

Cell-based therapeutic products: potency assay development and application

Potency is a critical quality attribute of biological products, defined by the US FDA as the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result. Ideally, a potency assay will leverage the product's mechanism of action. Alternatively, the assay may focus on a therapeutically relevant biological activity. The absence of rigorous mechanistic data for the majority of cell-based therapeutics currently in the process research pipeline has impeded efforts to design and validate indices of product potency. Development of a systematic battery of parallel functional assays that, taken together, can address all potential mechanisms of action believed to be relevant for the product platform is recommended. Such an approach is especially important during preclinical development. Here, we summarize the principal and unique challenges facing the development of functionally relevant and rigorous potency assays for cell-based therapeutics. We present perspectives regarding potency assay development for these products as illustrated by our experiences in process R&D of cryopreserved hepatocytes (Incara Pharmaceuticals) and selected renal cells (Tengion).

Keywords: bioassay • critical quality attribute • potency • process research

Potency as a critical quality attribute of cell-based therapeutics

At present, the regulatory infrastructure for the characterization of therapeutic products is focused around small molecule chemical compounds and biologicals, including natural and recombinant sourced proteins. The focus of medicinal product development pipelines continues a divergence away from leveraging the cell as a platform for manufacture toward an understanding of the cell in its totality as the final product. In so doing, it is critical that paradigms for quality and supply chain management, process and manufacturing controls, toxicological and pathologic analysis and evaluation of product bioactivity in preclinical animal studies and later clinical trials are sufficiently malleable to accommodate the evolving class of novel regenerative medicine (RM) biotherapeutics, previously inconceivable during the foundation of

these frameworks. An alternate possibility would involve reformulation of existing policies and regulatory infrastructure to remain germane, permitting the relevant regulatory organizations to efficiently execute on their objective to expedite authorization of benign and practical medicinal product candidates while avoiding onerous surveillance of the emerging RM industry.

With this outcome in mind, US FDA expectations for analytic characterization of cell-based therapeutic products identify four principal critical quality attributes (CQA): identity, purity, safety and potency [1]. Identity assessment is mandatory to demonstrate that bioactive cellular components are contained within the product. Purity data are used to establish that detrimental elements of any nature are either nonexistent or negligible in the product. Examples of detrimental elements include cellular contaminants and

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processing chemicals. Safety determines that the bio-burden, which includes contaminating fungi, viruses, bacteria and protozoa, is also nonexistent. Included in this grouping is the absence of any potential to induce tumors. Lastly, potency is intended to measure the product's relevant therapeutic biological activity (Figure 1) [2,3].

Increasingly, as additional RM products are currently being initiated into the clinical trials pipeline [4,5], there is a substantial unmet requirement within the RM industry for consistency of definition suitable to both the industry and regulatory agencies. To date, efforts by industry working groups toward establishment of a cohesive and universally acceptable set of analytical technologies for potency determination of RM products have been of limited value and usefulness to the broader RM industry, generally placing much greater importance on identifying and optimizing analytic methodologies aptly suited for stem cell products in development, principally mesenchymal and hematopoietic stem cells (MSC and HSC) [6].

Regardless of these efforts, clarification relevant to the application of CQA criteria for cell-based products other than MSC/HSC remains forthcoming. In particular, as mechanistic paradigms for bioactivity of stem-cell-based product candidates increasingly emphasize paracrine and other 'action at a distance' pathways that are not unique to stem cells, potency assay criteria

must be sufficiently expansive to incorporate products that leverage therapeutically bioactive cell populations other than stem cells. Here, we cite an example from Incara's development of a cryopreserved human hepatocyte product candidate to provide historical perspective on the development of potency as a CQA for cell-based therapies. Taken together with the more recent development of Tengion's selected renal cells (SRC), currently in Phase I trials, these product candidates represent a new paradigm of nonstem cell-based cellular biotherapeutics. Our intent is to use these products as illustrative models for both regulatory agencies and industry working groups in the RM field to better provide guidance and direction relevant to the development and application of potency assays for these novel products.

Potency

Potency is a CQA of biological products. The FDA defines potency as 'the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result (21 CFR 600.3[s]).' Furthermore, FDA requires that 'tests for potency shall consist of either *in vitro* or *in vivo* tests, or both, which have been specifically designed for each product so as to indicate its potency in a manner adequate to satisfy the

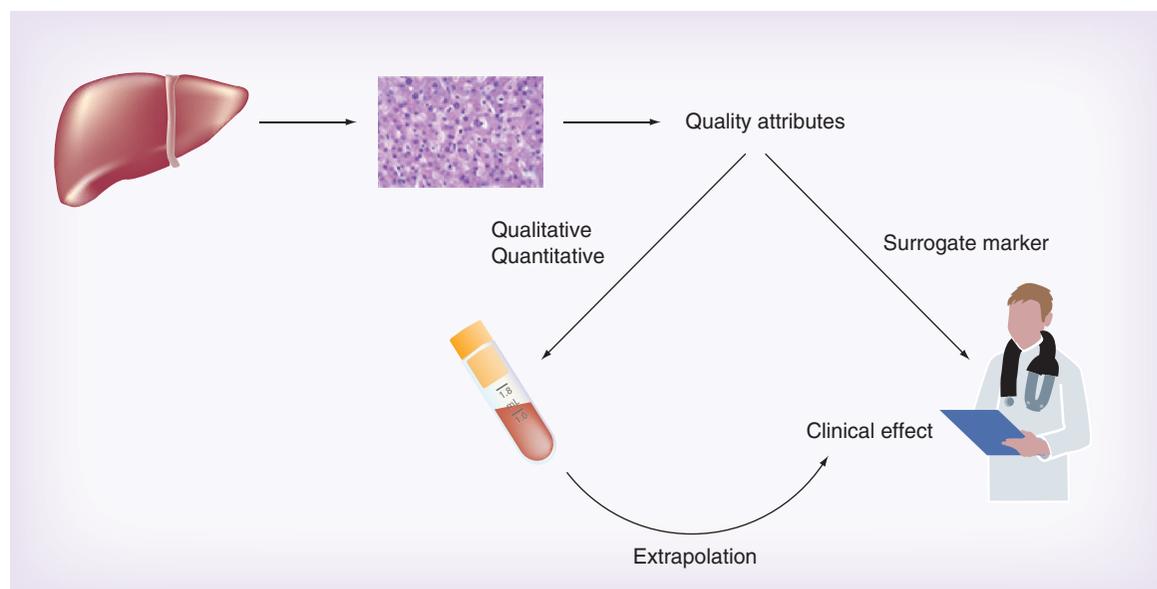


Figure 1. Potency assay design, objectives and implementation. The goal of the potency assay is to utilize quality cell attributes in the measurement of biological activity(s) relevant for treatment efficacy and safety in a patient as shown in this diagram; donated liver unsuitable for whole organ transplantation is rendered to a single cell suspension following enzymatic digestion under cGMP conditions. Quality attributes such as viability and morphology are assessed and recorded. *In vitro* quantitative/quantitative analyses (i.e., ureagenesis, clearance of 7-ethoxycoumarin, UDP-glucuronosyltransferase activity and sulfotransferase activity) are used to demonstrate bioactivity believed to be relevant to *in vivo* mechanism of action. Extrapolation of the *in vitro* data to the *in vivo* situation may then serve as a surrogate marker for the observed clinical effect.

interpretation of potency, as defined above, given by the definition of 600.3(s). As an outcome of their inherent character, cell-based therapeutics present intrinsically distinctive difficulties for development and application of potency assays. Such difficulties include the heterogeneous character of starting material, usually human tissue- or organ-based materials from autologous or allogeneic sources and of the putative product candidate; the product uniqueness and associated absence of understanding with regard to potential mechanism(s) of action (MOA[s]) and *in vivo* biodistribution; the lack of consistent and well-defined standards of reference and controls and restrictions associated with the obtainability and consistency of product for the potency testing itself [7].

