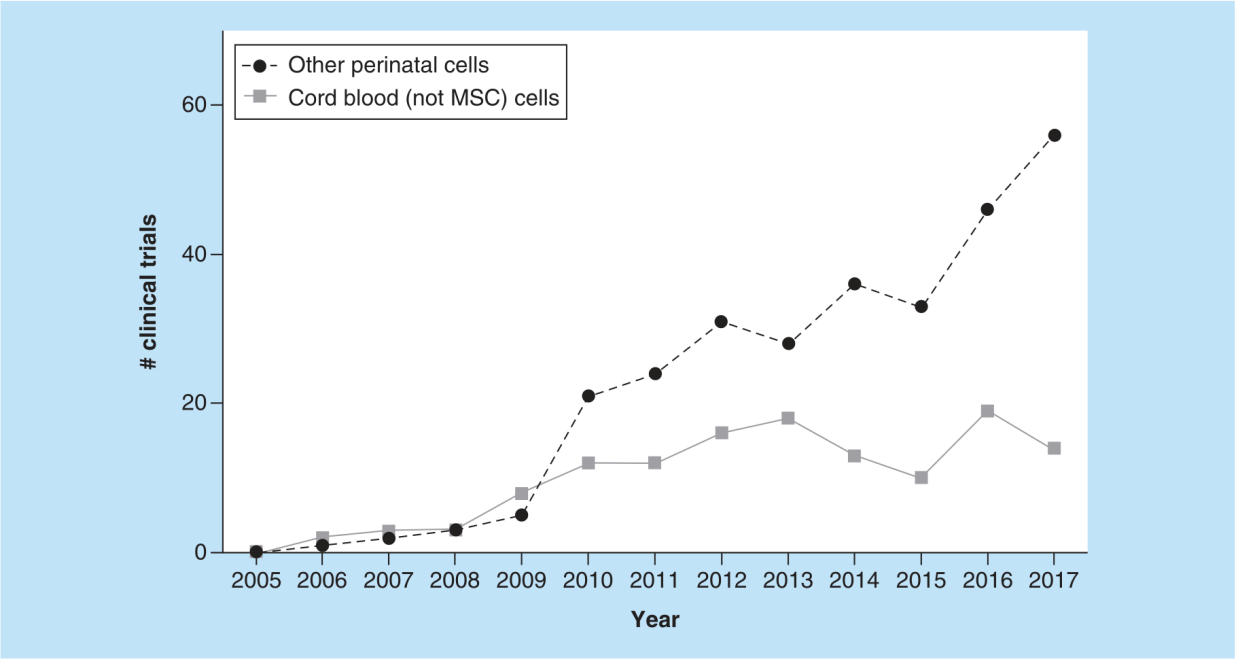

Figure 1. Trends in the field of cell and gene therapy with perinatal cells.
MSC: Mesenchymal stem cells.

(21%), and neurological (15%), cardiovascular (10%), orthopedic (9%) and liver diseases (9%). It should be noted that autoimmune disorders include conditions that have disparate symptoms but share auto-immune etiology, such as Crohn's disease, multiple sclerosis, psoriasis, rheumatoid arthritis, systemic lupus erythematosus and ulcerative colitis.

Thus, in the cord blood field advanced cell therapy clinical trials have been focused almost entirely on addressing two specific issues: engraftment during transplant and repair of acquired or degenerative neurologic damage. In contrast, perinatal MSCs have been trialed for almost any condition that might benefit from the immunomodulatory and anti-inflammatory properties of MSCs. This leads to the question of which trials, if any, have reached late phase and might culminate in an approved cell therapy product.

Through the end of 2017, 25 clinical trials of perinatal advanced cell therapies have reached Phase III or higher. Three companies have been responsible for all of the postmarket follow-up trials: Medipost, Gamida Cell and Osiris. Since each of these companies has a very different cell therapy manufacturing technology, we briefly review their product pipelines.

Medipost of South Korea has sponsored at least 17 advanced cell therapy clinical trials with perinatal cells so far, either alone or in collaborations, at locations in South Korea or the USA. In 2012 they achieved market approval in South Korea of their product Cartistem® for degenerative arthritis, and they are currently seeking approval of products Pneumostem® for bronchopulmonary dysplasia or hemorrhagic stroke, and Neurostem®-AD for Alzheimer's disease. The active cell type in all Medipost products is MSCs that are isolated from allogeneic cord blood and then expanded in culture.

