Biological Characterization of a Human Mesenchymal Stromal Cell Product

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Thesis structure and declaration

Thesis Title: Biological Characterisation of a hMSC product.

This thesis begins with a summary and then a comprehensive introduction. There are three distinct results chapters, each written with a short introduction, methods, results, discussion and conclusion. In **Chapter 1** we provide a guidance framework for investigators planning to submit investigational medicinal product application to support the human use of their stem cell product.

Chapter 2 characterizes the preclinical toxicology profile of a human mesenchymal stem cell (hMSC) product in support of its use for the treatment of critical limb ischaemia (CLI) patients.

In **Chapter 3,** we investigated the biodistrbutive fate of good manufacturing practice (GMP) grade mesenchymal stromal cells (MSCs) in immunodeficient mice.

In **Chapter 4,** we investigated the use of a series of assays to measure the bioactivity of our hMSC product. The overall aim of this study was to develop functional assays to characterize the angiogenic potential of our MSCs.

Finally, in **Chapter 5** we summarize each chapter of this thesis.

I, **Michael Creane**, declare that I have not obtained a previous qualification from NUI, Galway or elsewhere based upon any of the work contained in this thesis. I both conducted the experiments presented and wrote the thesis under the supervision of Professor Timothy O'Brien. There are a few exceptions clearly indicated at the beginning of each chapter in the 'contributors' section.

Abbreviations

ADME	Absorption, distribution, metabolism and excretion
Alb	Albumin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
Ang-2	Angiopoetin
ASPA	Animal scientific procedures act
ATMP	Advanced therapy medicinal product
Baso	Basophils
BM	Bone marrow
BSA	Bovine serum albumin
Ca	Calcium
CL	Chloride
CHOL	Cholesterol
CLI	Critical limb ischaemia
CM	Conditioned medium
Crea	Creatinine
CPK	Creatine phosphokinase
CT	Cell therapy
CTP	Cellular therapy product
DAB	Diaminobenzidine
DAPI	4’6-diamidino-2-phenylindole
DIl	1,1’Dioctadecyl-3,3,3’3’-tetramethylindocarbocyanine perchlorate
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco’s phosphate buffered saline
EBM	Endothelial basal media
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGM	Endothelial growth media
ELISA	Enzyme-linked immunosorbent assay
EMA	European medicines agency
Eos	Eosinophils
EtOH	Ethanol

EUDRA CT	European Union drug regulating authorities clinical trials
FBS	Foetal bovine serum
FDA	Food & Drug administration
FGF	Fibroblast growth factor
FGF-2	Fibroblast growth factor-2
FIH	First in human
FISH	Fluorescent in situ hybridization
GFP	Green fluorescence protein
GLP	Good laboratory practice
GLU	Glucose
GMP	Good manufacture practice
H&E	Hematoxylin and Eosin
hDNA	Human DNA
Hb	Haemoglobin
Hct	Haematocrit
hMSCs	Human mesenchymal stromal cells
HPRA	Health products regulatory authority
HUVECs	Human umbilical vein endothelial cells
IB	Investigator brochure
IGFBP-2	Insulin-like growth factor binding protein-2
IGFBP-3	Insulin-like growth factor binding protein-3
Il-8	Interleukin-8
IM	Intramuscular
IMP	Investigational medicinal product
IMPD	Investigational medicinal product dossier
IND	Investigational new drug
ISCT	International society for cellular therapy
IUR	Isotropic uniform random
IVC	Individually ventilated cages
K	Potassium
Ki-67	Protein; cellular marker for proliferation
LDH	Lactate dehydrogenase
LDI	Laser doppler imaging
LUC	Large unstained cells
MAPCs	Multipotent adult progenitor cells

MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
mDNA	Mouse DNA
MMPs	Matrix metallopreteinase
MNCs	Mononuclear cells
MOA	Mechanism of action
Mono	Monocytes
MPCs	Mesodermal progenitor cells
MRI	Magnetic resonance imaging
MSC	Mesenchymal stromal/stem cells
Na	Sodium
Neut	Neutrophils
Phos	Phosphorus
PlGF	Placental growth factor
Plt	Platelets
PK/PD	Pharmacokinetic/Pharmacodynamic
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cell count
ROA	Route of administration
RCF	Relative centrifugal force
Ret	Reticulocytes
SOP	Standard operating procedure
SD	Standard deviation
SPECT	Single-photon emission computed tomography
TG	Trigylcerides
TIMP-1	Tissue inhibitor of metalloproteinases
uPA	Urinary type plasminogen activator
VEGF-C	Vascular endothelial growth factor C
WBC	White blood cell count

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This thesis represents not only my most recent work in the lab and at my keyboard, it is a milestone of more than one decade at NUI, Galway. From undergraduate un-denominated Science, through to a Masters in Regenerative Medicine and finally to PhD research, it has been a journey akin to Paolo Coelho's 'The Alchemist'.

Every new pursuit requires entering uncharted territory and often it is easy to become overwhelmed by the many unknowns we face ahead of us. We are torn between our decisions and indecisions, constantly searching for answers to impossible questions. The irony is, by spending my days taking an external, microscopic view of the world – searching for answers relating to the very fabric of life and science – I have, unintentionally, been able to reflect internally where I have found some answers of my own. Both in my work, and in my personal life I have learned about how curious choices can lead to growth, change and evolution and how these are weaved into the fabric of our own personal reality. This understanding, I believe, has allowed me to become a better version of myself and I now see the world in terms of what I would like to see happen, and not what actually does. I've also come to understand that one can study, read, and listen for eternity but the full experience comes when one takes action – to have the confidence to stand up and make an impact. And, once you're done aiming, pull the trigger.

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Abstract

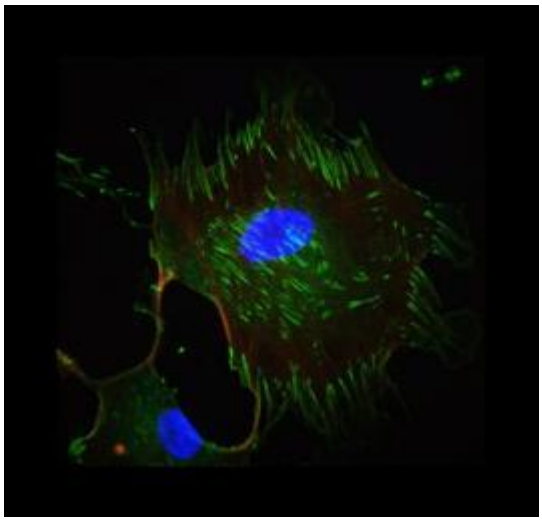
The translation of stem cell research into a therapeutic setting presents with it many scientific, logistic, financial and regulatory challenges for academic scientists. This thesis identifies some key areas that one must consider in the development of a cell therapy product intended for testing in humans. The data described herein formed the preclinical safety package of an investigator brochure that was submitted to the Health Products Regulatory Authority to support the clinical testing of a mesenchymal stromal cell product in 'no option for revascularization' critical limb ischaemia patients.

‘‘Whatever the mind of man
can conceive and believe,
it can achieve’’

Napoleon Hill

Chapter 1: Translating stem cell research to the clinic: A primer on translational considerations for your first stem cell protocol

ABSTRACT



Over the last two decades, a new therapeutic paradigm has emerged which has changed the way in which debilitating diseases may be treated in the future. Instead of using small molecule drugs and devices to help ameliorate the symptoms of disease, the therapeutic power of cells may be harnessed to help regenerate and cure diseases which currently represent major unmet medical needs.

Advancements in the scientific knowledge of stem cell biology, along with highly encouraging pre-clinical proof of concept studies in the last number of years has served as a launch pad for testing such therapeutics in humans with life threatening diseases. However, translating basic research findings into human therapy has not been straightforward and has presented many scientific, clinical and regulatory challenges for scientists and clinicians.

In this introduction we provide a guidance framework for investigators planning to submit an investigational medicinal product application to support the human use of their stem cell product. Important considerations are given to the design and planning of a meaningful preclinical safety program and a general overview of the required regulatory process is provided. Furthermore, important trial parameters and design features of the early phase clinical studies which must be considered before regulatory submission of such studies are highlighted.

Contributors to this work:

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INTRODUCTION

Stem cell therapy has enormous potential to alleviate human suffering and to provide solutions to conditions with current unmet medical needs. The number of clinical indications for use of these cells and the powerful therapeutic properties has produced a groundswell of interest by physicians around the world to translate scientific discoveries into patient benefit. Used as drugs, stem cells are required to follow the regulatory pathway of pharmaceuticals into the clinic. Using stem cells as drugs are often investigator initiated protocols and as such, investigators need to be aware of these regulatory pathways from the earliest stage of the translational process. Furthermore, the nature of cells as drugs is more complex and the translational pathway to development will require special considerations. The purpose of this review is to act as a primer for physicians that want their laboratory-based discoveries in stem cell therapy to be translated to clinical trials, to encourage investigators to consider the required regulatory steps from the earliest stage of the translational process, and to improve the efficiency of translation of these important discoveries.

REGULATORY AGENCIES AS GATE KEEPERS TO TRANSLATIONAL SCIENCE

No stem cell trial can proceed without first review and approval by regulatory authorities. Regulatory agencies and investigators share a similar goal – to bring safe, efficacious novel therapies to patients. These authorities provide critical, independent assessment of a protocol to determine if the protocol meets the requirements to reduce risk to patients. To maximize the efficiency of translating discoveries into practice, physicians and scientists must understand how regulatory agencies assess new applications. These agencies must assess the current evidence of the potential for safely testing a novel therapy to determine the risk regarding its use in humans. Safe use in humans takes into consideration the drug (its manufacture, purity, and potency), its route of administration, and the potential adverse effects in the environment of the disease to be treated. The inherent paradox in new drug development is the combination of the assessment of drug safety in the context where it has never been used. This paradox is managed by measuring the strength of the supporting data (pre-clinical data and related human clinical trials) in relation to the risk of potential harm (known or unknown) to the patient. It is reasonable (ethically and morally) to allow a greater risk to those patients with few therapeutic options and a poor prognosis. Understanding and mitigating this risk is the responsibility of the investigator during the application to regulatory agencies and effectively addressing the risk through proper pre-clinical studies and

identification of appropriate patient populations can catalyse approval of the protocol. For example, sequential patient enrolment where a cohort of patients is put on the trial and observed for adverse events before the next patient for a consecutive group prior to open enrolment can reduce the overall risk to patients in the protocol. Other risk mitigation strategies include careful pre-clinical studies that carefully mimic the clinical trial, and careful inclusion criteria that describe a patient population with as uniform a prognosis as possible.

GENERAL COMMENTS ON WHEN IT IS APPROPRIATE TO PROGRESS TO FIRST IN HUMAN STUDIES

There is controversy in the stem cell field concerning the amount of basic scientific knowledge required before clinical trials should occur (Davidson, 2010). While it is true that stem cell therapeutic mechanisms are unknown or hypothetical, many drugs currently in use for decades also lack detailed understanding of the mechanism of action. Investigators should consider the current knowledge concerning the mechanism of action, the alternative treatment options, and the severity of the underlying illness and the safety profile of the investigative drug – if known. Final criteria for exposing patients to the risks of these new therapies should be a balance between the knowledge (including mechanism of action and safety profile) of the drug (or its bioequivalent), an evaluation of the potential alternative therapies, and the ability of the investigator to adequately monitor for drug related adverse events.

RISK ASSESSMENT BASED ON CLINICAL PROTOCOL

Key to appropriate risk management, is the characterization and understanding of the patient prognosis. Protocols should be designed to identify a specific patient population with as few co-morbidities as possible. A narrowly defined patient population must be weighed against patient accrual, but generally, the trial should be performed in a well-defined population with a predictable clinical course. Ultimately, the risk of any drug is measured within the patient. **Establishment of drug safety in a patient cohort is the foundation of all future trials of the drug**. Understanding the pathology for a specific patient population allows for accurate attribution of adverse events to the study drug. Allowance of a broad spectrum of patients into safety trials can markedly complicate this attribution. Complex patient populations therefore require more sophisticated pre-clinical safety data to accompany the application as well as larger trials and sophisticated measures of attribution. As it is ethical for patients with extremely poor prognosis and no alternative acceptable therapies to assume more potential

risk in the evaluation of new drugs, a balance must be struck during the identification of the appropriate patient populations. A uniform patient population with predictable prognosis and few alternative treatment options would be ideal. However, patients with extremely advanced disease may not allow the time to evaluate the safety of the drug in the form of drug mediated disease progression, or may experience disease-mediated co-morbidities that prevent gathering firm evidence of safety. Together, the characterization of the patient population and development of a scheme to capture potential adverse events are the first key steps in determining the type of pre-clinical data required prior to submission.

RISKS ASSOCIATED WITH DRUG MANUFACTURING

Stem cells used in the clinic are drugs and therefore must be manufactured as drugs. The manufacture of cell-based medicinal products must be carefully designed and validated to ensure product consistency and traceability. Control and management of manufacturing and quality-control testing are carried out according to Good Manufacturing Practice (GMP) requirements (U.S. FDA, 2008). GMP practices include document control, standard operating procedures (SOPs), trained personnel, qualified reagents and equipment, and complete provenance of the manufacture of the drug. Prior to their use, drugs must be screened for purity and potency. Purity in stem cells is usually based on phenotype characterization by flow cytometry. Potency testing is used to confirm that the cell based drug is biologically active and capable of producing the desired biological effect (Bravery et al., 2013) Potency is usually based on the association of the phenotype characterization with in vitro activity such as immune suppression, cell differentiation etc. (Bravery et al., 2013). Data demonstrating consistent manufacturing in the patient population is required. The drugs must also be assessed to assure that no additional risks to the patients (via bacterial contamination or malignant transformation of the product) were introduced during manufacturing. Together, these elements (purity, potency, lack of additional risk factors etc.) make up the release criteria necessary for any stem cell medicinals to be administered to patients. Ideally, all data supporting the application to the regulatory agency should be generated identically to the product intended to be administered to humans i.e. cells manufactured using the same SOPs, materials, cell sources and meeting identical release criteria (Frey-Vasconcells et al., 2012). While this may not be possible for all demonstrations of pre-clinical efficacy, it should certainly be applied to toxicology studies. This raises the issues of whether toxicology studies should be undertaken using human cells in immunocompromised animals or in a xenogeneic transplant to animals or using animal cells. If cell products are generated in an identical

fashion to the clinical product to be used the former approach will be necessary. Additionally, if minor changes to process occur after efficacy or toxicology studies are complete, and these do not affect the product, an exception may be sought. Finally, unique attributes associated with using cells such as release criteria, storage requirements, shipping and shelf life (among others), require special focused attention to assure the investigator and regulatory agencies that patients in a clinical trial will be treated with the same drug.

SMALL MOLECULE DRUGS VS. CELL THERAPEUTIC

Although in some aspects they are quite similar first in human (FIH) trials using stem cells differ substantially to the typical FIH trials for small molecule drugs (Au et al., 2012). Unlike stem cells, small molecule drugs are composed of one active ingredient that works on a single target of action. Drugs often have a stable pharmacokinetic/pharmacodynamic (PK/PD) profile in vivo and results are easier to interpret due to the presence of well-defined reference standards. In contrast to single modality drugs, stem cell products are complex and can contain multiple active ingredients that work through multiple parallel mechanistic pathways (Bravery et. al., 2013). Furthermore, stem cells are living organisms that can produce responses in a complex multimodal manner depending on the environmental conditions encountered (Bravery et. al., 2013). For example, this is the case with adherent stem cell populations where it is understood that they exert therapeutic mechanisms via trophic mechanisms. It is understood that these trophic pathways are highly responsive to the microenvironment and are dynamic over time. Therefore, it is not the same dominant mechanistic pathway in which the cells will work every time. It is this inherent property of the cell therapy which makes it very difficult to define reference standards (Bravery & French, 2014) and suitable assays of potency within the field (Bravery et. al., 2013)

Multiple other differences also exist between the two types of products. For example, in contrast to small molecule drugs, stem cells are a living product and therefore are administered to the patient without undergoing terminal sterilization. Furthermore, differences in the absorption, distribution, metabolism and excretion (ADME) profile of the stem cell product after administration remain unclear. In the case of small molecule drugs breakdown/decay of drug concentrations can be monitored as opposed to stem cell products where unchecked cell proliferation may occur and go unmonitored. As a result, measurements of cell dose may be far less accurate than that for small molecule drugs. Furthermore, the anatomical site of administration proposed for the delivery of the stem cells may require surgical intervention, or the use of a novel cell delivery device to ensure adequate delivery of

cells. Such procedures pose further safety risks. If novel cell delivery devices are required, safety tests must be carried out to ensure that the material of the device in contact with the patient does not elicit a harmful biological reaction. Furthermore, the investigator must ensure that the device is sufficient at delivering the stem cell product without damaging the delivery system or the product itself. As stem cells exhibit sensitivities to both chemical and physical stimuli the investigator must also ensure that correct cell identity is maintained after the product is passed through the device. Another safety concern is the vulnerability of the administration site (brain/spinal cord). Each implantation site will have different degrees of toxicity associated with cell delivery. In some cases, it may be necessary to re-access the implantation site in order for product removal in the event of potential adverse reactions. This will depend on the individual product specifics and disease indication to be treated.

WHAT CONSTITUTES ACCEPTABLE DATA TO SUPPORT CLINICAL STUDIES?

The reviewed literature can be used as a resource in collecting data to support clinical trials. Papers that describe other clinical trials with specific details on the safety of the drug can and should be used to support arguments justifying the safe use in humans. It is important to be aware that other trials will likely differ in the dose, route of administration, disease indication or important differences in the drug used. When identifying data in publications used to support the argument for their use in humans, it is important to critically evaluate the differences in the composition, purity and potency of the drug used. In cell therapy, this can be difficult as the descriptions of the manufacture and evaluation of the drugs can be rather superficial. Data supporting a related but non equivalent drug will likely be considered irrelevant. However, in situations such as the use of mesenchymal stromal cells, characterization of the cells as per the International Society for Cellular Therapy (ISCT) criteria (Dominici et al., 2006), should allow the use of supporting data from the literature. Another important issue to consider is the type of animal model used to support a regulatory submission. Animal efficacy studies and animal toxicology studies are significantly different in the number included in the study, the doses tested, and ancillary (histology, non-target organ involvement). Efficacy studies rarely support the safe use of the drug, but are only used for the justification for the logical testing of the drug in humans. Animal models of efficacy are justified if the pre-clinical efficacy studies were meant to represent highly predictive models of efficacy in humans or to provide a rationale to why it is reasonable to progress to human studies. An ideal scenario would be one where pre-clinical data are truly predictive but

this is rarely if ever the case. It is important to note that in the United States (US), animal studies of efficacy are required for testing novel drugs in paediatric patients.

It is important that academic clinicians engaged in the translation of stem cell research to the clinic are cognisant of the requirements of the translational process from the beginning of the research programme. The science required to allow the clinical use of a novel drug is expected to be held to the most rigorous of standards. A typical mistake of a new investigator is to expect that scientific data published in peer reviewed journals automatically qualifies as data supporting a clinical trial; often it is not. Pre-written protocols for every step of the study, data provenance, equipment validation, reagents, animals, and supplies provenance (recording all lot and catalogue numbers and expiration dates, and methods to assure they have been handled or stored properly prior to use), biometric monitoring of animals prior to treatment, animal study randomization, written description of any deviation from the pre-written protocols, and an independent assessment of the data collected are some of the few considerations to be taken to produce data in support of the use of any new drug in humans. These requirements are typical of the Good Laboratory Practice (GLP) used in the pharmaceutical industry (U.S. FDA, 2013). GLP is important for laboratories performing pre-clinical toxicology studies as it ensures that a system is put in place of documentation and SOPs that would allow for the entire study reconstruction once the final report has been written. While not an absolute requirement for data in support of a clinical trial, every effort should be made to adhere to or approximate GLP principles. Strong self-evaluation of the lab and its practices (and the appropriate changes needed) is absolutely required to save the investigator time and money during the acquisition of the required preclinical data and to prevent potential repeat of the experiments due to failed rigor.

UNIQUE CONSIDERATIONS OF PRE-CLINICAL STUDIES RELATING TO CELL PRODUCTS

Cell products are guided essentially by the same regulations applied to standard pharmaceuticals. However, cells used as drugs are often culture derived. Cells in culture are populations derived from a complex starting material that self-purify during expansion. The culture process must be robust enough to result in a product with consistent purity and stability. The basis for evaluating the rigor of the safety studies in animals stems directly from the data associated with the reproducibility of the purity, stability and potency of the cells generated by the protocol. Thus, safety data in support of clinical studies should be produced

with a cell culture protocol that meets GMP processing with sufficient supportive data to assure consistency of the production of the drug (cells).

In the cases that the actual product cannot be used for pre-clinical studies, then every effort should be made to assure that the pre-clinical studies are done with equivalent cells. In the case of using published data in support of clinical trials, one must prove drug equivalency. Proving equivalency is difficult and complicated by often inadequate description of manufacturing in published manuscripts. Without equivalency, the drugs cannot be assumed to be functionally or toxicologically similar.

In early studies, animal models are often used to evaluate specific safety concerns regarding the drug or route of injection. Animal safety studies should not be confused with efficacy studies. Safety studies must be specifically designed to sufficiently address the question of safety. One complication of toxicology of stem cell products is whether a human based cell therapy or animal based should be used in these studies. If one needs to use the final product, then the former approach will be necessary. This may necessitate the use of immunocompromised animals or a xenogeneic approach would be necessary. There is no absolute guidance on which approach is optimal and this will need to be considered by the investigator and discussed with the regulator.

One of the challenges in the choice of pre-clinical animal models is the limited nature of the relevance of many of these models to the human situation. Some models may share similar features in anatomical terms (e.g. pig heart) but rarely are the pathology and pathogenesis identical. For example, in translational research of critical limb ischemia, small animal models of acute hindlimb ischemia are relatively poor surrogates for older humans with a history of hypertension, hyperlipidaemia, diabetes mellitus and cigarette smoking for many decades. In addition, dose equivalency is problematic in animals. For pharmaceutical drugs the dose size is merely adjusted based on animal weight. There is little evidence that the therapeutic value of cells as drugs relates to a correlative increase in dose. Likely, cells used as drugs meet some threshold of activity with little benefit from additional cells. Ultimately, cell dosage will require empirical evaluation in humans and the trials should reflect this. Finally, the route of injection has implications for the safety of the approach. It is logical that 300 million cells injected intramuscularly have a different safety profile than those cells injected into the carotid artery. Delivery into tissues using needles, catheters, or adherence to matrices needs to be carefully evaluated (and potentially also evaluated for safety) prior to trial initiation.

Finally, it is critical for investigators to realize that in cases of a halted clinical trial due to the safety of the underlying drug, it is likely that all data in support of the clinical trial will be audited. The rigor required by adherence to GLP will provide the assurance that a review of the data, methods and conclusions will withstand an arduous audit.

OVERSIGHT OF PRECLINICAL STUDIES USED TO SUPPORT FIH STEM CELL CLINICAL TRIALS

Progression of cell therapy (CT) products to first in human (FIH) testing requires review and approval by the regulatory authorities. The development of a regulatory plan as early as possible in the product development process is fundamental to the successful introduction of the CT into clinical testing. The investigator should become aware of the regulations surrounding their CT product and understand how the regulatory agencies assess new applications.

The EMA and FDA have published guidelines on how to build a safety profile of the CT product by using a risk-based approach (EMA, 2013). The guidelines provide an understanding of a risk-based approach to the safety evaluation of CT products in preclinical studies. Furthermore, they define risks and risk factors as qualitative and quantitative characteristics of the CT product that contributes to its specific risk after administration. Tumour formation, unwanted tissue formation due to aberrant differentiation and migration of cells to unwanted areas are all risks associated with the administration of a CT product (EMA, 2013). Building an adequate risk profile requires the accumulation of as much information as possible of the potential risks and risk factors associated with the CT product. In order to do this, it is important for the investigators to gain an understanding of the potential benefit as well as the potential untoward effects surrounding their CT product before testing in the clinic. This understanding of the risk-benefit ratio of the CT product can only be obtained from well-designed pre-clinical studies in accordance with the identification of the appropriate patient population.

The regulations surrounding the development of CT products proposed for testing in FIH trials involves several steps, each of which must satisfy both a scientific and a regulatory need. In this section, I describe the regulatory process associated with developing a CT product in Ireland and provide guidance on how to design preclinical studies that will help first time investigators build a risk profile of their CT product.

HEALTH PRODUCTS REGULATORY AUTHORITY

Clinical testing using CT products in Europe are regulated under the Clinical Trial and Advanced Therapy Medicinal Product (ATMP) Directives and require the submission of a national clinical trial application. The health products regulatory authority (HPRA) represents the national competent authority and regulates cell therapy testing in Ireland. The HPRA are responsible for GMP facility licensing and clinical trial authorization, followed by inspection of compliance and pharmacovigilance of cell therapies. Several key steps govern the regulatory process attached to a CT product. Any clinical testing of a CT must be performed under an investigational medicinal product application. Therefore prior to clinical testing the investigator must submit an Investigational Medicinal Product (IMP) application to the HPRA. Following review of this application the HPRA will ensure that the proposed clinical testing of the CT product has an acceptable risk-benefit ratio. For example, the HPRA assesses the application and determines whether the testing of the product subjects participants to an unreasonable risk. The review also ensures that the rights of research participants are being protected. In parallel, the clinical trial application is reviewed by an institutional research ethics committee. It should also be noted that the clinical trial application can only be submitted after a GMP license has been obtained. The Irish regulator does not allow parallel submissions of a request for a GMP license and the proposed clinical trial (HPRA, 2015). Prior to submitting an application to the HPRA the investigator must obtain a EUDRA CT (European Union Drug Regulating Authorities Clinical Trials) number (HPRA, 2015). EUDRA is a clinical trial database set up by the European Medicines agency. Each clinical trial being performed in the European Union must have a EudraCT number. The investigator must include this number with their clinical trial application.

INVESTIGATIONAL MEDICINAL PRODUCT APPLICATION

The IMP application is the formal submission that notifies the regulatory body of the investigators intent to evaluate the investigational product in a clinical setting. In this application the investigator will make a case to the regulatory agency of why their medicinal product should be tested in humans. The document must provide a suitable risk-benefit analysis of the medicinal agent proposed for human testing. Claims must be data driven and provide evidence that the product is safe for testing in human subjects with any risks counterbalanced by the disease severity and the potential benefits of the medicinal product administration. An IMP application characteristically is composed of 3 folders of information:

general information folder, protocol related folder, and IMP-related folder. The clinical trial protocol, investigational medical product dossier and the investigator brochure are contained within the IMP related folder and represent the major components of the application. The requirements for these components will be discussed in this chapter.

MAJOR COMPONENTS OF THE IMP APPLICATION

Clinical Trial Protocol

The clinical trial protocol is a critical component of the CT product development program. The protocol should clearly describe the proposed clinical trials; objective, methods, design and general organization. For FIH trials the protocol will focus mainly on the elements that are critical to the safety evaluation, for example safety assessments, toxicity monitoring, study stopping rules, recording and reporting of adverse events (FDA, 1998). Furthermore, the protocol will contain the proposed enrolment criteria; inclusion and exclusion criteria, details of the risks associated with the proposed disease and anticipated disease related adverse events should be considered (FDA, 1998). As mentioned earlier a risk-benefit analysis of the CT product is generated based on the preclinical work. Therefore, any knowledge of potential toxicities of the CT product observed in the preclinical studies should be clearly highlighted in the protocol. The route of administration will need to be carefully considered and the composition of the final product as detailed in the investigational medicinal product dossier (IMPD) will need to be defined and considered throughout the translational pathway.

Investigational Medicinal Product Dossier

The investigational medicinal product dossier (IMPD) provides a definition of the proposed clinical product and a detailed summary of the CT product manufacturing process. This component of the IMP application provides all of the information regarding the infrastructure of the manufacturing facility, processes, organization, quality management, materials and management of supplies that are used in the manufacture of the CT product. Essential constituents of the IMPD section are the manufacturing standard operating procedures such as isolation and seeding and expansion to harvest etc. Furthermore, safety and quality testing, donor screening, certificates of analysis of all reagents used, product stability, environmental monitoring and specifications of equipment will be included.

The Investigator Brochure

This document is used by the investigator to provide a rationale for the clinical testing in humans based on the preclinical program. Since a large body of work completed in this thesis was included in an investigator brochure submitted to the HPRA to support FIH testing of human mesenchymal stem cells in critical limb ischaemia patients, the investigator brochure content will be described in some detail.

The investigator brochure (IB) is a document containing a compilation of all the clinical and preclinical data of the product that is proposed for testing in the human disease population. Typical information included in the investigator brochure includes clinical and preclinical review of the literature. The IB should contain the following elements:

IB Summary/Abstract: The summary is essentially an abstract of the document. The summary describes the known information of the product relevant to the clinical stage of development. This section should summarize all the characterization data; physicochemical (growth factor secretion, viability, phenotypic markers) and biological (toxicology, efficacy, biodistribution) and clinical data of the proposed test item.

Introduction: This section introduces the test item under consideration for clinical testing to the regulatory body. For the CT products a clear description of the cell type being used should be provided along with a clear rationale for performing the clinical research using this CT product. The introduction should be closed with a line describing the approach in which the investigator has undertaken to evaluate the investigational product.

Test article description: This section describes the test item being proposed for human testing. A brief description of the test item along with its physical and chemical properties should be provided. Information regarding the product formulation, storage concentration and conditions e.g. ($2x10^6$/ml per cryobag stored in the liquid phase of liquid nitrogen temperature range -140 to -196°C) should also be provided in this section.

Pre-clinical studies: This section contains the results of all the preclinical studies using the CT product and is split into three parts (1) preclinical efficacy (2) preclinical biodistribution (3) preclinical toxicology. At the end the data from each of these sections should be summarized to describe all the relevant findings from each of three sections. The relevance of the findings from each section to the CT product and possible unwanted effects in humans must be discussed. Furthermore, emphasis must be placed on the relevance of the information provided by these studies to the proposed human dosing. Typical data produced by these studies should inform the investigator of the nature, frequency, and severity of the efficacious

or toxic effects; the time of onset and duration of these effects; and dose response information. To ensure clarity, it is recommended that all data is presented in tabular format listing both summary animal data along with data on individual animals. A detailed description of what to consider when designing these studies will be provided later on in this document.

Clinical data: This clinical data section should provide in depth discussion of the known effects of the product for example pharmacokinetics, safety, dose response and efficacy. This section will contain two parts (1) pharmacokinetics and metabolism in humans and (2) safety and efficacy in humans. This data is often generated from previously completed clinical trials in humans. However, first time applications, where no previous clinical data on the investigational CT product has been obtained, an extensive review of the clinical literature will be provided.

Overall summary: In this section the investigator should demonstrate an understanding of the potential adverse events, risks and precautions associated with the proposed clinical trial. This understanding should be supported by the information provided from pre-clinical and clinical data of the investigational product. Furthermore, where appropriate previously published preclinical and clinical reports on related CT products can be used to support the proposal. It should be stressed that this section should not be a summary of the observations in the preclinical and clinical studies but rather a conclusion of the investigator's evaluation of the data and conclusions based on that evaluation. The investigator's conclusion must be data driven and should be directed to build a case to the regulatory body of why the investigational product should be tested for the proposed clinical indication.

