STEM CELL
applications and opportunities in drug discovery

The high attrition rate of late stage drug candidates has led the pharmaceutical and biotech industries to a reassess the *in vitro* models used to select and validate drug targets and subsequent lead molecules. Traditional cell lines from animals, tumours, or genetic transformation have been the industry's workhorses over the years, yet efficacy data from testing drug candidates in these 'abnormal' cells have significantly contributed to a lack of translation to the clinic. Human stem cells with their potential to differentiate *in vitro* into various specialised cell types have become a new and important tool for drug discovery partners. As stem cell are deployed into this 'new age' of drug discovery, many practical considerations are considered to develop reproducible assays that will replace those using traditional cell lines and significantly improve the level of confidence pre-clinically to increase the rate of future success in the clinic.

By Dr Mark A. Thiede

Global drug discovery partners including academia and biotechnology and pharmaceutical industries envision the 'stem cell' as leading a metamorphosis of how cell-based assays are used to improve the level of success in the discovery of new 'cellular' and molecular drugs to serve unmet medical needs. Stem cells have utility as therapeutics as chronicled in the decades of successful hematopoietic stem cell transplants and the recent approval of mesenchymal stem cells for the treatment of graft vs host disease (GVHD). While cell therapies using multi-potent adult stem cell have shown success, their application to human disease is limited due to a restricted differentiation potential. The greatest therapeutic potential lies in the 'pluripotent' human embryonic stem cell (hESCs), which has the potential to create all cells of the body, and therefore be used to treat complex medical challenges such as spinal cord injury, the loss of pancreatic beta cells (diabetes), neurons (Alzheimer's) and cardiac tissue due to infarct. The recent demonstration that human somatic cells such as skin fibroblast could be reprogrammed into 'embryonic stem cell-like' induced pluripotent stem cells (iPSCs) is likely to revolutionise how we exploit broad genetic backgrounds of pluripotent stem cells to create scalable iPSCs cells from patients to link human disease to drug discovery and possibly cellular therapy. Researchers in the growing field of regenerative medicine are exploring pathways to pharmacologically target stem cells *in situ* to augment their regenerative potential. As we await breakthroughs that will bring hESC or hiPSCs to the routine drug discovery screening laboratory, available multipotent adult stem cells are being used to create

human cell types for novel assay development with the hope that they will help improve the future rate of success.

Over the past decade, the lack of pharmacology and safety in late stage drug candidates has plagued the industry and this has called for creating better *in vitro* (and *in vivo*) models to reduce attrition. Modern pharmaceutical research widely deploys tumour-derived or engineered 'immortalised' cells from human and animal sources to screen compounds, facilitate SAR and advance lead molecules through the pipeline. Such cell lines bring a high value in scalability and convenience, while providing relative confidence in the pharmacology and safety of lead compounds. Major advantages of immortalised cells is their uniformity and expandability so cell banks can be created to complete screens of large compound libraries (**Table 1**). However, these 'abnormal' cell models may in part be to blame for the high rate of late stage portfolio attrition throughout the industry. Cultures of specialised primary cells such as human umbilical endothelial cells (HUVC), keratinocytes and hepatocytes are used in drug discovery but restricted expandability limits the use of one cell lot in high throughput screens. Stem cells provide a 'normal' human cell model which we hope will increase confidence in drug targets and pharmacology (**Table 1**). This article will focus on the applications and technical challenges of turning human adult stem cells into diverse cellular tools in drug discovery.

Adult stem cells

Cultures of tumour cells or cells immortalised through genetic manipulation have provided a readily expandable, hardy and reproducible cell source for broad assay development. Like immortalised cells, stem cells applied to drug discovery need to retain their phenotype after culture expansion to minimise variability while meeting the cell needs of large scale screens. Human adult stem cells can be isolated from a spectrum of human tissues (**Table 2**), expanded in culture and retain the ability to differentiate into a number of important specialised cell types. Not all adult stem cells are created equal. Stem cells isolated from tissues such as liver, adipose and muscle possess a relatively limited ability to expand and when expanded lose their potential to differentiation.