Gamida Cell of Israel is seeking approval for their product, which is manufactured from allogeneic cord blood $CD133^+$ cells that are cultured with the small molecule nicotinamide, and is called NiCord® when used for hematologic malignancies or CordIn™ when the indication is hemoglobinopathies. They have sponsored at least ten trials at multinational locations and were the first stem cell transplant product to receive the US FDA Breakthrough Therapy Designation.

Osiris Therapeutics of the USA self-launched their Grafix® wound dressing in 2011, and this product is included in follow-up studies conducted by wound registries. Grafix contains viable cryopreserved placental membranes; two versions of the product are Grafix Prime (amniotic membrane) and Grafix Core (chorionic membrane). Ironically, there are dozens more products manufactured from the amniotic membrane of the placenta that are marketed as wound dressings, but the majority of them do not contain live cells and therefore are not tracked by our database of advanced cell therapy clinical trials.

A few nations continue to lead the development of cell therapy products that rely on perinatal cells. We noted [1] that over the decade 2005–2015, only three countries accounted for 79% of the 281 advanced cell therapy trials with perinatal cells: China (36%), the USA (30%) and South Korea (12%). Since then, during the years 2016–2017, an additional 137 trials were registered to run at hospitals and clinics located in China (52%), the USA (18%), South Korea (7%) and elsewhere or multinational locations (23%). As before, nearly 80% of the trials are in the leading three nations, but recently over half the registered trials are in China.

At CellTrials.org [3] we noticed an odd coincidence: cumulative through the end of 2017, the worldwide number of advanced cell therapy trials with perinatal cells, which is 417, is very similar to the cumulative worldwide number of clinical trials with T-cells modified by Chimeric Antigen Receptors (CAR-T), which is 421. In recent years the success of CAR-T therapy has garnered enormous media attention. This illustrates that it only takes one dramatically successful study to propel a research area from relative obscurity into the limelight. We think that there is also a feedback pattern where regulatory approvals catalyze the growth of further clinical trials. Thus, the 2012 approval of Cartistem contributed to the growth of trials with perinatal MSCs, just as the 2017 approvals of CAR-T products Kymriah and Yescarta catalyzed that field. While CAR-T trials primarily target patients that have recently developed hematological malignancies, where the number of patients diagnosed per year is less than 0.2 million in the United States; by comparison, any one of the top five indications treated by trials of perinatal MSCs could be applied to millions of patients in the United States that have chronic conditions in those categories.

Based on the pipelines of clinical trials and regulatory approvals, we predict that more products manufactured from cord blood will achieve regulatory approval in the next couple of years. We also predict that the use of perinatal MSCs will continue to grow relative to MSCs from bone marrow or adipose tissue as a source of MSCs in clinical trials, both due to the relative ease of harvesting MSCs from perinatal sources as well as the greater proliferation ability of perinatal MSCs [4]. We predict that the use of placental membranes as a wound product will continue to grow, but most of these products will probably be decellularized and terminally sterilized, so that they are more accessible as an off-the-shelf therapy in the developing world.

Financial & competing interests disclosure

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Editorial

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Bioanalysis

Complexity and diversity of bioanalytical support for gene therapy modalities

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Ex vivo and *in vivo* gene therapy (GTx)-based methodologies for treatment of various human conditions are experiencing a clear and dynamic renaissance, with several therapeutic modalities and platforms evaluated preclinically or already in patients. A significant number of gene therapies are developed to treat various cancers or rare genetic diseases. These revolutionary and potentially disease-modifying approaches to medical intervention are intended to work through recruitment of elements of the immune system to attack cancer cells or to correct the root of the underlying condition caused by a single gene defect with a single or limited number of treatments.

Although development of the viral vector delivery *in vivo* GTx modality was initiated almost a generation ago, only recently, and mainly due to the advent of adeno-associated virus [AAV]-based technology, has it shown a strong and real potential for practical application. AAVs are ssDNA containing, relatively small, nonenveloped parvoviruses that can be constructed to carry up to 5 kb transgene payloads [1]. AAVs are commonly viewed as nonpathogenic and can infect dividing and quiescent postmitotic cells in the absence of a helper virus to form an episomally maintained genome. A number of natural serotypes of AAVs that can infect human cells have been identified, with additional designer serotypes actively in development [2]. AAVs therefore offer a significant opportunity for an effective *in vivo* GTx intervention; an opportunity that has been very actively and broadly pursued by academia and industry investigators.