If possible, potency testing needs to be instituted on the product as it is delivered for administration, with all its associated components. For cell-based therapeutics, this may include biomaterials such as hydrogels used to formulate the cellular active biological ingredient prior to administration. The potency of any cell-based biological product is generally assessed by using a quantitative biological assay (bioassay) that quantifies product activity as determined by its unique ability to mediate a defined, biological outcome. Bioassays are exemplified by the application of living biological platforms (*in vitro* tissue or organ cultures, *in vivo* animal and cell cultures) to demonstrate the bioactivity of a medicinal biotherapeutic candidate product. On the other hand, analytical assays assess the pertinent characteristics of a candidate product external to a living biologic platform, as with FACS, ELISA and PCR for example. It is not unusual for these two groups of assays to be implemented together to satisfactorily assess the potency of a cell-based therapeutic [7].

Cell count and viability are important components of the definition of potency. However, regulatory guidelines regarding the relevance of cell count/viability for evaluation of product potency have been subject to ongoing modification. Previous FDA recommendations for evaluation of the purity and potency of hematopoietic progenitor cells and cord (HPC-C) specified the following combination of analytical assays [8]:

- Total nucleated cell (TNC) count, generated by manual or automated cell counting. HPC-C TNC $\geq 1.7 \times 10^7$ TNC/kg intended recipient body weight;
- Cell viability assayed by dye exclusion (trypan blue/7-aminoactinomycin) $\geq 85\%$ of TNC in HPC-C final product prior to cryopreservation;
- CD34⁺ expression assayed by flow cytometry. %TNC CD34⁺ $\geq 0.25\%$ TNC in HPC-C final product prior to cryopreservation.

This specific example notwithstanding, none of the above criteria measure biological activity, the salient feature of potency assays. To this end, FDA currently recommends that the ideal potency assay has the characteristics outlined in Table 1. Examples of single, validated potency assays that meet these criteria include HALO-96 PQR for HSC and the LUMENESC-96 PQR for MSC [9]. These assays leverage proliferative potential as an index of bioactivity common to both HSC and MSC. The MOA of these assays is presented in detail below in the section “Establishment of reference standards and controls”. However, for most other cases, the chances of any single potency assay alone presenting a complete profile are slim. To address this difficulty, FDA recommends assembly of an assay matrix, as illustrated in Table 1. The amalgamation of assays within the assay matrix, when taken together, serves to address the FDA’s regulations for potency assays in its entirety. The more successful matrices employ a diverse variety of both biologic and analytical technologies.

With these considerations in mind, the FDA recommends an iterative, tactical approach toward instituting and executing potency assays, with an appreciation that assay practices will not be static and are expected to progress with the phase of product development as an established database of product functionality and putative MOA is created over time. With this objective as a desired outcome, multiple bioassays exploring the most expansive range of product features and (putative) MOA should be investigated in parallel beginning with the initial stages of process research of the product. Product data including the proteomic, immunological, secretomic, transcriptomic (including miRNA), biophysical and other product characteristics should be brought together on each product lot within a single database. Cross-indexing these results with *in vivo* studies on MOA together with earlier published investigations on MOA of related cellular biotherapeutics will generally highlight possible routes for potency assay implementation. The FDA has stated it will ‘allow for considerable flexibility in determining the appropriate measure of potency for each product,’ which suggests that the agency will accept a variety of assay types, provided the assay can be rationally justified as pertinent. However, FDA has communicated certain key considerations for establishing whether a potency assay is appropriate for release of cell-based therapeutic products [1]. These recommendations are outlined below:

- If possible, a potency assay should be based on an established feature of the product’s MOA, as demonstrated via *in vivo* experimentation.

Table 1. Matrix approach to potency assay development.

Potency characteristic	Bioassay	Assay 1	Assay 2	Assay 3
Demonstration of bioactivity relevant to mechanism of action	X			X
Appropriate for lot release	X	X	X	
Specific to the product	X		X	X
Quantitative	X	X	X	
Can demonstrate lot-to-lot consistency	X	X	X	X
Validated: meets predefined acceptance/rejection criteria	X	X	X	X
Compared to reference controls	X		X	X

Data taken from [10].

For example, current models for MOA of MSC emphasize paracrine bioactivity to define the experimentally observable therapeutic impact of MSC at a distance without significant levels of directed differentiation and site-specific engraftment [11]. In support of this, preclinical rat models of inflammatory organ failure demonstrate increased survival and changes in the severity of inflammation when MSC-derived conditioned media and/or MSC cellular lysate are given. In conjunction with these observations, serum IL-10 levels also increase. Mimicking the effect can be achieved by treatment of peripheral blood mononuclear cells *in vitro* with MSC-derived conditioned media. In so doing, the elemental basis of an MSC potency assay for this specific therapeutic indication is produced. Once this is achieved, the absolute amounts of secreted IL-10 resulting from treating peripheral blood mononuclear cells *in vitro* with MSC-derived conditioned media may then be quantified by ELISA [12].

- Alternatively, the assay should focus on a therapeutically relevant biological activity.

Tengion’s primary renal cell-based therapeutic, SRC, currently in Phase I clinical evaluation for chronic kidney disease (CKD), is believed to operate at least in part by locally engrafting and regenerating new tubule-like elements within unhealthy kidney. The potency of the biological product may therefore be evaluated at least in part by *in vitro* assays measuring tubulogenic potential: the ability of product elements to assemble into tube or sphere-like elements spontaneously upon extended growth in 3D hydrogels [13]. When measuring the number of tube/sphere-like elements, normalization relative to the number of microscope fields scored, volume of the hydrogel, number of cells etc. is advised. Additionally, SRC is understood to operate through directly or indirectly facilitating endogenous populations of stem and progenitor cells to migrate and by enabling *de novo* angiogenesis and modulation of both apoptosis and

fibrosis within the diseased kidney [14,15]. All of these putative MOA(s) may be leveraged for development of potency assays.

- Development of a systematic battery of parallel functional assays that, taken together, can address all potential MOA(s) believed to be relevant for the product platform is recommended.

Such an approach is especially important during preclinical development when little may be known regarding MOA of the product. As part of the progressive potency assay implementation policy recommended by FDA, this matrix of functional assays is expected to evolve during the developmental phase of the product. As more information becomes available regarding the likely MOA of the product, bioassays that become less relevant may be dropped from the potency assay matrix [7]. Using SRC as an illustrative example, the potency assay development matrix follows these principles as shown in Table 2, which links potential bioassays to the idealized FDA characteristics in Table 1.