Two of the best characterised and exploited adult stem cell preparations are the CD34+ hematopoietic stem cell (HSC) and mesenchymal stem cell (MSC) isolated from human bone marrow. Using specific cytokine cocktails and culture conditions, HSCs can be differentiated into all types of blood cells. However, undifferentiated HSCs expand poorly *in vitro* limiting their use of HSCs in large scale screens. Bone marrow-derived mesenchymal stem cells (BM-MSCs) are multipotent cells isolated from wide spectrum tissues including adipose, umbilical cord, lung, heart, brain muscle and other tissues. BM-MSCs are readily expanded *in vitro* and can be differentiated into adipocytes, osteocytes, chondrocytes and to a less extent muscle cells and neurons; cells types relevant to discovery programmes in osteoporosis, obesity, diabetes, metabolic disorders and joint disease. Therefore, BM-MSCs are an attractive stem cell platform with which to develop screening assays for new target and drug candidate discovery.

MSCs have been isolated from the stromal vascular fraction of different tissues such as fat and such cells may differentiate into cells that are more reflective of the tissue of origin. Since the expansion of such MSC cultures are more restricted they are best suited for secondary screens. MSC isolated from the 'Wharton jelly' of human umbilical cords demonstrate a differentiation potential similar to BM-MSCs and a greater potential to expand *in vitro*, and may become the choice of sources of MSCs for larger scale needs.

BM-MSCs obtained from healthy adults purchased as frozen aliquots of early passage (passage 2) cells are widely used to study adipogenesis,

CELL TYPE	GENOTYPE	GROWTH IN CULTURE	PHYSIOLOGICAL RESPONSE	SCALABILITY	CELL DIVERSITY	VARIABILITY
Tumour or transformed cell line	Abnormal	Abnormal	Abnormal	Essentially Limitless	Tissue type restricted	Change over time
Stem cells	Normal	Normal	Uniform	ESC>MSC> Tissue-specific	Unrestricted	Uniform

Table 1

Table 2

ADULT STEM CELL	TISSUE	SCALABILITY	IVD
Embryonic stem cells	Blastocytes	Unlimited	++++++
Induced Pluripotent cells (iPSCs)	Reprogrammed somatic cell	Unlimited	++++++
Hematopoietic stem cells (HSCs)	Bone marrow Blood following cytokine (ie G-CSF) treatment Umbilical cord blood	Limited	++++
Mesenchymal stem cell (MSCs)	Bone marrow	Good	++
Multipotent adult progenitor cells (MAPC)	Bone marrow, brain	Fair	++++
Amnion stem cell	Amnion membrane	Good	+++
Umbilical cord stem cell	Wharton jelly	Very good	+++
Neural stem cell	Brain	Limited	+
Cardiac muscle stem cell	Heart	Limited	+
Skeletal muscle stem cell	Muscle biopsy	Limited	++
Liver stem cell	Liver	Limited	+
Adipose stem cell	Adipose	Fair	+++
Pancreas progenitor	Pancreatic ducts	Limited	+
Skin	Hair follicle	Good	++
Epithelial stem cell	Intestinal crypts	Good	+

osteogenesis and chondrogenesis. Inherent lot to lot variability in the proliferation and differentiation potential of BM-MSCs necessitates initial screening to identify lots that differentiate along at least three different lineages. Prior to initiating an IVD screen, selected lots of BM-MSCs are expanded to the desired passage and cryopreserved to create a cell bank that can match the cell numbers and consistency of the traditional cell lines established for screening.

Scalability for IVD

BM-MSCs can undergo 50 doublings without complete loss of differentiation potential, however in practice the differentiation potential significantly decreases as cells undergo extensive proliferation (**Figure 1**). In the model shown in **Figure 2**, one million BM-MSCs at passage 2 can be expanded ~200-fold after four additional passages to generate enough cultures in 96-well plates to test up to 500,000 compounds in duplicate. In practice this model has produced >300 million passage 5 BM-MSCs from ~15 million passage 2 cells. To initiate an IVD screen, cryopreserved BM-MSCs are recovered through one passage and assayed in cells at passage 7 when the cells retain robust differentiation potential.