Chimeric antigen receptor expressing T-cells (CAR-T) CTx modality is an example of *ex-vivo* GTx that represents highly successful and promising approach to treat various types of oncological conditions [3,4]. Two CAR-T-based products have received required regulatory approvals (YESCART, KYMRIAH) [5,6]. Genetically modified T-cells are designed to carry a CAR protein that binds to a tumor cell expressed target [3,4]. Additional intracellular domains are added as part of the CAR construct in order to increase CAR-T cytotoxic potential. Such complexity is achieved by employing various *ex vivo* GTx techniques, which modify patient or donor T-cells with either lentiviral, onco-retroviral systems or by applying nonviral gene transfer methodologies [3,7–9].

As one might expect, the high complexity of GTx modalities aligns with a significant increase in expectations and requirements for the bioanalytical evaluations requested in support of nonclinical and clinical studies. The diversity and broad nature of questions, methods and reported data are noteworthy. In the case of GTx modality, therapeutic and bioanalytical support may include detection of the viral genome in circulation or in tissues (particularly during nonclinical evaluation), expression of transgene and transgene protein and/or its activity, evaluation of binding and neutralizing antibody-based as well as cellular immune responses against viral capsid and transgene protein [10–14]. Safety monitoring may require evaluation of GTx genome integration, shedding and infectivity of the virus post dose [15,16].

To enable adequate bioanalytical support, there is a clear expectation for access to a broad range of analytical platforms and methodologies. Notably, a range of bioanalytical methods may be applied to address a given objective. For example, expression of transgene may be evaluated by detection of mRNA, transgene protein by either ligand binding assay (LBA) or LC–MS protocol or by detecting corresponding biologic activity. This diversity is in stark contrast with bioanalytical expectations for a typical protein-based biotherapeutic or a small molecular weight chemical compound.

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The diversity of analytical platforms applied in support of GTx and CTx modalities cuts across many platforms and often includes PCR and LC–MS based protocols, LBA and various cell-based methodologies [10,17]. Access to laboratories with a broad range of bioanalytical capabilities is therefore highly desirable to ensure successful support of a GTx or CTx modality therapeutic.

Complexity of questions and methodologies is paralleled by the complexity of the regulatory landscape that is clearly in development and still forming. Guidelines have been published by the US and European agencies to reflect on the general principles of GTx and CTx development. These cover topics related to nonclinical, clinical and post-approval phases of development [12–14,18–20]. Separate regulatory documents have been made available to present regulatory agencies' position on treatment of specific indications, including retinal, hemophilia and rare diseases [21–23]. In some cases, researchers may be able to apply agency recommendations made for protein-based biotherapeutics. For example, evaluation of anti-transgene protein and possibly anti-viral capsid immunogenicity responses may be conducted based on the recommendations described in previously issued US FDA and European Medicines Agency guidelines [24,25]. Evaluation of anti-GTx immunity also has a strong connection with a likely needed companion diagnostic or similar test designed to detect pre-existing anti-viral capsid antibodies, which can be requested in order to determine whether a patient can be admitted for the treatment. Such connection between pre- and post-approval phases of therapeutic development is quite unique to the GTx modality. The regulatory landscape may be incomplete and lacking some of the guidelines at this point. Such perceived gaps include lack of guidelines that would describe regulatory expectations for bioanalysis of transgene protein expression by either LC–MS or other methods and guidelines on the value and methodologies to conduct cellular immune response assessment. Industry white papers are available to describe methods for detection of cellular immunity [26]. A general position of regulatory agencies may be available although with a limited description of bioanalysis specific questions, for example in the guidance documents describing shedding and infectivity evaluations [15,16].

Focusing on the viral vector delivery GTx and to understand current clinical development landscape of the modality, information available on the ClinicalTrials.gov website was searched in the early 2019 based on the following criteria [27]:

- Keywords applied: 'gene therapy' AND 'virus' + 'AAV' OR 'adeno' OR 'HSV' OR 'lentivirus';
- Items excluded: cell-based therapy studies, antiviral treatment-focused studies;
- To report on the indications and viral vector type, only studies with unique therapeutic were retained.

The search produced 173 unique items (studies) with a significant majority (~72%) registered as early Phase (I/II) clinical investigations. Several studies were identified as Phase III or II/III (total ~10%) and a very limited number were listed as Phase IV (~1%). The majority (>75%) of the individual sponsors were found to conduct between one and three studies each with only a handful of sponsors supporting greater than six studies at the time data were collected. In the vast majority of cases (~75%), GTx therapeutics were AAV based, including AAV2, 5, 8 and 9 or a recombinantly produced AAV serotypes. Other viral vectors, including adenovirus and herpes simplex virus, were fond to be investigated for treatment of oncology conditions.