Potency surrogates

A bioassay is understood, by definition, to involve the leveraging of living systems, generally (but not always) cell cultures, to establish a relevant functional index of the potency of the product candidate. It is commonly appreciated that the application of living experimental platforms is concomitant with particular methodological difficulties that may be circumvented through the use of an assay matrix or analytic assay as a proxy indicator of biologic functionality. This necessitates the determination of an unambiguous and quantitatively meaningful correlative relationship between bioactivity and the nonbiologic analytic assay. At the end, the analytic assay can be applied as a convenient substitute to the initial bioassay for quantification of product potency. To illustrate, amounts of the cytokines CXCL5, IL-8 and VEGF from conditioned media derived from human multipotent adult

progenitor cells (MAPC) cultures have been successfully leveraged as proxy markers of potency indicative of the angiogenic biological activity of MAPC as demonstrated in an *in vitro* human umbilical vein endothelial cell (HUVEC) based angiogenesis assay [16]. By association of cytokine levels needed to catalyze tube formation *in vitro* with levels observed in conditioned media from production runs, detection and identification of these factors was acknowledged as a proxy marker of potency with established pass/fail benchmarks. *De novo* angiogenesis is postulated to be at least one possible MOA that may explain how MAPC functions therapeutically in animal models of myocardial infarct [17].

Establishment of reference standards & controls

As is the case with all assays, establishment of stringent negative and positive controls is essential to have confidence in both the assay and interpretation of the results. A salient prerequisite for all potency assays is the expected potential to specifically fail any sub-potent product or subpotent materials unconnected to the candidate product. For example, the *in vitro* tubulogenesis assay under development for evaluating potency of SRC will specifically fail unrelated cell lines as well as SRC product lots indicated to be sub-potent by alternate assays [BASU J & LUDLOW JW, UNPUBLISHED DATA]. Potency assays for negative controls can be established by the intentional degradation of the product by treatment with toxins, aging, mechanical damage, presentation of external environmental stress etc. The reference standard needs to be a sample of the same type as the product that serves as an acceptable comparator for the potency assay, allowing for the designation of a potency ratio as the biological activity of the product candidate compared to the reference standard. Reference standards may, and hopefully should, incorporate clinical lots or preclinical study materials that are well established, or often-used cell lines that mimic the biological activity of the candidate product. Reference standards are also critical for establishing dose–response relationships, although it should be noted that not all potency assays are amenable to the creation of dose–response curves (see examples in [7]).

It may not always be possible to secure well-determined reference standards for a desired product candidate, especially for those product candidates at the preclinical phase of product development. To address this difficulty, a reference standard may be established gradually over an extended period of time as iteratively additional data are acquired from additional clinical material. These data can then be combined to formulate an overall reference standard suitable for

Table 2. Matrix-based development of potency bioassays for selected renal cells.

Bioassay	MOA	Product specific?	Quantifiable?	Time frame (h)	Methodology	Potency characteristics (from Table 1)
<i>In vitro</i> tubulogenesis	Regeneration of functional kidney structures <i>in vivo</i>	Yes	Yes	24	3D hydrogel culture	1, 3, 4
<i>In vitro</i> angiogenesis	Regeneration of new vasculature	Yes	Yes	3–5	Assembly of tubular structures from HUVEC	1, 4, 5
miRNA expression	Exosome-mediated action at a distance	Yes	Yes	24	qRT-PCR	1, 2, 4, 5, 7
Stimulation of cell migration	Mobilization of native progenitor populations	Yes	Yes	24	Transwell migration of product toward defined growth factor	1, 3, 4
Secretion of VEGF and KIM1	Paracrine action at a distance	Yes	Yes	12	ELISA analysis of conditioned media	2, 5, 6, 7
Modulation of TGF- β -induced EMT of HK2 cells	Amelioration of fibrosis	Yes	Yes	24	qRT-PCR analysis of epithelial and mesenchymal markers in HK2 cells exposed to TGF- β and product-derived conditioned media	1, 4

EMT: Epithelial–mesenchymal transition; HUVEC: Human umbilical vein endothelial cell; MOA: Mechanism of action; qRT-PCR: Quantitative real-time PCR. Data taken from [7].

use. Needless to say, this approach will be nominally helpful during nascent implementation of the potency assay, but will serve to be increasingly more meaningful as time progresses. Otherwise, a reference standard may be randomly nominated solely for the objective of filling the need for the particular bioassay, with all subsequent results compared to the reference standard. As an illustrative example, the potency of HSC can be evaluated through measurement of increasing intracellular ATP (iATP) with increasing HSC concentration in the presence of stimulatory growth factors [9]. The slope of this dose–response curve is indexed as a measure of potency – HSC with greater proliferative potential (defined here as the measure of potency and equivalent to engraftment potential) have steeper dose–response curves relative to an arbitrarily designated reference standard. The potency of the reference standard by definition is always 1. A potency ratio greater than 1 indicates that less of the product is needed to elicit the same response as the reference standard. As with the potency ratio for a drug, the potency ratio for a cell-based therapeutic indicates the dosage (see Figure 1A and B in [18]).

At the present time, it is not possible to create a global reference standard for any cell-based therapy, simply because there are not enough cells available. As an alternative, primary, secondary and even tertiary reference standards of the same material-type as the product to be assayed may be established in-house. The critical point is that for comparison of potency data across and within product lots and laboratories to be meaningful, it is essential that the assay used be the same and be calibrated, standardized and validated. In short, internal standards and controls need to be included to calibrate and standardize the assay.

Work-flow integration

Ideally, the potency assay(s) can be executed on the day of product release, or, alternatively, while the product is in route to the intended recipient. Compounding factors for cell-based therapeutics may include the requirement for product administration within hours of formulation, leaving little time for rigorous evaluation of product potency. Additionally, lot sizes may be limited to a single dose of cells re-suspended in a relatively small volume, permitting sampling of minimal material, thus limiting the ability to test potency.

Ease of use & assay time frames

The widespread reliance on cell counting and cellular viability assays such as trypan blue exclusion and MTT/Presto-Blue (Presto-Blue is a commercially sourced colorimetric reagent used to evaluate cellular metabolism) colorimetry as an index of product

potency is in no small part a function of the simplicity and short time frames associated with execution of such assays. Application of surrogate analytic assays in place of biological potency assays serves to considerably simplify assay execution and streamline time frames, criteria that are especially significant when considering assays suitable for release of product lots.

Cell count/viability relationship to potency

It should now be clear that TNC/viability is woefully inadequate as a cell product potency marker in the absence of supporting bioassay data specifically leveraging putative MOA. Cellular elements may very well be ‘viable’ as established by TNC/MTT type assays, but dramatically lowered in their bioactivity. For example, viable human MSC culture potency as measured by the widely accepted tri-lineage differentiation assay is generally reduced with continued passage or with increased donor age [19]. This observation notwithstanding, assessment of TNC/viability is typically required to permit communication of product potency in the form functional unit per viable cell, and as such needs to be included as a constituent analytic assay within the matrix of assays supporting the potency assay matrix, as shown in the following illustrative example based on Tengion’s SRC product candidate.

Potency testing for SRC

Cellular functionality of SRC is currently assessed by ability of SRC to express VEGF. Expression of other functional markers such as KIM1 is also assessed for information and potential linkage to MOA and potency in later stages of development. Potency or ‘fitness for use’ of the final product is established using a combination of the presence of a defined concentration of viable renal cells and demonstration of cellular activity in the final product. SRC in the final product must be functional as indicated by metabolism of Presto-Blue. While this analytic assay measures non-specific cellular activity, this assay, when combined with a specified renal cell concentration of the required phenotype and cellular functionality demonstrated by expression of VEGF, is *currently* proposed to provide adequate information regarding the potency or ‘fitness for use’ of the final product for Phase I (Tables 3 & 4).