Making The Investigational Medicinal Product Application

When all of the above-mentioned documents are completed the investigator can file the application for FIH testing. Once the full IMP application is submitted and validated, the HPRA has 30 days to assess and respond formally to the submission. Written acceptance will be sent to the investigator authorizing the trial under specific conditions or will provide the grounds for non-acceptance. In the case of non-acceptance, a formal response will be received from the HPRA detailing the issues of concern. The investigator should respond within 30 days or within the timeframe agreed by the HPRA. The response will then be reviewed by a clinical trial subcommittee and within 90 days from the original application date, a written response either accepting or rejecting the request for authorization will be provided to the investigator.

Regulatory Interaction – pre submission meeting

The task of developing a CT product with well-characterized therapeutic properties is an innovative and iterative process that is built through the creation, collection and assimilation of scientific data and regulatory standards. Many first time investigators are unfamiliar with the challenges associated with translating a new CT product to clinical studies. Unfamiliarity with the various processes often means investigators will underestimate the time, regulatory challenges and costs associated with bridging the CT product from research laboratory into early phase clinical trials. Therefore, for clinical trials where investigators are planning to use CT products most regulatory agencies will strongly recommend that the investigators request a pre submission meeting. Pre submission meetings are most useful for investigators planning to test a novel product or for inexperienced investigators. As the regulatory agency only has 30 days to respond after the initial submission it is necessary that the application fulfil the agency requirement before the adequacy of the application can be assessed. For this reason, it is necessary that all queries raised are clarified and resolved before the submission. For each new product the regulatory agency will provide one formal pre-submission meeting. This meeting will mainly focus on the preclinical studies, cell manufacturing and clinical trial design. In order for this meeting to be useful it is strongly suggested that the investigator prepare questions and data beforehand. The investigator must note that it is important to provide the regulatory agency with sufficient information in order for them to provide appropriate feedback.

GENERAL CONSIDERATIONS WHEN DESIGNING PRECLINICAL STUDIES USING CELL THERAPY PRODUCTS

Preclinical testing is required for all new investigational products prior to their testing in humans. Unlike for small molecule drugs there exist no “set in stone” guidelines on how to adequately test a CT product. The properties of stem cells, including their differentiation and proliferation potential present safety concerns that are very different from those of small molecule drugs. The inherent ability of a stem cell to persist or alter its function depending on intra or extracellular signals from the microenvironment makes safety assessment more complex than that of well-defined small molecule drugs. Furthermore, safety is determined based on the individual product attributes. It is becoming more evident that great heterogeneity and complexities exist amongst different CT products from different

laboratories for example characteristics (cell type/allogeneic or autologous/manufacturing/formulation/ROA). The heterogeneity of each individual product therefore prevents the use of a "one size fits all" model for preclinical testing. As a result, CT associated pre-clinical studies should be carried out on a case-by-case basis and the preclinical studies design should be tailored to the individual specifics of the CT product with focus on the indication to be treated (Cavagnaro et al., 2002; U.S. FDA 2010).

Although the pre-clinical studies are recommended to be carried out using a case by case approach there still exists a set of common questions which must be addressed in preclinical studies e.g. Does the product have biological activity? Does it elicit a biological response in a disease model? What is the durability of the response? How long is needed to assess the effect of the treatment and the durability of the treatments effect?

After a meaningful measure of biological activity and efficacy has been recognized, toxicology studies should be performed in order to characterize any undesired effects of the product. Undesired effects may represent local toxicities due to the product at the injection site or systemic toxicities due to cell migration outside of the tissue of interest. Tumorigenicity or differentiation may occur at the administration site or in distant areas to which the product migrates. To further assess the migratory potential of the cells after administration adequate and meaningful biodistribution studies should be conducted. Dose response studies are recommended to determine the effect of different variations of cell numbers and their therapeutic effect on the target tissue. The overall purpose of designing the preclinical studies is to enable the investigator to choose a dose which is safe and effective for use in the human study.

Preclinical Safety Studies for Cell Therapy Products

Due to the risk-benefit profile not being favourable for healthy volunteers, investigators must begin the safety and pharmcodynamic evaluations of their CT products in diseased patients. Prior to human studies however pre-clinical studies are required in animal models (U.S. FDA, 2013). Preclinical toxicology and biodistribution studies are required to determine if the CT product produces untoward effects. As the data generated from these studies will inform the investigator whether the CT product has an acceptable risk-benefit profile to justify clinical testing it is imperative that they are designed and executed correctly. In the next section, important parameters in the design of their preclinical toxicology and biodistribution studies will be discussed.

Optimal Cell Product Used for Preclinical Safety Testing

Preclinical toxicology and biodistribution studies are conducted in order to characterize the safety profile of the CT product prior to testing in humans. As a result, wherever possible the intended human CT product should be used for testing in these studies (U.S. FDA, 2010). In particular, it is necessary to have product manufactured in an identical manner to the trial product and preferably under GMP should be used. In cases where the intended human product is not available for testing, regulatory guidelines suggest that analogous CT product may be used (U.S. FDA,2010). Analogous cellular products may be derived from animals but the regulator will require equivalency studies if this approach is taken. Furthermore, any manufacturing differences that exist between the analogous product and the human product must be well described and documented. Many limitations exist when using analogous products and should be considered prior to designing the preclinical studies. For example, where rodent cells are used as the analogous product; generation of cells from rodents are often pooled from multiple donors whereas in the human situation cells are isolated and expanded from one donor. Furthermore, characterization of the cell phenotype is often more difficult in rodents due to heterogeneity of the cells and lack of specific antibodies. Cells isolated from rodents often require the use of equine and bovine serum to grow where human cells can be cultured in serum free media or supplemented with bovine serum alone. Furthermore, rodent cells require extensive culturing times to obtain a clean cell population and are more prone to genetic abnormalities in comparison to their human counterparts (Zhou et al., 2006) Such differences make comparisons very difficult. For these reasons preliminary studies using the analogous products must be carried out to ensure an adequate level of homology to the intended clinical product prior to conducting the pivotal preclinical studies. From extensive interactions with HPRA, human cells are preferred for toxicology studies and ideally these should be manufactured under GMP using an identical process to that which will be used for the manufacture of the clinical product.

Choosing An Animal Model

Using healthy or diseased animals is another factor the investigator must consider. Safety studies often use healthy, diseased or injured animals however there is significant debate on what represents the most appropriate model for preclinical testing. However, the choice is generally driven by the investigators need to test a specific hypothesis. From a regulatory perspective there is no standard animal model selection. Whatever species/model chosen by

the investigator, healthy or diseased, for testing, a full scientific justification of the choice should be provided. Where the intended human product is used for testing then studies will generally be performed in immunodeficient rodents.

If immunodeficient animals are not available as in the cases where studies use larger animals, then immunosuppression may be used (Frey-Vasconcells et al, 2012). However, where immunodeficient animals are used for human cell transplantation it should be acknowledged that inadequately defined host tissue response may mask the full functionality of the cell as the microenvironment for the engrafted cells will never be equivalent to the environment which exists in a human immunocompetent tissue. Moreover, therapeutic molecules secreted by the human cells may not be fully pharmacologically active in these animals or may have unexpected interactions with the signalling pathways in the animal. Furthermore, the interaction between the human cell secretome and the animal's downstream pathways may not be the same as what is seen in humans and this may adversely affect the physiology and behaviour of the animal. For these reasons, it is therefore recommended that pilot efficacy studies are first carried out to assess if the test article displays activity in the animal model. This is important in order to enable the assessment of the potential benefit and untoward effects of the CT product. Often testing on two or more models is required to make the decision on which is best to use.

Preclinical Toxicology

The nature and degree of adverse effects can vary largely depending on the type of CT product being tested. Nonetheless there are three types of data sets that toxicology department should provide to the investigator to enable them to generate a risk-benefit analysis of their CT product: (1) Is there any overt toxicity as evidenced by clinical signs and micro and macroscopic injury? (2) Is there any surrogate measures of toxicity? E.g. increase/decrease in blood serum proteins or enzymes. (3) What are the pharmacodynamics of the CT product?

As mentioned earlier the toxicology should be designed and carried out in a manner that mimics as closely as possible the human scenario. CT products can be administered via multiple different routes of administration. As cell therapies are novel sometimes they require the use of novel delivery systems. For the toxicology studies the CT product should be delivered via the intended clinical route and using the intended clinical delivery system (U.S. FDA, 2010) . If a delivery system is required to deliver the cells, then it is recommended that investigator first carries out some in vitro tests to characterize the delivery system prior to starting the pivotal studies (U.S. FDA, 2010). The tests must demonstrate that the system can

deliver the CT product successfully through the apparatus without damaging the system itself and also the CT product. In vitro models that closely mimic the clinical protocol should be developed to assess cell viability/metabolic activity (U.S. FDA, 2010). Due to the nature of cells to aggregate and adhere, especially in lower flow rates, cell adherence to the device should be carefully assessed (U.S. FDA, 2010). Cells are highly sensitive entities and with variable responses to even subtle physical or chemical stimuli. For this reason, tests should be carried out to ensure that the cells have not been altered and that they still maintain their biological activity after being passed through the system.

Once this in vitro data regarding the delivery device is obtained pilot studies using an appropriate animal model (small or large animal depending on the device) should be designed. These studies will enable the assessment of the delivery system deployment, retrieval and delivery of the CT product. The pilot animal studies should answer the following device specific questions: (1) Does the patient contacting material of the delivery system evoke a harmful biological response? Furthermore, is there any damage to the delivery site, surrounding vasculature or adjacent tissue structure after the system is deployed or retrieved? (2) What are the delivery systems handling characteristics? Is the device flexible or are there difficulties inserting and removing the system? Is it compatible with other devices and systems for example if x-ray guidance is needed how radiopaque is it? (3) Does the delivery system become damaged after repeated administrations and simulated use?

Pivotal Toxicology Study Design

Toxicity testing of the CT is performed on equal numbers of both male and female animals. The data is segregated in accordance with gender for statistical purposes and is classically presented as number of animals per gender per group. In general, the toxicology studies use 5-10 animals per gender per group; per time point, however, this will vary depending on the design of the study (Frey Vasconcells et al., 2012). More animals may need to be included to account for deaths that may occur during the longer time points. Furthermore, if the investigator choses to use diseased animal models, then attrition due to the disease or the procedure must be considered to ensure a sufficient amount of animals to meet the study endpoints. Additionally, the investigator must consider the animal numbers based on the measures in which they would like to assess at each time point. For example, if mice are being used in clinical pathology, testing may be limited due blood sample volume. Furthermore, the small blood volume in a mouse does not make it possible to conduct

hematology and serum chemistry analysis from the same mouse. As a result, two separate groups should be allocated for hematologic and serum chemistry testing.

Toxicology studies will generally include multiple different safety measurements at different time points. If diseased animals are used both efficacy and safety measurements can be evaluated in the same study. Typically, toxicity measurements include general health evaluation signs, clinical pathology and histopathological analysis. Measures of food consumption, body weight loss/gain and clinical signs, assess the general well-being of the animals. Changes in these measurements can serve as early indicators of toxicity occurring within the animal. Clinical pathology (hematology and biochemistry) will offer surrogate measures of toxicity of the test article. Hematology can provide information regarding the status of the animals hematopoietic and immune system whereas serum chemistry will provide data regarding the functional status of major organs for example liver and kidneys etc. Urinalysis can also be performed however sample collection can be quite difficult depending on the animal species used. The number of clinical pathology assessments is determined based on the animal model. Due to a greater blood volume in larger animals, serial phlebotomy measurements can be carried out thus enabling multiple clinical pathology assessments over different time points. However due to small blood volumes of small animals such as mice interim blood sampling is not feasible and therefore blood sampling is collected only at terminal sacrifice. One of the key aspects in the safety assessment of a CT product is delivery toxicity. Anatomic pathology evaluations via macroscopic and microscopic examination of the on target off target tissues/organs are necessary to determine the propensity of the CT product to form ectopic tissue or tumours. While H&E staining of sections can detect a bolus injection of cells it cannot determine the detection of small numbers of migrating cells. As a result, immunohistochemical staining should be incorporated into the protocol. Such staining will not only help determine if the cell persist or proliferate within the tissue section but can also provide data on the cells ability to differentiate into the desired or undesired cell type. Where the intended human product is used Ki-67 and proliferating cell nuclear antigen represent useful markers to assess cellular proliferation. In the case where ectopic tissue or tumours have been discovered, human nuclear antigen or human mitochondrial antigen can be used to determine whether the tumour is derived from human organ or a spontaneous endogenous tumour which are commonly associated with immunodeficient models. While immunostaining represents an important research tool for assessing cellular differentiation and proliferation many limitations exist when developing a staining protocol. Consideration should be given to whether the human specific antibodies are compatible with the research species model. For example, the use of

albino and immune deficient mice are more useful for 3,3'-diaminobenzidine (DAB) substrate detection due to lack of pigmentation in the tissues. DAB is the substrate used for colour detection and the lack of pigmentation in the tissues of these animals eradicates the use of bleaching (Baker et al., 2015). Bleaching is a process required in immunostaining, which is often needed to remove the naturally occurring melanin pigment. This process can often interfere with the immunostaining of key markers and thus skew data interpretation. This highlights that the choice of mouse model may facilitate a better evaluation of the CT product by facilitating suitable reagent selection that will ultimately result in less background and non-specific staining.

Tissue List And Tissue Handling

Histopathological evaluation accounts for the majority of the cost in the preclinical assessment of CT products in toxicology studies. The cost significantly increases with progression from wet tissues to fixed paraffin blocks to stained slides. The use of tissue triaging is recommended by (Baker et al., 2015). Tissue triaging entails dividing the tissue into multiple different pieces that can be used for different sets of analysis e.g. electron microscopy, histological or biochemical analysis. The use of tissue triaging and appropriate selection of tissues and tissue sections can have significant impact on the final cost without impacting negatively on the feasibility of the study.

The tissue list to be selected is designed using a tier-based system. The tier based approach is designed based on the intended route of administration, the location of the draining lymph nodes, the already known or proposed biodistribution of the product and finally the CT proposed mechanism of action (Baker et al., 2015). The injection site and draining lymph nodes are the most obvious first choices. The potential of the CT product migrating from the administration site requires the evaluation of the well perfused tissues and organs such as the kidney, liver, spleen and lungs. If the mechanism of action of the CT product is through the secretion of growth factors and cytokines, then the collection of proximal and closely related tissues must be collected. Finally, if any abnormal masses are observed these must be removed and analysed to exclude that tumorigenicity of the CT product. Data from biodistribution study if available is useful when refining the tissue list. Standard procedure is to use 5 μm hematoxylin and eosin to evaluate the chosen organs and tissues. However, the use of such sections can be problematic when trying to evaluate the distribution of cells in larger volume organs such as the liver. Serial sectioning of large organs in a bid to find the transplanted cells is impractical and can be compared to finding a needle in a haystack.

Furthermore, serial sections through the whole tissue will provide the need to obtain multiple slides in order to avoid permanent tissue loss. Unstained tissues will require storage in slide boxes and archiving, which will significantly increase costs. To avoid this, the integration of a correct tissue collection with an integrated stereological approach should be designed and incorporated into the protocol. A stereological approach will ensure a proper tissue sampling approach and will facilitate the construction of whole tissue interfaces using smaller tissue samples thus increasing the practicality of the approach and at the same time minimizing tissue loss (Baker et al., 2015; Dockery et al 2007). To give an example, the use of stainless steel tissue matrices can be used to obtain tissue segments at 0.5 or 1mm intervals, which can then be embedded individually into serially labelled blocks. Single sections from each block can then be obtained and evaluated in a practical cost effective manner with minimal tissue loss.

The use of phased tissue examination should also be considered. Cells that display weak kinetics and don't persist for long after administration are less likely to become a long-term hazard. The use of quantitative PCR (qPCR) for the detection of cells to track the survival or disappearance of such cells in pilot studies may justify a reduction in time point for further studies. Providing the qPCR assay is highly sensitive and validated to the specific cell type, the absence of a negative signal in non-target tissues at earlier time points may provide a justification for a less arduous tissue examination in later time points.

While the author acknowledges that first time investigators may not be fully aware of the techniques suggested above, it is suggested that investigators ensure the involvement of the toxicologic pathologist in the earliest stages of the study design. Toxicologic pathologists are highly trained and early involvement in the design of the study protocol may save time, reduce cost and ensure the design of translational ready studies of your CT product.

Preclinical Biodistribution

Biodistribution is a multifaceted issue not only relating to the cellular localization and migration but also survival and differentiation status of transplanted cells (Goldring et al., 2011). Determining the distributive fate of a cell therapy product after administration is an essential part of characterizing its biosafety profile (Sensebe et al., 2013). These studies provide the investigator with valuable information about the CT by helping the investigator to understand where the transplanted cells can be found after administration. By knowing where the cells go not only helps the investigator establish potential points of toxicity but also provides data on the affinity of the cells to the intended target of action. Due to the limited

biodistribution data in humans, preclinical biodistribution remains one of the most important data sets in characterizing the risk and risk factors of the CT product. Due to the complex biological diversity and other associated issues with CT products, a risk-based approach should be conducted and the biodistribution studies should be designed with the clinical indication in mind (EMA, 2013). As mentioned earlier safety of CT product is determined based on the individual product characteristics. Therefore, as with the toxicology studies wherever possible, the biodistribution studies should be carried out using the intended human product under good laboratory practice conditions.

Before designing the biodistribution study multiple factors must be considered. Considerations include the animal model, route of administration, and frequency of dosing.

An ideal method to assess biodistribution should, be compliant with regulatory standards, be non-invasive, enable longitudinal cell detection and be highly sensitive to allow for the detection of small numbers of cells located deep within the host tissue free of artifact. At present there is no single method that satisfies all of these requirements. Whichever assay or method of detection that is chosen, a detailed description of the sensitivity of the method, and its limits of detection, must be provided by the investigator.

Various approaches such as immunohistochemistry, whole animal sectioning, nanoparticle labelling of cells quantitative polymerase chain reaction and a wealth of in vivo imaging modalities have been used by labs worldwide to assess the biodistribution of their CT products. All biodistribution assays have limitations in terms of sensitivity and limits of detection. Nonetheless the investigator must choose the appropriate method of detection based upon both the characteristics of the products and methods that are readily available.

Microscopic visualization of histological samples has been widely used to detect the presence of the transplanted cells in pre-clinical models. Labelling of CT products can be achieved using a variety of cell labelling techniques, such as membrane dyes (PKH26, DII) or nuclear dyes (Hoechst 33342, bromodeoxyuridine, DAPI [4’6-diamidino-2-phenylindole]) genetic labelling and the use of nanoparticles. While cell tracking using histological techniques is very useful for providing results regarding cell viability, differentiation status and location of the CT product, many limitations of using this approach exist (Terrovitis et al., 2010). Quantitative data from histological techniques can be variable and subject to sampling error. For example, often only a few sections with a small number of fields of view are taken from the tissues being analysed. As a result, careful consideration must be given to developing a stereological approach (Baker et al., 2015; Dockery et al. 2007) . Where dyes are used to stain the cells intercalating dyes e.g. DAPI, may exert a toxic effect on the surrounding cells. In

addition, where Hoechst 33342 nuclear stain is being utilized in the staining protocol consideration must be given as Hoechst 33342 can be released by dead cells and, as a result, stain adjacent cells and become phagocytosed by tissue macrophages, therefore, resulting in false positive data (Terrovitis et al., 2010). Furthermore, direct labelling of cells using such dyes can be subject to dilution after the cell proliferates leading to loss of signal over time (Terrovitis et al., 2010). While genetic labelling of cells can circumvent this, simple transfection and adenoviral vectors cannot always confer stable expression of the transgene. As a result, cell loss cannot be confirmed as a result of the absence of reporter gene detection (Terrovitis et al., 2010). Fluorescent in situ hybridization (FISH) method is another method of cell detection via microscopy that uses probes that enable the detection of targets that are stably expressed within the cell. FISH is an advantageous technique, as it does not require cell labelling to detect the transplanted cells. Furthermore, as the target cell's specific sequences are stable within the cell they are not subject to silencing. Nonetheless similar to histological microscopic techniques FISH is labour intensive and subject to artifact (false positives/negatives). As highlighted earlier, the choice of albino strains or immunodeficient models may help reduce the chances of increased background staining therefore helping in DAB detection process in otherwise naturally pigmented tissues of the brain and retina (Baker et al., 2015). Therefore, if these techniques are used, careful validation of the staining protocol to determine the appropriate controls to be used thus ensuring sensitivity is needed to absolute specificity of the technique.

While all of these techniques have their advantages and disadvantages they all share the generic limitation of the necessity for animal euthanasia for tissue harvest which limits longitudinal cell tracking. For these reasons investigators often opt for the use of non-invasive in vivo imaging methods to assess their products distribution potential. Magnetic resonance imaging (MRI), bioluminescence imaging, spect photon imaging tomography (SPECT) and positron emission tomography (PET) represent methods of in vivo imaging systems used to assess cellular distribution (Terrovitis et al. 2010). While the use of these techniques has many advantages such as longitudinal tracking of cells with sensitive limits of detection, using these techniques can raise two important questions. Firstly, these techniques require the modification of cells in order to determine the distributive potential of the CT product e.g. addition of reporter gene for bioluminescence, radiolabelling of cells for SPECT and PET and incorporation of iron particles into the cells for visualization with MRI (Sensebe et al., 2013; Terrovitis et al., 2010). Regulatory agencies suggest that the cells used in the preclinical studies represent the actual product or close surrogates of the intended final product (U.S. FDA, 2013). In the case where labels are added to the cells how does this relate to the clinical

product? Moreover, will the addition of the cell label effect the function of the cell and thus affect its biodistributive potential? Chang et al reported impairments of the chondrogenic and osteogenic differentiation of human mesenchymal stem cells after cell labelling with iron oxide nanoparticles. In addition, the immunogenicity of GFP as a cell label is well documented. Furthermore, Yang et al further confirmed the immunogenic potential of GFP labelled cells using in vivo imaging and histological analysis. Where these techniques are used direct interactions with the regulatory body should be considered in order reach a consensus on the characterization of the extraneous phenotypes to determine the impact they will have on the true assessment of the biodistribution of your CT product.

Quantitative PCR using human Alu represents the gold standard for assessing the biodistribution of the CT products. While qPCR does not allow for the longitudinal follow up of the fate of the transplanted cells, it bypasses the drawbacks associated with cell labelling. QPCR using human Alu represents the most sensitive and reliable method available to investigators evaluating their intended clinical products in xenotransplatation models. The presence or absence of the highly repeated species-specific hAlu sequence is evaluated via qPCR from extracted DNA from organs and tissues of the injected and non-injected animals. As whole organs are processed and homogenized this technique is not subject to sampling errors. As no labelling of cells is required the intended human product can be used when using this method. Furthermore, as the genetic information is stable within the cells, information regarding cellular proliferation can also be provided using this technique.

Pivotal Biodistribution Study Design

Once the method of biodistribution has been chosen it is recommended that pilot studies should be performed before carrying out the pivotal biodistribution study. By the end of the pilot studies the investigator must be clear regarding the methods sensitivity and its limits of detection. Pilot studies can be used to highlight potential areas of concern and thus enable the investigator to finalize the biodistribution protocol prior to the pivotal study initiation. All documentation regarding the pilot studies and method development should accompany the final study report.

Biodistribution studies can be performed in healthy diseased or injured animals as appropriate for the CT product. Similar to toxicity testing, biodistribution studies should be carried out on both male and female animals and data should be presented as animal number per sex per group (U.S. FDA, 2010) The number of animals per group depends on the species being tested and number of time points required. However, in practice, biodistribution studies are

generally carried out on 5-10 rodents per sex per time point (Frey-Vasconcells et al., 2012). The study design should incorporate both acute and chronic time-points to enable the examination of the presence or absence of the transplanted CT product.

As highlighted above there are many questions and challenges associated with preclinical testing of a CT product. To design a study that satisfies the scientific and regulatory need is an onerous task. Each and every CT product is unique in terms of formulation, cell type, manufacturing process and route of administration and poses its own risk. For this reason, each preclinical program must be tailored to that individual CT product with careful consideration being given to its intended clinical application (U.S. FDA, 2013). Without doubt as the field emerges and more and more CT products become tested in the clinic more information will become available regarding the translational pathway that will give investigators a better understanding of more appropriate preclinical strategies and associated regulatory expectations. The regulatory agency and the investigator share the same goals in particular to bring therapeutic CT products to market for the value of patients. Effective interactions between the investigators and the regulatory agencies are fundamental to effectively navigating through the regulatory process and thus progressing the CT product from the laboratory into early phase clinical trials.

PHASE 1 CLINICAL TRIAL CONSIDERATIONS

The primary objective of a phase 1 trial is safety assessment, providing mainly information regarding dosage safety and the presence or absence of adverse reactions. Phase 1 or FIH trials can also provide valuable secondary data such as information on issues of feasibility of administration and also on the drug's biological activity. Such data can be used to design subsequent trials. The following information has been adapted from the US FDA guidelines (U.S. FDA, 2013) and will highlight the points that must be taken into consideration by investigators when designing early FIH clinical trials of cellular therapy products.

Dose exploration: FIH studies can be designed to explore and assess varying dose ranges. Maximum tolerated doses can be explored where the product is being used for life threatening diseases in which some toxicities are anticipated and can be adequately justified. However, for cases in which minimal toxicity is expected, the dose to be explored is one that can be used to decipher ranges in which the product will produce its maximum biological and therapeutic potential. In stem cell therapy trials an additional factor for consideration is the

limits on dose production with a focus on establishing a safety profile for the dose that is most feasible to produce.

Feasibility and Delivery: Cell therapy products can sometimes require state of the art devices and novel procedures in order to maximize cell delivery. FIH trials can be used to discover any technical issues associated with such procedures.

Efficacy Assessment: Although safety is the primary objective, preliminary data on the product's efficacy can be assessed. Although most FIH will not include a sample size great enough to truly assess the product's activity, suggestions of efficacy as a result of the treatment will provide encouragement to strengthen the scientific rationale to proceed to a phase 2 trial. Caution must be exercised however, as phase 1 trials will not include controls.

Choosing a Study Population: FIH trials are associated with potential risk of unanticipated side effects for the patients. Therefore, the correct patient choice for such trials is very important. Choosing a patient population can be difficult. However, the trials objective is to select a patient population in which there is a reasonable balance between potential risks and benefits whilst also accomplishing the scientific objectives of the study.

As with all clinical trials patient safety is always a major concern and this is specifically true in particular for FIH trials. The possibility of persistent or permanent side effects coupled with invasive procedures for product delivery deems such trials unfavourable for healthy volunteers. The risk-benefit ratio is not optimal for healthy volunteers and therefore the use of healthy volunteers is not acceptable for FIH cell therapy trials.

Patients with severe disease states may be more suitable for FIH investigational cell therapy trials as the risk-benefit ratio may be more acceptable. Despite this, the selection of the correct study population that will provide interpretable data involves several considerations. Patients suffering from more advanced stages of the disease may tend to experience adverse events not due to the therapy but as a result of the disease progression. Adverse events as such can lead to difficulty in interpreting efficacy and safety data. However, it may be unacceptable to recruit patients with less severe disease states. If 'no option' patients are to be included, it is important to have ensured fully that all their treatment options have been fully explored and evaluated and such information is recorded carefully. The optimal patient selection criteria for FIH trials would be those with predictable prognosis, no viable therapeutic alternatives with sufficient time before significant morbidity of mortality occurs.

Dose Selection: Pre-clinical strategies can be used to generate sufficient information on whether a specific starting dose has an acceptable risk level or not. However, dose

extrapolation using the allometric scaling method may be less precise than for those of small molecule drugs. Furthermore, pharmacokinetic and pharmacodynamics for cell based drugs may also not be as straightforward to assess and it may be difficult to extrapolate from small animals to humans. It is recommended that, if available, previous clinical data produced using cell based drugs, even if by a different route of administration, should be used to help justify a starting dose for the trial.

Dose Frequency: In most early phase trials the administration of the treatment is a single, once off, dose. Cell drugs differ from small molecule drugs where they are administered, metabolized in the liver and then cleared from the body. However, this is not the case for cell based drugs as often such products once administered have the ability to persist within the body and may have a duration of activity longer than expected. As a consequence, repeated dosing may not be prudent until pertinent information regarding the toxicity and duration of activity of the cells has been obtained.

Dose Escalation: Staggering of drug administration is recommended where no previous human experience has been obtained with the specific dose in question. In the interest of patient safety, staggering of the treatment minimizes the number of patients who are at risk of the unknown side effects of the drugs. Staggering of the treatment is most often between cohorts. For dose escalation studies treatment groups can be completed sequentially beginning with the lowest dose first. Data should be reviewed by the Data Monitoring and Safety Board prior to escalation. The choice of staggering interval between subjects should be chosen in such a way that both acute and subacute adverse events can be monitored. Information on the time course in which acute and subacute adverse events may occur can be obtained from pre-clinical animal data and previous experience in humans if possible. Furthermore, consideration of the duration of the products biological activity should be considered also when choosing the length of the staggering interval.

Patient Specific Products: Cell therapy products are classified as either autologous or allogeneic products. Autologous products involve harvest of cells and re-administration to the same individual. In contrast, allogeneic products are obtained from a selected donor who will ordinarily be healthy and multiple doses may be manufactured for receipt by a number of individuals.

Since cell therapies can take a considerable amount of time to manufacture after collection, the patient's condition must be taken into consideration. Take, for example, that the patient satisfied the enrolment criteria at the point of cell collection, however in the time it has taken to manufacture the drug the patient's disease status has worsened in a manner in which now

does not make him/her eligible to have any further participation within the trial. To account for such circumstances it is recommended that the trials enrolment criteria contain a set of standards to ensure selection of patients that will still be eligible for participation after the manufacturing process is complete. An alternative option is that the patients, at the time of administration, must satisfy an independent set of criteria before they can be deemed fit to receive the product.

Another issue that can be encountered upon product manufacture is failure to successfully generate a product that can be used for administration for the recipient. It is important to consider that the patient's characteristics can influence such issues. For example, the disease and age of the patients may be a predictor of a poor cell yield or cell expansion upon ex vivo culture. It can be argued that likelihood of manufacturing success or failure should be addressed in the batch runs included in the investigational medicinal product dossier (IMPD) in the investigational new drug (IND) application prior to the trial approval. However, such studies offer high risk with low benefits and little or no incentive for the donors to provide such data. It is optimal to address these manufacturing questions as part of the phase 1 trial and the data gathered from this can be used to design later phase trials. Furthermore, this data will highlight to the investigator a set of subject selection criteria that is needed to minimize manufacturing failure.

Safety monitoring and follow up to mitigate risk to the patients: Safety monitoring in the FIH trial will depend on the anticipated adverse events associated with the specific product. Preclinical toxicology studies should provide sufficient data to help in the choice of safety and monitoring tests that must be carried out to assess both anticipated and unanticipated safety concerns. Common safety tests include general examination and recording of symptoms, blood chemistry, blood hematology and or echocardiography if cardiotoxicity is a concern. Immunology tests may also be required if the product is allogeneic or poses an autoimmunity risk. However, aside from the general safety tests, specific tests and monitoring related to product specific anticipated events should be considered. Such tests should be carefully chosen once the capabilities of the monitoring tools and analytic methods available at the trial site have been reviewed. Taking this into consideration implementation of specific safety and monitoring procedures relevant to the stem cell product should be implemented prior to the trial initiation. For example, immunological assays such as cell and humoral responses should be evaluated if immunogenicity of the product is a safety concern. If suitable assays to assess this have not yet been developed, then retention of the baseline and treatment plasma or serum should be considered. This will enable sample evaluation when such assays are made available. It is also suggested that attempts to evaluate product

persistence or biological activity are carried out. Such assessments can be made at the region of administration or from the site of the product's proposed therapeutic activity. This may only be possible if a biopsy can be easily obtained. In addition, protocols can be put in place that if death of a patient occurs within the trial, appropriate post mortem studies can be carried out on tissues/organs to assess persistence or migration of the product [9]. Furthermore, if applicable to the trial site imaging studies can be used to monitor any ectopic formation or aberrant cell activity.