BM-MSCs offer a valuable new tool to explore molecular and cellular regulation of progenitor proliferation, commitment and eventual specialisation. During the past decades, scientists have created differentiation cocktails composed of critical factors needed to induce lineage-specific differentiation. One well-defined IVD system is reflected in the complex cytokines/growth factor combinations used to differentiate HSCs along pathways to the different types of blood cells. BM-MSCs are quite amenable to IVD as well-established growth and differentiation media are available commercially. Some lots of hMSCs double

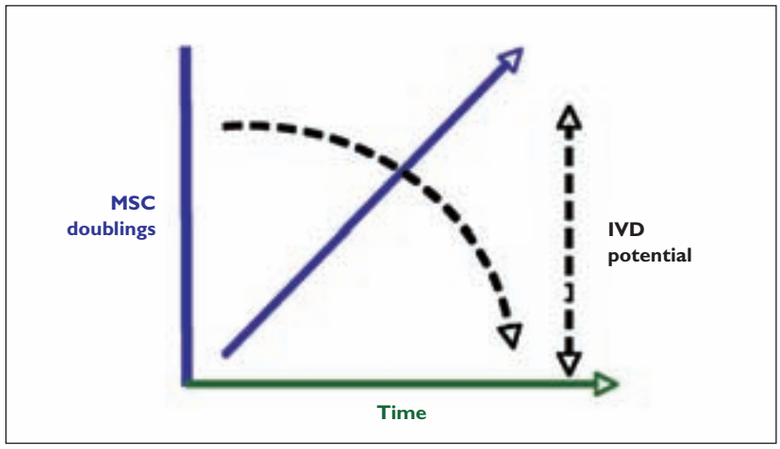
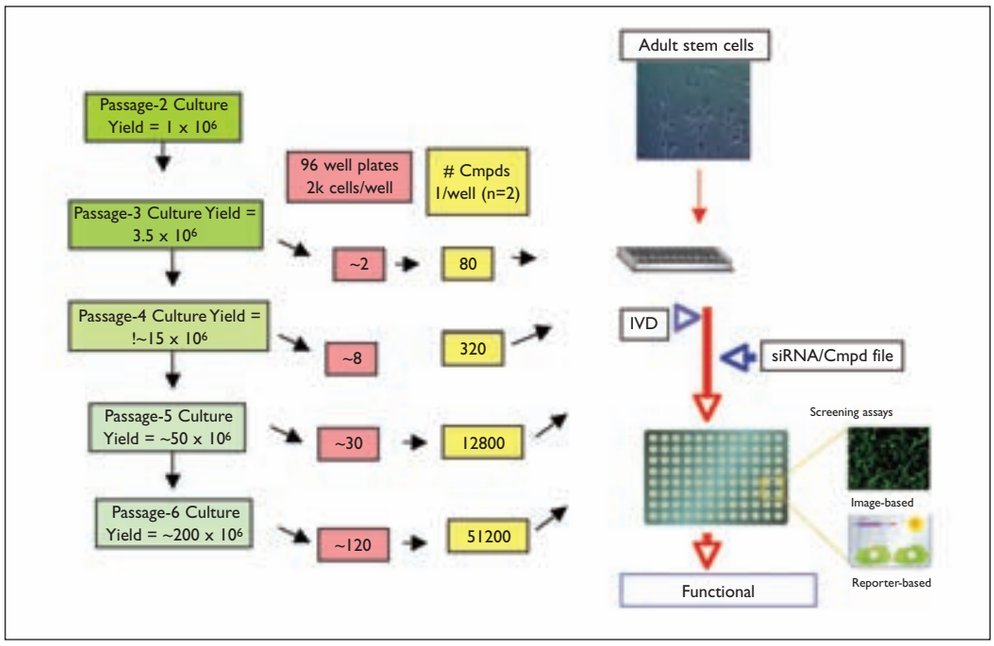


Figure 1
Relationship between proliferation and *in vitro* differentiation potential of MSCs

more rapidly than others or show a bias for differentiation along one of three pathways. While IVD of BM-MSCs can be predictable, like most primary cell culture, there is an inherent donor-to-donor variability in the proliferations and differentiation of the cells. Therefore, MSCs lots must be screened to identify those with the propensity to deliver the desired cell profile. The biological reason or relevance for the measurable difference in biological response among MSC lots remains unclear, but it can greatly influence how MSC will be used for IVD screens. Biological diversity of stem cells obtained from individuals with broad age, ethnic and health backgrounds and the researcher's ability to model disease states on normal cells *in vitro* provide unique ability to use stem cell IVD to screen compound or gene silencing libraries in disease relevant assays.