Case study: cryopreserved human liver cells for transplantation – historical perspective

In the earlier days of cell therapy, Incara Pharmaceuticals developed a cell product consisting of cryopreserved human liver cells for transplantation to treat patients suffering with cirrhosis and end-stage liver disease who had an estimated life expectancy of approximately 6–18 months. Back in 2002, just as

Table 3. Release criteria for selected renal cells: illustrative example

Quality attribute	Biological/analytical assay	Pass/fail criteria
SRC phenotype (identity, purity)	Flow cytometry, immunocytochemistry, cytokeratin CK18, GGT, aquaporin 2	≥90% positive; for information only
SRC viability	Trypan blue	≥70%
Potency (fitness for use)	Cell concentration: calculation	100 × 10 ⁶ ± 20% per ml of SRC product
	SRC product volume	≥8.5 ml
	Cell function: Presto Blue	≥0.73 µg/ml (fluorescence)
	VEGF	≥0.35 ng/ml (ELISA)
	Kim-1	For information only (ELISA)

SRC: Selected renal cell.

today, there were well established testing techniques to evaluate the nature of pharmaceutical compounds within isolated cells and tissues, which are referred to by the acronym ADMET or ADME Tox (adsorption, distribution, metabolism, excretion, toxicity). Cell therapy products behave very differently than pharmaceutical compounds. As such, what works for pharmacologic and toxicologic studies on 'small molecule' drug compound development does not generally work for cellular therapeutics. The major reason is that these products contain live cells that cannot be adsorbed by other cultured cells in a manner that is comparable to pharmaceutical compound adsorption. Since they are not adsorbed, metabolism and excretion in the classical sense of these *in vitro* assays does not take place. Failing to fit into the ADMET paradigm also raised the question of how to assess the potency of this cell product. During pre-investigational new drug (IND) meetings with the FDA, the agency worked closely with Incara to come to an agreement on the types of testing that would be deemed acceptable and informative before the product was allowed in the clinic. It was agreed that for this product, in keeping with the agency directives, information was to be obtained through numerous studies of different types, with the expectation of combining these various experimental systems to assemble a dataset of sufficient comprehensiveness

allowing for sound decisions regarding the conduct of human clinical trial studies. Incara collected data on cell viability, morphology, phenotype, function (a precursor to potency) and how well the cells tolerated the freeze–thaw cycle during cryopreservation. In 2002, after a 30-day review cycle, the FDA allowed Incara's IND. This IND was the foundation for CytoNet's current IND to treat children aged 0–5 years who are suffering from ornithine transcarbamylase deficiency, carbamoyl phosphate synthetase I (CPS I) deficiency or citrullinemia with cryopreserved human liver cells.

Incara's product overview

The product is a sterile, cryopreserved dissociated liver cell suspension derived from donated cadaveric adult or pediatric human livers. The manufacturing process does not involve immunoselection or cell expansion. The cells are suspended in a cryo-buffer, which contains HypoThermosol® (40–80% v/v), human serum AB (10% v/v), DMSO (10% v/v) and sterile RPMI 1640 without phenol red (0–40% v/v). The cell suspension is packaged at a fill volume of 3 ml, and a nominal concentration of 3 million total cells/ml in 33-ml capacity cryo-bags. The product is to be thawed at the clinic and diluted 10-fold with Plasma-Lyte®-A prior to administration to patients. All release testing on the final product, including sterility, is to be

Table 4. Illustrative potency assay data from renal biopsies (n = 6).

Renal biopsy		1	2	3	4	5	6
Potency (fitness for use)	Concentration: ×10 ⁶ per ml of SRC	82.3	89.9	97.0	102.5	105.2	101.4
	Cell function: Presto Blue (µg/ml)	4.75	75.1	8.71	5.81	5.73	3.32
	VEGF (ng/ml)	2.04	1.91	1.33	1.66	1.63	1.40
	Kim-1 (ng/ml)	1.26	1.19	0.73	0.49	0.42	0.54

SRC: Selected renal cell.

Table 5. Summary of testing values required for product release.

Test	Test method	Limits
Cell concentration	Trypan blue exclusion	$3 \times 10^7 \pm 20\%$ cells/ml ($2.4 - 3.6 \times 10^7$ cells/ml)
Viability	Trypan blue exclusion	$\geq 65\%$
Characterization/identity		
% CD45 ⁺	Flow cytometry	$\leq 20\%$
% CD3 ⁺	Flow cytometry	$\leq 5\%$
% Albumin ⁺	Flow cytometry	$\geq 70\%$
Potency		
Rate of ureagenesis	Average rate of urea nitrogen production	Report results
Overall metabolic clearance of 7-EC	Average rate of formation of total 7-EC metabolites	Report results
UDP-glucuronosyltransferase activity	Average rate of formation of 7-hydroxycoumarin glucuronide	Report results
Sulfotransferase activity	Average rate of formation of 7-hydroxycoumarin sulfate	Report results
Sterility	USP <71>/CFR 610.12	No growth
Endotoxins	USP <85>	≤ 35 EU/ml

7-EC: 7-ethoxycoumarin.

conducted on post-thaw samples from each donor prior to release and shipment of supplies to the clinic.

The cell suspension is designed to be infused through and inserted into the patient's femoral artery and advanced to the splenic artery. It is anticipated that some of the transplanted cells will engraft in the spleen; others will advance through the splenic vein into the portal circulation and engraft in the liver. Based on animal studies and human studies of other liver cell suspensions, performed by other investigators, once engrafted, the cells will function and perhaps proliferate. The anticipated benefit to patients is improved liver function that may improve symptoms of end-stage liver disease, reduce certain complications, decrease hospitalizations, delay or obviate the need for a whole liver organ transplant, prolong life or otherwise improve quality of life. The patients will have to be placed on immunosuppressive therapy shortly before cell transplantation, as this is an allogeneic cell product.

Cell count & product viability

Cell count and viability are essential metrics for most viable cell-based products. These measurements are necessary to ensure the integrity of the cellular active substance, the consistency of the product (across multiple batches) and content of the final product (assuming a link between cell dose and efficacy). In addition, there may be a cell-type dependency on number and viability associated with product safety, as a threshold

number of dead and dying cells in the population may be deleterious.

In vitro cell characterization/identity

The tentative specifications for albumin positive, CD45-positive and CD3-positive cells are set at ≥ 70 , ≤ 20 and $\leq 5\%$, respectively. These values were selected after review of the data generated from development batches, with attention to methods limitations. In the case of CD3-positive cells, consideration was also given to literature reports of amounts given safely to patients in transplantations conducted by other investigators [20].

The percent albumin, CD45- and CD3-positive cells are determined by flow cytometry. These assays involve the staining of cells with appropriate fluorescent-labeled antibodies to specific proteins, followed by measurement of fluorescence and comparison with controls. Percent positive cells are determined based on the percentage of individual cells, which fluoresce above the background established by the control samples (a positive gate is set above the background fluorescence indicated by the control samples). All results are reported as a percentage of total cells. Acceptable levels of precision have been demonstrated for these flow cytometry methods.

In the case of the albumin assay, staining for the protein albumin is intended to identify hepatocytes, as only hepatocytes are capable of albumin production. For this assay, the results are significantly affected by the high degree of autofluorescence of the variable liver cell

mixtures. The cells in the product, especially larger cells, autofluoresce across a wide range of the fluorescence spectrum. The signal generated by the albumin antibody stain is not great enough to clearly distinguish all positively stained cells from the background signal. As a result of this low signal-to-noise ratio, the data reported from the assay represent the minimum number of albumin positive cells in the sample, since there are invariably a number of albumin positive cells not included in the positive gate. Efforts to improve the signal-to-noise ratio have been unsuccessful, and the assay will be updated or replaced if a more accurate assay becomes available. For this reason, the assay is considered to be a qualitative identification test, which assures the origin and character of at least a certain minimum proportion of the product, while not accurately measuring the percentage of hepatocytes as defined by albumin staining.