Stem cell therapies are still in the experimental phase and therefore uncertainties still remain regarding the frequency or severity of adverse events. The inclusion of trial stopping rules into the protocol can enable the investigator to control the number of patients that are put at risk particularly if safety concerns start to arise early in the trial. Stopping rules define the number of events or unexpected deaths necessary to put a temporary halt to trial enrolment or dosing. The inclusion of stopping rules does not imply that if such events occur that the trial will be terminated but it allows for the trial to be temporarily suspended until an adequate assessment of the situation has occurred. This can be beneficial to the trial as the correct assessment can enable the revision of the clinical trial protocol in a manner which benefits the safety and welfare of the patient. For example, revisions of the trial protocol may be made after the assessment to the exclude individuals who are more susceptible or at high risk of developing adverse events.

To further reduce the risk for patients enrolled in the trial suitable follow up protocols are suggested to be incorporated into the trial design. Pre-clinical studies, familiarity with the disease and expertise with the stem cell product will help choose a suitable follow up time. In the event that the patient fails to receive the product i.e. disease severity worsens and now the patient fails to meet the inclusion criteria, a suitable follow up protocol must be in place that allows for the risk assessment of the cell harvesting procedure or any subsequent preparation that the patient may have received in preparation for the trial. It is recommended that long term patient monitoring is integral to the trial design. Long term follow-up visits are not required to be as detailed as the initial safety assessments provided in the trial. In some instances, brief phone calls to the patient may be sufficient to obtain the required information. Long term monitoring usually will focus on post-trial patient survival and frequency of adverse events.

FINAL STATEMENT OF WORKING WITH REGULATORY AGENCIES

As with all new therapeutic strategies, our information regarding the biological effect of stem cells is limited and therefore methods to assess safety and efficacy need to be constantly expanded. Adherent stem cell populations such as hMSCs are often portrayed as stem cells that are a well understood, homogenous population of cells that exhibit predictable properties. Although they are the most extensively studied and characterized cell type, great diversity exists in how investigators have defined and manufactured these cells. Major differences in terms of cell sourcing, product manufacture and cell surface marker expression exist amongst different laboratories (Au et al., 2012). In addition, differences in the in vitro and in vivo bioactivity of the cells can vary depending on the donor source. With immense speculation surrounding the field and pressure to deliver effective therapies to patients, product quality and consistency is of upmost importance. Identification of parameters important to the cell safety and efficacy is important to ensure quality. Development of assays and screening for stem cell specific markers early in product development will help build our knowledge about the in vitro and in vivo bioactivity of the cell product. The discovery of biological markers that can predict the intended biologic effect which can then be correlated with a beneficial clinical response are essential. Once identified these parameters can be controlled for in a manner in which the product can be manufactured with a high degree of quality and consistency.

The investment in the development and refinement of new and existing technologies is on-going in the stem cell field. The development of more advanced preclinical models such as humanized mice and replacement of the use of animal-derived sera in the culture media with safer alternatives such as human platelet lysate is facilitating the development of safer stem cell products.

Regulatory agencies around the world are interested in promoting the safe and effective investigation of novel therapies. The investigator should not consider that they are working alone or in an antagonistic manner on their novel therapies. We strongly recommend when planning to apply for FIH stem cell trials that the investigator becomes familiar with the country specific process, reads and follows all guidance documents, and engages with the agencies early in the development of their process. In addition, the discipline required with FIH trials is worthy of pursuit and involves a close interaction between academic investigators, industry and regulators. Progress in the therapeutic use of cell based therapies require investigators to have the skills to navigate the regulatory environment, develop

appropriately designed clinical trials, and consistently manufacture this new class of exciting therapies.

REFERENCES

AU, P., HURSH, D. A., LIM, A., MOOS, M. C., JR. , OH, S. S., SCHNEIDER, B. S. & WITTEN, C. M. 2012. FDA oversight of cell therapy clinical trials. *Sci Transl Med,* 4**,** 149fs31.

BAKER, J. F. & ASSAF, B. T. 2015. Preclinical study design for evaluation of stem cell-derived cellular therapy products: a pathologist's perspective. *Toxicol Pathol,* 43**,** 126-31.

BRAVERY, C. A. & FRENCH, A. 2014. Reference materials for cellular therapeutics. *Cytotherapy,* 16**,** 1187-96.

BRAVERY, C. A., CARMEN, J., FONG, T., OPREA, W., HOOGENDOORN, K. H., WODA, J., BURGER, S. R., ROWLEY, J. A., BONYHADI, M. L. & VAN'T HOF, W. 2013. Potency assay development for cellular therapy products: an ISCT∗ review of the requirements and experiences in the industry. *Cytotherapy,* 15**,** 9-19.e9.

CHANG, Y. K., LIU, Y. P., HO, J. H., HSU, S. C. & LEE, O. K. 2012. Amine-surface-modified superparamagnetic iron oxide nanoparticles interfere with differentiation of human mesenchymal stem cells. *J Orthop Res,* 30**,** 1499-506.

DAVISON, A. C. 2010. Brave pioneers or clinical cowboys? *Cell Stem Cell,* 6**,** 504-5.

DOCKERY, P. & FRAHER, J. 2007. The quantification of vascular beds: a stereological approach. *Exp Mol Pathol,* 82**,** 110-20.

DOMINICI, M., LE BLANC, K., MUELLER, I., SLAPER-CORTENBACH, I., MARINI, F., KRAUSE, D., DEANS, R., KEATING, A., PROCKOP, D. & HORWITZ, E. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy,* 8**,** 315-7.

EUROPEAN MEDICINES AGENCY, COMMITTEE FOR ADVANCED THERAPIES. 2007. Guidline on the risk based approach according to annex I, part IV of directive 2001/83/EC applied to advanced therapy medicinal products. Retrieved from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500139748.pdf

FREY-VASCONCELLS, J., WHITTLESEY, K. J., BAUM, E. & FEIGAL, E. G. 2012. Translation of stem cell research: points to consider in designing preclinical animal studies. *Stem Cells Transl Med,* 1**,** 353-8.

FREY-VASCONCELLS, J., WHITTLESEY, K. J., BAUM, E. & FEIGAL, E. G. 2012. Translation of stem cell research: points to consider in designing preclinical animal studies. *Stem Cells Transl Med,* 1**,** 353-8.

GOLDRING, C. E., DUFFY, P. A., BENVENISTY, N., ANDREWS, P. W., BEN-DAVID, U., EAKINS, R., FRENCH, N., HANLEY, N. A., KELLY, L., KITTERINGHAM, N. R., KURTH, J., LADENHEIM, D., LAVERTY, H., MCBLANE, J., NARAYANAN, G., PATEL, S., REINHARDT, J., ROSSI, A., SHARPE, M. & PARK, B. K. 2011. Assessing the safety of stem cell therapeutics. *Cell Stem Cell,* 8**,** 618-28.

HEALTH PRODUCT REGULATORY AUTHORITY 2015. Guide to clinical trial apllications. Retrieved from: https://www.hpra.ie/docs/default-source/publications-forms/guidance-documents/aut-g0001-guide-to-clinical-trial-applications-v9.pdf?sfvrsn=25.

SENSEBE, L. & FLEURY-CAPPELLESSO, S. 2013. Biodistribution of mesenchymal stem/stromal cells in a preclinical setting. *Stem Cells Int,* 2013**,** 678063.

SONG, P., XIE, Z., GUO, L., WANG, C., XIE, W. & WU, Y. 2012. Human genome-specific real-time PCR method for sensitive detection and reproducible quantitation of human cells in mice. *Stem Cell Rev,* 8**,** 1155-62.

TERROVITIS, J. V., SMITH, R. R. & MARBAN, E. 2010. Assessment and optimization of cell engraftment after transplantation into the heart. *Circ Res,* 106**,** 479-94.

U.S DEPARTMENT OF HEALTH AND HUMAN SERVICES, FOOD AND DRUG ADMINISTRATION.2008. Guidance for Industry, CGMP for Phase 1 Investigational Drugs (2008). Retrieved from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070273.pdf

U.S DEPARTMENT OF HEALTH AND HUMAN SERVICES, FOOD AND DRUG ADMINISTRATION.2013. Good Laboratory Practices for Nonclinical Laboratory Studies . Available at http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?cfrpart=58&showfr=1. Accessed May 19, 2014

U.S DEPARTMENT OF HEALTH AND HUMAN SERVICES, FOOD AND DRUG ADMINISTRATION.2013. Guidance for Industry, Considerations for the Design of Early-Phase Clinical Trials of Cellular and Gene Therapy Products. Retrieved from: http://www.fda.gov/downloads/biologicsbloodvaccines/guidancecompliancegulatoryinformation/guidances/cellularandgenetherapy/ucm359073.pdf. Accessed May 19, 2014

U.S DEPARTMENT OF HEALTH AND HUMAN SERVICES, FOOD AND DRUG ADMINISTRATION.2010. Cellular therapy for cardiac disease. Retrieved from: http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/UCM164345.pdf

U.S DEPARTMENT OF HEALTH AND HUMAN SERVICES, FOOD AND DRUG ADMINISTRATION.2013. Guidance for Industry, Preclincal assessment of investigational cellular and gene therapy products. Retrieved from http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceCompliance

nceRegulatoryInformation/Guidances/CellularandGeneTherapy/UCM376521.pdf

U.S DEPARTMENT OF HEALTH AND HUMAN SERVICES, FOOD AND DRUG ADMINISTRATION 1996. Guidance for industry: E6 good clinical practice: consolidated guidance. Retrieved from: http://www.fda.gov/downloads/Drugs/.../Guidances/ucm073122.pdf

VON BAHR, L., BATSI, I., MOLL, G. Moll, G., HAGG M., SZAKOS, A., SUNBERG B., UZUNEL, M., RINGDEN O.,LE BLANC, K. 2012. Analysis of Tissue Following Mesenchymal Stromal Cell Therapy In Humans Indicates Limited Long-Term Engraftment and No Ectopic Tissue Formation. Stem Cells 2012, **30**: 1575-1578.

YANG, Z., WANG, Y., LI, Y., LIU, Q., ZENG, Q. & XU, X. 2014. Options for tracking GFP-labeled transplanted myoblasts using in vivo fluorescence imaging: implications for tracking stem cell fate. *BMC Biotechnol,* 14**,** 55.

ZHOU, Y. F., BOSCH-MARCE, M., OKUYAMA, H., KRISHNAMACHARY, B., KIMURA, H., ZHANG, L., HUSO, D. L. & SEMENZA, G. L. 2006. Spontaneous transformation of cultured mouse bone marrow-derived stromal cells. *Cancer Res,* 66**,** 10849-54.

AIMS AND OUTLINE OF PRESENT THESIS

The overall aim of this thesis was to generate preclinical data to support an IMP application to allow for the clinical testing of human MSCs in 'no option for revascularisation' critical limb ischaemia patients.

Chapter 2 – Toxicology

In this chapter, we investigated preclinical safety profile of a GMP grade MSC product.

Objective – To investigate the toxicological potential of our MSCs immunodeficient mice.

The aims of the study were:

- *Aim 1* – To address safety parameters with direct administration of MSCs into thigh musculature.
- *Aim 2* – To assess the induction of any local or systemic toxicities after the MSC administration.
- *Aim 3* - To assess the capacity of the MSCs to form ectopic tissue or tumors.

Chapter 3 – Biodistribution

In this chapter, we investigated the biodistributive fate of GMP grade MSCs in immunodeficient mice.

Objective – To develop a novel, accurate, reproducible and quantitative, qPCR-based method of tracking the biodistribution of human MSCs in immuodeficient mice.

The aims of this study were:

- *Aim 1* – To design and validate qPCR assay specific for hAlu in mixed hDNA and mDNA samples.
- *Aim 2* - To test the linear range of the assay e.g. defining the upper and lower limits of the assay.
- *Aim 3* – To assess the biodistribution of a MSC product.

Chapter 4 – Towards A Potency Assay

In this chapter, we used a series of assays to measure the bioactivity of a MSC product.

Objective - To optimize a panel of functional assays that could be used to characterize the angiogenic potential of a MSC product.

The aims of this study were:

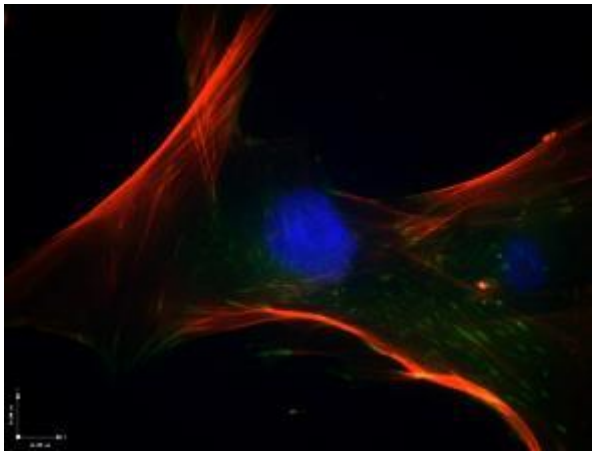
- *Aim 1* – Optimization of in vitro and in vivo assays of angiogenesis.
- *Aim 2* – To assess the angiogenic potential of different human MSC donors.
- *Aim 3* – To screen for targets or biomarkers that may be important for MSC mediated angiogenesis.

Chapter 5 – Thesis Summary

In this chapter, we provide a summary of the results of this thesis.

Chapter 2: A Three Month Toxicology Study of Human Bone Marrow Derived Mesenchymal Stromal Cells Administered Once by the Intramuscular Route to Immunodeficient Mice.

ABSTRACT



Critical limb ischaemia represents the severest manifestation of peripheral arterial disease and is a major unmet medical need. This disease occurs when the arterial blood supply within the limb fails to meet the metabolic demands of the resting muscle or tissue resulting in chronic ischaemic rest pain and/or tissue necrosis. Human mesenchymal stromal cells, termed hMSCs, represent an exciting therapeutic modality for the treatment of this disease due to their immunomodulatory and tissue reparative functions. The aim of the study was to assess the preclinical safety profile of human bone hMSCs in support of their use as a treatment for critical limb ischaemia. A toxicology study was carried out in immunodeficient mice who received a single dose of $3x10^5$ hMSCs via the intramuscular route. No significant clinical signs, haematological, biochemical or histopathological changes were found in the hMSC-treated mice in comparison to the controls. These results highlight that the administration of hMSCs at this dose was well tolerated and not associated with any toxicity including aberrant differentiation or tumorigenesis. This data set supported the initiation of a phase 1b first in human study in "no option" for revascularization patients with critical limb ischaemia.

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Centre for Cell Manufacturing in Ireland (CCMI) contributed to the generation of the GMP grade cells used in this study.

Charles River Laboratories contributed execution of the GLP toxicology study.

INTRODUCTION

Atherosclerotic peripheral arterial disease (PAD) represents one of the most prevalent non-communicable diseases in the world today. CLI, the most advanced form of PAD is associated with high morbidity and mortality. CLI does not symbolize a single pathophysiologic process but is caused by a multitude of different pathogenic mechanisms such as, atherosclerosis; arteritides, hypercoagable states, cardioembolisms and lower limb graft bypass failure (Adam et al., 2005; Gupta and Losordo., 2011). CLI is a disease that presents in patients with great heterogeneity but ultimately occurs when the arterial blood supply within the limb fails to meet the metabolic demands of the resting muscle or tissue, resulting in chronic ischaemic rest pain and/or tissue necrosis, often leading to amputation (Gupta and Losordo., 2011). Furthermore, CLI is associated with an alarmingly high fatality rate as a result of ischemic cardiovascular events with higher mortality rates similar to, if not worse than, other serious medical conditions (Armstrong et al, 2007). With over 200 million estimated to be suffering from PAD and with reports documenting an increased prevalence of CLI it is almost a certainty that this disease burden will continue to rise in the absence of preventative measures (Fowkes et al, 2013; Hirsch et al, 2013) – Impacting negatively on both global health and the economy.

The most widely studied CT and nearest to becoming clinically translated to CLI patients is MSCs. Although MSCs full mechanism of action remains unclear, what has been realised is that these cells act as growth factor/cytokine factories and in this way augment the tissue repair and regeneration process. Collateral blood vessel development remains the therapeutic and prognostic determinant in CLI patients. The damage in the limbs that caused due to ischaemia in CLI patients is complex and tissue reparation will require multiple different mechanisms. Thus far, the use of single agents such as the delivery of growth factors and genes has failed to demonstrate efficacy in pivotal human trials. To address this unmet medical need, investigators have explored the administration of MSCs via multiple different routes of administration and the preclinical data has been very promising. Several groups have shown that MSCs can improve perfusion and augment recovery in mice with hindlimb ischemia (Kinnaird et al., 2004; Gremmels et al, 2013; Smadja et al. 2012; Kinnaird et al., Leroux et al, 2010.). Recently Bortolotti *et al* compared the therapeutic properties of three MSC populations and showed that each MSC type provided therapeutic benefit after hindlimb ischemia. However, they showed that MSCs derived from the bone marrow were the most effective at restoring tissue perfusion. Bone marrow MSCs superior therapeutic activity, in comparison to the other MSC types was associated with increased cytokine/growth factor production (TGF-β, PDGF-β and MMP9), longer engraftment and increased smooth muscle

migration (Bortolotti et al., 2016). Several other groups including our own have shown that MSCs secrete large amounts of angiogenic cytokines and growth factors which provide trophic support for neoendothelium, aid in endothelial precursor cell recruitment, extracellular matrix remodelling and promote neovascularization by augmenting angiogenesis and arteriogenesis (Kwon et al., 2014; Smadja et al., 2012.; Hung et al, 2007).

The complex nature of CT products requires great scientific effort in order to ensure successful translation form bench to bedside. Every step in the development of a CT product requires complete scientific rigour from manufacturing processes to preclinical testing to clinical administration. While establishing efficacy is critical to CT product development, the successful implementation of these therapies will also heavily rely on resolving potential safety concerns both pre-clinically and clinically. Delivery toxicity and histological efficacy represent the two most important safety aspects of CT products. Clinical studies testing MSCs for CLI have shown an encouraging safety profile (Das et al., 2013; Lasala et al., 2011, Lu et al. 2011). However, in spite of this data, regulatory guidelines suggest that preclinical safety evaluations must be carried out on a "case by case" basis for all new investigational products prior to clinical testing (U.S. FDA, 2013). These guidelines suggest that safety is determined based on the individual products attributes and due to the heterogeneity of each CT product (manufacturing, cell type, mechanism of action) the safety of each new product must be evaluated individually (U.S. FDA, 2013).

In this chapter, we present the design and the results of our preclinical safety study that was submitted to the HPRA as part of an IMP application to support the clinical testing of our human MSC product. This study assessed key safety concerns surrounding local or systemic toxicities after the direct administration of the MSCs via the intended clinical route of administration. To further maximize the clinical applicability, the cells used in this study were manufactured under GMP conditions.

SHORT TERM TOXICITY STUDY

METHODS

Animals, Cell preparation, Treatments and Experimental Procedures

Animals

Male and Female SCID mice (strain C.B-17/lcrHsd-prkdc^{scid}) were obtained from Harlan laboratories (UK) and maintained on standard chow. They were allowed free access to drinking water supplied to each cage via polyethylene bottles with stainless steel sipper tubes. During the study period mice were individually housed in individually ventilated cages (IVC) with wood shavings as a bedding material.

Cell preparation

Bone marrow human MSCs (hMSCs) were prepared in a GMP like manner. Cells were tested for mycoplasma and stored in the vapour phase of the liquid nitrogen tank until use. Prior to cell injection hMSCs were thawed, counted using trypan blue exclusion and re-suspended in 50µl of surgical saline.

Route of administration

Because the intramuscular (IM) route of administration corresponds to the route projected in the proposed clinical trial, it was selected as the method to administer the dose. Human MSCs and saline were injected into the left thigh musculature using a 30-gauge needle.

Cell Dose

In the clinical trial design, the proposed maximum cell dose that was going to be administed to the patient was $70x10^6$ MSCs. Taking that the average weight of an adult male is approximately 70 kilograms (kg) we calculated a dose that would be suitable taking into consideration the weight of the mouse.

$70x10^6$ hMSCs administered to a 70kg adult male would approximately equal to 30,000 hMSCs to a 30g mouse when scaling from mouse to human.

For our injections into the mice in this study, we chose to increase this figure of 30,000 tenfold to $3x10^5$ as this represents 10 times the maximum dose that we proposed to administer in the trial.

Study Design

This toxicity study included four male and three females per group (Figure 1A). Group one received an IM injection of 50 µl of saline. Group two received an IM injection of $3x10^5$ hMSC (Passage 2) suspended in 50 µl of surgical saline. For the two groups the total injection volume was divided into two injection sites (25 µl per site) in the left thigh musculature. Animals were randomly assigned to study groups using computer generated randomisation. All animals were monitored for any abnormal clinical signs post injection for example changes in fur, piloerection, unusual respiratory patterns, diarrhoea etc.

Biochemistry

Blood for biochemistry was collected prior to euthanasia. Blood was drawn from all mice via cardiac puncture. At least 150-200µl serum was collected into non-coated tubes. The tubes were stored at -80°C until transported to the analytical laboratory.

Necropsy and Tissue Handling

Complete necropsy and macroscopic examinations were performed on all treated and control animals. The muscles samples were collected and fixed in 10% neutral buffered formalin.

The injection site (left thigh musculature) was trimmed in the middle on both sides (right and left) approx. 2-3mm from the middle section, dehydrated in graded ethanol, cleared in xylene, embedded in paraffin wax, and sectioned to approximately 5µm thickness. All of the prepared sections were stained with hematoxylin and eosin (H&E) and examined microscopically by two pathologists.

Statistical Analysis

Where appropriate, a Student's t-test was used to determine differences across groups for body weight and blood biochemistry.

3 MONTH TOXICOLOGY STUDY CONDUCTED UNDER GOOD LABORATORY PRACTICES

Note: As toxicology studies used to supported the clinical testing of MSC products must be carried out in accordance with GLP we therefore opted to outsource the the longer term toxixology study to Charles River Preclinical Services that were based in Edinburgh. Although this study was conducted in Charles Rivers, I designed the study protocol with the aid of Prof O'Brien. Furthermore, I was present during the procedures and helped manage and co-ordinate the study.

METHODS

Manufacturing Process of the Bone Marrow Derived MSCs

Human MSCs were isolated from adult bone marrow and cultured expanded in accordance with local ethical approval and GMP regulations. Upon receipt, the bone marrow aspirate was washed with Dulbecco's Phosphate Buffered Saline (DPBS) and centrifuged at 900g. A 4% acetic acid wash was performed on a sample of the marrow to lyse the red blood cells and enable an accurate mononuclear cell (MNC) count. MNCs, plated at 40-50 million per $175cm^2$ were cultured expanded in monolayer with complete medium (αMEM supplemented with 10% selected foetal bovine serum [FBS]) in 5% carbon dioxide (CO_2) at 37°C. On day 3, cell culture medium was removed and 100% fresh medium was added to the culture. On day 5, the cultures were washed with DPBS to remove non-adherent cells and fresh complete medium was added to each flask. When the monolayer reached 80-90% confluence, the adherent cells were washed with DPBS and detached from the culture plastic with 0.25% trypsin/EDTA. The dissociated cells were centrifuged at 400g for 5 minutes. The resultant pellet was re-suspended in complete fresh medium and the cellular yield determined. hMSCs were further sub-cultured in triple flasks through two passages (Figure 1). hMSCs were cryopreserved at a dose of $2x10^6$ per ml in FBS combined with 10% dimethyl sulfoxide.

The cells were harvested, and suspended in a final formulation of 10% dimethyl sulfoxide (DMSO) and 4.5% human serum albumin (HSA). The hMSCs were filled into 1.8ml cryovials at a final dosage of $2x10^6$ /ml and cryopreserved at -150°C storage following controlled rate freezing at -1°C/min from room temperature to -80°C.

Following cryopreservation, the hMSCs product was tested for mycoplasma, endotoxin, sterility and viability. The cells also underwent karyology and immunophenotyping.

For immunophenotype characterization, the cells were stained with antibodies for MSC-positive markers CD73, CD90, CD105 and for negative markers CD45, CD34, Cd11b, CD19, and HLA-DR. The immune phenotype test specifications were set as ≥90% for all positive markers and ≤5% for all negative markers.

Upon meeting the release criteria, the hMSCs were then shipped to Charles River Laboratories Preclinical Services, Tranent (PCS-EDI), Edinburgh, EH33 2NE, UK on dry ice where they were received and stored in liquid nitrogen until the initiation of the toxicology study.

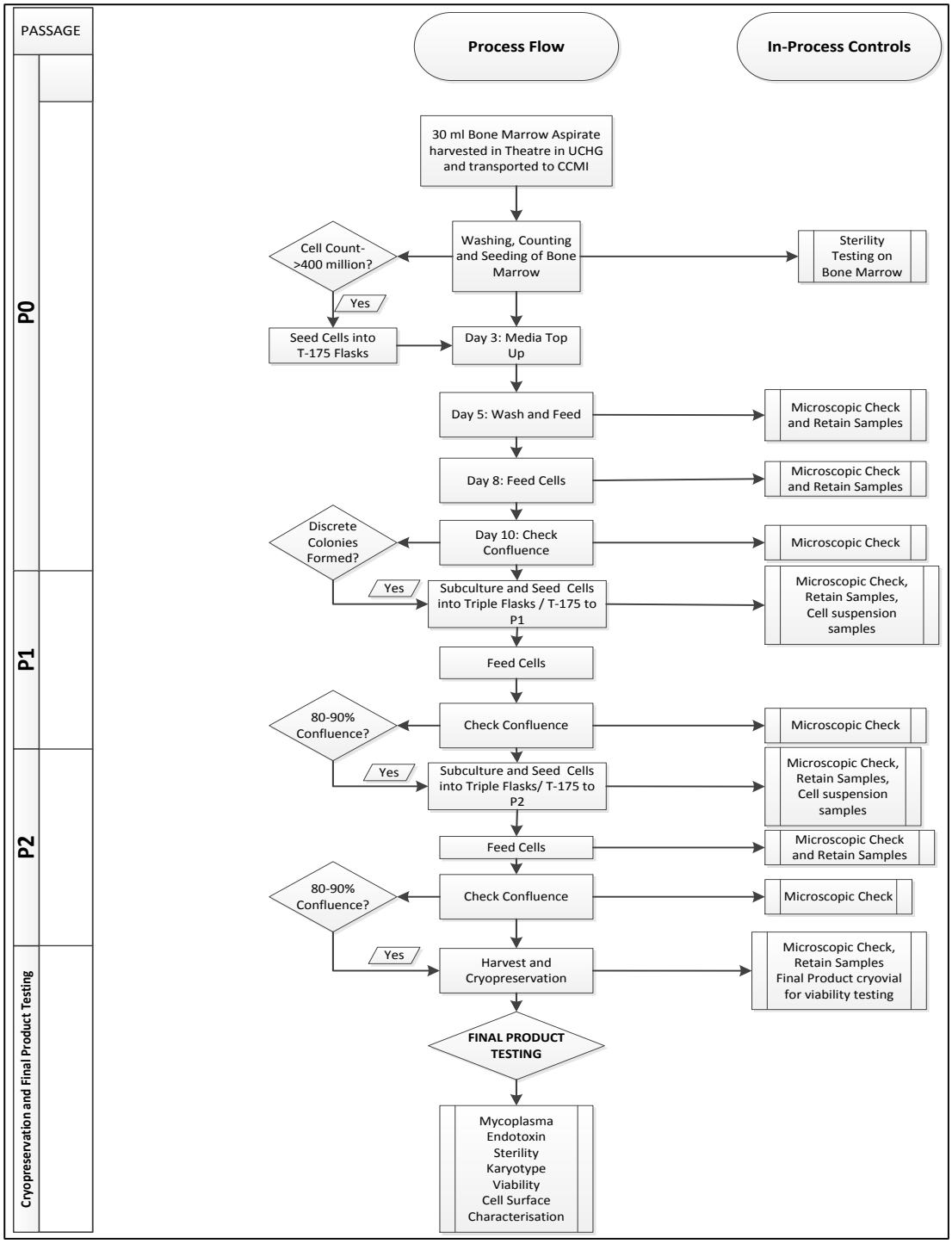


Figure 1: Process flow for the GMP manufacturing of hMSC used for the toxicology study.

Animals, Treatments, and Experimental Procedures

Animal care and administration of the hMSCs were conducted in Charles River preclinical services, a GLP certified site. Approval was obtained under the Animal Scientific Procedures Act (ASPA) 1986 by the Home Office in Scotland before initiation of the study.

Male and female BALB/c nude mice (Hsd-*Foxn1*^{nu}) were obtained from Harlan Ltd, (Oxon, United Kingdom) and maintained on a Teklad Rodent Diet 2919. The diet and water was provided *ad libitum* except during designated procedures. During the acclimation period and study duration, animals were housed in a limited access rodent facility and kept in groups of 2 or 3 per cage in appropriately sized polycarbonate/polypropylene cages with stainless steel grid tops and solid bottoms. Each cage was fitted with a filter top and had sterilized white wood shavings. However, the bedding material was changed during the study to help reduce eye irritation in the mice. The mice were allowed a *ca*. 2-week acclimation period to the Charles River facility conditions (19°C - 23°C, 40% - 85% relative humidity and a 12 hour light/dark cycle) prior to inclusion in the study.

hMSC Preparation

hMSCs were thawed and prepared immediately before injection. The cells were removed from liquid nitrogen, thawed in a 37°C water bath and transferred directly into tubes containing the 4ml of saline vehicle. The cryovial was washed once to ensure that all cells were removed. A cell count was performed using the trypan blue (Sigma Aldrich) exclusion method. A cell suspension containing a total number $3x10^5$cells/ml was transferred into 15 ml conical tubes and centrifuged at 400g for 5 minutes at room temperature. The supernatant from the centrifuged cells was discarded and the pellet of hMSCs was re-suspended in 150 µl of saline vehicle. The cell suspension was mixed well and transferred to a sterile cryovial. The cell suspension was transferred to three insulin syringes. 50 µl of cell suspension was aspirated into 3 syringes. The hMSCs were administered to the animals within 2 hours of re-suspension in saline.

hMSC Transplantation

The intramuscular route of administration corresponds to the anticipated route in the projected forthcoming clinical trial and therefore was selected as the method of delivery. hMSCs were administered at a dose of $3x10^5$ cells in 150 µl per animal. The total volume was divided between 3 injection sites (50 µl per site), two in the thigh and one in the calf on the right leg.

Each injection was administered over *ca.* 1 to 3 seconds. The control animals received 3 injections of 150 µl of saline in a similar manner. Animals in each group were subjected to termination at 3 months after the hMSCs administration.

Toxicology Study Design

The study included 8 male and 8 female mice per group (Table 1). Animals in each group were subjected to study termination at 3 months after the hMSCs dosing. Animals were weighed and randomly assigned to the 2 treatment groups (Table1).