The IVD of stem cells is a gradual process that generally takes 2-3 weeks to produce a mature cell culture following addition of an 'induction cocktail'. BM-MSCs propagate in monolayer culture, however the efficient induction of some pathways such as cartilage formation requires the cells to be in a three-dimensional configuration as either a cell pellet or embedded in alginate gel. Such assays require very large numbers of BM-MSCs to execute. Significant donor-to-donor variability in BM-MCS may lead to cell lots that possess a bias for one lineage over the others. This can either preclude its use in broad pathway screens or provide a cell lot rich in a desired progenitor uniquely based on the need. MSCs under IVD conditions produce 'mixed' cell cultures composed of the desired specialised cells and an underlying population of early progenitors and a 'stroma' composed of undefined fibroblastic cells. After adipogenic differentiation, approximately 50% of the nucleated are mature lipid laden adipocytes. Current protocols for inducing stem cells along lineages such as neurons and cardiomyocytes remain relatively inefficient in producing the desired cell type and can produce only small numbers of the specialised cells. This creates unique technical challenges for establishing reproducible screening tools from such cell types. It is feasible to isolate the specialised cells from the culture but physical trauma associated with the cell

Figure 2
MSC expansion and deployment in IVD assay



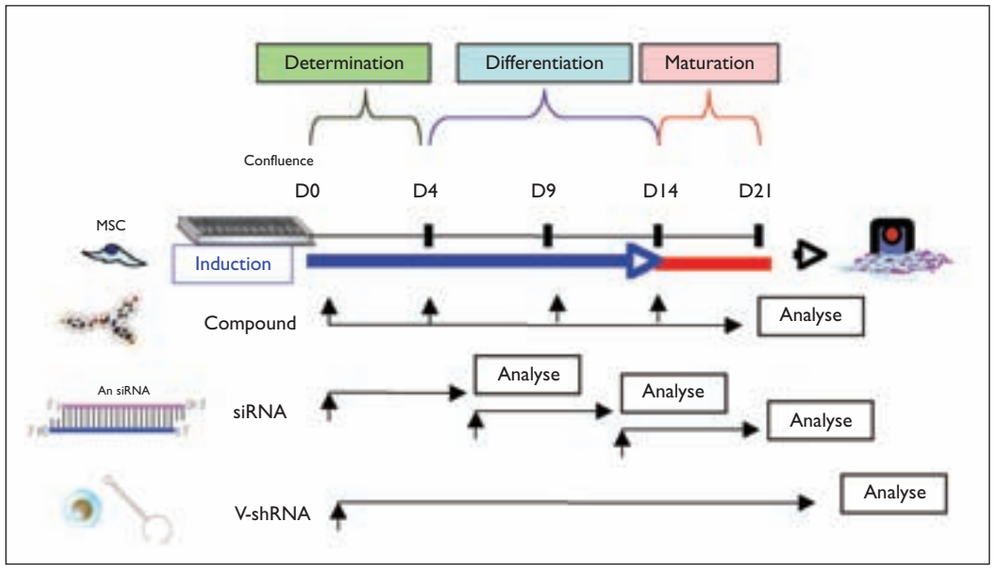


Figure 3
Target and drug discovery strategies during *in vitro* differentiation of MSCs

recovery is likely to compromise cell integrity and subsequent functional readouts. A mixed cell IVD culture may be desired as it provides cell-to-cell contact that may best reflect tissue milieu. The assay readouts from the IVD need to originate from the differentiating/differentiated cells in order to minimise contribution of the 'stroma' to the biological response.