In the CD45 and CD3 assay, CD45 is used as a marker for leukocyte common antigen, which will be present on lymphocytes (including T cells and B cells), monocytes, granulocytes and macrophages. These cells represent the group of cells most likely to be present in the product other than hepatocytes. CD3 is used as a marker for the T-cell receptor complex and is intended to further characterize the CD45-positive population.

In vitro potency assays

Two measures of hepatocyte potency are assessed. Ureagenesis was chosen to assess the cells' ability to convert ammonia to urea, which is an important clinical function that is lost during liver failure. In this assay, cells are incubated in suspension in a supportive environment

and in the presence of added NH_4Cl . At specific time points, aliquots of the suspension are removed and analyzed for urea nitrogen. For each time point, the amount of urea nitrogen produced is calculated based on the number of live cells present in the suspension, and the rate of production is determined. The average rate, expressed as $\text{nmol}/10^6$ live cells/h, is reported.

7-ethoxycoumarin (7-EC) metabolism was chosen to measure both microsomal cytochrome P-450-dependent phase I oxidation and coupled phase II conjugation reactions. The formation of 7-hydroxycoumarin (7-HC) is mediated by cytochromes P450 1A, 2A and 2B, and is indicative of phase I oxidation of 7-EC. Formation of 7-hydroxycoumarin glucuronide is mediated by UDP-glucuronosyltransferase, located in the endoplasmic reticulum, and formation of 7-hydroxycoumarin sulfate is catalyzed by the cytosolic enzyme sulfotransferase. Both of these reactions are indicative of phase II conjugation of 7-HC. The ability of the cells to perform these coupled metabolic functions is indicative of the overall health of the cells.

In the 7-EC metabolism assay, cells are incubated in suspension in a supportive environment in the presence of added 7-EC. At specific time points, aliquots of the suspension are removed and analyzed for all three metabolites (7-HC, 7-hydroxycoumarin glucuronide and 7-hydroxycoumarin sulfate). For each time point, the amount of each metabolite formed, as well as the sum of the three, is calculated. The rate of formation is determined for each of the phase II conjugation metabolites, as well as for the total of all three metabolites (calculation of the rate of 7-HC formation would be

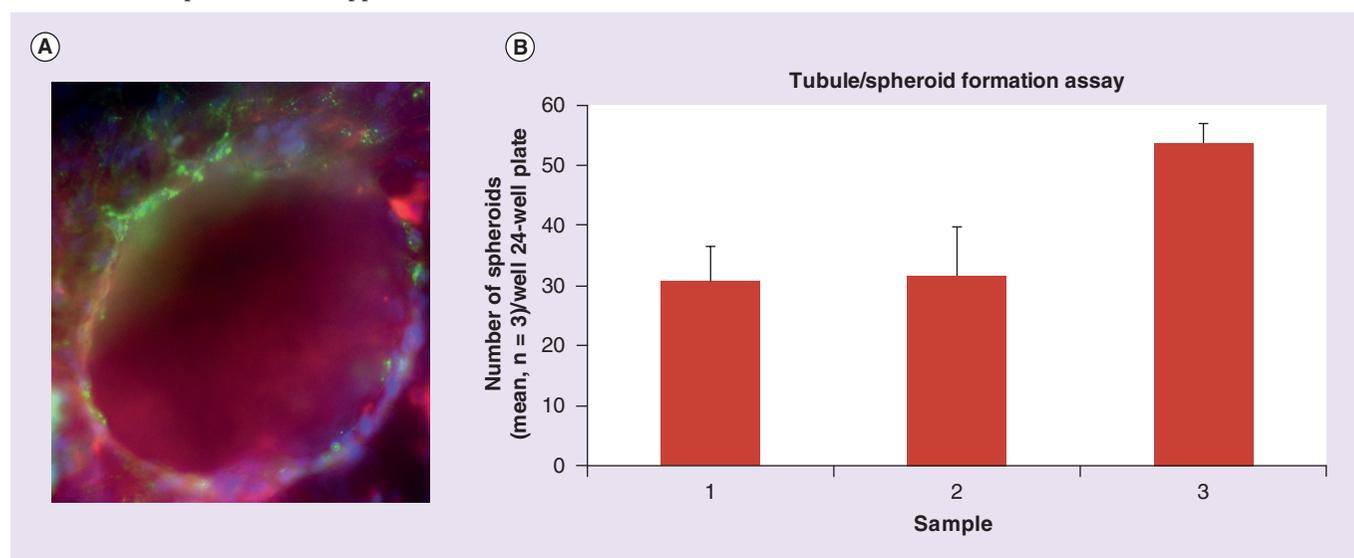


Figure 2. De novo tubulogenesis from selected renal cells. (A) The induction of tubules and organoids from renal cells grown in 3D culture may be used as a measure of functionality. In this example, the number of spheroids/tubules formed per well of a 24-well plate is leveraged as an indicator of product potency. Tubules (in cross-section) are functional as indicated by expression of aquaporin 1 (green) and cytokeratins (red). Both proteins are markers of renal cell functionality. DNA is in blue. Magnification: $\times 10$. **(B)** Quantitation of tubule/organoid formation at 24 h after assay initiation.

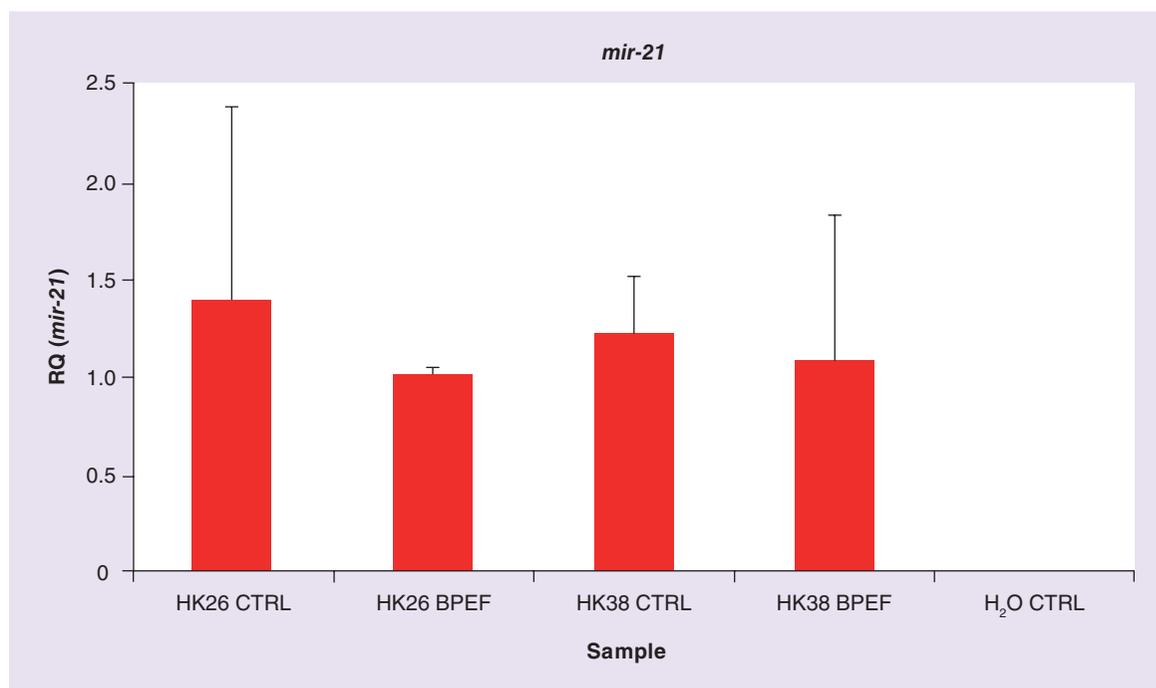


Figure 3. Quantitative real-time PCR expression analysis of *mir-21* expression from selected renal cell samples HK26 and HK38 in control and bovine pituitary extract-free media. *mir-21* is a miRNA potentially involved in antagonizing the NF- κ B signaling pathway. Evaluation of *mir-21* expression levels may be leveraged as an indicator of cellular functionality linked to therapeutic mechanism of action. The relative expression (compared to an arbitrarily selected house-keeping transcript) levels (RQ) of the miRNA are shown on the y-axis. BPEF: Bovine pituitary extract-free; CTRL: Control.