Approximately a half of the ongoing studies were designed to investigate GTx-based treatment of rare diseases, including hemophilia A and B, muscular dystrophies, mucopolysaccharidosis and Leber hereditary optic neuropathy. Oncological conditions were listed as the primary indication in approximately 20% of protocols. Importantly, nonhereditary conditions such as Alzheimer's disease, Parkinson's disease, heart failure, cardiomyopathy, myocardial ischemia or rheumatoid arthritis also appear on the list of potential GTx modality-based indications [28–30].

It is reasonable to expect that the number of GTx and CTx-based therapeutics will continue to grow quickly in the future. Significant complexity and diversity of bioanalytical support expectations related to *in vivo* and *ex vivo* GTx modalities require concerted and expedited effort to provide an aligned position accepted by the regulatory, industrial and academic participants. Many considerations related to the bioanalytical support of GTx and CTx modalities remain in development and will require additional clarification. Better understanding of required end points, methods and analytical platforms to use and expectations for the generated data will be key in ensuring successful advancement of the modalities.

Financial & competing interests disclosure

Boris Gorovits is employed by Pfizer, Inc., which is involved in development of gene therapy products. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Editorial

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Bioanalysis

Hybrid LC–MS as a powerful tool for supporting protein bioanalysis in gene and cell therapies

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Gene and cell therapies are promising new therapeutic modalities for treatment of inherited and acquired diseases [1]. In gene therapy, exogenous DNA or RNA are introduced into targeted cells via a viral vector or a nonviral carrier to override the faulty gene and restore its normal function. In cell therapy (for example, chimeric antigen receptor T cell [CAR-T]), autologous or allogenic immune cells engineered *ex vivo* to express target receptors and immune modulators are infused into humans to induce immune responses and kill cancer cells. Both modalities have demonstrated a promising curative potential after a single treatment and hundreds of drug candidates are currently under evaluation in clinical trials.

To develop the complex modalities, a repertoire of genomic, ligand-binding assay (LBA) and cell-based assay are generally necessary for assessing biodistribution, persistence, efficacy and immunogenicity of the therapeutic products – with the bioanalytical support focusing on the transgene products, key effectors of the therapies and other related biomarkers. In particular, a number of bioanalytical challenges have emerged which are associated with quantification of transgene expressed proteins and the protein biomarkers in preclinical studies which call for specific, selective and sensitive assay solutions.

Hybrid LC–MS is a methodology that selectively extracts protein analytes based on immunoaffinity and measures the enriched analytes after proteolytic digestion. In the method, it monitors the protein surrogate signature peptides by LC–MS using stable isotope labeled proteins or peptides as internal standards. The method has demonstrated high selectivity and sensitivity and has been broadly used for the bioanalysis of protein biotherapeutics and biomarkers [2–5] and is thus uniquely positioned as a powerful tool to complement the common LBA assays for protein bioanalysis in supporting gene and cell therapies.

Transgene proteins

In gene and cell therapies, lentiviral or adeno-associated virus vectors are engineered to deliver the target transgenes. The viral biodistribution and transduction efficiencies are evaluated by measuring the viral vector in the targeted matrices at the DNA and mRNA level using quantitative polymerase chain reaction and reverse transcriptase quantitative polymerase chain reaction methods [6]. Efficacies are generally determined by measurement of disease biomarkers, levels of transgene expressed proteins and their corresponding activities [7–10]. In their early discovery, the lead constructs from gene therapy or CAR-Ts needed to have the translational proteins characterized and quantified to check the intended post-translational processing in the targeted cell and associated tissue microenvironment. This is particularly important for transgenes with dual-vector or polycistronic expression designs. The assessment helps select candidates with appropriate transduction, transcription, translation and minimal or no unintended protein modifications for reducing underlying immunogenic risks. Furthermore, the direct measurement of transgene proteins in clinical studies can be critical to characterize pharmacokinetic properties for selecting optimal dosage and building a pharmacokinetic/pharmacodynamic relationship for clinical development [11].