		Treatment		
Group	No. of animals per group	Test Material	Dose (cells/150 µL)	Study period
Male	8	Saline	0	3 months
Female	8	Saline	0	3 months
Male	8	hMSC	300,000	3 months
Female	8	hMSC	300,000	3 months

Table 1: Experimental study design – 3-month toxicology study.

Food Consumption

Food consumption was quantitatively measured once weekly throughout the study. Measurements were started at -1 week and the last food consumption was carried out prior to the scheduled study termination. Surviving animals were euthanized by CO_2 asphyxiation prior to scheduled necropsy.

Haematology and Biochemistry

Blood was collected from the vena cava at necropsy. Blood samples were collected, in ethylenediaminetetraacetic acid (EDTA) coated tubes for haematology and lithium heparin tubes for clinical chemistry. Animals were euthanized via CO_2 asphyxiation followed by exsanguination. Blood samples were obtained via the vena cava using a hypodermic needle and a plastic syringe. Animals were fasted for 4 hours prior to their scheduled necropsy.

Necropsy

Animals were subjected to a complete necropsy examination, which included evaluation of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs and tissues.

Representative samples from the following tissues and organs were collected and fixed in 10% neutral buffered formalin: administration site (right leg with the foot attached); aorta; femur; sternum; bone marrow (femur and sternum), brain; cervix; epididymides; eyes; gallbladder; adrenal glands; harderian glands; lacrimnal glands; mammary glands; pituitary glands; salivary glands; seminal vesicle; thyroid gland; gut-associated lymphoid tissue; heart, kidneys; caecum; colon; rectum; liver; lung; mandibular lymph node; mesenteric lymph node; inguinal lymph node, skeletal muscle (left leg); nasal cavity; sciatic nerve (left leg); oesophagus; ovaries; oviducts; pancreas; skin; duodenum; ileum; jejunum; spinal cord; stomach; thymus; tongue; trachea; ureters; urinary bladder; uterus and vagina. The optic nerves were fixed in Davidson's fixative and the testes in modified Davidson's fixative. In addition, any organ/tissue with gross macroscopic change(s) was collected recorded and fixed in 10% neutral buffered formalin.

Tissues were trimmed, embedded with paraffin, sectioned, mounted on glass slides and stained with hematoxylin and eosin. Bone marrow smears were stained with May Grünwald Giemsa staining. The injection sites (right thigh and calf musculature) were removed after whole leg fixation and samples were labelled from 1-5. The thigh muscle was cut into 3 equal parts and labelled from 1 – 3. Injection site 1 = proximal thigh; injection site 2 = middle thigh; injection site 3 = distal thigh. The calf muscle was also dissected and cut into 2 equal pieces and labelled 4 and 5. Injection site 4 = proximal calf; injection site 5 distal calf. Each injection site form the right leg was embedded in separate paraffin wax blocks (see appendix). One 5µm section was cut from each block, stained with H&E and evaluated microscopically.

The muscle from the left limb served as an internal control for each mouse. Briefly, the thigh muscle was dissected and labelled form 1 – 3 as above. One 5µm section was obtained from injection site 2 (middle thigh) was then cut, stained with hematoxyylin and eosin and evaluated microscopically.

Lesion Grading

Histopathological changes such as infiltration, inflammation, degeneration, atrophy, hyperplasia, haemorrhaging, plasmaycytosis and edema were scored using a semi-quantitative grading of five grades (0 - 4); 0 = no visible lesion, 1 = minimal change, 2 = mild change, 3 = moderate change, 4 = marked change.

Body and Organ weights

Males and females were randomized separately and body weights were measured at randomization, on the day of injection and once weekly thereafter for the study duration. The group body weights of the animals were compared to ensure homogeneity.

Organ weights were taken at scheduled necropsy after blood sampling from all the surviving animals. Paired organs were reported together and the terminal body weights were used for organ weight analysis.

Statistical Analysis

Body weights, food consumption, haematology and clinical chemistry (females only due to insufficient data in the male animals) were analysed for homogeneity of variance using the 'F Max' test. The group variances were homogenous, therefore a parametric ANOVA was used and pairwise comparisons were made using Fisher's F protected LSD method via Student's t test.

RESULTS OF SHORT TERM TOXICITY STUDY

Clinical observations

No mortality in reaction to the treatment occurred in any of the mice prior to the scheduled study termination. Furthermore, there were no changes observed in any of the male or female MSC treated or control animals (Figure 2B).

Biochemistry

Due to red blood contamination, the serum of 8 mice were discarded. Figure 2C represents the blood biochemistries from individual male and female mice in the saline and the hMSC treated groups. There was no treatment related effects on the biochemical parameters measured in this study.

A

Experimental Study Design

Group	No animals per group	Test material	Dose (cells/50 µl)	Study period
1	4 males and 3 females	Saline	0	7 days
2	4 males and 3 females	hMSCs	300,000	7 days

B

Parameter	7-Day Toxicity Study	
	Saline Injection	hMSC Injection
Males		
Terminal body Weight (g)	33.1 (3.10)	31.9 (3.74)
Females		
Terminal body Weight (g))	25.8 (3.30)	24.7 (4.08)

Note: values are mean (SD).

C

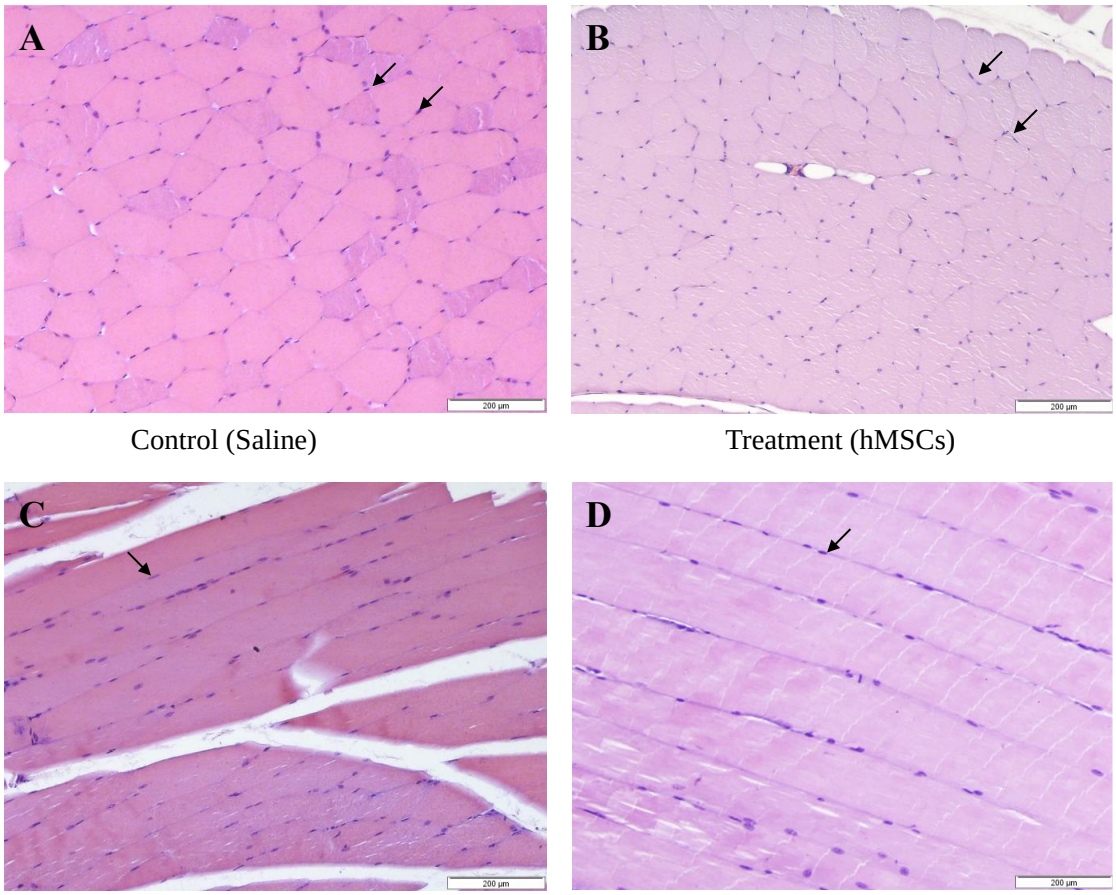
7-Day Toxicity Study - Blood Biochemistry

Group / sex		**Glu mmol/L**	**Urea mmol/L**	**Alb g/L**	**ALT U/L**	**LDH U/L**	**Chol mmol/L**	**Tg mmol/L**
Male Vehicle	Mean	17.25	6.22	30.15	30.1	586	3.29	0.96
	SD	0.81	1.22	0.21	9.48	253.14	0.08	0.49
	n	2	2	2	2	2	2	2
Male hMSC	Mean	16.27	7.84	31.3	24.03	351.25	3.88	1.07
	SD	1.54	1.22	0.68	3.83	97.72	0.13	0.13
	n	4	4	4	4	4	4	4
Female Vehicle	Mean	15.76	6.82	34.3	23.2	349	2.45	1.28
	SD	-	-	-	-	-	-	-
	n	1	1	1	1	1	1	1
Female hMSCs	Mean	19.22	10.3	32.5	47.8	377	2.55	0.44
	SD	-	-	-	-	-	-	-
	n	1	1	1	1	1	1	1

Figure 2: 7-Day Toxicity Study – incl. Experimental Study Design & Blood Chemistry. (A) 7-day toxicity study experimental design. (B) Mean weights of male and female SCID mice in the 7-day toxicity study following intramuscular injections with hMSCs or saline. (C) Selected biochemistry analyses of SCID mice in the 7-day toxicity study following intramuscular injections with hMSCs or saline. *Abbreviations: GLU, glucose; ALB, albumin; ALT, alanine aminotransferase; TG, triglycerides; CHOL, cholesterol; LDH, lactate dehydrogenase.*

Macroscopic and Histopathological findings

No macroscopic abnormalities were present at necropsy in any of the saline or hMSC treated mouse limbs after 7 days (Figure 3). Evaluation of the thigh muscles of the animals (saline and hMSC treated) sacrificed at 7 days by H&E staining confirmed that there was no inflammation, no significant muscle atrophy and no tumours present in any muscles of the males or females in the saline or hMSC treated groups.



Control (Saline) Treatment (hMSCs)

Control (Saline) Treatment (hMSCs)

Figure 3. Representative H&E stained transverse and longitudinal sections of SCID mouse thigh muscles 7 days after intramuscular injections with saline and hMSCs. Transverse (A) and Longitudinal (C) muscle sections after intramuscular injections with 50µl of saline (10x magnification). Transverse (B) and longitudinal (D) muscle sections after intramuscular injections with $3x10^5$ hMSCs (10x magnification). (A, B) Muscle fibres exhibit normal polygonal shape and are characteristically showing one or more peripheral nuclei (arrows). (C, D) Nuclei (arrows) are aligned in defined rows with eosinophilic muscle fibres in between. (A-D) No inflammation, significant muscle atrophy or tumours present in any of the treatment or controls muscle upon microscopic examination.

Summary of Short Term Toxicology Study

In view of our reported findings that under the conditions of this study, we can conclude that the administration of hMSCs following a single dose of $3x10^5$ cells/50µl was not associated with adverse effects. No muscular toxicity was detected macroscopically or microscopically.

RESULTS OF 3 MONTH GLP TOXICOLOGY STUDY

Unscheduled Death

One animal was found dead in its cage prior to terminal necropsy. The death occurred in the saline treated group and was not test article related. Nonetheless, the animal was subject to necropsy and the full tissue list was obtained.

Clinical observations

There were no test-item related clinical signs post dose or throughout the 3-month study period (Table 2). Both hMSCs and saline treated animals demonstrated transient inflammation (swollen eyes) of the eyes. This issue was due to the type of bedding used in the cages and as a result both the bedding and nestle material was changed. Other occasional observations included skin redness (hMSC and vehicle males) and overgrown toe-nails (hMSCs and vehicle females).

Observation	Male Vehicle	Male hMSCs	Female Vehicle	Female hMSCs
Number of Animals	16	16	16	16
Terminal Kill	15	16	16	16
Unscheduled Death: Found Dead	1	-	-	-
Bulging in eye(s)	1	-	1	-
Partially closed both eyes	1	-	-	-
Eye(s): damaged, clear, pale, opaque, discharge	5	7	6	3
Lesion: dorsal abdomen, dorsal neck, dorsal surface, dorsal thorax, extremeties, head, ventral abdomen, ventral thorax, whole body	7	5	1	1
Lid(s): encrusted, inflamed, swollen	10	16	7	16
Scabs: dorsal abdomen, dorsal surface, tail, ventral abdomen, whole body	1	3	-	-
Tail, nodules	-	-	1	-
Mass subcutaneous left ventral abdomen	-	1	-	-
Skin:redness, scabbing, discoloured, pale	3	1	-	-
Perigenital swelling	-	1	-	-
Toe nails overgrown	-	1	5	10
Vagina, opaque discharge	-	-	-	1

- = not applicable

Table 2: Incidence of clinical observations in hMSCs and vehicle control over the 97-day study period.

Food Consumption

Food consumption measurements of the hMSCs treated groups were similar to those of the vehicle control group throughout the 3-month study duration (Table 3).

Group / sex		Day 0	7	14	21	28	35	42	49	56	63	70	77	84	91	97
Male Vehicle	Mean	3.5	3.7	3.7	3.6	3.8	3.9	4.2	3.8	4.1	4.1	4.2	4.2	4.2	4.4	4.9
	SD	0.4	0.5	0.4	0.3	0.4	0.4	0.9	0.5	0.4	0.2	0.2	0.5	0.5	0.3	1
	n	6	6	6	6	6	6	6	6	6	5	5	6	6	6	6
Male hMSCs	Mean	3.5	3.6	3.8	3.7	3.8	3.9	4	3.8	4.1	4.1	4.3	4.3	4.3	4.4	4.3
	SD	0.1	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.5	0.3	0.4	0.4
	n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Female Vehicle	Mean	3	3.2	3.2	3.2	3.3	3.4	3.3	3.3	3.7	3.5	3.6	3.5	3.5	3.7	3.5
	SD	0.3	0.2	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.4	0.2
	n	4	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Female hMSCs	Mean	3.1	3.2	3.2	3.3	3.3	3.3	3.3	3.4	3.6	3.6	3.7	3.5	3.5	3.8	3.7
	SD	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.1
	n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6

Significantly different from Group 1:a=p<0.5, b= p<0.01, c= p<0.0

Table 3: Group mean values of food consumption in hMSCs and vehicle control nude mice over 97-day study period.

Body weights

There were no test item related findings on body weight gain and final body weight over the study period in either male or female animals (Table 4). Although group mean body weights were increased in hMSC treated males, compared with control males (Table 4), for most of the study, this observation was considered incidental due to the fact that hMSC injected mice body weights were slightly greater than control mice values from pre-trial (Day -7).

Group / Sex		Day -7	0	7	14	21	28	35	42	49	56	63	70	77	84	91	97	Change 0-97
Male Vehicle	Mean	20.4	21.3	21.9	22.1	22.8	23.4	23.6	23.7	24.7	24.3	25.1	25.1	25.5	25.5	25.9	26.2	4.9
	SD	1.2	1.3	0.9	1	0.8	1	0.9	0.9	0.9	0.9	1	1	0.9	1.1	1.1	1.1	1
	n	16	16	16	16	16	16	16	16	16	16	16	16	15	15	15	15	15
Male hMSCs	Mean	21.5**	21.6	22.6*	23.0*	23.6*	24	24.2	24.3	25.2	25.3**	25.5	25.7	26.4*	26.4*	26.1	26.6	5
	SD	0.9	1.1	1	1	1	1.1	1.1	1	1	1	0.9	1	1	1.1	1.2	1	1.2
	n	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16
Female Vehicle	Mean	17.2	17.9	17.7	18.7	19	19.4	19.7	20.1	20.4	20.1	20.8	20.9	20.8	21.5	21.3	21.6	3.7
	SD	1	0.8	0.8	0.8	0.8	0.9	0.8	1	1	0.9	0.9	1	1	1.1	0.9	1.1	0.9
	n	15	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16
Female hMSCs	Mean	16.8	17.5	17.8	18.7	19.1	19.4	19.6	20	20.4	20.1	20.8	21	21.1	21.6	21.4	21.6	4.1
	SD	0.8	0.6	0.6	0.6	0.6	0.7	0.7	0.8	0.8	0.8	0.9	0.8	0.9	1	1	0.9	0.9
	n	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16

Statistical difference from control mean expressed as p values:*p<0.05, **p<0.005.

Table 4: Group mean body weights of nude mice in hMSCs and vehicle control over the 97-day study period.

Organ weights

Absolute organ weights are presented in Table 5. One statistically significant difference versus the control was noted. A decrease ($p < 0.01$) in the mean lung weight was noted in the hMSC treated male when compared to the control.

Organ weight as % body weight is also presented in Table 5. Two statistically significant differences versus the control were noted. A decrease ($p<0.05$) in the mean brain weight and a decrease ($p<0.01$) in the mean lung weight were noted in the hMSC treated male when compared to the control.

	Treatment	
Parameter	Vehicle	hMSCs
Males		
Terminal Body Weight (g)	24.9 (1.6)	25.5 (1.2)
Organ Weight (g)		
Adrenals	0.0048 (0.0005)	0.00042 (0.0008)
Brain	0.43 (0.02)	0.42 (0.06)
Epididymides	0.0727 (0.0049)	0.0773 (0.00097)
Heart	0.16 (0.01)	0.18 (0.02)
Kidneys	0.513 (0.026)	0.531 (0.027)
Liver	1.58 (0.11)	1.58 (0.10)
Lung	0.25 (0.04)	0.19 (0.01)**
Prostate	0.010 (0.003)	0.011 (0.003)
Spleen	0.133 (0.012)	0.121 (0.032)
Testes	0.21 (0.01)	0.21 (0.02)
Thymus	0.010 (0.003)	0.010 (0.005)
Organ to Body Weight Ratio (mg organ weight/g body weight)		
Adrenals	0.01921 (0.00235)	0.01637 (0.00316)
Brain	1.744 (0.069)	1.653 (0.078)*
Epididymides	0.2928 (0.01223)	0.30290 (0.03295)
Heart	0.639 (0.049)	0.689 (0.106)
Kidneys	2.0650 (0.0536)	2.0844 (0.1049)
Liver	6.380 (0.405)	6.196 (0.251)
Lung	1.021 (0.200)	0.726 (0.056)**
Prostate	0.0388 (0.0115)	0.0413 (0.0129)
Spleen	0.5382 (0.0562)	0.4725 (0.1129)
Testes	0.834 (0.039)	0.811 (0.095)
Thymus	0.0402 (0.0129)	0.0386 (0.0202)
Females		
Terminal Body Weight (g)	20.9 (0.8)	20.4 (0.9)
Organ Weight (g)		
Adrenals	0.0099 (0.0014)	0.0101 (0.0022)
Brain	0.43 (0.02)	0.43 (0.04)
Heart	0.12 (0.01)	0.11 (0.01)
Kidneys	0.316 (0.019)	0.317 (0.017)
Liver	1.22 (0.07)	1.18 (0.09)
Lung	0.18 (0.03)	0.17 (0.03)
Ovaries	0.0118 (0.0026)	0.0101 (0.0015)
Spleen	0.177 (0.036)	0.161 (0.033)
Thymus	0.007 (0.002)	0.007 (0.002)
Uterus	0.14 (0.03)	0.12 (0.04)
Organ to Body Weight Ratio (mg organ weight/g body weight)		
Adrenals	0.04751 (0.00539)	0.04939 (0.00894)
Brain	2.049 (0.052)	2.133 (0.223)
Heart	0.552 (0.075)	0.547 (0.032)
Kidneys	1.5136 (0.0758)	1.5556 (0.0795)
Liver	5.850 (0.242)	5.764 (0.315)
Lung	0.868 (0.1696)	0.822 (0.132)
Ovaries	0.05628 (0.01032)	0.0496 (0.00812)
Spleen	0.8498 (0.1696)	0.7864 (0.1461)
Thymus	0.0354 (0.0111)	0.0338 (0.0102)
Uterus	0.678 (0.133)	0.589 (0.174)

Values represent Mean (Standard Deviation). Statistical difference from control mean expressed as p values:*p<0.05, **p<0.005.

Table 5: Absolute organ weights and organ to body weight ratio of male and female nude mice following the injection of hMSCs or vehicle control.

Haematology and Biochemistry

Due to issues surrounding the blood collection from mice and sample quality some parameters could not be read in the haematology and biochemistry in both the treatment and the control mice. Missing data was due to insufficient sample collection or clotting of the sample.

In the samples that were analysed there were no changes in haematology evaluations attributable to the administration of hMSCs (Table 6). Clinical chemistry evaluations indicated a twofold increase in plasma creatine phosphokinase after 3 months in male and female hMSC injected mice in comparison to the control mice. However, the increase was not statistically significant (Table 7).

Group/ Sex		Hb g/dL	RBC x10^12/L	Hct L/L	MCH fL	MCV fL	MCHC g/dL	RDW %	Reti %	Ret x10^9/L	WBC x10^9/L	Neut x10^9/L	Lymph x10^9/L	Mono x10^9/L	Eos x10^9/L	Baso x10^9/L	LUC x10^9/L	Plat x10^9/L
Male Vehicle	Mean	19.3	12.22	0.623	15.8	51	30.9	14.1	2.9	352	5.7	1.12	3.51	0.16	0.7	0.21	0.02	-
	SD	2.1	1.16	0.055	0.2	0.3	0.7	0.1	0.1	27	0.84	0.02	0.58	0.06	0.23	0.04	0.01	-
	n	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0
Male hMSCs	Mean	18.2	11.89	0.586	15.3	49.3	30.9	14.1	3.2	381	11.58	3.06	7.17	0.5	0.63	0.18	0.06	662
	SD	0.8	0.13	0.001	0.5	0.7	1.4	0.1	0.6	69	5.06	1.26	3	0.22	0.45	0.1	0.04	132
	n	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Female Vehicle	Mean	18.4	11.54	0.579	15.9	50.2	31.7	13.2	3	344	7.21	1.42	4.99	0.3	0.34	0.12	0.05	698
	SD	0.7	0.34	0.015	0.2	0.7	0.7	0.3	0.6	74	2.48	0.56	1.93	0.11	0.14	0.06	0.02	391
	n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	5
Female hMSCs	Mean	17.9	11.19	0.571	16.1	51.1	31.4	13.5	3.1	342	6.57	1.5	4.16	0.35	0.43	0.09	0.05	778
	SD	0.3	0.18	0.013	0.2	0.8	0.4	0.3	0.8	87	4.41	0.9	3.47	0.17	0.23	0.05	0.04	196
	n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	4

Table 6: Selected haematology analyses of male and female nude mice following the injection of hMSCs or vehicle control. *Abbreviations: Hb, haemoglobin; RBC, red blood cell count; Hct, haematocrit; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; RDW, red blood cell distribution width; Ret, reticulocytes; WBC, white blood cell count; Neut, neutrophils; Lymph, lymphocytes; Mono; monocytes; Eos, eosinophils; Baso, basophils; LUC, large unstained cells; Plat, platelets.*

Group / sex		ALP U/L	ALT U/L	LDH U/L	CPK U/L	Urea mmol/L	Glu mmol/L	T.Bil µmol/L	Chol mmol/L	Alb g/L	Na mmol/L	K mmol/L	Cl mmol/L	Phos mmol/L	Ca mmol/L	Crea µmol/L
Male Vehicle	Mean	96	21	170	70	4.3	16.61	1.9	4.3	33	151	9.3	108	3.15	2.77	20
	SD	-	3	-	7	-	0.21	0.2	0.9	1	4	0.7	3	-	0.06	2
	n	1	2	1	3	1	2	2	2	2	8	4	8	1	2	4
Male hMSC	Mean	-	35	-	126	-	10.05	-	-	31	152	9.5	111	-	2.72	18
	SD	-	10	-	42	-	3.60	-	-	3	3	0.4	4	-	0.04	0
	n	-	4	-	4	-	3	-	-	3	7	4	7	-	2	4
Female Vehicle	Mean	-	24	-	84	6.7	14.41	2.6	2.9	37	153	8.8	113	3.01	2.86	19
	SD	-	2	-	31	-	0.59	-	-	1	3	0.9	2	-	-	2
	n	-	4	-	5	1	2	1	1	2	8	7	8	1	1	6
Female hMSCs	Mean	-	30	-	192	7.2	12.45	1.9	3.0	36	150	8.7	112	-	2.72	19
	SD	-	9	-	175	-	5.38	0.1	0.4	1	4	1	1	-	0.1	1
	n	-	3	-	3	1	3	2	2	3	7	5	7	0	2	4

Table 7: Selected biochemistry analyses of male and female nude mice following the injection of hMSCs or vehicle control. *Abbreviations: ALP; alkaline phosphatase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CPK, creatine phosphokinase; Urea, urea nitogen; Glu, glucose; T.Bil, total bilirubin; Chol, cholesterol; Alb, albumin; Na, sodium; K, potassium; Cl, chloride; Phos, phosphorus; Ca, calcium; Crea; creatinine.*

Macroscopic and Histopathological Findings

Macroscopic findings at necropsy are summarized in Table 8. One animal in the male hMSCs group developed a subcutaneous mass which was determined histologically as an abscess of the preputial gland and was therefore non treatment related. Dark foci and mild to moderate discolouration consistent with haemorrhaging was observed in both male and female treated and control animals (Table 8). These lesions were an expected find and were considered as artefacts of the euthanasia procedure (CO_2 inhalation) (Fawell et al., 1972; Burkholder et al., 2010).

Overall, no treatment related effects were observed in any histological slides analysed (Table 9 and Table 10). Any microscopic findings observed were considered incidental; of the nature that would be commonly observed in this age and strain of nude mouse. The findings were of similar frequency and severity in both control and treated animals and were therefore considered unrelated to the administration of the hMSCs (Table 9 and Table 10). All histopathology was carried out under GLP by a board certified veterinary pathologist.

	Sex / Group			
	Male Vehicle	Male hMSCs	Female Vehicle	Female hMSCs
Number of animals neropsied	8	8	8	8
Organ/tissue				
Eye				
Opacity, one/both	-	-	2	4
Lung				
Foci, dark	1	2	1	3
Discolouration, mottled	2	3	1	1
Discolouration, dark	2	1	1	-
Lymph Node (inguinal)				
Enlargement, one/both	1	1	-	-
Lymph Node (madibular)				
Enlargement	-	-	1	1
Mass				
Subcutaneous mass	-	1	-	-
Salivary Gland				
Area, dark, right	1	-	-	-
Skin and Subcutis				
Abnormal consistency	1	-	-	-
Scab	1	3	1	-
Nodule	-	-	1	-
Spleen				
Enlargement	-	-	2	1
Systemic Condition				
Autolysed	1	-	-	-
Trachea				
Fluid accumulation, pale	2	-	-	-
Uterus				
Dilation, both horns	-	-	-	1

Table 8: Macroscopic findings at necropsy.

Histopathological Findings Mean Severity (Number Affected/Total Number of Animals)		
	Treatment	
Organ/tissue	Male Vehicle	Male hMSCs
Brain		
Chroid plexus, mononuclear inflitration	0.1 (1/7)	(0/8)
Epididymis, monuclear iniltration	(0/7)	0.1 (1/8)
Oesophaghus, degenerataton	0.1 (1/7)	(0/8)
Eye		
Cornea, hyperplasia	0.3 (2/7)	(0/8)
Cornea, neutrophilic infiltration	0.3 (2/7)	0.5 (4/8)
Cornea, ulceration	0.1 (1/7)	(0/8)
Preputial gland, abscess	(0/7)	0.1 (1/8)
Prostate, mononuclear cell infiltration	0.1 (1/7)	(0/8)
Salivary gland, necrosis	0.1 (1/7)	(0/8)
Seminal vesicle, mononuclear cell infiltration	0.1 (1/7)	(0/8)
Heart, cardiomyopathy	0.1 (1/7)	(0/8)
Lung, acute haemorrhage	1.0 (7/7)	1.0 (8/8)
Inguinal lymph node, edema	0.1 (1/7)	0.1 (1/8)
Skeletal muscle, mononuclear cell infiltration	0.1 (1/7)	(0/8)
Sciatic nerve, mixed cell infiltration	0.1 (1/7)	(0/8)
Injection site, thigh, myofiber degeneration	0.1 (1/7)	(0/8)
Injection site, calf, mononuclear cell infliltration	0.1 (1/7)	(0/8)
Skin		
ulceration	0.1 (1/7)	(0/8)
hyperplasia	0.1 (1/7)	0.1 (1/8)
serocellular crust	0.1 (1/7)	0.3 (2/8)
inflammation, neutrophilic	(0/7)	0.1 (1/8)
Small intestine		
ilieum, acute haemorrhage	0.1 (1/7)	0.8 (6/8)
Stomach, mononuclear cell infiltration	0.1 (1/7)	0.3 (2/8)
Thymus, rudiment	0.3 (2/7)	0.3 (2/8)

Table 9: Histopathology findings in the male nude mice following the injection of hMSCs or vehicle control.

Histopathological Findings Mean Severity (Number Affected/Total Number of Animals)		
	Treatment	
Organ/tissue	Female Vehicle	Female hMSCs
Brain		
Subrachoroid space,, mononuclear inflitration	(0/8)	0.1 (1/8)
Eye		
Cornea, hyperplasia	(0/8)	0.3 (2/8)
Cornea, neutrophilic infiltration	0.6 (5/8)	0.6 (5/8)
Gall bladder, cyst	(0/8)	0.1 (1/8)
Adrenal gland, spindle cell hyperplasia	(0/8)	(0/8)
Lacriminal gland		
atrophy	0.1 (1/8)	0.1 (1/8)
mononuclear cell infiltration	0.5 (4/8)	0.3 (2/8)
Salivary Gland, mononuclear infiltration	(0/8)	0.1 (1/8)
Kidney, cyst	0.1 (1/8)	(0/8)
Lung	0.1 (1/7)	0.1 (1/8)
acute haemorrhage	0.6 (5/8)	0.4 (3/8)
mononuclear cell infiltration	0.1 (1/8)	0.1 (1/8)
Mandibular lymph node, plasmacytosis	0.3 (2/8)	0.1 (1/8)
Skeletal muscle mononuclear cell infiltration	0.1 (1/8)	(0/8)
Ovary, follicle depletion	(0/8)	0.1 (1/8)
Injection site, thigh, perineurial mixed cell infiltration	0.1 (1/8)	(0/8)
Injection site, calf		
foreign material	(0/8)	0.1 (1/8)
mixed cell infiltration	(0/8)	0.1 (1/8)
Skin		
ulceration	0.1 (1/8)	(0/8)
hyperplasia	0.1 (1/8)	(0/8)
mononuclear cell infiltration	0.3 (2/8)	0.6 (5/8)
Spleen, plasmacytosis	0.3 (0/8)	0.1 (1/8)
Thymus, rudiment	0.1 (1/8)	(0/8)
Trachea, mononuclear cell infiltration	0.1 (1/8)	(0/8)
Uterus, glandular ectasia	(0/8)	0.1 (1/8)
Vagina, mononuclear cell infiltration	(0/8)	0.1 (1/8)

Table 10: Histopathology findings in the female nude mice following the injection of hMSCs or vehicle control.

DISCUSSION

Revascularization constitutes the main target in the treatment of CLI patients. As the proposed mechanism of MSC action is through the restoration of tissue reperfusion by the stimulation of neovasculature by means of biological bypass and by augmenting blood flow in pre-existing arteries and arterioles, they may prove more effective than former approaches used to treat CLI.