meaningless since the product is the substrate for the phase II reactions and is quickly consumed). The average rates of 7-hydroxycoumarin glucuronide and 7-hydroxycoumarin sulfate formation, expressed as nmol/10⁶ live cells/h, are reported. In addition, the average rate of overall metabolic clearance of 7-EC is reported as the rate of formation of total metabolites, again expressed as nmol/10⁶ live cells/h.

Potency

According to the FDA, potency measurements are necessary for product characterization testing, comparability studies and stability protocols, which are used to establish that a consistently manufactured product is administered during all phases of clinical investigation. A biological product has specific challenges to successfully meeting this requirement, including specific properties of the product that may be unique to that product, and/or limitations on the technical capabilities to measure certain unique properties. Owing to the biological complexity of this liver cell product, it was felt that one assay is insufficient to measure the product attribute(s) that indicates potency, as defined by the specific ability to effect a given result. As such, an alternative approach was used to develop multiple complementary assays that measure different product characteristics (i.e., ureagenesis, metabolic clearance

and enzymatic activity) associated with quality, consistency and stability. Predicting how well the transplanted cells will rescue a failing liver may be inferred using a two-step process. First, *in vitro* potency values, as described above, may be correlated with engraftment, survival and proliferation in animals following intrasplenic injection. In this way, minimums for ureogenesis, 7-EC clearance, UDP-glucuronosyltransferase and sulfotransferase activities may be established and used to assess the probability of the transplanted cells taking up residence in the host liver. Since residency is deemed crucial for reconstitution of function in the diseased liver, this *in vitro* potency data can then be correlated to efficacy assessments in human patients, which include standard liver function and coagulation tests. In this way, clinical study results may be used to establish correlations between the product's clinical efficacy and potency measurements, which can be used for subsequent product lot release, stability and/or comparability studies (Table 5).

Case study: matrix-based approach to potency assay development. Perspectives from SRC – a current, Phase I product candidate

SRC is a primary renal tubular epithelial cell-based therapeutic intended to supplement renal function in

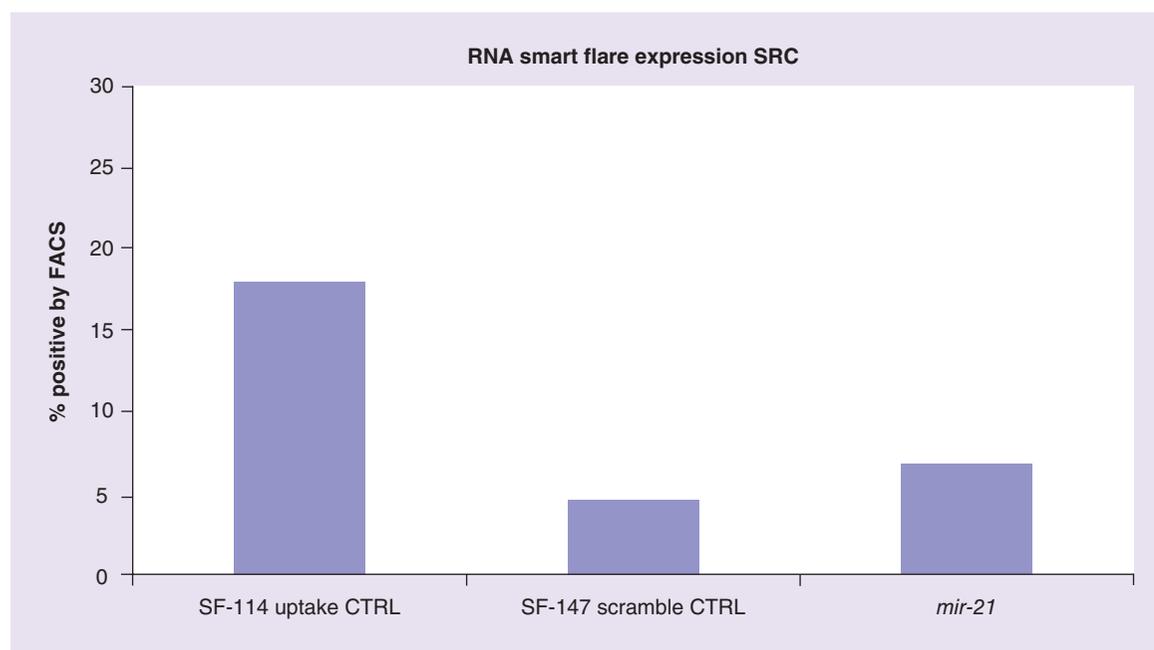


Figure 4. Smartflare™ expression of *mir-21* in living selected renal cells. *mir-21* is a miRNA potentially involved in antagonizing the NF- κ B signaling pathway. Evaluation of *mir-21* expression levels may be leveraged as an indicator of cellular functionality linked to therapeutic mechanism of action. Left bar: positive control for probe uptake; center bar: negative control for probe uptake; right bar: SRC product sample. Y-axis indicates the percentage of cells positive for each labeled miRNA as observed by FACS. CTRL: Control; SRC: Selected renal cell.

patients with end-stage renal disease [14,15,21]. At time of publication, SRC is in Phase I clinical trials in Europe and USA with limited characterization and mechanistic data available. As outlined previously, ‘potency testing/fitness for use’ for SRC is currently established through a combination of the presence of a defined concentration of viable renal cells and demonstration of cellular activity (secretion of VEGF) in the final product. This assay is unlikely to be acceptable at subsequent stages of product development (Phase II/III). With this in mind, this case study presents a ‘real-world’ example of a potency bioassay matrix currently under active development. As such, most assays have been established only as proof-of-concept, with reference standards and acceptance/rejection criteria yet to be determined. This is nonetheless reasonable for an early phase product candidate. Development of these potency bioassays has been guided by the following general principles:

- A known or potential MOA for the product candidate should be leveraged wherever possible;
- Assays should clearly discriminate subpotent and unrelated materials away from the product candidate;
- All assays should be quantifiable wherever possible, use only small amounts of the product and must be

completed before product release or before patient implantation (ideally within 24 h postformulation).