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Specifically, the hybrid LC–MS based transgene protein bioanalysis adds values to the multifacet drug discovery process. First, in preclinical pharmacology and toxicology study support, it allows for distinguishing the human transgene protein from the endogenous protein of animal species [12], while the matrix interference and antibody cross reactivity make the bioanalysis of the two forms difficult using conventional LBA assays due to the high protein homology across species. Second, the transgene products are frequently structural, membrane or low-solubility proteins which require a high level of detergents for protein extraction. The detergent rich samples are longer compatible with typical LBA assays but still amenable with hybrid LC–MS assays. For example, in the bioanalysis of translated dystrophin protein [12], Neubert *et al.* used 5% sodium dodecyl sulfate to extract the protein and proteolytically digested prior to immune enrichment by antipeptide antibodies for online LC–MS analysis. Third, the structural integrity of transgene proteins needs to be characterized in order to confirm the intended expression of the various engineered proteins, such as truncated proteins like mini dystrophin [10], mutated proteins, isoforms or multimeric proteins. The simultaneous monitoring of signature peptides from both N- and C-termini of the transgene protein can be utilized for both identification and quantification. Moreover, for polycistronic expressions commonly seen in cell therapies, the intended cleavages of the fused recombinant proteins could be assessed using signature peptides from all component proteins by multiplexing hybrid LC–MS.

Biomarkers

Biomarkers are critical in evaluating the efficacy, toxicity and patient stratification of drug candidates during their development. In cell therapies, CAR-T cells are engineered to target tumor cell specific antigens such as CD19 in B-cell acute lymphoblastic leukemia. Quantitative assessment of tumor surface antigen density, tissue distribution and potential instability due to expression perturbation or tumor proteolytic environment changes could facilitate evaluation of drug dose response and persistence which eventually will help project efficacious doses. Tumor cells are known to secret a high level of antigens before, during or after a drug treatment, resulting in drug resistance and modification of the drug distribution and pharmacokinetics [13,14]. Although soluble targets may not neutralize CAR-T at the *in vitro* setting or in animal studies [15], evaluating target shedding at the molecular level is critical in understanding target engagements in order to design drugs that are less prone to target-mediated drug-resistance. In addition, special biomarker strategies are often needed to monitor targeted tissues and specific cell populations (CAR-T) in prolonged durations for gene and cell therapies [16].

Hybrid LC–MS has been proven a power tool in the quantitative measurement of protein biomarkers [3]. Both membrane and humoral therapeutic targets could be measured for cell membrane receptor density as well as shed target concentrations in circulation. Multiple forms of a shed target could be generated by varied tissue proteolytic environment or truncations at different domains on the surface target. The identification and quantification of the multiple forms of the shed target are important to assess their potential impacts on the distribution, kinetics, efficacy and toxicity of the cell therapeutics. Using hybrid LC–MS and multiple epitope specific antibodies, the species associated with the shed target could be captured, identified and quantified by their relative concentrations. Besides therapeutic targets, other biomarkers such as disease protein biomarkers can also be measured by the hybrid LC–MS to help evaluate on pharmacodynamics and efficacy of the therapies. For example, Tau protein is a biomarker for neural degenerative diseases such as Alzheimer disease. McAvoy *et al.* validated an assay to measure total concentrations of six Tau isoforms in human cerebrospinal fluid by immunoprecipitation and detection of a common surrogate peptide sequence [17]. In gene and cell therapies, due to its nature of highly targeted delivery of the drug, the biomarker measurement could be conducted on local relevant tissues or cell clusters with the benefit of avoiding systemic dilutions for enhanced sensitivity of detection. For example, Sato *et al.* measured with high sensitivity Tau proteins in neurons derived from induced pluripotent stem cells that are from patients and reprogrammed to a pluripotent state as cell-replacement therapies for Alzheimer disease and age-related macular degeneration [18]. The hybrid LC–MS methodology was also able to provide high spatial resolutions for different Tau protein forms which helped to assess the progression of the disease after the treatment.

Conclusion

Excellent opportunities lie in the bioanalytical support of gene and cell therapy using hybrid LC–MS based technologies. The high specificity, reproducibility, multiplexing capabilities, less stringency on reagent requirements have made the hybrid LC–MS assays well suited for assessment of transgene products and key therapeutic biomarkers in diverse matrices and testing species for studies of long durations. In addition, the method has enabled to differentiate construct variants and endogenous counterparts in different species, and impact assessment of tissue

specific environment for overall translation efficiencies, specificity and post-translation processing of the transgene products. We believe its role in supporting the drug discovery and development for gene and cell therapies will become increasingly important and more widely accepted.

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