Recent clinical studies have demonstrated that the administration of MSCs to patients with CLI is safe [(Das et al., 2013; Lasala et al., 2011, Lu et al. 2011). Given these results and encouraging preclinical data, MSCs have become a relevant CT for testing in CLI patients. From a regulatory perspective, safety concerns will represent a barrier to the translation of MSCs to a successful clinical product. Delivery toxicities (due to novel delivery techniques), acute immunogenicity and tumour formation are the main toxicity endpoints of concern to regulatory agencies. Extensive interactions were held with the regulator prior to submission of the clinical trial dossier. It was clear to us, in spite of the widespread use of MSCs in clinical trials for multiple indications globally, that the regulator in Ireland would require a toxicology undertaken with the product which we intended to use in our clinical trial. While we had performed in house toxicology testing and despite demonstrating no local toxicity after the cell injection the regulator would not accept this data as sufficient to support the IMP application.

Clinical testing using CT products in Europe are regulated under the Clinical Trial Directive and require national clinical trial applications. The HPRA represent the national competent authority and are responsible for clinical trial authorization, inspection of compliance and pharmacovigilance of CTs. As we wanted to test our hMSC product in CLI patients we organized a meeting with representatives from the HPRA in order to develop an effective regulatory strategy. After meeting with the HPRA, there was agreement that our proposed MSC product was an ATMP and deemed this IMP application as ‘the first of its kind’ in Ireland. Furthermore, since there were no precedents to learn from, they suggested that extensive preclinical safety studies were needed to support the IMP application. Despite, data from our own in house toxicology study in combination with a wealth of preclinical and clinical data in the literature demonstrating safety of hMSCs, the HPRA stated that this data could not be used to support the intended medicinal product application from a safety perspective as equivalency had not been shown for all products. Therefore, they suggested to us to conduct toxicology and biodistribution studies under GLP, testing the intended GMP manufactured clinical product, and that the grounds for approving the trial would be largely based on the receipt of these study reports. The toxicology study reported here was conducted in accordance with GLP to investigate the potential toxicity of our hMSC product after intramuscular delivery and contributes to the harmonization of regulatory requirements for hMSC therapy in Ireland.

Our choice of animal model was guided by our regulatory body. The HPRA recommended that our toxicology studies should be completed using our intended medicinal product manufactured under GMP conditions and that this product should be administered via the route to be used in the proposed clinical study. For this reason, the toxicity study was conducted in BALB/c nude mice as this mouse is immunodeficient and therefore provides an environment that is permissive to human cell survival with the avoidance of confounding xenogeneic effects. Furthermore, it is a sensitive model for the assessment of stem cell grafts with limits of detection as low as 20 tumorigenic cells (Lawrenz et al., 2004). Our overall aim was to demonstrate the safety and tolerability of the hMSCs product via the intramuscular route in this model. Both aspects of this model enabled the evaluation of safety concerns surrounding the administration of our CT product e.g. local and systemic toxicity and biodistribution.

Several preclinical parameters were evaluated in order to investigate the safety and tolerability of our hMSC administration. Firstly, our data showed no impact of the proposed therapy on the general well-being of the animal. All animals showed consistent body weight (Table 4) and food consumption (Table 3) measurements across the hMSC and saline treated mice. Furthermore, no test item related clinical signs after the hMSC administration or at any time throughout the 3-month study duration were observed as measured by the clinical parameters set in this study (Table 2). No haematologic abnormalities were observed and animals who received hMSCs presented the same white blood cell count, red blood cell count, haemoglobin concentration, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration (Table 6). No abnormal readings were observed in the core serum chemistry tests performed (Table 7). Biochemical tests were normal in the hMSC treated animals. Furthermore, hMSC did not impact on hepatocellular health as ALT and AST levels were comparable between the treatment and control groups. In spite of blood sampling and processing issues overall no treatment related changes were observed in any of the blood tests performed.

Local toxicities at the injection site such as altered tissue function, tumour formation, cell differentiation to unwanted cell types at the administration sites of the CT product may be caused by the product interacting adversely with host tissue environment or the degradation of the product itself or it components. Furthermore, migration of the CT product outside of the target tissue represents a major safety risk and may lead to systemic toxicities such as immune mediated toxicities or ectopic tissue formation in the distant organs. In order to assess local and systemic toxicities histopathological analysis was performed on the injection site and selected organs (Table 9 and 10). The histopathology conducted in this study did not uncover any tumours, ectopic tissue formation, or major inflammatory response that was associated with our MSC product. Although differences were noted in the absolute organ and organ to body weight

ratios in the lungs of the hMSC treated groups this data when evaluated with the histopathology data and taking into consideration the method of euthanasia, was deemed an expected finding. CO_2 inhalation represents an established method of euthanasia in rodents. Nonetheless, failure of the operator to maintain a ratio of 70% CO_2 / 30% oxgen (O_2) in the euthanasia chamber can induce haemorrhaging within the parenchyma of the lungs (Fawell et al., 1972; Burkholder et al., 2010).

In summary, in view of our reported findings and under the conditions of this study, we can report that the administration GMP grade hMSCs via intramuscular injection at a single dose of $3x10^5$ cells per mouse (*ca.* 15million cell/kg) was well tolerated with no indication of any malignancy systemically or locally.

Based on the toxicity data presented in this chapter and in accordance with our biodistribution study (**Chapter 3**), historical efficacy data and the literature, the risk-benefit of our hMSC product was considered acceptable and cleared by HPRA to initiate a phase 1b dose escalation study in 'no option for revascularization' patients with CLI. The first in human study plans to collect extensive safety data on the hMSC product and is thus expected to provide valuable information on the clinical suitability of increasing doses hMSCs for CLI patients.

Future perspective

This chapter describes the results of the toxicity study that was required in combination with our biodistribution data (**Chapter 3**) to allow our hMSC product to be tested clinically in 'no option for revascularization' patients. The results from this study demonstrated that the administration of 9 times the maximal proposed clinical dose via the intended clinical route of administration did not cause adverse clinical observations or systemic toxicities. Furthermore, the transplanted cells showed no evidence of tumour formation at the injection site or any other organs tested. In addition, this study provided us with important biological characterization data which can be used in the future to help further refine our hMSC product. Because published toxicology studies using MSCs are rare, the objective of this chapter was to provide the reader with the authors own experiences to help aid first time investigators in the development of their novel therapies.

REFERENCES

ADAM, D. J., BEARD, J. D., CLEVELAND, T., BELL, J., BRADBURY, A. W., FORBES, J. F., FOWKES, F. G., GILLEPSIE, I., RUCKLEY, C. V., RAAB, G. & STORKEY, H. 2005. Bypass versus angioplasty in severe ischaemia of the leg (BASIL): multicentre, randomised controlled trial. *Lancet,* 366**,** 1925-34.

ARMSTRONG, D. G., WROBEL, J. & ROBBINS, J. M. 2007. Guest Editorial: are diabetes-related wounds and amputations worse than cancer? *Int Wound J,* 4**,** 286-7.

BORTOLOTTI, F., UKOVICH, L., RAZBAN, V., MARTINELLI, V., RUOZI, G., PELOS, B., DORE, F., GIACCA, M. & ZACCHIGNA, S. 2015. In vivo therapeutic potential of mesenchymal stromal cells depends on the source and the isolation procedure. *Stem Cell Reports,* 4**,** 332-9

BURKHOLDER, T. H., NIEL, L., WEED, J. L., BRINSTER, L. R., BACHER, J. D. & FOLTZ, C. J. 2010. Comparison of carbon dioxide and argon euthanasia: effects on behavior, heart rate, and respiratory lesions in rats. *J Am Assoc Lab Anim Sci,* 49**,** 448-53.

DAS, A. K., BIN ABDULLAH, B. J., DHILLON, S. S., VIJANARI, A., ANOOP, C. H. & GUPTA, P. K. 2013. Intra-arterial allogeneic mesenchymal stem cells for critical limb ischemia are safe and efficacious: report of a phase I study. *World J Surg,* 37**,** 915-22.

FAWELL, J. K., THOMSON, C. & COOKE, L. 1972. Respiratory artefact produced by carbon dioxide and pentobarbitone sodium euthanasia in rats. *Lab Anim,* 6**,** 321-6.

FOWKES, F. G., RUDAN, D., RUDAN, I., ABOYANS, V., DENENBERG, J. O., MCDERMOTT , M. M., NORMAN, P. E., SAMPSON, U. K., WILLIAMS, L. J., MENSAH, G. A. & CRIQUI, M. H. 2013. Comparison of global estimates of prevalence and risk factors for peripheral artery disease in 2000 and 2010: a systematic review and analysis. *Lancet,* 382**,** 1329-40.

GREMMELS, H., FLEDDERUS, J. O., TERAA, M. & VERHAAR, M. C. 2013. Mesenchymal stromal cells for the treatment of critical limb ischemia: context and perspective. *Stem Cell Res Ther,* 4**,** 140.

GUPTA, R. & LOSORDO, D. W. 2011. Cell therapy for critical limb ischemia: moving forward one step at a time. *Circ Cardiovasc Interv,* 4**,** 2-5.

HIRSCH, A. T. & DUVAL, S. 2013. The global pandemic of peripheral artery disease. *Lancet,* 382**,** 1312-4.

HUNG, S. C., POCHAMPALLY, R. R., CHEN, S. C., HSU, S. C. & PROCKOP, D. J. 2007. Angiogenic effects of human multipotent stromal cell conditioned medium activate the

PI3K-Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. *Stem Cells,* 25**,** 2363-70.

KINNAIRD, T., STABILE, E., BURNETT, M. S., SHOU, M., LEE, C. W., BARR, S., FUCHS, S. & EPSTEIN, S. E. 2004b. Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation,* 109**,** 1543-9.

LASALA, G. P., SILVA, J. A., KUSNICK, B. A. & MINGUELL, J. J. 2011. Combination stem cell therapy for the treatment of medically refractory coronary ischemia: a Phase I study. *Cardiovasc Revasc Med,* 12**,** 29-34.

LAWRENZ, B., SCHILLER, H., WILLBOLD, E., RUEDIGER, M., MUHS, A. & ESSER, S. 2004. Highly sensitive biosafety model for stem-cell-derived grafts. *Cytotherapy,* 6**,** 212-22.

LEROUX, L., DESCAMPS, B., TOJAIS, N. F., SEGUY, B., OSES, P., MOREAU, C., DARET, D., IVANOVIC, Z., BOIRON, J. M., LAMAZIERE, J. M., DUFOURCQ, P., COUFFINHAL, T. & DUPLAA, C. 2010. Hypoxia preconditioned mesenchymal stem cells improve vascular and skeletal muscle fiber regeneration after ischemia through a Wnt4-dependent pathway. *Mol Ther,* 18**,** 1545-52.

LU, D., CHEN, B., LIANG, Z., DENG, W., JIANG, Y., LI, S., XU, J., WU, Q., ZHANG, Z., XIE, B. & CHEN, S. 2011. Comparison of bone marrow mesenchymal stem cells with bone marrow-derived mononuclear cells for treatment of diabetic critical limb ischemia and foot ulcer: a double-blind, randomized, controlled trial. *Diabetes Res Clin Pract,* 92**,** 26-36.

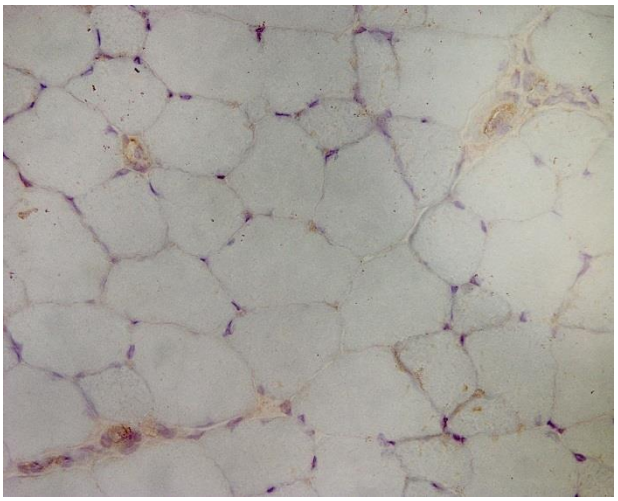
MOON, M. H., KIM, S. Y., KIM, Y. J., KIM, S. J., LEE, J. B., BAE, Y. C., SUNG, S. M. & JUNG, J. S. 2006. Human adipose tissue-derived mesenchymal stem cells improve postnatal neovascularization in a mouse model of hindlimb ischemia. *Cell Physiol Biochem,* 17**,** 279-90.

SMADJA, D. M., D'AUDIGIER, C., GUERIN, C. L., MAUGE, L., DIZIER, B., SILVESTRE, J. S., DAL CORTIVO, L., GAUSSEM, P. & EMMERICH, J. 2012. Angiogenic potential of BM MSCs derived from patients with critical leg ischemia. *Bone Marrow Transplant,* 47**,** 997-1000.

U.S DEPARTMENT OF HEALTH AND HUMAN SERVICES, FOOD AND DRUG ADMINISTRATION.2013. Guidance for Industry,Preclincal assessment of investigational cellular and gene therapy products. Retrieved from http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatory Information/Guidances/CellularandGeneTherapy/UCM376521.pdf.

Chapter 3: Biodistribution and Retention of Locally Administered Human Mesenchymal Stromal Cells: Quantitative PCR-Based Detection of Human DNA in Murine Organs.

ABSTRACT



Determining the distributive fate of a cell therapy product after administration is an essential part of characterizing its biosafety profile. Therefore, regulatory guidelines stipulate that biodistribution assays are a requirement prior to advancing a cell therapy to the clinic. Here, the development of a highly sensitive quantitative PCR based method of tracking the biodistribution of human mesenchymal stromal cells in mice is described. A primer-probe based qPCR assay was developed to detect and quantify human Alu sequences in a heterogeneous sample of human DNA and murine DNA from whole organ genomic DNA extracts. The assay measures the amount of human genomic DNA by amplifying a human Alu repeat sequence, thus enabling the detection of 1 human cell in $1.5x10^6$ heterogeneous cells. Using this assay we investigated the biodistribution of $3x10^5$ intramuscularly injected hMSCs in BALB/c nude mice. Genomic DNA was extracted from murine organs and hAlu sequences were quantified by qPCR analysis. After 3 months hDNA ranging from 5.32 - 11.79% was detected only at the injection sites and not in the distal tissues of the mice. This assay is a reproducible, sensitive and accepted by the HPRA as a method of detecting human DNA in rodent and lapine models.

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Centre for Cell Manufacturing in Ireland (CCMI) contributed to the generation of the GMP grade cells used in this study.

This chapter in full has been submitted for publication in Cytotherapy. The paper is currently under review.

INTRODUCTION

Determining the distributive fate of CT products after administration is an essential part of characterizing the product's mechanism of action (MOA) and biosafety profile. The therapeutic cell's phenotype, efficacy and migratory potential are influenced by the formulation of the CT product as well as by the route of administration (ROA) and the micro-environment in which the cells reside in the host. Concerns surrounding the *in vivo* acquisition of cellular autonomy resulting in ectopic tissue formation prompt regulatory authorities to require stringent pre-clinical investigations into the biodistribution of the administered CT (U.S. FDA, 2013).

For CT products it is vital that reproducible, sensitive, quantitative assays are developed and applied to evaluate the persistence and distribution of cells after administration. Regulatory guidelines stipulate that CT product safety is determined using risk-based approaches such that the assays developed to determine biosafety for the intended host consider and directly address any risks posed to the intended host (U.S. FDA, 2013; Bailey et al., 2014; Frey-Vasconcells et al., 2012; Sensebe & Fleury-Cappellesso, 2014). Unlike small molecule pharmaceuticals, the biological complexity of living cells does not make them suitable for routine absorption, distribution metabolism and excretion (ADME) and pharmacokinetic testing (U.S. FDA, 2013). As a result, biodistribution assays are a regulatory requirement for advancing CT to the clinic. Biodistribution studies can provide data on CT product localization or migration over time as well as *in vivo* survival and differentiation in the case of progenitor cell-based CT (Sensebe & Fleury-Cappellesso, 2014).

The biodistributive profile of a CT product has safety and efficacy implications, addressing questions such as: Are the cells reaching the reparative site of interest in the host? Are they engrafting in numbers sufficient to elicit the desired response? How long do they persist in the host? Laboratories worldwide have used a wide range of techniques in attempts to determine the distribution of transplanted cells (Naumova et al. 2014; Terrovitis et al., 2010). Microscopic visualization of histological samples has been widely used to detect the presence of the transplanted cells in pre-clinical models using a variety of cell labelling techniques, such as membrane dyes (PKH26, DII [1,1'Dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate]) or nuclear dyes (Hoechst 33342, bromodeoxyuridine, DAPI [4'6-diamidino-2-phenylindole]) (Terrovitis et al., 2010). However, cellular labelling methodologies are susceptible to dilution with cellular division reducing the label below the limits of detection (Terrovitis et al., 2010). Furthermore, the labour intensive histological techniques required to locate the CT *in vivo* can be subject to sampling error, leading to variability and thus reduced sensitivity and reliability of the results (Terrovitis et al., 2010).

Genetic modification of the intended CT can allow for the identification of the administered cells without concern about dilution of the label. However, the required use of cellular labelling molecules can have consequences on CT product functions and may potentially alter the biodistributive fate of the cells (Sensebe & Fleury-Cappellesso, 2014; Wolfs et al., 2013). Modification of the pre-clinical CT product with genes encoding proteins which can be visualized via microscopy (green fluorescent protein, enhanced green fluorescent protein, red fluorescent protein, yellow fluorescent protein, B-galactosidase and mCherry) can provide quantitative information about cellular location and survival, however gene silencing in long-term studies may result in a decrease in signal with time (Terrovitis et al., 2010). Furthermore, autofluorescence within the tissues of interest and uptake of the fluorescent protein by adjacent cells, such as macrophages, can result in false positives thus compromising the accuracy of the results (Terrovitis et al., 2010). Newer technologies are emerging in which non-invasive imaging can provide real time *in vivo* tracking of the transplanted CT (Naumova et al. 2014; Wolfs et al., 2013; Dwyer et al., 2011; Nguyen et al., 2014). Such imaging modalities are exciting as they enable the investigators to obtain dynamic measurements of cellular viability and location after administration. However, their application relies upon suboptimal extensive cell labelling. This strategy may not be optimal as the regulatory authority require that the preclinical studies to support the first in human application must be completed using the final cell product intended for human use (U.S. FDA, 2013; Bailey et al., 2014).

Real time, quantitative PCR (qPCR) is a relatively inexpensive technique that bypasses the disadvantages associated with other cellular detection methods (Terrovitis et al., 2010). qPCR enables the accurate and sensitive detection of transplanted cells via their cell-specific DNA sequences within the whole host organ, minimalizing sampling errors (Terrovitis et al., 2010; Prigent et al., 2015). The Alu sequence remains the marker of choice when assessing the biodistribution of transplanted cells in xenogenic models, due to genomic repetition and species specificity. The human Alu (hAlu) sequence can be amplified and quantified by qPCR from genomic DNA (gDNA) with a high degree of accuracy (Terrovitis et al., 2010; Prigent et al., 2015).

Here we describe the development of an accurate, reproducible, quantitative and inexpensive qPCR-based method of tracking the biodistributive fate of human cells in xenogenic models. The assay is a primer-probe based PCR assay using custom-made primers to detect and quantify the hAlu sequences in a heterogeneous sample of human DNA (hDNA) and murine DNA (mDNA) from whole organ gDNA extracts. The assay enables the quantification of human gDNA by amplifying a hAlu repeat sequence, with a sensitivity to detect the DNA equivalent of 1 human cell in $1.5x10^6$ heterogeneous cells

MATERIALS & METHODS

Human Bone Marrow Mesenchymal Stromal Cell Isolation and Culture

Human hMSCS were isolated from adult bone marrow and cultured expanded in accordance with local ethical approval and regulatory body-approved GMP protocols. Upon receipt, the bone marrow aspirate was washed with DPBS and centrifuged at 900g. A 4% acetic acid wash was performed on a sample of the marrow to lyse the red blood cells and enable an accurate MNC count. MNCs, plated at 40-50 million per 175cm^2 were cultured expanded in monolayer with complete medium (αMEM supplemented with 10% FBS) in 5% CO_2 at 37°C. On day 3, cell culture medium was replaced and fresh medium was added to the culture. On day 5, the cultures were washed with DPBS to remove non-adherent cells and fresh complete medium was added to each flask. When the monolayer reached 80-90% confluence, the adherent cells were washed with DPBS and detached from the culture plastic with 0.25% tryspin/EDTA. The dissociated cells were centrifuged at 400g for 5 minutes. The resultant pellet was resuspended in complete fresh medium and the cellular yield determined. hMSCs were further sub-cultured in triple flasks through two passages. hMSCs were cryopreserved at of a dose of 2x10^6 per ml in FBS combined with 10% dimethyl sulfoxide.

Animal Husbandry

Animal care and administration of the hMSCs were conducted in Charles River, a GLP certified site. Approval was obtained under ASPA 1986 by the Home Office in Scotland before initiation of the study. Male and female BALB/c Nude mice (Hsd-*Foxn1*^{nu}) were obtained from Harlan UK Ltd, (Oxon, UK) and maintained on a Teklad Rodent Diet 2919. The diet and water were provided *ad libitium* except during designated procedures. During the acclimation period and study duration, animals were housed in a limited access rodent facility and kept in groups of 2 or 3 per cage in appropriately sized polycarbonate/polypropylene cages with stainless steel grid tops and solid bottoms. Each cage was fitted with a filter top and had sterilized white wood shavings. The mice were allowed a *ca* 2-week acclimation period to the Charles River facility conditions (19°C - 23°C, 40% - 85% relative humidity and a twelve-hour light/dark cycle) prior to inclusion in the study.

hMSC Preparation

Bone marrow derived hMSCs were thawed and prepared immediately before injection. The cells were removed from liquid nitrogen, thawed in a 37°C water bath and transferred directly into tubes containing 4ml of saline vehicle The cryovial was washed once to ensure that all cells

were removed. A cell count was performed using the trypan blue (Sigma Aldrich) exclusion method. A cell suspension containing 300,000 cells was transferred into 15 ml conical tubes and centrifuged at 400g for 5 minutes at room temperature. The supernatant from the centrifuged cells was discarded and the pellet of hMSCs was resuspended in 150 µl of saline vehicle. The cell suspension was mixed well and transferred to a sterile cryovial then to to three insulin syringes containing 50 µl each. The hMSCs were administered to the animals within 2 hours of resuspension in saline.

hMSC Transplantation

hMSCs were administered at a dose of 300,000 cells in 150 µl per animal. The total volume was divided between 3 injection sites (50 µl per site), two in the thigh and one in the calf on the right leg. Each injection was administered over ca 1 to 3 seconds. The control animals received 3 injections containing a total of 150 µl of saline in a similar manner. Animals in each group were subjected to termination at 3 months after the hMSCs administration.

Necropsy and Tissue Collection

The following tissues, in the following order, were harvested from all animals at necropsy: liver, kidneys, heart, lungs, brain and spleen. To harvest the injection site, the complete right leg was collected, without the foot attached, including the lateral head of gastrocnemius muscle, semitendinosus muscle, semimembranosus muscle, adductor muscle and the calf muscle. Tissues were collected into RNAse-free 1.5ml Eppendorfs frozen in liquid nitrogen and stored at -80ºC. When required, the tissues were removed from -80°C and placed on ice to thaw. Using a sterile pipette tip, the tissues were removed from the tube and weighed on a small sterile tissue culture dish. The right thigh and calf regions (injection sites) were dissected and similarly weighed.

gDNA Extraction and Quantification

Due to the sensitivity of this assay to detect hDNA, heightened measures to avoid hDNA contamination were required, such as a dedicated set of pipettes, frequent changing of gloves, the donning of protective eyewear, utilization of sterile disposables and filtered pipette tips. To gain an accurate, representative profile of hDNA content in each organ, the full organ was homogenized. Briefly, the organs were placed on a 100 µm cell filter strainer (Fischerbrand) and mechanically dissociated using a sterile pestle (Becton, Dickinson Company) before gDNA

isolation (Bioline). The manufacturer's protocol was then scaled based on the weight of each organ, adding a proportional volume of lysis buffer (Bioline) and proteinase k (20 mg/ml, Bioline) to each organ. The tissue suspension was incubated in a shaker at 55°C for 12-20 hours. Lysis buffer (Bioline) was then added to each tissue digest, followed by further incubation for 10 minutes at 70°C. A volume of the tissue lysate corresponding to 25 mg tissue was placed in the DNA spin column. The silica membrane with bound DNA was washed and the DNA eluted by adding 50 µl of elution buffer preheated to 70°C in a 3-minute incubation. The elution step was repeated to generate 100 µl of pooled eluted DNA. For a detailed protocol please refer to the supplementary data.

Human-Specific Alu qPCR Primers

In an effort to determine the most efficient and sensitive qPCR methodology to detect hDNA, qPCR primers were designed for both SYBR-based and primer-probe based qPCR assays targeting the unique human-specific sequence of the Alu repeat (Figure 4). The forward primer for the SYBR qPCR assay (Sigma Aldrich) annealed upstream of the human specific hAlu sequence (5'-CGC CTG TAA TCC AGC TAC TC-3') while the reverse primer annealed primarily within the hAlu-specific sequence (5'-ATC TCG GCT CAC TGC AAC-3') ensuring amplification of only hAlu sequences and not Alu of the murine host (Figure 4A). For the primer-probe based assay the forward primer (Applied Biosystems) was designed to anneal upstream of the human specific Alu sequence (5'-TGG TGG CTC TCT CCT GTA AT-3') and the reverse primer designed to primarily anneal within the human specific Alu sequence (5'-GAT CTC GGC TCA CTG CAA C-3'). The probe was designed to bind between the two primers (5'-TGA GGC AGG AGA ATC GCT TGA ACC-3') upstream of the hAlu specific sequence (Figure 4B). In comparison to the state of the art McBride *et al. (2003)* publication, the forward primer utilized was 5'- CAT GGT GAA ACC CCG TCT CTA – 3' along with the reverse primer 5'-GCC TCA GCC TCC CGA GTA G-3' and probe 5'- FAM- ATT AGC CGG GCG TGG TGG CG-TAMRA-3' (Figure 4C).

A

GGCTGGGCGTGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGTGGG
TGGATCACCTGAGGTCAGGAGTTCAAGACCAGCCTGGCCAACATGGTGAAACCCCG
TCTCTACTAAAAATACAAAAATTAGCCGGGCGTGGTGGCGCGCGCCTGTAATCCCA
GCTACTCGGGAGGCTGAGGCAGGAGAATCGCTTGAACCCGGGAGGTGGAG**GTTGC**
AGTGAGCCGAGATCGCGCCACTGCACTCCAGCCTGGGCGACAGAGCGAGACTCC
GTCTCA

B

GGCTGGGCGTGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGTGGG
TGGATCACCTGAGGTCAGGAGTTCAAGACCAGCCTGGCCAACATGGTGAAACCCCG
TCTCTACTAAAAATACAAAAATTAGCCGGGCGTGGTGGCGCGCGCCTGTAATCCCA
GCTACTCGGGAGGCTGAGGCAGGAGAATCGCTTGAACCCGGGAGGTGGAG**GTTGC**
AGTGAGCCGAGATCGCGCCACTGCACTCCAGCCTGGGCGACAGAGCGAGACTCC
GTCTCA

C

GGCTGGGCGTGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGTGGG
TGGATCACCTGAGGTCAGGAGTTCAAGACCAGCCTGGCCAACATGGTGAAACCCCG
TCTCTACTAAAAATACAAAAATTAGCCGGGCGTGGTGGCGCGCGCCTGTAATCCCA
GCTACTCGGGAGGCTGAGGCAGGAGAATCGCTTGAACCCGGGAGGTGGAGG**TTGC**
AGTGAGCCGAGATCGCGCCACTGCACTCCAGCCTGGGCGACAGAGCGAGACTCC
GTCTCA

Figure 4: Primer sequence alignment with the hAlu genomic repeat. Pictorial representation of hAlu repeats with the SYBR Green primer (A) and primer-probe (B) alignments described herein, compared to the previously published primer-probe sequences (McBride et al., 2003) (C), demonstrates a primer design targeting the human-specific sequence (Nelson et al., 1989) within the Alu DNA repeat (bold). In all assays, the forward primer (dashed underscore) binds in a generic region of the Alu DNA sequence. The probe (underscored) binds between the forward and reverse primers (B, C) to a xeno-conserved Alu sequence. The reverse primer, however, is targeted (A, B) to the human conserved sequence for specificity (double underscore) while in previously published reports it binds in a generic region of the hAlu repeat, resulting in reduced specificity (C).

DNA Quantification and qPCR

The concentration of gDNA isolated from each murine organ was quantified using a Quant-iT PicoGreen dsDNA assay according to the manufacturer's instructions (Invitrogen). For the SYBR Green technique, qPCR was performed in a volume of 25 µl that contained 12.5 µl of qPCR Sensimix (Bioline), 0.5 µl of forward primer, 0.5 µl reverse primer (Sigma Aldrich) and 200ng of target template gDNA diluted in water. For the primer-probe based technique, qPCR was performed in a volume of 20 µl that contained 10 µl of qPCR master mix (Applied Biosystems), 1 µl of primer-probe solution (FAM-MGB, Applied Biosystems) and 100 ng of target template gDNA diluted to the final volume in water.

The SYBR Green PCR reactions were incubated at 95°C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds followed by 60°C for 1 minute. For the primer-probe reaction, the PCR samples were incubated at 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. All qPCR assays were performed in duplicate and the average values presented. qPCR assays were performed using StepOne Plus real time PCR machines (Applied Biosystems). StepOne software was used to calculate Ct values for standards and samples using the automatic setting of baseline and threshold. Standard curves were generated by adding 10 fold serial dilutions (200ng-0ng) of hDNA on each PCR plate where a difference of 1.5 Cts below that of the negative (0ng) control was required to determine the lowest end of the assay's dynamic range.

Calculating hMSC Equivalents from hDNA Weight

By scatter plotting the log 10 of the standard concentrations versus the CT values and determining the equation of the best fit line, the number of human cell equivalents in each qPCR well was calculated. After mathematically correcting for sampling and dilutions, the human cell equivalent per 25 mg tissue was scaled according to the organ weight to give the total number of human cell equivalents contained within the murine organ.

The human and mouse haploid genomes each contain approximately $3.3x10^9$ bp DNA (Alberts et al., 2002). This value assumes all cells are diploid, although the authors acknowledge that a percentage of cells will be undergoing DNA synthesis, mitosis or cytokinesis. To calculate the mass of the diploid genome, $3.3x10^9$ bp was multiplied by 1.096^{-21}g/bp and multiplied by two (representing the conversion to a diploid genome), thus the total mass of the human or murine diploid genome is 6.6 pg. Therefore, a qPCR well containing 100 ng of gDNA was considered the equivalent of profiling 15,152 cells.