FDA recommends construction of an assay matrix, with multiple, parallel functional assays leveraging known or possible MOA(s) as part of an overall policy

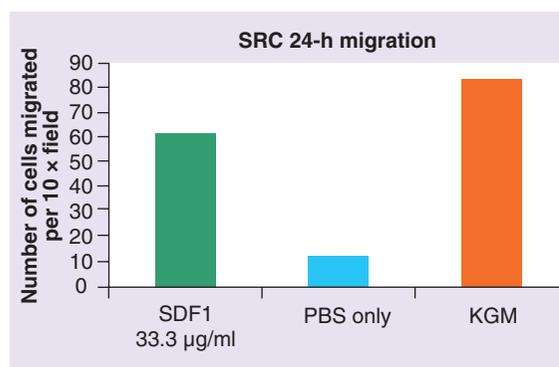


Figure 5. Migration of selected renal cell through a hydrogel barrier in response to SDF1 over a set time window (24 h). The SDF1/CXCR4 signaling axis is known to be involved in mediating cellular migration across an endothelial barrier (see [30] for additional details). Left bar: experimental; center bar: negative control; right bar: positive control (KGM). Y-axis represents the number of cells observed to be migrating in a 10x microscope field. KGM: Keratinocyte growth medium; PBS: Phosphate-buffered saline; SRC: Selected renal cell.

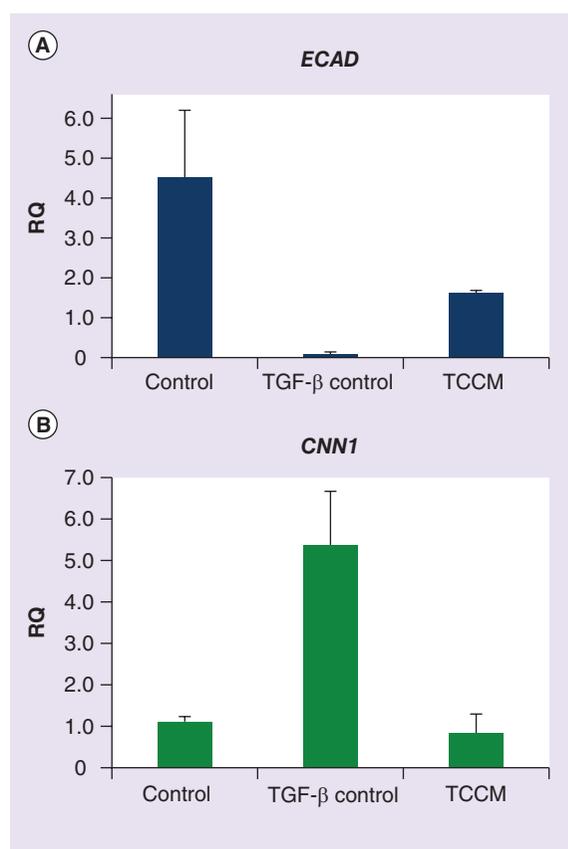


Figure 6. Epithelial–mesenchymal transition is an established mechanism for induction of renal disease.

The ability of a therapeutic product candidate to ameliorate disease-associated fibrosis may be measured by its ability to interfere with onset of epithelial–mesenchymal transition (EMT) [15]. A PCR-based assay monitoring expression of epithelial and mesenchymal transcripts may be used as functional index of product potency [15]. Quantitative real-time PCR analysis of (A) *ECAD* and (B) *CNN1* expression in HK2. HK2 cells present an epithelial phenotype, characterized by robust expression of *ECAD* and minimal expression of *CNN1* (left bars). Treatment with TGF- β (10 ng/ml) reverses this expression pattern (center bar), representing an *in vitro* example of EMT. This EMT event can be reversed in part by treatment with conditioned media derived from the product (right bar), providing evidence of product potency related to a therapeutic mechanism of action. The relative expression (compared to an arbitrarily selected house-keeping transcript) levels (RQ) of the RNA are shown on the y-axis. TCCM: Tissue culture-derived conditioned media.

of iterative, progressive, potency assay development. The expectation is that one or two lead assays will eventually emerge to become the principal potency assay candidates as incrementally more data on product characterization and MOA are accumulated as the product candidate progresses through later phases of the product development cycle. Examples of bioassays currently under active development for SRC potency are presented below.

Example 1

- **MOA:** Induction of new kidney tubules contributes to regeneration of tubular functionality in chronic diseased kidney.
- **Assay:** Spontaneous autoassembly of product-sourced cell constituent into organoids and tube-like elements following culture in 3D hydrogels.
- **Developmental phase:** Preclinical.
- **Methods and illustrative results:** An important functional property of the SRC cell population is the ability to self-assemble into discrete 3D cellular composites, including organoids, spheroids and tubule-like elements within 24 h of growth within collagen hydrogels. These self-assembled aggregates strongly express key functional markers associated with tubular epithelial cells, such as aquaporin 1 (green) and cytokeratins (red) shown below (left). The induction of organoids and tubule-like elements can be quantifiably indexed as demonstrated in Figure 2.

Additional requirements for development as potency bioassay: Reference standards and quantitative acceptance/rejection criteria are currently being defined.

Example 2

- **MOA:** Paracrine action-at-a-distance includes secretion of VEGF and KIM1 regenerative growth factors and miRNAs such as *mir-21*.
- **Assay:** qRT-PCR/FACs quantitation of *mir-21* generation by the cell population.
- **Developmental phase:** Preclinical.
- **Methods and illustrative results:** Expression of VEGF and KIM1 regenerative growth factors is the current potency bioassay for release of SRC. However, there is increasing evidence that miRNAs can mediate intercell signaling via integration within microvesicles/exosomes and epitomize a putative MOA that may explain the ‘action-at-a-distance’ element of numerous cellular biologics, including SRC [22]. We have made the following observations on the potential role of miRNAs in mediating SRC therapeutic bioactivity:
 - *In vivo* evidence of MOA: The NF- κ B signaling network is established to be increasingly and systematically activated within the rat CKD models generated by 5/6 nephrectomy. Intrarenal injection of SRC has been previously shown to decrease NF- κ B nuclear localization within these preclinical small animal models.

- *In vitro* bioassay recapitulating *in vivo* mechanistic pathway: TNF- α -induced NF- κ B stimulation of the proximal tubule-sourced cell type HK2 can be attenuated by conditioned media from SRC.
- Development of *mir-21* as a surrogate potency biomarker: Upon analyzing the miRNA contained within exosomes sourced from SRC generated conditioned media by ultracentrifugation, PCR arrays of established miRNA elements were able to identify several miRNA species that are putative antagonists of NF- κ B signaling. This includes *mir-21*. As such, it has been put forth that *mir-21* is a potential surrogate potency biomarker for cell-sourced bioactivity secondary to amelioration of NF- κ B signaling [BRUCE A ET AL. SELECTED REGENERATIVE RENAL CELLS MODULATE DISEASE PROGRESSION IN RODENT MODELS OF CHRONIC KIDNEY DISEASE THROUGH ATTENUATION OF PRO-INFLAMMATORY NF- κ B PATHWAY AND ENHANCEMENT OF TUBULAR CELL PROLIFERATION (2014), SUBMITTED] [23].

mir-21 expression may be evaluated by qRT-PCR methods as in the following example establishing consistency of expression between two SRC samples, HK26 and HK38, grown in control and bovine pituitary extract-free media (Figure 3). Real-time quantification of *mir-21* expression can be achieved in living cells by application of Smartflare™ probes [24]. Application of the Smartflare system allows miRNAs under development as potency surrogate biomarkers to be reliably quantified within hours of product formulation as shown (Figure 4).

Additional requirements for development as potency bioassay: Reference standards and quantitative acceptance/rejection criteria are currently being defined for *mir-21*. The suitability of other miRNA candidates involved in NF- κ B signaling for development as potency surrogates is also being examined.