IVD of stem cells is being deployed to identify druggable gene targets (ie receptors, enzymes, etc) critical to cell proliferation, differentiation and function. The IVD system is amenable to target inactivation by RNA interference (RNAi) and the use of microRNA has become the current research tool of choice for studying the effect of gene silencing on biological processes. RNAi via selective degradation of target mRNA (and subsequent protein expression) is accomplished by exposing cells to either a pool of synthetic siRNAs or virus-delivered short hairpin RNA (shRNA) against a specific mRNA species. A preliminary knowledge of gene expression patterns during the 2-3 week IVD period is an invaluable roadmap for efficient RNAi screening. Patterns of gene expression provide key information to study the effect of gene silencing or small molecule pathway inhibitors on the cell fate and function and gene expression patterns can facilitate selection of gene silencer panel and pathway inhibitors to use in a screen. During IVD, cell processes such as lipid accumulation or mineralisation are changing over days and exposures may require the siRNA or compound to be added over several media changes (**Figure 3**). The delivery of synthetic siRNA pools to MSCs is most efficient in pre-confluent undifferentiated cultures, and under IVD conditions these molecules remain with the cell for up to seven days and can identify genes involved in differentiation or early cell maturation. Viral delivery of shRNA to the MSC offers continuous exposure to RNAi. Large scale IVD screens with vast numbers of siRNA or shRNA are poorly monitored for the overall efficiency of uptake and action on target from culture to culture. For instance, screening a library of more than 1,000 pools of synthetic siRNAs against druggable targets requires considerable faith in the delivery system and the action of the siRNA once in the cells as there is no clear means to monitor efficiency of siRNA delivery or shRNA expression or the subsequent action (degradation) on target mRNA or the depletion of target protein during the time course of exposure. Efficient uptake of synthetic siRNA by stem cells or committed progenitors requires a liposome or nanoparticle based carrier, and pilot studies with a fluorescent siRNA, show the fluorescence to be retained in the cell for up to seven days. This short half-life allows examination of the impact of siRNA during specific timeframes of IVD. Unfortunately, the efficient delivery of siRNAs to specialised cells such as adipocytes to explore impact on adipocyte function remains a technical challenge that can perhaps be overcome with virus mediated delivery of shRNA to MSCs. While the relatively high costs of virus-mediated shRNA delivery is significantly higher on a per gene basis than pools of synthetic siRNA, the potential longer duration of action allows for gene silencing throughout the IVD timeframe.

Readouts

As with immortalised cell-based assays, the stem cell-based IVD assays bring considerable value in the drug discovery process, but this value is only as good as the dependability and relevance of the readout to the programme goal. Early commitment to lineage differentiation (ie reduce pre-adipocyte formation is measured by via expression of FABP4 mRNA (Q-PCR) or protein (Immuno-Fluorescence in cell arrays) within 4-7 days of adding induction media. Lipid accumulation by maturing adipocytes is measured fluorometrically following staining of the cultures between days 14 and 21 with the neutral lipid Nile Red. Programmes in obesity and diabetes now apply cultures of mature human adipocytes to measure insulin signalling, glucose uptake, lipolysis and adipokine release. Alkaline Phosphatase (ALP), an early marker of osteogenesis is measured on day seven while on day 16 mineral deposited in the culture can be extracted and calcium measured colorimetrically or the cell layer can be stained and quantified by high content array analysis. MSC-based pellet or micromas cultures are a useful surrogate of cartilage formation, which can be used to identify modulators of early chondrogenic differentiation. Cartilage formation is of great interest for the repair of cartilage defects and treatment of osteoarthritis and can be measured in a three-dimensional 'pellet' culture by measuring SOX-9 expression or the release of MIA/CD-RAP into the media between after 7-21 days. Cartilage formation is confirmed histochemically by measuring proteoglycan or collagen type 2 in sections of pellets after day 21 in pellet culture. The success of IVD in target and drug candidate discovery is highly dependent on the ability of the stem cell and the IVD method to produce reproducibility and robustness of the desired cell populations.

Conclusions

Over the years, cell lines have become invaluable tools to drug/target discovery and the follow up validation process. However, a general lack of translation of the pharmacology in these cells within the clinic setting has played a significant role in the high level of failure of late stage drug candidates. The value of IVD as a target or drug discovery tool is greatly dependent upon cell quality, the reproducibility of the delivery system and the sensitivity and relevance of the analytical readouts. The hope of stem cells is to provide unique 'normal' human cell types that will increase target and pathway relevance to disease process earlier in the costly drug discovery and development process to improve our chance of success. Target discovery programmes have an array of new tools to apply to the cell culture, including gene silencing technologies and a growing library of selective chemical pathway inhibitors to impact prosecution of druggable targets (ie enzymes, receptors). The true power of IVD of human stem cells in drug discovery lies in the quality and diversity of available stem cell cultures, the breadth of the IVD conditions to create human target cells and disease-relevant assay readouts. To impact the drug discovery process it is up to the researcher to develop strong molecular tools and bring these elements together to identify and confirm new drug targets and generate pharmacology packages with strong translation to the clinical setting. DDW

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