RESULTS

Specificity of the hAlu Primers: SYBR Green Vs Primer-Probe

To confirm primer specificity for hDNA in a heterogeneous solution with mouse DNA (mDNA) and determine the most sensitive qPCR methodology, both SYBR and primer-probe qPCRs were conducted (Figure 5). Positive control samples contained 100 ng hDNA and 100 ng mDNA while negative control samples contained 0 ng hDNA and 200 ng mDNA. In the SYBR Green-based assay, the presence of hDNA (red amplification line, Figure 5A) resulted in a Ct value of 7.893 while the negative sample (yellow amplification line, Figure 5A) generated a Ct value of 28.62. When using a primer-probe based assay, the presence of hDNA (purple amplification line, Figure 5B) resulted in a Ct value of 16.80 whilst the absence of hDNA (pink amplification line Figure 5B) resulted in a Ct of 36.60. In both the SYBR and primer-probe assays, a distinct difference was observed between the positive and negative samples, demonstrating amplification of the hAlu sequence. However, the SYBR based assay had notably higher Ct values in the negative sample, indicating increased background as compared to the primer-probe based assay. It was therefore determined that the primer-probe based assay would be utilized in biodistributive analysis as the low background signal enables greater qPCR sensitivity.

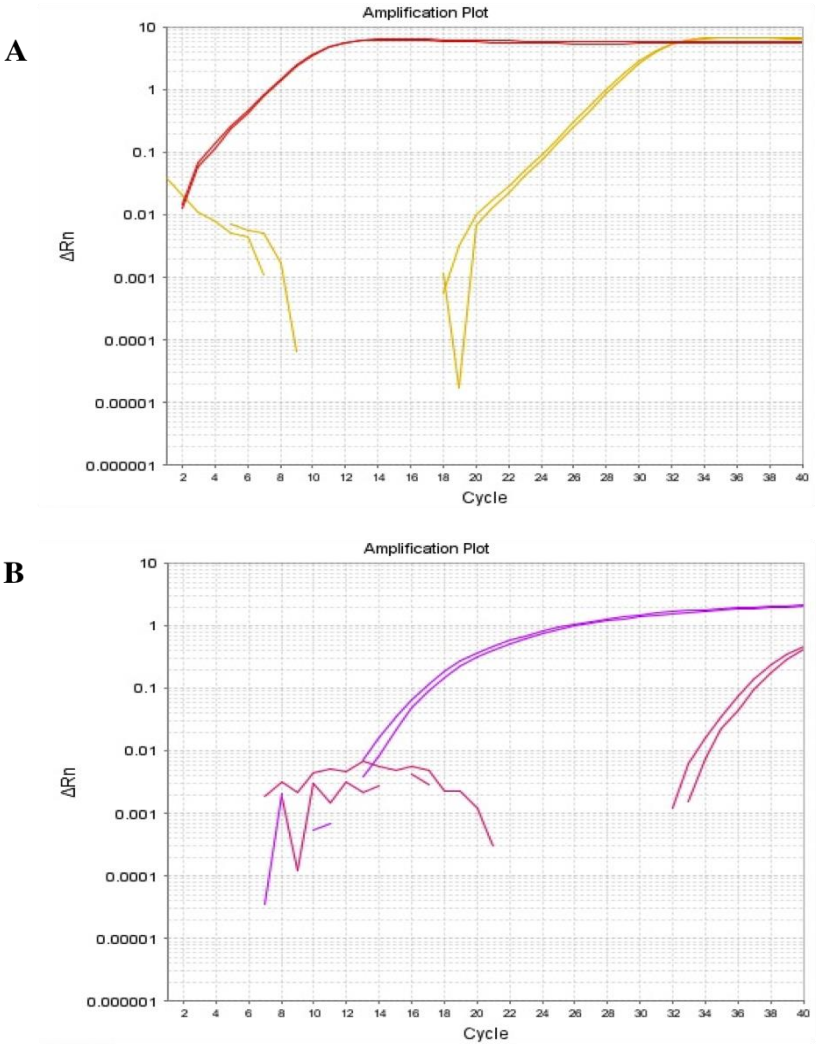


Figure 5: Representative examples of real time qPCR amplification curves demonstrating the specificity of hAlu primers employed in SYBR Green and primer-probe assays. PCR reactions containing templates of 200 ng hDNA with 200 ng mDNA (positive control) or 400 ng mDNA (negative control) were assayed in SYBR Green (A) or primer-probe qPCR assays (B). DNA amplification was clearly detected in samples containing hDNA (red amplification curves) with slight background amplification observed in PCR samples containing only mDNA (yellow line in A, pink line in B). A notable reduction in background signal, visualized as a shift to the right of the negative control amplification curves, was observed when using primer-probe assays as compared to SYBR green assays.

To ensure that the mouse organ gDNA isolation methodology did not result in the retention of residual chemicals with potential to inhibit the qPCR reaction, serial dilutions of commercially obtained hDNA were analysed by qPCR in the presence or absence of 200ng of isolated organ mDNA (Table 11).

Mixed Human and Mouse gDNA Templates: Average Ct Values

Standard hDNA (ng)	- mDNA	+ mDNA
200	16.44 (0.05)	16.52 (0.03)
20	18.69 (0.00)	18.67 (0.05)
2	21.94 (0.20)	22.16 (0.10)
0.2	25.49 (0.03)	26.30 (0.76)
0.02	29.22 (0.05)	29.61 (0.03)
0.002	32.86 (0.03)	32.87 (0.06)
0.00002	35.23 (0.10)	35.26 (0.17)
0	36.17 (0.32)	36.87 (0.09)

Note: Values are mean of two replicates (standard deviation).

Table 11: hAlu primers amplify specifically hDNA in the presence of mDNA. Serial dilutions of genomic hDNA created in the presence of 0ng or 200ng of mDNA demonstrate the species-specific amplification of hAlu and not mAlu. Comparable Ct values with and without the addition of mDNA to the sample indicates that the hAlu sequence supports the qPCR reaction while the mDNA is not amplified. Values are mean of 2 technical replicates (standard deviation).

It was observed that the highest point on the standard curve, containing 200 ng of hDNA, was saturated rendering it indistinguishable from the adjacent point on the standard curve containing 10-fold less DNA. Similarly, the lowest point on the standard curve (containing 0.0002 ng hDNA) was indistinguishable from the negative control (0 ng hDNA) by 1.5 Cts. The samples containing 20 – 0.002 ng hDNA, as anticipated, resulted in a reliable reduction of 3.3 Cts with each ten-fold dilution of hDNA in the presence or absence of mDNA, confirming both the absence of non-specific background amplification of murine Alu sequences as well as the absence of residual inhibitory chemicals from the gDNA extraction protocol. Additional analysis of serially diluted hDNA assayed in the presence of 200 ng of rat or rabbit gDNA confirmed primer specificity for hDNA sequences as there is no increase in Ct values in the presence of xenogeneic gDNA (Table 12).

Mixed Human, Rat and Rabbit gDNA Templates: Average Ct Values

Standard	Standard hDNA	200ng Rat DNA	200ng Rabbit DNA
200	16.62 (0.09)	16. 41 (0.01)	16.54 (0.45)
20	17.05 (0.53)	17.35 (0.01)	17.32 (0.01)
2	20.33 (0.42)	20.77 (0.32)	20.68 (0.01)
0.2	23.53 (0.08)	23.99 (0.09)	24.01 (0.08)
0.02	27.27 (0.15)	27.69 (0.09)	27.67 (0.20)
0.002	31.10 (0.39)	31.15 (0.03)	31.37 (0.04)
0.0002	33.93 (0.07)	34.25 (0.32)	34.02 (0.08)
0	35.73 (0.99)	35.93 (0.03)	35.89 (0.05)

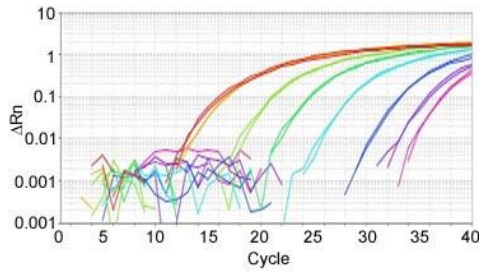
Note: Values are mean of two replicates (standard deviation).

Table 12: hAlu primers amplify hDNA and not rat or rabbit gDNA. Serial dilutions of genomic hDNA alone or in combination with 200ng of rat or rabbit DNA resulted in comparable Ct values, demonstrating the specific qPCR amplification of hAlu and not rat or rabbit Alu sequences.

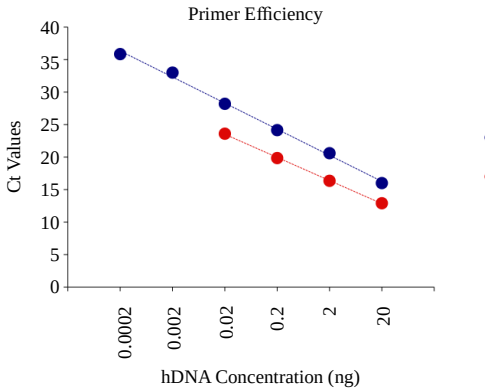
Efficiency and Sensitivity of the hAlu Primer-Probe Combination

To compare the efficiency of the newly developed primer-probe combination with the current state-of-the-art in identifying hDNA in heterogeneous gDNA samples, a standard curve was created (Figure 6). qPCR was performed on tenfold dilutions of hDNA from 200 ng to 0.0002 ng of hDNA using either the presence of the primer-probe sequence described in Figure 6A or in the presence of primer-probe set from McBride *et al (2003)* (Figure 6B). The resultant Ct values were logarithmically graphed to calculate primer efficiency (Figure 6C). Both the Mc Bride and currently presented primer-probe combinations exhibited comparable efficiency at 98.0 and 99.4% respectively.

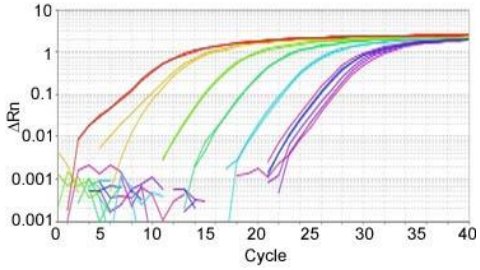
A



C



B



D

qPCR Standard Curve Ct Values

hDNA (ng)	Cellular Equivalent	Ct	McBride Ct
200	30303	15.40	12.45
20	3030	16.00	12.90
2	303	20.60	16.35
0.2	30	24.10	19.85
0.02	3	28.20	23.60
0.002	0.3	33.00	27.20
0.0002	0.03	35.80	28.10
0	0	37.60	27.70

Figure 6: The linear range and comparative efficacy of primer-probe based qPCR reactions. The efficiency of two primer-probe combinations were compared by amplifying hDNA standard curves ranging from 200 ng to 0.0002 ng of hDNA per qPCR well. The amplification plots of the in house custom primer-probe (A) and the state-of-the-art McBride primer-probe combination (B) indicate a positive signal in both assays in the presence of hDNA with the anticipated 3.3 Ct reduction with a 10-fold decrease in DNA concentration. Standard curves derived from the mean Ct values in (A) and (B) were plotted against the $\log_{10}$ fold change in hDNA concentration (C), presented in tabular format in D, to calculate a comparable 98% efficiency of the state-of-the-art McBride primer-probe combination (red dot plot) or 99% with the in house primer-probe (blue dot plot) protocol.

The primer-probe combination presented in this chapter produced less background signal compared to the McBride primer-probe combination, as demonstrated by the increased negative control Ct values (purple amplification curves in Figures 6A and 6B). This reduction in background signal enabled a distinction between the negative controls and samples containing 0.002-0.0002 ng hDNA, samples that were indistinguishable from the negative control when using the McBride *et al. (2003)* primers. Therefore, the currently described primer-probe combination is 100 times more sensitive than the current state of the art (Figure 6C). By converting hDNA weight to a cellular equivalent, the McBride primer-probe combinations detected the DNA equivalent of 0.02 human cells while the in house primer-probe combination extended the assay's range of detection to the DNA equivalent of 0.0002 human cells (1 human cell in a mixture of 1.5 x 10^6cells). (Figure 6D).

Biodistribution of hMSCs after Intramuscular Administration to Nude Mice

In support of hMSC-based CT translation to the clinic, the pre-clinical biodistribution and retention of hMSCs was evaluated 3 months after intramuscular administration to BALB/c Nude mice. The 3-month time-point was chosen to coincide with parallel acute exposure toxicology studies. Control animals (n=5 male; n=5 female) received intramuscular injections of saline vehicle alone while hMSC treated animals (n=5 male; n=5 female) received intramuscular injections of saline with 300,000 hMSCs. Three months after local administration, eight critical organs were harvested, their gDNA extracted and qPCR analysis executed (Table 13). The resultant Ct values ranged from 34.42 to 35.36 in control males 34.35 to 36.30 in control females. Ct values higher than the 0.002 ng standard were interpreted as background and were deemed non-detectable (ND). There is a low but real signal in the absence of human DNA, but in every case it is lower than the lowest standard used. As such it is considered ND.

Organ Ct Values: Male and Female Group Mean Values

Group/sex		Thigh	Calf	Heart	Lung	Brain	Liver	Kidney	Spleen
Control Male	Mean	34.74	34.53	34.99	35.20	34.42	35.04	35.03	35.36
	SD	1.14	1.44	1.43	1.19	1.48	0.76	0.63	1.21
	% DNA	ND	ND	ND	ND	ND	ND	ND	ND
hMSC Male	Mean	28.74	26.83	35.85	35.65	35.21	35.77	35.94	35.87
	SD	1.47	1.91	1.19	0.69	0.16	0.73	0.99	1.22
	% DNA	7.78	7.85	ND	ND	ND	ND	ND	ND
Control Female	Mean	34.35	34.45	34.42	35.78	35.21	35.23	35.81	36.30
	SD	0.56	0.78	1.02	0.49	1.14	0.97	0.52	0.77
	% DNA	ND	ND	ND	ND	ND	ND	ND	ND
hMSC Female	Mean	30.27	28.67	35.75	36.23	35.90	36.05	36.14	36.19
	SD	1.32	1.78	0.80	1.41	0.70	0.66	0.57	1.61
	% DNA	11.79	5.32	ND	ND	ND	ND	ND	ND

Note: Any Ct value above the 0.002 ng standard is outside the limits of detection in this assay and is therefore interpreted non detectable (ND).

Table 13: Biodistribution of bone marrow derived hMSCs 3 months after intramuscular injection into BALB/c nude mice. The presence or absence of hDNA in murine organs was assayed by primer-probe qPCR analysis of gDNA extracted from male or female BALB/c nude thigh muscle, calf muscle, heart, lungs, brain, liver, kidneys and spleen. With n=5 mice in each group, the Ct values were combined and the mean $\pm$ SD calculated. No hDNA was observed in the untreated male or female animals.

In cell treated animals, Ct values in male and female animals ranged from 26.83 to 35.94 and 28.67 to 36.23, respectively. Samples from the heart, lung, brain, liver, kidney and spleen were negative for hDNA in treated animals of both sexes. However, in both sexes, hDNA was detected in the thigh and calf samples, the sites of cell injection, in hMSC administered groups. Male thigh and calf samples retained the DNA equivalent of 7.78% and 7.85% of administered human cells respectively while female thigh and calf samples retained the DNA equivalent of 11.79% and 5.32% of administered human cells respectively.

DISCUSSION

Patient safety is a paramount consideration when developing CT products. Early investment in the biological characterization of the therapeutic cell's phenotype, activity and migration upon administration is essential to ensure that the CT product is of a high quality, safe and efficacious when applied clinically. More specifically, it is critical to know where the CT product resides upon administration to ensure complimentary toxicity assessments are conducted. Although the biologic safety of MSCs has been confirmed in clinical trials (Guiducci et al., 2010; Lee et al., 2012), the lack of sufficient techniques to track cells after administration in humans means that the biodistribution of the transplanted cells remains largely unknown. As a result, regulatory

agencies are now requiring preclinical evaluation of CT biodistribution as a prerequisite to first in human (FIH) trial initiation (U.S. FDA, 2013; Bailey et al., 2014; Bailey, 2012).

To date, there is no universally accepted methodology to quantitatively assess the biodistribution of a CT *in vivo*. Various methods such as imaging modalities, immunohistochemistry and flow cytometry have been used to assess the *in vivo* distribution of transplanted cells in multiple pre-clinical models (Terrovitis et al., 2010; Wolfs et al., 2013; Dwyer et al., 2011; Nguyen et al., 2014; Prigent et al., 2015). However, the sensitivity and qualitative nature of such assays to detect a CT *in vivo* remains a concern. qPCR quantification of hAlu sequences represents one of the most efficient and sensitive techniques currently available.

Here we describe the development of a versatile qPCR assay capable of amplifying the highly repetitive human-specific Alu DNA sequence (Nelson et al., 1989) in the presence of mouse, rat or rabbit gDNA. Due to its highly repetitive nature, targeting the Alu sequence will enable the detection of fragments of one cell in a xenogenic tissue sample. Although many groups have described PCR-based techniques for the detection of human cells in xenotransplantation systems, each qPCR protocol differs in its degree of sensitivity. Using the primer targeting strategy described herein with SYBR Green qPCR detection, we were able to detect l hMSC in 100 murine cells, superior to the detection limits reported by Song *et al. (2012)* of 1 human cells in 20 murine cells, but inferior to those described by Toupet *et al. (2013)* and Pringent *et al. (2015)*, detecting 1 adipose derived hMSC in 41,000 murine cells or 1 hMSC in 200,000 murine cells, respectively. Although used to detect circulating human tumour cells, the SYBR Green-based protocol described by Schneider et al (2002) was far superior to our initial experimentation, detecting 1 human cell equivalent in $1x10^6$ murine cells.

By developing this human specific Alu-targeting strategy into a more sensitive primer-probe based qPCR protocol, we were able to reduce the assay background fluorescence and thereby enable the detection of 1 human cell equivalent in $1.5x10^6$ murine cells. With comparable methodology, Alcoser *et al (2011)* and Ramot *et al (2009)* described the detection of 1 human tumour cell in 149 murine cells or the identification of 1 placental-derived human stromal cell in 99,950 murine cells, respectively. Most recently, Priest *et al* published the quantification of 1.4 human embryonic cell-derived oligodendrocytes in a heterogeneous mixture of $1.5x10^6$ cells following their direct administration to the rat spinal cord (Priest et al., 2015). However, the publication by McBride *et al. (2003)* was of primary interest as it most closely reflected our intended application of quantifying the biodistribution of hMSCs in a murine model. We therefore compared the primer-probe combination developed herein with the sensitivity and efficiency of the state-of-the-art sequences described by McBride *et al. (2003)*. Although both

assays retained comparable, high levels of efficiency, they differed largely in sensitivity. The protocol described herein surpassed the previously described detection of 1 hMSC in 20,000 cells by detecting 1 hMSC equivalent in $1.5x10^6$ murine cells, advancing the state-of-the-art of qPCR-based biodistribution assays beyond all previously published protocols (McBride et al., 2003; Toupet et al., 2013; Ramot et al., 2009; Becker et al., 2002; MacIsaac et al., 2012; Schneider et al., 2002). It is hypothesized this increase in sensitivity is a result of the differences in primer alignment. The primers described in this manuscript specifically target the human specific sequence in the Alu repeat, while the McBride primers do not.

In support of a regulatory submission for FIH testing, it is advised to evaluate the intended human product in genetically immunodeficient models as this creates an immunotolerant environment for the human cellular component (U.S. FDA, 2013; Bailey et al., 2014; Bailey, 2012). In this study, immunodeficient BALB/c Nude mice received an intramuscular administration of clinical grade, GMP produced hMSCs via the ROA intended for a proposed FIH study. Three months subsequent to hMSC administration, the critical organs were harvested and qPCR analysis conducted to localize and quantify the persisting hDNA. Within the limits of detection of this assay, no hDNA was detected in the brain, heart, lungs, kidneys, spleen or liver of animals that received hMSCs, indicating the CT product does not migrate and reside in these satellite sites. Moreover, the data demonstrate that after 3 months, small quantities of DNA derived from the hMSCs were retained within the muscle, at the site of administration. 7.78-11.79% of DNA from the administered hMSCs was retained in the thigh, while 5.32-7.85% was detected in the calf, similar to the cellular retention profiles previously reported in similar studies (Toupet et al., 2013; Ramot et al., 2009).

Upon identifying persisting CT DNA, the critical concern is the viability, safety and function of the residual cells. Are they alive and active, residual and senescent, or is the assay detecting CT that has been engulfed by local macrophages? The reliable quantification of hDNA isolated from dead cells is unlikely as gDNA degradation by caspases and DNAses occurs nearly immediately upon phagocytosis (McIlroy et al., 2000; Enari et al., 1998; Odaka & Mizuochi, 1999). Further, data from Schneider *et al* (2002) support the theory that qPCR for hAlu amplifies DNA from live cells by showing a direct correlation with hAlu intensity and human cell proliferation marker Ki-67 from murine gDNA extracts. Similarly, Prigent *et al* (2015) demonstrate that targeting hAlu sequences by qPCR provides quantification of live cells by showing a positive correlation between their qPCR data and histological localization of human cells actively transcribing a transgene. Therefore, it is here hypothesized that hDNA identified in a murine organ sample was isolated from a viable hMSC. However to fully confirm that the DNA detected is coming from viable MSCs the cells would need to be selected via cell sorting and cultured ex vivo.

Herein, we have developed an inexpensive, sensitive and Irish regulatory body-accepted qPCR methodology to track unmodified bone marrow derived hMSCs in mouse, rat and rabbit models. This assay is advantageous as it can provide accurate and precise quantification of small amounts of hDNA with a high degree of sensitivity. The qPCR assay described in this methods paper can therefore be used a universally standardized method of quantitatively evaluating human CT engraftment, persistence and proliferation in support of FIH CT products.

REFERENCES

ALBERT, B. JOHNSON, A., LEWIS, J., RAFF, M., ROBERT, K., WALTER, P. 2002. *Biology of the Cell*, 4th edn. Garland Science, New York, pp 191-234

ALCOSER, S. Y., KIMMEL, D. J., BORGEL, S. D., CARTER, J. P., DOUGHERTY, K. M. & HOLLINGSHEAD, M. G. 2011. Real-time PCR-based assay to quantify the relative amount of human and mouse tissue present in tumor xenografts. *BMC Biotechnol,* 11**,** 124.

BAILEY, A. M. 2012. Balancing tissue and tumor formation in regenerative medicine. *Sci Transl Med,* 4**,** 147fs28.

BAILEY, A. M., MENDICINO, M. & AU, P. 2014. An FDA perspective on preclinical development of cell-based regenerative medicine products. *Nat Biotechnol,* 32**,** 721-3.

BECKER, M., NITSCHE, A., NEUMANN, C., AUMANN, J., JUNGHAHN, I. & FICHTNER, I. 2002. Sensitive PCR method for the detection and real-time quantification of human cells in xenotransplantation systems. *Br J Cancer,* 87**,** 1328-35.

DWYER, R. M., RYAN, J., HAVELIN, R. J., MORRIS, J. C., MILLER, B. W., LIU, Z., FLAVIN, R., O'FLATHARTA, C., FOLEY, M. J., BARRETT, H. H., MURPHY, J. M., BARRY, F. P., O'BRIEN, T. & KERIN, M. J. 2011. Mesenchymal Stem Cell-mediated delivery of the sodium iodide symporter supports radionuclide imaging and treatment of breast cancer. *Stem Cells,* 29**,** 1149-57.

ENARI, M., SAKAHIRA, H., YOKOYAMA, H., OKAWA, K., IWAMATSU, A. & NAGATA, S. 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature,* 391**,** 43-50.

FREY-VASCONCELLS, J., WHITTLESEY, K. J., BAUM, E. & FEIGAL, E. G. 2012. Translation of stem cell research: points to consider in designing preclinical animal studies. *Stem Cells Transl Med,* 1**,** 353-8.

GUIDUCCI, S., PORTA, F., SACCARDI, R., GUIDI, S., IBBA-MANNESCHI, L., MANETTI, M., MAZZANTI, B., DAL POZZO, S., MILIA, A. F., BELLANDO-RANDONE, S., MINIATI, I., FIORI, G., FONTANA, R., AMANZI, L., BRASCHI, F., BOSI, A. & MATUCCI-CERINIC, M. 2010. Autologous mesenchymal stem cells foster revascularization of ischemic limbs in systemic sclerosis: a case report. *Ann Intern Med,* 153**,** 650-4.

LEE, H. C., AN, S. G., LEE, H. W., PARK, J. S., CHA, K. S., HONG, T. J., PARK, J. H., LEE, S. Y., KIM, S. P., KIM, Y. D., CHUNG, S. W., BAE, Y. C., SHIN, Y. B., KIM, J. I. & JUNG, J. S. 2012. Safety and effect of adipose tissue-derived stem cell implantation in patients with critical limb ischemia: a pilot study. *Circ J,* 76**,** 1750-60.

MACISAAC, Z. M., SHANG, H., AGRAWAL, H., YANG, N., PARKER, A. & KATZ, A. J. 2012. Long-term in-vivo tumorigenic assessment of human culture-expanded adipose stromal/stem cells. *Exp Cell Res,* 318**,** 416-23.

MCBRIDE, C., GAUPP, D. & PHINNEY, D. G. 2003. Quantifying levels of transplanted murine and human mesenchymal stem cells in vivo by real-time PCR. *Cytotherapy,* 5**,** 7-18.

MCILROY, D., TANAKA, M., SAKAHIRA, H., FUKUYAMA, H., SUZUKI, M., YAMAMURA, K., OHSAWA, Y., UCHIYAMA, Y. & NAGATA, S. 2000. An auxiliary mode of apoptotic DNA fragmentation provided by phagocytes. *Genes Dev,* 14**,** 549-58.

NAUMOVA, A. V., MODO, M., MOORE, A., MURRY, C. E. & FRANK, J. A. 2014. Clinical imaging in regenerative medicine. *Nat Biotechnol,* 32**,** 804-18.

NELSON, D. L., LEDBETTER, S. A., CORBO, L., VICTORIA, M. F., RAMIREZ-SOLIS, R., WEBSTER, T. D., LEDBETTER, D. H. & CASKEY, C. T. 1989. Alu polymerase chain reaction: a method for rapid isolation of human-specific sequences from complex DNA sources. *Proc Natl Acad Sci U S A,* 86**,** 6686-90.

NGUYEN, P. K., RIEGLER, J. & WU, J. C. 2014. Stem cell imaging: from bench to bedside. *Cell Stem Cell,* 14**,** 431-44

ODAKA, C. & MIZUOCHI, T. 1999. Role of macrophage lysosomal enzymes in the degradation of nucleosomes of apoptotic cells. *J Immunol,* 163**,** 5346-52.

PRIGENT, J., HERRERO, A., AMBROISE, J., SMETS, F., DEBLANDRE, G. A. & SOKAL, E. M. 2015. Human Progenitor Cell Quantification After Xenotransplantation in Rat and Mouse Models by a Sensitive qPCR Assay. *Cell Transplant,* 24**,** 1639-52.

RAMOT, Y., MEIRON, M., TOREN, A., STEINER, M. & NYSKA, A. 2009. Safety and biodistribution profile of placental-derived mesenchymal stromal cells (PLX-PAD) following intramuscular delivery. *Toxicol Pathol,* 37**,** 606-16.

SCHNEIDER, T., OSL, F., FRIESS, T., STOCKINGER, H. & SCHEUER, W. V. 2002. Quantification of human Alu sequences by real-time PCR--an improved method to measure therapeutic efficacy of anti-metastatic drugs in human xenotransplants. *Clin Exp Metastasis,* 19**,** 571-82.

SENSEBE, L. & FLEURY-CAPPELLESSO, S. 2013. Biodistribution of mesenchymal stem/stromal cells in a preclinical setting. *Stem Cells Int,* 2013**,** 678063.

SONG, P., XIE, Z., GUO, L., WANG, C., XIE, W. & WU, Y. 2012. Human genome-specific real-time PCR method for sensitive detection and reproducible quantitation of human cells in mice. *Stem Cell Rev,* 8**,** 1155-62.

TERROVITIS, J. V., SMITH, R. R. & MARBAN, E. 2010. Assessment and optimization of cell engraftment after transplantation into the heart. *Circ Res,* 106**,** 479-94.

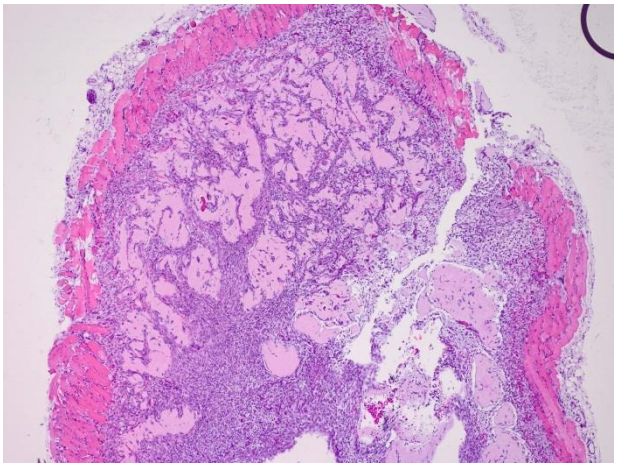
TOUPET, K., MAUMUS, M., PEYRAFITTE, J. A., BOURIN, P., VAN LENT, P. L., FERREIRA, R., ORSETTI, B., PIROT, N., CASTEILLA, L., JORGENSEN, C. & NOEL, D. 2013. Long-term detection of human adipose-derived mesenchymal stem cells after intraarticular injection in SCID mice. *Arthritis Rheum,* 65**,** 1786-94.

U.S DEPARTMENT OF HEALTH AND HUMAN SERVICES, FOOD AND DRUG ADMINISTRATION.2013. Guidance for Industry,Preclincal assessment of investigational cellular and gene therapy products. Retrieved from http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/UCM376521.pdf

WOLFS, E., STRUYS, T., NOTELAERS, T., ROBERTS, S. J., SOHNI, A., BORMANS, G., VAN LAERE, K., LUYTEN, F. P., GHEYSENS, O., LAMBRICHTS, I., VERFAILLIE, C. M. & DEROOSE, C. M. 2013. 18F-FDG labeling of mesenchymal stem cells and multipotent adult progenitor cells for PET imaging: effects on ultrastructure and differentiation capacity. *J Nucl Med,* 54**,** 447-54.

Chapter 4: Towards A Potency Assay

ABSTRACT



Characterization of the cell and its active substance is a fundamental part of the cell products development. A comprehensive understanding of the CT products properties and active substance is important in order to control its quality and therefore its safety and its efficacy. It is important to develop assays that provide measurements of the products biological activity in order to verify that a product of consistent quality is being manufactured. This is essential because where there is product inconsistency it is unreasonable to expect consistent clinical results. Therefore, the regulator suggests that the potency tests be developed in order to set lot release criteria to verify the cell manufacturing process. The potency assay development process begins as part of the characterization of the product's active ingredients, which occurs during the early preclinical development stage. In this chapter we describe the early stages of the process of development of a series of assays that we used in order to measure the potency or bioactivity of our hMSC product.

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INTRODUCTION

Human MSCs are regulated as ATMPs because they represent a product that is more than minimally manipulated, they are utilized in a manner that is not homologous to their natural function and their primary effects depend on the metabolic activity of living cells. In Europe MSCs are therefore regulated as biologics under European Regulation number 1394/2007 (EMA, 2007). The regulatory guidelines specify that any biological product intended for human use must be pure, potent, safe and effective (U.S. FDA, 2011).

Potency is defined as "the specific ability or capacity of the product as indicated by appropriate laboratory tests or by adequacy of controlled clinical data obtained through the administration of the product in a manner intended to effect a given result." (U.S. FDA, 2011). In both the Europe Union and the United States, potency evaluation of each batch of licensed CT product is a legal requirement. As new CT advances through clinical trials there has been an increased awareness of the need for the development of diagnostic assays to assess the bioactivity/potency of the CT products.