Example 3

- **MOA:** Host-derived progenitor cell migration and mobilization along the SDF1–CXCR4 axis.
- **Assay:** Migration of labeled product-derived cell sample within a defined time frame through a barrier of hydrogel in response to SDF1.
- **Methods and illustrative results:** The SDF1/CXCR4 axis is a well-defined and understood mechanism for recruitment and relocation of host-sourced stem cells [25]. Movement of fluorescently-labeled SRC within a pre-established period of time across a hydrogel membrane toward a gradient of SDF1 has been established as a measure of the potency of SRC, as shown in Figure 5.

Additional requirements for development as potency bioassay: Dose–response curves correlating number of product-derived cells migrating through the hydrogel barrier relative to concentrations of SDF1 will be used to define reference standards and acceptance/rejection criteria.

SDF1 exposure and concomitant upregulation of CXCR4 in the product: Expression of CXCR4 in response to SDF1 is also quantifiable, subject to dose–response optimization and may be further developed as a separate potency bioassay (data not shown).

Example 4

- **MOA:** Fibrosis modulation by interference with epithelial–mesenchymal transition (EMT) in diseased kidney.

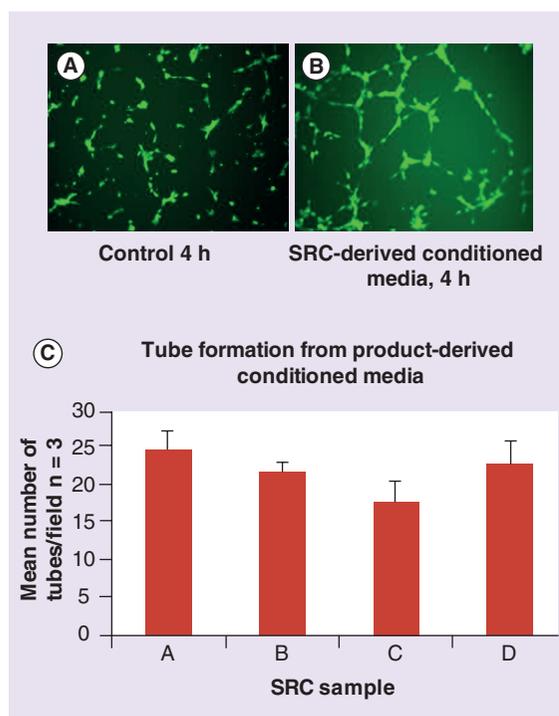


Figure 7. Human umbilical vein endothelial cells growing in hydrogel cultures will self-organize into 2D tube-like structures in response to trophic factors [16]. Therefore, this property may be leveraged as a measure of the potency of a product candidate. ‘Control’ refers to human umbilical vein endothelial cells (HUVECs) treated with unconditioned media. Control HUVECs fail to effectively assemble 2D tubules when compared to HUVECs exposed to SRC-derived conditioned media over a 4-h time period. **(A & B)** Self-organization of HUVECs into tubular structures in response to proangiogenic factors in SRC-derived conditioned media. **(C)** Quantitation of tube formation. SRC: Selected renal cell.

- **Assay:** Reduction of TGF- β -induced EMT in HK2 cells, as quantified through analysis of *ECAD* (E-cadherin) and *CNN1* (calponin) by qRT-PCR.
- **Methods and illustrative results:** The progression of tubulointerstitial fibrosis as CKD develops is linked with TGF- β -mediated EMT of epithelial tubular cell populations [26]. Also, a reduction in TGF- β pathways was observed in an *in vivo* preclinical rat model of developing CKD where animal survival was prolonged and the renal functionality enhanced through application of SRC [27]. The HK2 human proximal tubular cell line is a well-established *in vitro* model system to evaluate the modulatory properties of biologicals on TGF- β -generated EMT [28]. TGF- β -induced EMT in HK2 cells is examined by quantitating the relative expression of *ECAD* (epithelial) and *CNN1* (mesenchymal) markers. As shown in Figure 6, SRC-derived conditioned media (TCCM) is observed to reverse the TGF- β -induced upregulation of *CNN1* expression and downregulation of *ECAD* expression.

Additional requirements for development as potency bioassay: Reference standards and quantitative acceptance/rejection criteria are currently being defined.

Example 5

- **MOA:** *De novo* angiogenesis.
- **Assay:** HUVEC (endothelial cells) self-organization into 2D tube-like elements upon exposure to conditioned media sourced from the product.
- **Methods and illustrative results:** Product may act partly by facilitating the *de novo* assembly of blood vessels through proangiogenic factor secretion (VEGF). Although absolute amounts of VEGF in the extracellular milieu may be evaluated as an element of product cell character through ELISA or related assays, such measures do not discriminate between functional and nonfunctional variants of secreted VEGF. A HUVEC-based assay has been leveraged to evaluate expression of functional VEGF secreted by SRC as an indicator of SRC cell functionality, bioactivity and potency. In this assay, tubular network organization from HUVEC in 2D culture upon application of SRC-derived conditioned media can be applied as an appropriate cellular assay for functional VEGF generated by SRC as shown in Figure 7.

Additional requirements for development as potency bioassay: Reference standards and

quantitative acceptance/rejection criteria are currently being defined. Data from the angiogenesis assay may be generated within 3–5 h of assay initiation. Note that amounts of VEGF in the conditioned media measured through ELISA can be used as a surrogate potency marker for the angiogenesis bioassay.

Future perspective

Functionally relevant potency bioassay development is contingent upon clear and unambiguous definition of product's MOA. To date, our understanding of how RM product candidates catalyze regenerative outcomes remains largely speculative. Paracrine 'action-at-a-distance' modalities may well be relevant and form the basis of the current generation of potency bioassays, which leverage secretion and bioactivity of salient cytokines and growth factors such as VEGF and HGF. Nevertheless, to definitively establish a causal relationship, the product must be modified to establish a genetic null mutant for that particular growth factor. This of course leads to the requirement to demonstrate bioequivalence between the modified and the original product candidate, only possible through an expensive and time-consuming series of *in vivo* preclinical studies. Escape from this predicament will be made possible by evaluating multiple candidate products with a series of well-defined and carefully controlled characterization studies leveraging secretomic, proteomic, transcriptomic and other '-omic' platforms to demonstrate that genetic modification of the product candidate does not significantly impact its biologic properties. Keep in mind that what will be acceptable for novel RM biotherapeutics will also impact biosimilars, which are biological products similar to an already approved biological product for which there is no clinically meaningful differences between the two in terms of the safety, purity and potency [29]. Through a new approval pathway, such biosimilars will cite a biological product that is already approved by the FDA as a reference product.

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Executive summary**Cell count & product viability**

- Cell viability and number alone are not adequate measurements of the potency of regenerative medicine products, but must be incorporated into the final index to enable potency to be quantitatively expressed.

Potency

- The definition of potency for cell-based therapeutic products is as follows: (Functional bioactivity of product candidate/unit viable cell)/(Functional bioactivity of reference standard/unit viable cell).

If possible, a potency assay should be based on an established feature of the product's mechanism of action, as demonstrated via *in vivo* experimentation

- Ideally, the biological assay(s) used to measure product potency should be based on the established mechanism of action of the product or some other biological activity associated with clinical outcomes.

Development of a systematic battery of parallel functional assays that, taken together, can address all potential mechanism(s) of action believed to be relevant for the product platform is recommended

- US FDA recommends implementation of a sequential, matrix-based approach to potency assay development directly linked to phase of product development.

Case studies

- Incara's cryopreserved human hepatocytes and Tengion's selected renal cells are illustrative examples from a historical and current perspective on how potency bioassays should be developed.

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