The mechanism of action varies immensely amongst different CT products. Factors contributing to this variability are; cell source, inherent cell characteristics, manufacturing process, mode of administration, and conditions in which the CT product is exposed to in vivo (Bailey et al., 2014). Therefore, elucidating the CT therapeutic MOA and defining tests to measure its bioactivity is a very challenging process. As the therapeutic action of the CT product is dependent on multiple biological mechanistic pathways, developing a single test to predict clinical efficacy/bioactivity may not be feasible. Instead of one test, a matrix of tests measuring different attributes of the CT product may be required (U.S. FDA, 2011; Pritchett and Little, 2012). The European Medicines Agency and the Food and Drug Administration (FDA) have recognized the difficulties and inherent challenges associated with potency assay development for CT products and adopted a flexible but still rigorous regulatory approach. Due to the multimodal MOA the regulators do not require a potency test that measures or reflects all of the products' attributes. Nonetheless they do require that an assay or matrix of assays are developed to measure one or more of the relevant biological therapeutic properties and this measure should provide consistency across clinical production batches (EMA, 2011; U.S. FDA, 2011). For this reason, potency testing should begin as early as possible. The FDA guidance document recommends to the implementation of a progressive potency strategy (U.S. FDA, 2011). In this situation, the regulator suggests that the potency tests be developed over the course of the preclinical and clinical studies with the assay becoming more defined with increased understanding and knowledge of the product and its biological functions. As potency measurements are primarily interrelated with product characterization, the potency assay development process begins as part of the characterization of the product's active ingredients

(PAS 93, 2011), which occurs during the early preclinical development stage. Optimization and development of the assay, or matrix of assays, advances incrementally during clinical development. While quantitative assays are not required for phase 1 trials, for phase 2 some candidate assays should be identified and evaluated (U.S. FDA 2011; Pritchett and Little, 2012). Fully validated potency assays are required after pivotal clinical trials are complete. It must be acknowledged; through all the development stages of the CT product's lifecycle the assay can only be defined as a potency assay once it has been correlated with clinical data obtained from pivotal phase 3 clinical trials (Porat et al., 2015).

Reperfusion therapy such as angioplasty and bypass surgery is the standard of care that vascular surgeons use in restoring blood flow to ischemic tissues in patients with CLI (Spaltro et al, 2015). Using these therapies blood flow is adequately restored through large to medium sized blood vessels. The proposed therapeutic mechanism of action of MSC based tissue perfusion in CLI patients is the restoration of tissue reperfusion by means of biological bypass through the stimulation of new vasculature and thereby augmenting blood flow in pre-existing arteries and arterioles. The biological process underlying this therapeutic activity of MSCs includes angiogenesis. Angiogenesis can be described as the sprouting of blood vessels from already pre-existing blood vessels (Watt et al., 2013). This is a complex process that begins with basement membrane degradation and mural cell detachment from the small vessels (Watt et al, 2013). The next step is characterized by endothelial cell proliferation and migration. Endothelial tip cells invade to form filopodia and lamelipodia in response to cues from growth factors and cytokines (Watt et al, 2013). Stalk cells that are located behind the tip cells proliferate to extend the blood vessel length and form an extracellular matrix, junctions and vessel lumens (Watt et al, 2013). The endothelial tip cells then anatomize with the stalk cells and vessel maturation begins. The final process occurs when recruited mural cells come in contact with the newly formed blood vessel resulting in vessel stabilisation (Watt et al, 2013). Once the vessel is stabilized through the final deposition of extracellular matrix the blood flow recommences (Watt et al, 2013).

Before embarking on the development of a potency assay the investigator must develop an appropriate strategy in order to choose read outs relevant to the cell therapy product (Bravery et al, 2013). This strategy should be constructed based on an understanding of the processes that are central to the proposed mechanism of action of the product (U.S. FDA 2011; Bravery et al, 2013; Porat et al, 2015). As we believe angiogenesis is central to the therapeutic benefit of our MSC product our potency target represented this process. In this chapter the early stages of the development of a series of assays used to measure the bioactivity of our hMSCs is described. Using a human umbilical endothelial vein cell (HUVEC) tube formation assay the capacity of hMSC conditioned medium (CM) to induce tubule formation in vitro was demonstrated. To provide confidence in our in vitro assay at predicting the angiogenic activity of our cells, an in

vivo matrigel plug assay on 5 selected MSC donors was undertaken and compared with the in vitro results. After demonstrating an angiogenic response in vivo, a potential association with increasing levels of VEGF and increased blood vessel density was observed. Furthermore, secretome analysis was performed to mimic the drug discovery process to screen our angiogenic donor cells in order to elucidate future novel targets that may be central to MSC mediated angiogenesis.

METHODS

Cell Culture

HMSCs were isolated and maintained in culture as described in previous chapters. Briefly,hMSCS were isolated from healthy adult bone marrow and cultured expanded in accordance with local ethical committee approval. Upon receipt, the bone marrow aspirate was washed with DPBS and centrifuged at 900g. A 4% acetic acid wash was performed on a sample of the marrow to lyse the red blood cells and enable an accurate MNC count. MNCs, plated at 40-50 million per 175cm^2 were cultured expanded in monolayer with complete medium (αMEM supplemented with 10% selected foetal bovine serum [FBS]) in 5% CO_2 at 37°C. On day 3, fresh medium was added to the culture. On day 5, the cultures were washed with DPBS to remove non-adherent cells and fresh complete medium was added to each flask. When the monolayer reached 80-90% confluence, the adherent cells were washed with DPBS and detached from the culture plastic with 0.25% tryspin/EDTA. The dissociated cells were centrifuged at 400g for 5 minutes. The resultant pellet was re-suspended in complete fresh medium and the cellular yield determined. hMSCs were further sub-cultured in T-175 flasks through two and three passages. hMSCs were cryopreserved at a dose of 1x10^6 per ml in FBS combined with 10% dimethyl sulfoxide.

HUVECs were cultured in endothelial basal media (EBM) supplemented with a growth factor bullet kit supplied by the manufacture. After the addition of the growth factor bullet kit to the media it can then be classified as endothelial growth media (EGM). Media was changed the day after cell seeding and every other day thereafter. HUVECs between passage 7 – 10 were used for the in vitro angiogenesis assays.

Preparation of Conditioned Media From hMSC Donors

MSCs ($1x10^6$ per T-175 flask) were seeded into a flask-containing αMEM. When the cells were 80-90% confluent the cell culture medium was changed and the cells (passage 2) were placed back into an incubator at 37°C, 5% CO_2 to generate conditioned medium (CM). After 24 hours the CM was collected, spun down at 400 RCF for 5 minutes and the supernatant was collected, aliquoted and stored at -80°C.

In Vitro Angiogenesis Assay

Growth factor reduced matrigel (Corning life sciences) was thawed on ice at 4°C overnight. 110µl of matrigel was distributed to the inner wells of a pre-cooled 48-well tissue culture plate and allowed to solidify for approximately 1 hour at 37°C. The addition of matrigel to the plate was done over ice and using pre-cooled P-200 pipette tips. HUVECs were washed with DPBS followed by a brief rinse with 0.25% trypsin/EDTA. The cells were then placed into an incubator at 37°C. After 5 minutes the cells were recovered using EGM and spun down at 400 RCF for 5 minutes. Cells were re-suspended in DPBS and counted using a haemocytometer. HUVECS were added to the hMSCs CM and other experimental conditions and controls at a concentration of 12,500 cells/ml/well. A total of 500µl of cells suspension was added to each well. Each sample of the control and treatment groups was assayed in triplicate.

VEGF ELISA

CM from 5 hMSC donors was collected after 24 hours. VEGF was quantified using a commercial enzyme linked immunosorbent assay (ELISA) in accordance with the manufacturers instructions (R&D Systems, DVE00).

Pilot Matrigel Plug Study

To standardize the in vivo matrigel assay a pilot study using 4 mice (N=2 per group) was completed using FGF-2 and PlGF at concentrations already determined in our in vitro matrigel assay. Briefly, each mouse was injected in the dorsal air sac with 300 µl of matrigel containing 300 ng/ml FGF-2 or PlGF 50 ng/ml and matrigel alone (negative control). After 7 days the mice were euthanized by CO_2 asphyxiation and the matrigel plugs were excised, photographed and fixed in 10% neutral buffered formalin for 24 hours. After 24 hours the samples were processed using standard histological procedures (Leica ASP3000) embedded in paraffin wax and stained with H&E.

Hematoxylin and Eosin Staining

De-waxing was performed by immersing the batch of slides in two sequential baths of xylene (10 minutes each). Xylene was removed by two submersions in 100% ethanol (EtOH) for 2 min each. Samples were re-hydrated by sequential baths for 2 minutes each in 95% EtOH, 70% EtOH, 50% EtOH followed by water. Slides were then immersed in Mayers hematoxylin (Sigma) solution for 6 min to stain the nuclei dark, and then washed under a running tap water for 4 min in order for bluing of the nuclei to occur. Slides were then placed in Eosin (with 0.5% glacial acetic acid) (Sigma) for 2 minutes to stain cytoplasm in red. Slides were quickly rinsed in tap water and subjected to successive dehydration in sequential alcohols (50%, 70%, 95%, 100%, 100%) for 2 minutes each. Finally, the slides were then immersed in two final baths of xylene (10 minutes each) removed and then cover slipped using slides with xylene-based mounting media (DPX, Sigma). Slides were then filed away into storage boxes and stored at room temperature. This process was repeated until all slides were stained.

Pivotal Matrigel Plug Study

Cryopreserved hMSCs were thawed and prepared immediately before injection. The cells were removed from liquid nitrogen, thawed in a 37°C water bath and transferred directly into tubes containing 4 ml of saline vehicle. The cryovial was washed once to ensure that all cells were removed. Cell viability was determined by trypan blue (Sigma Aldrich) exclusion staining. Cells were counted using a hemocytometer. The cell suspension was transferred into 15 ml conical tube and centrifuged at 400g for 5 minutes at room temperature. After washing the cells, cells were suspended in matrigel to a concentration of $1x10^6$ cell/ml (300 µl matrigel containing $3x10^5$ hMSCs). For a positive control, 500ng/ml of fibroblast growth factor was suspended into matrigel, while matrigel alone was used as a control. A total of 5 mice were injected representing one mouse per donor. Each mouse had its own positive and negative control to account for the inter mouse variability at endogenously stimulating blood vessels. Each mouse was injected in the dorsal air sac with 300 µl of matrigel containing 500ng/ml FGF, $3x10^5$ hMSCs and matrigel alone. After 12 days the mice were euthanized and the matrigel plugs were excised and fixed in 10% neutral buffered formalin for 24 hours. After 24 hours the samples were processed using standard histological procedures (Leica ASP3000) and embedded in paraffin wax.

STEREOLOGICAL METHODS AND FORMULAE

The integration of stereological methods into any study design is essential to ensure proper sampling of tissues and generation of whole tissue interfaces that adequately represent the tissue (Dockery et al., 2007). For this reason, a stereological approach was applied to the sectioning procedure of our matrigel plugs. Stereology was performed in order to determine the minimum field of view required to estimate the entire matrigel thickness and also to evaluate neo-angiogenesis in our excised matrigels.

Estimation of the Minimal Number of Fields of View

In order to obtain the minimum number of fields of view required to obtain statisically relevant data from the histological blood vessel quantifcation, a pilot study on one matrigel plug was completed. Briefly, 5µm tissue sections were taken every 100µm. Each slide was then stained with H&E. Images of 10 random fields of view per slide were captured at 20x magnification (Olympus light Microscope) and an unbiased stereological grid was applied. Data was collected on the cumulative number of blood vessels per mm^2 (Cf Na mm^2) and field of views were plotted in a graph. . A +/- 5% range was calculated from the last value of Cf Na mm^2**.** The minimum number of fields of view required to obtain statisically relevant data was represented by the first value that fell within the +/- 5% range (red dotted line).

Estimation of Angiogenesis using a Stereological Approach.

A stereological approach was applied to our sectioned and stained matrigel plug slides in order to obtain information regarding the volume and distribution of the blood vessel network contained within it. The three parameters that we chose to measure were number of blood vessels per mm^2 (Cf vessels), radial diffusion distance (R_{diff}), and length density (L_v).

An unbiased counting grid (459.3µm and 740.5µm) was overlaid on the images and a blinded observer carried out blood vessel quantification. For quantification only blood vessels that touched the left and bottom side of the grid were excluded from the counts (N vessels). The associated test grid was calculated by multiplying the amount of cross points touching tissue (PTs tissue) by the area of the square estimated by the calibrated scale bar on the image.

Length density (L_v) is a parameter measure that provides information regarding the length of the blood vessel per unit volume of tissue and is estimated by multiplying the number of profiles on a selection per unit area (Q/A) by 2. (Dockery et al., 2007; Garcia et al., 2007). The length

density of the blood vessels quantified within the grid on the H&E stained sections was calculated using the following formula.

$$L_V = \frac{L}{V} = 2 \times \frac{Q}{A} \qquad \text{(Eq. 1)}$$

The radial diffusion distance (R_{diff}) provides an estimate the cylindrical zone of diffusion around another blood vessel. R_{diff} was another important parameter which we were interested in quantifying as it provided us with quantitative information regarding the distance between blood vessels. Using the value obtained from the L_v and applying the equation below, R_{dif} can be estimated.

$$R_{diff} = \frac{1}{\sqrt{\pi \times L_V}} \qquad \text{(Eq. 2)}$$

STATISTICAL ANALYSIS

Statistical analysis of the results was performed using Graphpad Prism (Graphpad Prism 5 Software). The results of multiple observations are presented mean ± standard deviation (SD). For multivariate data analysis, group differences were assessed with one-way analysis-of-variance (ANOVA) with correspondent multiple comparison test, which is specified in the legend of each figure. Differences were considered statistically significant when *p* values were lower than 0.05.

RESULTS

In Vitro Tube Formation Assay Standardization and Qualification

An extensive series of assays were performed in order to determine the optimal seeding density. We observed that 25,000 cells per well of 48 well coated with matrigel was the minimum amount of HUVECs needed to be seeded in order to produce the least amount of background tube formation within the negative control and enough to enable tube formation within the treatment and positive control groups. After standardization of the cell density, the dynamics of tube formation was observed in order to determine the optimal time for tube counting. The cells were seeded and incubated in EBM and EGM and were monitored over a period of 24 hours to observe tube formation (Figure 7). It was observed that the cells start to migrate towards each other from 1-3 hours. Furthermore, at 6 hours the tubes begin to mature to form a tubular

network (Figure 7). Fully mature tubule formation was observed at 18 hours (Figure 7) and therefore was chosen as the time point for analysis of all of the in vitro matrigel studies described in this chapter.

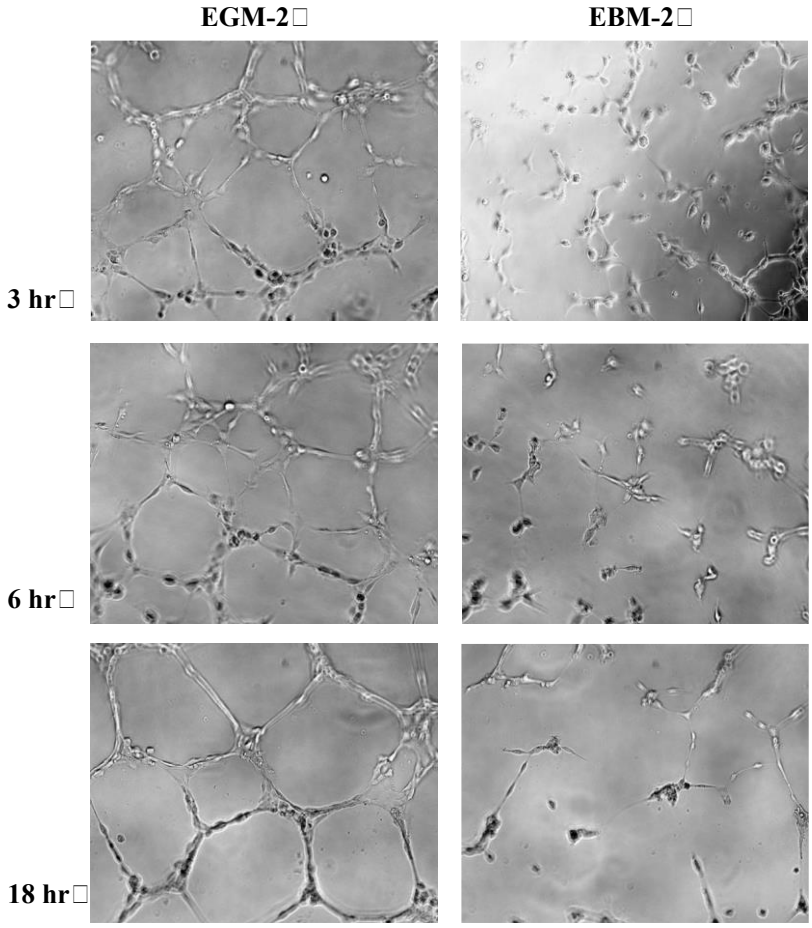
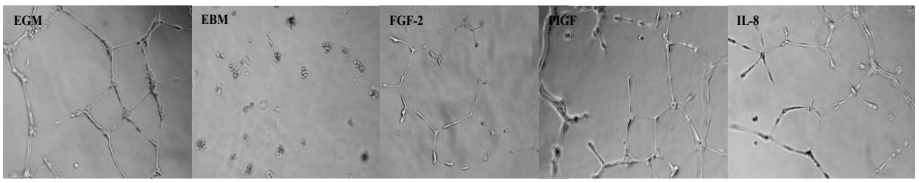


Figure 7: Dynamics of behavior of HUVEC tubule formation after plating on a matrigel basement membrane extract. Shown is the appearance of the HUVECS seeded at a density of 25,000/well 48 well plate on matrigel and incubated in endothelial growth media (EGM) and endothelial basal media (EBM). The cells initially attach in the first hour followed by migration towards each other between 1-3 hours. Capillary like tubules form by 6 hours and are fully mature by 18 hours. By 24 hours the cells detach from the matrix and the tubes break apart.

Upon determination of the optimal conditions for this assay we next proceeded to qualify the assay using known angiogenic agents. Fibroblast growth factor-2 (FGF-2), placental growth factor (PIGF) and interleukin-8 (Il-8), all of which are potent stimulators of angiogenesis, were added to EBM media and placed on the HUVECs on matrigel. In the absence of angiogenic stimulants (EBM) very few tubes were quantified (Figure 8A+B). However, in the presence of the single angiogenic factors, FGF-2 (300ng/ml), PlGF (50ng/ml), Il-8 (50ng/ml) and EGM a vast tubular network was observed and quantified. All angiogenic factors were significantly different to the EBM negative control at stimulating tubule formation.

A



B

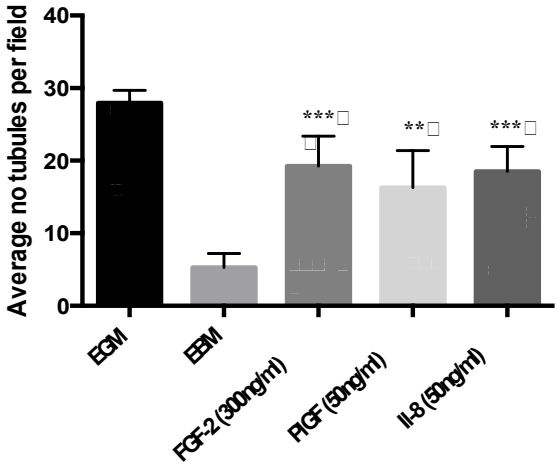


Figure 8: Standardization of in vitro matrigel assay. HUVEC tube formation to test the angiogenic potential of FGF-2, PlGF and IL-8 (A) Representative photomicrographs of in vitro angiogenesis induced by EGM, EBM, FGF-2, PlGF and IL-8 culture with HUVECs. (B) Average tubule number of tubes formed per field for each condition: endothelial growth factor media (EGM), endothelial basal media (EBM) and endothelial cell basal media containing FGF-2 (300ng/ml), PIGF (50ng/ml) and Il-8 (50ng/ml) n=4. Statistical significant difference by one-way ANOVA with Tukey's multiple comparison test. **p<0.0002 and ***p<0.0001 compared to EBM.

Dose Extrapolation from In Vitro Matrigel To In Vivo Matrigel.

To test if the observations observed in figure 8 could correlate with an in vivo response we tested the same concentration of FGF-2 and PlGF in an in vivo matrigel model (Figure 9). The matrigels containing the proteins were injected into the dorsal air sac of a BALB/c nude mouse and excised after 7 days. An abundant amount of blood vessels were observed in both PIGF and FGF treated animals (n=2 per group) Figure 9 below demonstrates the in vitro to in vivo correlation between the biological responses observed in vitro and in vivo using the same dose. At this point robust in vitro and in vivo assays of angiogenesis had been developed and optimized using known angiogenic factors and we were thus ready to proceed to using these assays with human bone marrow derived MSCs.

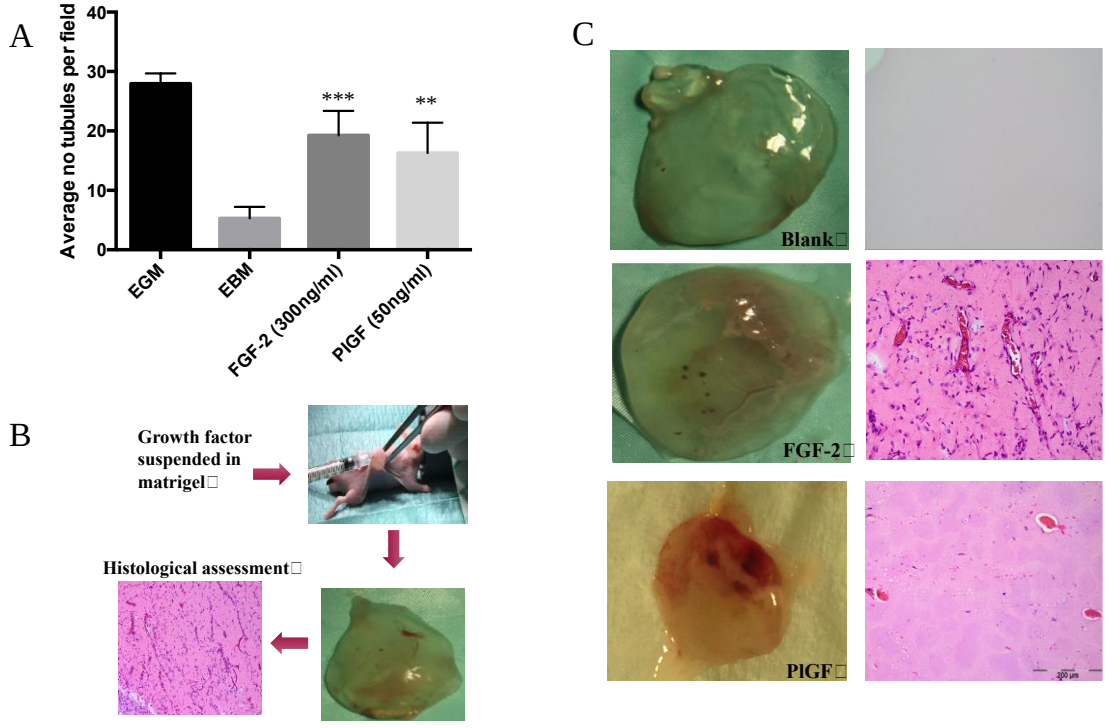


Figure 9: Dose extrapolation from in vitro to in vivo. (A) In vitro matrigel assay demonstrating the in vitro tubule count of FGF-2 (300ng/ml) and PlGF (50ng/ml) N=4. (B) Schematic of matrigel plug assay being used to qualify the angiogenic response of the in vitro doses of FGF-2 and PlGF (C) Gross images and corresponding photomicrographs of H&E stained 5 µm sections of the 7-day matrigel plugs that contained matrigel alone, FGF-2 (300ng/ml) and PlGF (50ng/ml). Stained sections demonstrate endogenous blood vessel infiltration in both the FGF-2 and PIGF but not in the control. **p<0.0002 and ***p<0.0001 compared to EBM.

hMSC Secrete Factors That Promote Angiogenesis In Vitro

HUVECS are very sensitive to the media in which they grow. In order to test if the MEM media used to generate conditioned media was capable of supporting angiogenesis, a pilot study was carried out using the in vitro matrigel assay. HUVECs were plated on growth factor reduced matrigel with the addition of basal MEM media with serum, MEM containing serum and growth factor bullet kit (contains numerous growth factors that are added to supplement the EGM media to ensure adequate HUVEC growth) and EGM for 18 hours. As MEM represents the media in which MSCs are cultured in, the addition of the growth factor bullet kit was to in a simplistic form to mimic MSCs mediated paracrine secretions. The average number of tubes formed per field of view was used as the measure of angiogenesis. The formation of a robust intercalated tube network was observed in the EGM or MEM containing growth factors, which was in stark contrast to the MEM basal media, which showed very little tube formation (Figure 10).

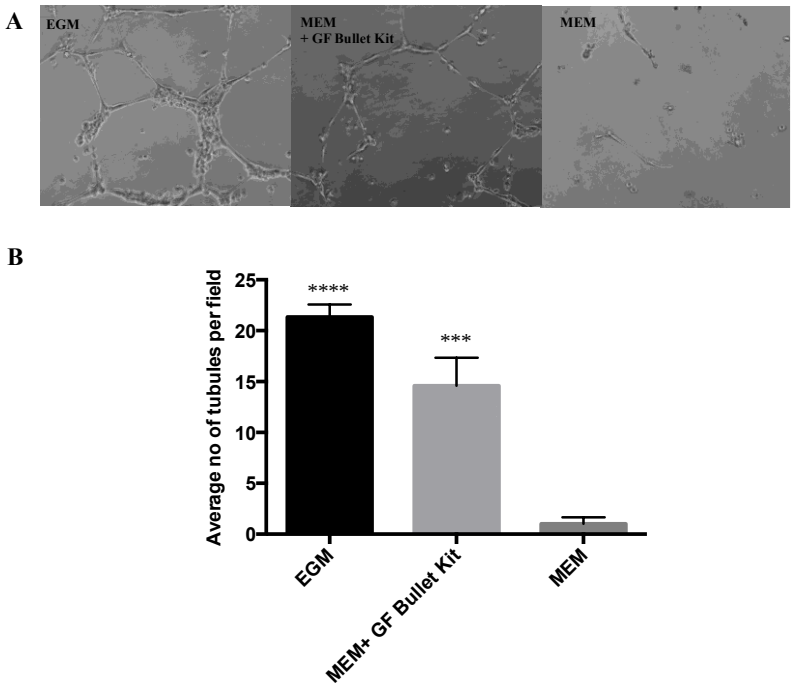
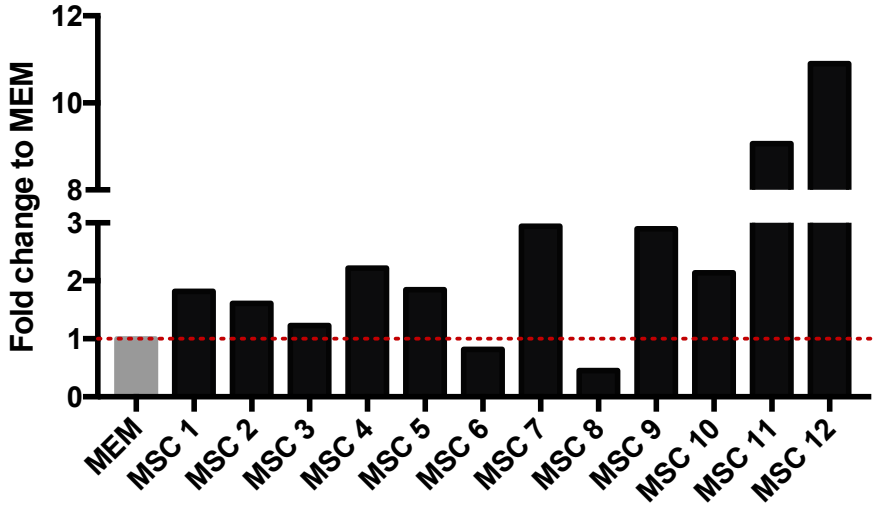


Figure 10: MEM media compatibility testing. (A) Representative photographs of HUVEC seeded on matrigel and incubated for 18hrs with EGM, MEM and MEM plus bullet kit. (B) In vitro tubule assay demonstrating that MEM media with the addition of growth factor bullet kit was superior at stimulating tubule formation over basal MEM but insufficient at inducing tube formation to the level seen by EGM. Statistical significant difference by one-way ANOVA with Tukey's multiple comparison test. **p<0.0002 and ***p<0.0001 compared to MEM.

This data suggests that MEM media in the presence of growth factors is supportive of angiogenesis in our in vitro tubule assay. As HUVECs were receptive to this media we therefore used it to generate CM from our hMSCs donors.

To test whether hMSC had the ability to secrete factors that can promote angiogenesis we used an in vitro matrigel assay using CM generated from the cells. hMSCs were thawed and plated into a T-175 tissue culture flask and grown under standard tissue culture condition. Upon reaching 80-90% confluency the media was changed in order to generate CM for 24 hours. hMSC represent a heterogeneous population of cells. Due to the inherent variability of each generated cell line we wanted to test the angiogenic potential of multiple different hMSC donors (N=12) (Figure 11). Angiogenesis was measured by counting the number of tubules per high powered field for each condition, MEM (negative control), MEM plus growth factor bullet kit, hMSC CM from 12 different donors. The angiogenic potential of CM from hMSC was confirmed. Figure 11 demonstrated the occurrence of donor to donor variability in angiogenic potential of our different MSC populations.

A



B

Experiment	Treatment	No of Tubules Per Field	P Value Compared To MEM	Fold Change To MEM
1	MEM	11.25 (3.96)	-	1
	EGM	28.92 (3.09)	0.0001	2.58
	MSC 1	20.5 (1.78)	0.0001	1.82
	MSC 2	18.08 (3.2)	0.0001	1.61
	MSC 3	13.83 (1.53)	NS	1.23
2	MEM	8.75 (1.77)	-	1.00
	EGM	25.38 (2.33)	0.0001	2.9
	MSC 4	19. 42 (3.29)	0.0001	2.22
	MSC 5	15.42 (2.68)	0.0001	1.85
3	MEM	9.583 (1.98)	-	1.00
	EGM	28.58 (2.30)	0.0001	2.98
	MSC 6	7.83 (2.92)	NS	0.82
	MSC 7	28.17 (4.53)	0.0001	2.94
	MSC 8	4.33 (4.141)	NS	0.45
	MSC 9	27.75 (4.07)	0.0001	2.90
	MSC 10	20.50 (4.01)	0.0001	2.14
4	MEM	2.13 (0.99)	-	1.00
	EGM	-	-	-
	MSC 11	19.25 (3.14)	0.0001	9.06
	MSC 12	23.17 (2.73)	0.0001	10.90

Figure 11: In vitro angiogenesis assay used to determine the angiogenic potential of conditioned media generated from different hMSC donors. 18-hour, 2-dimensional in vitro matrigel tubule assay illustrating the difference in angiogenic potential of conditioned medium for different MSC donors. (A) The data is represented as fold change to the neagtive control, MEM. (B) Average values of the number of tubules per field per MSC donor, MEM and EGM. One-way ANOVA, Dunett comparison test, $p<0.0001$ as compared to MEM.

IN VIVO MATRIGEL

We next compared our in vitro observations with an in vivo matrigel model as described above. Growth factor reduced matrigel was mixed with $3x10^5$ hMSCs. Five MSC donors were selected for in vivo testing based on their in vitro response in the matrigel assay. This experiment allowed us to compare a biological in vitro response with an in vivo response. 12 days after the hMSC/matrigel injection, nude mice were sacrificed and the matrigel plugs were removed, fixed, embedded in wax. To account for the inter animal variability in stimulating angiogenesis, each animal contained its own positive and negative control. For a negative control matrigel alone was administered. For a positive control FGF 500 ng/ml was mixed with matrigel and administered (Figure 13-17). Before the results of this study are discussed we will describe the results of a pilot stereological study used to determine the number of tissue sections required to enable a meaningful assessment of angiogenesis across each matrigel plug tested.

Estimation Of The Minimum number of Fields of View Required For Histological Analysis.

In order to apply the stereological method to enable blood vessel quantifications the samples must be orientated randomly. This means that the tissue sections produced from the embedded tissue must be in isotrophic uniform planes. Only in this way can it be ensured that the retrieved tissue from in vivo has an equal probability of being observed. Normally to account for this variable, tissues are randomly cut into pieces and each piece is then embedded into a circular wax mold. Istotrophy is generally achieved by re-embedding the tissues in spheres. The subsequent spheres are generally spun and re-embedded in larger paraffin blocks in order to ensure random orientation of the sample. As the matrigels removed from the mice were of circular shape we did not require this embedding technique. The circular nature of the matrigel structures ensured that each part of the structure had an equal probability of being sectioned.

When quantifying blood vessels, sampling a single “representative” section of the matrigel may not be representative of the entire matrigel and therefore may underestimate the number of blood vessels present within the structure. For that reason, a pilot study was undertaken to determine the minimal number of fields of view that were required for statistical significance using a confidence interval of 95%. A pilot set of data consisted of 5 representative sections (5 slides) from a matrigel of one test animal. Due to the size of each matrigel 10 fields of view per tissue section were chosen. Therefore, a total of 50 fields of view were included in the analysis.

Slide	Cf field of view	N Vessels	PTs Tissue	Cf Vessels	Cf Tissue	Cf Area	Cf Na mm2	
1	1	4	1	4	1	0.3401	11.7614	
	2	8	1	12	2	0.6802	17.6421	
	3	12	1	24	3	1.0203	23.5228	
	4	3	1	27	4	1.3604	19.8474	
	5	0	1	27	5	1.7005	15.8779	
	6	0	1	27	6	2.0406	13.2316	
	7	15	1	42	7	2.3807	17.6421	
	8	8	1	50	8	2.7208	18.3772	
	9	4	1	54	9	3.0609	17.6421	
	10	0	1	54	10	3.4010	15.8779	
2	11	16	1	70	11	3.7410	18.7113	
	12	14	1	84	12	4.0811	*20.5825*	*1st*
	13	16	1	100	13	4.4212	22.6181	
	14	7	1	107	14	4.7613	22.4727	
	15	9	1	116	15	5.1014	22.7387	
	16	0	1	116	16	5.4415	*21.3176*	
	17	2	1	118	17	5.7816	*20.4095*	
	18	9	1	127	18	6.1217	20.7458	
	19	10	1	137	19	6.4618	21.2015	
	20	0	1	137	20	6.8019	20.1414	
3	21	7	1	144	21	7.1420	20.1624	
	22	25	1	169	22	7.4821	22.5873	
	23	1	1	170	23	7.8222	21.7331	
	24	5	1	175	24	8.1623	21.4401	
	25	4	1	179	25	8.5024	21.0529	
	26	3	1	182	26	8.8425	20.5825	
	27	2	1	184	27	9.1826	20.0380	
	28	5	1	189	28	9.5227	19.8474	
	29	3	1	192	29	9.8628	19.4672	
	30	3	1	195	30	10.2029	19.1123	
4	31	13	1	208	31	10.5429	19.7288	
	32	35	1	243	32	10.8830	22.3283	
	33	7	1	250	33	11.2231	22.2754	
	34	3	1	253	34	11.5632	21.8797	
	35	8	1	261	35	11.9033	21.9266	
	36	4	1	265	36	12.2434	21.6443	
	37	3	1	268	37	12.5835	21.2977	
	38	9	1	277	38	12.9236	21.4336	
	39	9	1	286	39	13.2637	21.5626	
	40	3	1	289	40	13.6038	21.2441	
5	41	13	1	302	41	13.9439	21.6582	
	42	7	1	309	42	14.2840	21.6326	
	43	4	1	313	43	14.6241	21.4030	
	44	10	1	323	44	14.9642	21.5849	
	45	9	1	332	45	15.3043	21.6933	
	46	3	1	335	46	15.6444	21.4135	
	47	9	1	344	47	15.9845	21.5209	
	48	8	1	352	48	16.3246	21.5626	
	49	4	1	356	49	16.6647	21.3626	
	50	5	1	361	50	17.0048	**21.2294**	

5% of Total Cf Na mm2	1.0615
Total Cf Na mm2 + 5%	22.2908
Total Cf Na mm2 - 5%	20.1679

Table 14. Raw data displaying the method used to determine the cumulative number of blood vessels from the number of vessels counted from ten fields of view on five separate tissue sections from one pilot matrigel plug. The final Cf Na mm^2 value in bold represents the number of blood vessels per mm^2 of the total data set. A 5% confidence interval from this value has been calculated. The italicized value is the first to fall within the 5% confidence interval of the final Cf Na mm^2. It is understood from this data that sufficient sections have been recorded to limit statistically significant variance. *Abbreviations: Cf = cumulative values of each variable (e.g. fields of view, blood vessels, tissue, area), N vessels = Number of vessels counted per individual field of view, PTs Tissue = Points hitting tissue, Cf area = cumulative area, calculated by multiplying Cf Tissue by the area estimated from a calibrated scale bar from the image which equaled to 0.34009501 mm^2. Cf Na mm^2 = cumulative number of blood vessels per mm^2, which is calculated by dividing Cf vessel per Cf Area.*

The magnification of the field of view was chosen based on the minimum magnification necessary to allow for resolution of the object of interest. In this case, we wanted to observe blood vessels and therefore 20x magnification was determined as the optimal magnification to allow for counting of blood vessels. Table 14 we display the data collected of the number of blood vessels per mm^2 from 5 tissue sections. The number of blood vessels (N Vessels) falling within the counting grid and the crossing points falling on tissue (PTs Tissue) for 10 fields of view per section were counted. The cumulative number of blood vessels (Cf Vessels) is measured by expressing the sum of all the blood vessel counts (N Vessels total = 361) as a function of the sum of the areas of the counting grids (Cf tissue total = 17.0048). The associated area to test grid was calculated by multiplying the frame estimated from a calibrated scale bar from the image. A similar approach has been used by Garcia et al., 2007.

In Figure 12 the number of fields of view was plotted against the set of cumulative number of blood vessels per mm^2, which display the variance in the data set. The first value to fall in the +/- 5% range of the final cumulative number of blood vessels (Cf Vessels), is highligted in Table 14 and Figure 12, and it determines the minimum number of samples to be measured to ensure a 95% confidence interval. To achieve this value, as evidenced by the graph, we would need 12 fields of view (i.e. minimum of 2 slides). However, taking into account the variability observed in the number of blood vessels per mm^2 counted in the first 4 slides, we considered to use a number of 5 slides in the final analysis where that variability was considerably reduced. We found that one serially sectioned matrigel from start to finish (i.e. one 5μm section taken every 100μm) yielded 5 slides in total. As 5 slides are inside of the 95% confidence interval and the variability found was minimum we decided choose this number of slides for the final analysis

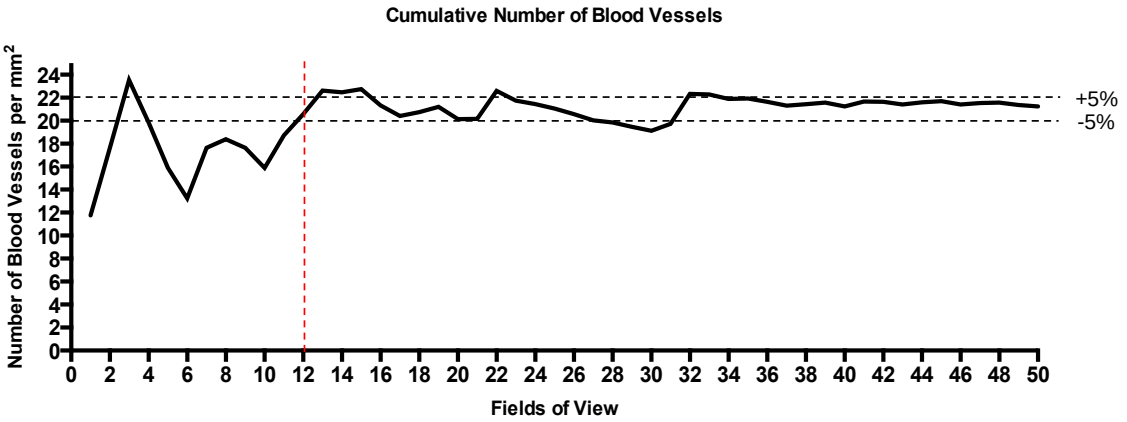


Figure 12. Cumulative number of blood vessels per mm^2 are plotted for each image and consecutive fields of view. As the variance in cumulative number of blood vessels diminishes, the graph is highlighted to show the point (vertical dotted line) at which the values are deviating less than ± 5% (horizontal dotted lines) of the final cumulative number of blood vessels.

hMSCs Stimulate Angiogenesis In Vivo

The in vitro angiogenic response of our MSC donors observed in vitro was observed in vivo (Figure 13-17). A total of five MSC donor lots were used for these in vivo experiments and were selected on the basis of their in vitro assay performance. Four of the donors demonstrated positive response in the in vitro tubule assay (Figure 13A,15A,16A,17A) and one MSC donor lot displayed weak angiogenic potential in vitro (Figure 14A). In the matrigel plug assay, vessel formation was increased in both FGF-2 control and four of the five MSC donors (Figure 13C, 15C, 16C and17C) when compared to the control (matrigel alone). Representative images of blood vessel density for each MSC lot are shown in Figure 13-17B. Furthermore, blood vessel radial diffusion distances were counted for each matrigel. To further examine the in vivo angiogenic effect of MSC donors, in vivo radial diffusion distances and blood vessel length density were calculated. Length density, which represents an estimation of the length of the blood vessels per unit volume and radial diffusion, representing the distance between blood vessels, were calculated using the equations described earlier in the methods section. Four of our hMSC donors responded equally and demonstrated reduced radial diffusion distances when compared to controls (Figure 13E, 15E, 16E, 17E). In addition, an increase in blood vessel length density (Figure 13D, 15D, 16D, 17D) was also observed in comparison to controls suggesting that not only did these MSC stimulate more blood vessels than the control but they also stimulated the growth of longer vessels.

In contrast to four “angiogenic” donors that displayed increases in vessel density, length density and smaller radial diffusions distances, one of our MSC donor lot (Lot 2) failed to display any

superiority over the controls in both our in vitro or in vivo assays. Based on these data we therefore classified this MSC donor lot as a "non angiogenic" donor (Figure 14).

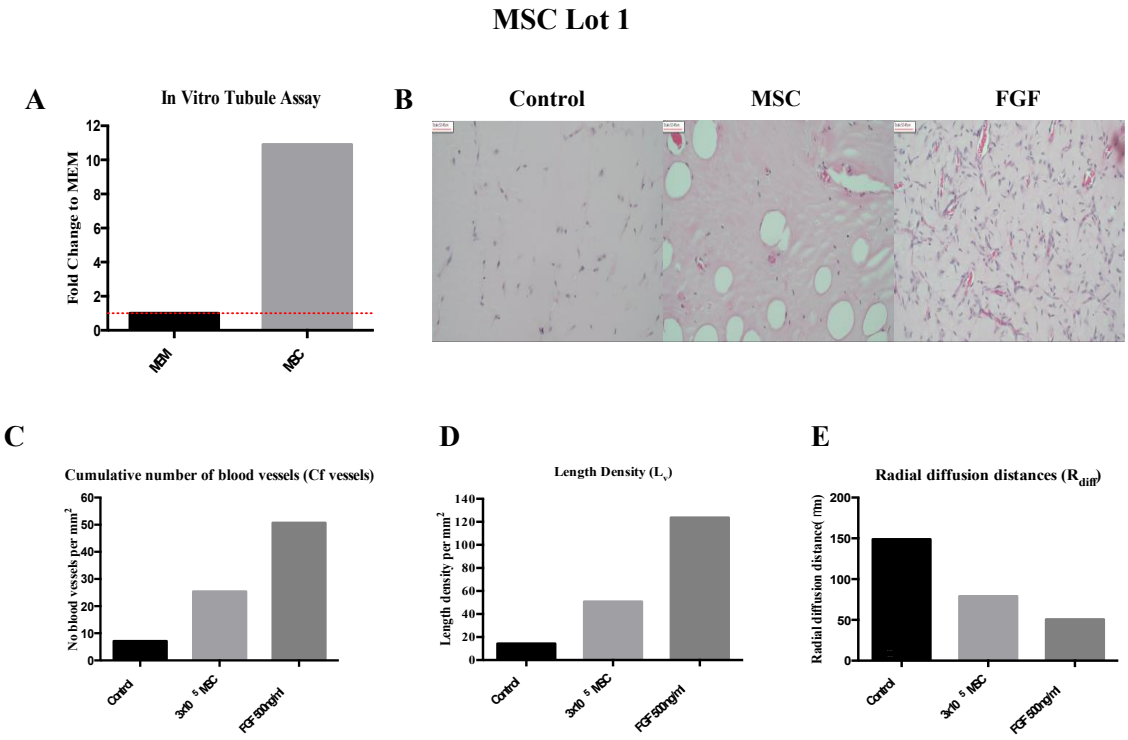


Figure 13: hMSC Lot 1 induces angiogenesis in vitro and in vivo. (A) In vitro tubule assay. Fold change in the number of tubes per field for each condition: EGM, MEM, hMSC-CM. (B) In vivo assessment of hMSC angiogenesis. Representative photomicrographs of hematoxylin and eosin stained 12-day matrigel plugs containing control (matrigel alone), FGF-2 (500ng/ml) and hMSC ($3x10^5$). Injection of matrigel containing, no treatment (control), FGF-2 or hMSCs resulted in increased scoring in cumulative blood vessels per mm^2 (C) and blood vessel length densities (D) in comparison to the control after 12 days. (E) Decreased values were obtained in the radial diffusion distances in the hMSC and FGF-2 groups in comparison to the control.

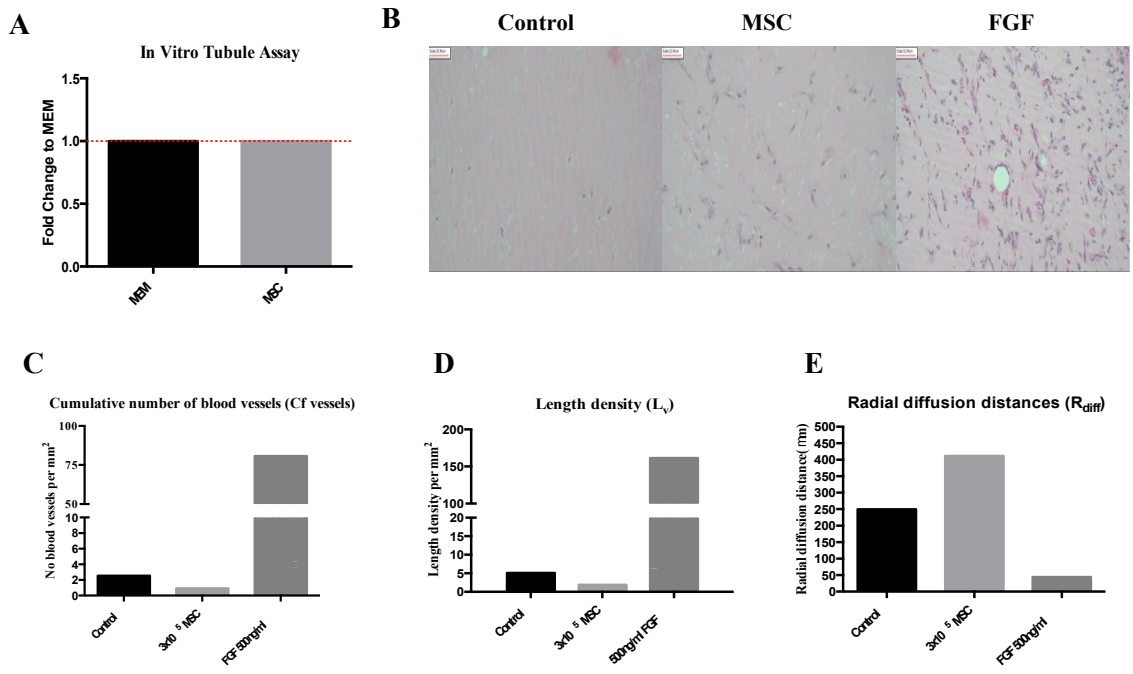


Figure 14: hMSC Lot 2 does not induce angiogenesis in vitro and in vivo. *(*A) In vitro tubule assay. Fold change in the number of tubes per field for each condition: EGM, MEM, hMSC-CM. (B) In vivo assessment of hMSC angiogenesis. Representative photomicrographs of hematoxylin and eosin stained 12-day matrigel plugs containing control (matrigel alone), FGF-2 (500ng/ml) and hMSC ($3x10^5$). Injection of matrigel containing, no treatment (control), FGF-2 or hMSCs resulted in decreased scoring in cumulative blood vessels per mm^2 (C) and blood vessel length densities (D) in comparison to the control after 12 days. (E) Increased values were obtained in the radial diffusion distances in the hMSC and MEM groups in comparison to the positive control (FGF).

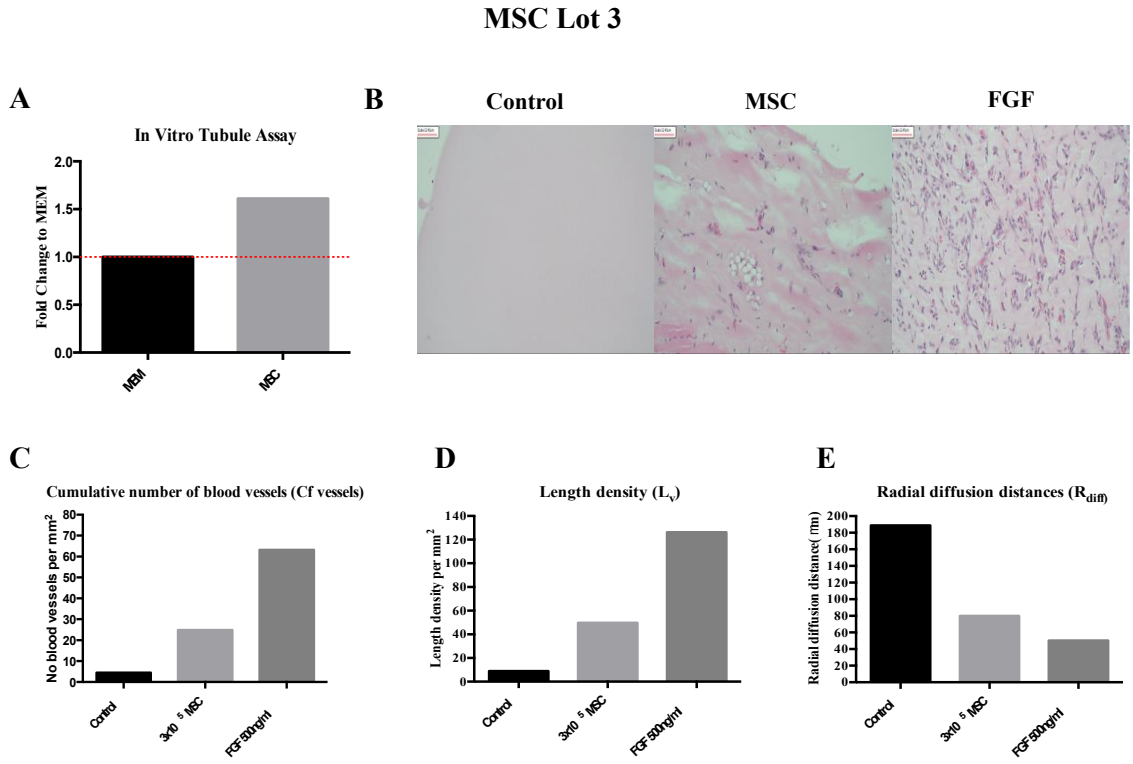


***Figure 15. hMSC Lot 3 induces angiogenesis in vitro and in vivo*.** (A) In vitro tubule assay. Fold change in the number of tubes per field for each condition: EGM, MEM, hMSC-CM. (B) In vivo assessment of hMSC angiogenesis. Representative photomicrographs of hematoxylin and eosin stained 12-day matrigel plugs containing control (matrigel alone), FGF-2 (500ng/ml) and hMSC ($3x10^5$). Injection of matrigel containing, no treatment (control), FGF-2 or hMSCs resulted in increased scoring in cumulative blood vessels per mm^2 (C) and blood vessel length densities (D) in comparison to the control after 12 days. (E) Decreased values were obtained in the radial diffusion distances in the hMSC and FGF-2 groups in comparison to the control.

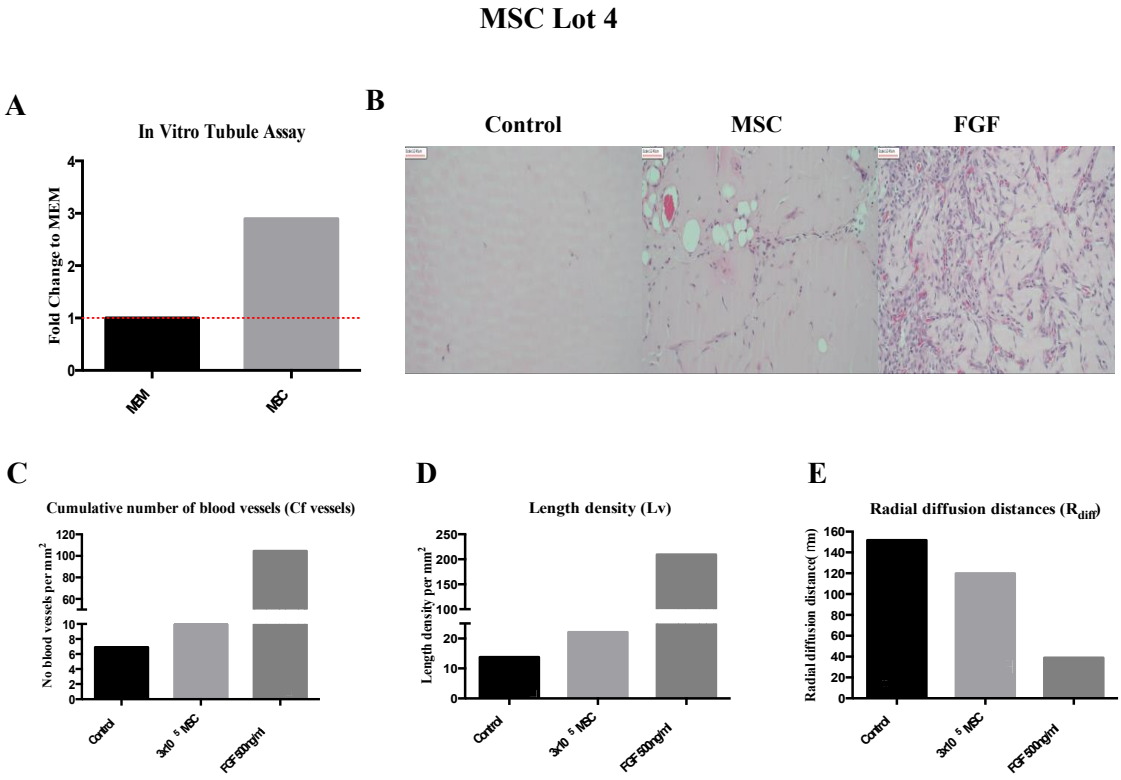


Figure 16: hMSC Lot 4 induces angiogenesis in vitro and in vivo. (A) In vitro tubule assay. Fold change in the number of tubes per field for each condition: EGM, MEM, hMSC-CM. (B) In vivo assessment of hMSC angiogenesis. Representative photomicrographs of hematoxylin and eosin stained 12-day matrigel plugs containing control (matrigel alone), FGF-2 (500ng/ml) and hMSC ($3x10^5$). Injection of matrigel containing, no treatment (control), FGF-2 or hMSCs resulted in increased scoring in cumulative blood vessels per mm^2 (C) and blood vessel length densities (D) in comparison to the control after 12 days. (E) Decreased values were obtained in the radial diffusion distances in the hMSC and FGF-2 groups in comparison to the control.

MSC LOT 5

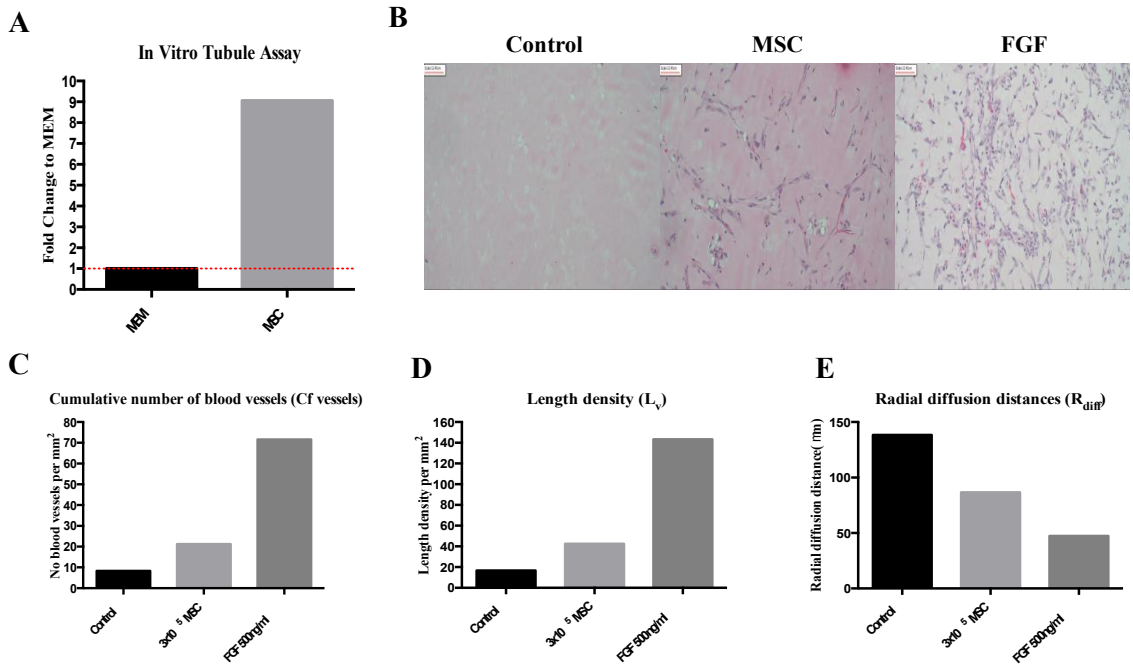
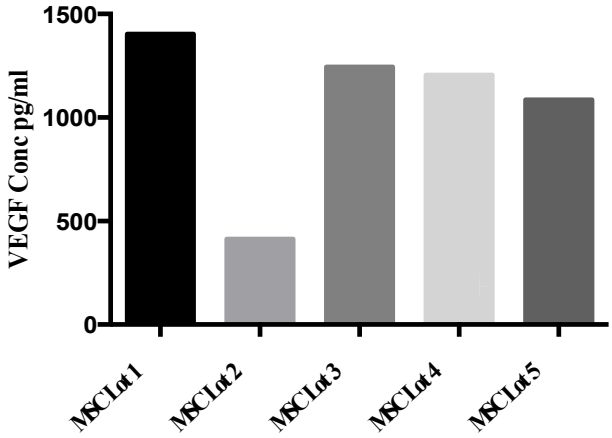


***Figure 17: hMSC Lot 5 induces angiogenesis in vitro and in vivo*.** (A) In vitro tubule assay. Fold change in the number of tubes per field for each condition: EGM, MEM, hMSC-CM. (B) In vivo assessment of hMSC angiogenesis. Representative photomicrographs of hematoxylin and eosin stained 12-day matrigel plugs containing control (matrigel alone), FGF-2 (500ng/ml) and hMSC ($3x10^5$). Injection of matrigel containing, no treatment (control), FGF-2 or hMSCs resulted in increased scoring in cumulative blood vessels per mm^2 (C) and blood vessel length densities (D) in comparison to the control after 12 days. (E) Decreased values were obtained in the radial diffusion distances in the hMSC and FGF-2 groups in comparison to the control.

VEGF ELISA

Studies have shown that MSCs augment angiogenesis and induce neovascularization through the secretion of pro angiogenic growth factors such as VEGF (Kinnaird et al., 2004, Bortolotti et al., 2015). Based on these observations of an important role for VEGF in stimulating angiogenesis, VEGF levels were examined in the CM of the hMSC donors. Furthermore, it was of interest to determine if increased levels of VEGF would be secreted in the angiogenic donors compared to the non-angiogenic donor. The secretion of VEGF in the CM was measured by ELISA and was found to be higher in 4 of the angiogenic donors as measured by positive angiogenic performances in the in vivo matrigel assays (Figure 18) and also previous in vitro assays. However in contrast VEGF concentration in the conditioned medium of MSC lot 2, the poorest angiogenic donor, was low (Figure 18).

A



B

MSC Lot	VEGF (pg/ml)	Cf Blood Vessels (1/mm2)	Lv Blood Vessels (1/mm2)
1	1403.30	25.41	79.15
2	412.54	0.94	411.28
3	1244.21	24.88	79.99
4	1205.10	11.06	119.98
5	1085.22	21.23	86.59

Figure 18. **MSC VEGF secretion.** (A) VEGF protein levels in the conditioned media of 5 MSC lots tested previously tested in in vitro tubule assays. (B) Comparison of VEGF levels in MSC conditioned medium with an in vivo angiogenic response**.** VEGF protein levels were quanitifed from the conditioned media of 5 MSC donors. The same MSC donors were tested in an in vivo matrigel angiogenesis assay and cumulative (Cf) number of blood vessels per mm^2 and length densities (Lv) per mm^2 were quantified using a stereological approach. VEGF protein levels were then compared with the Cf blood vessels per mm^2 and Lv blood vessel values.VEGF levels are mean values of MSC CM assayed in triplicate.

Analysis of MSC Secretome

In order to examine the secretome of MSCs a human angiogenesis array was performed. For consistency, the CM used for the angiogenesis array was the same that was tested in the in vitro angiogenesis assays. Secretome analysis was not performed on hMSC lot 1 as there was insufficient sample available. The data provided from the array revealed multiple different angiogenic factors secreted by the hMSCs (Figure 19). Quantitative determination of secreted factors associated with the "angiogenic" MSC phenotype and the "non-angiogenic" phenotype was assessed. Of these phenotypes we selected the most prominent factors secreted by the cells (Figure 20).

Fibroblast growth factor 7 (FGF-7), insulin-like growth factor binding protein 2 (IGFBP-2), insulin like growth factor binding protein 3 (IGFBP-2), serpin E1, tissue inhibitor of metalloproteinases (TIMP-1) were expressed at higher levels in the 3 angiogenic donors (MSC lot 3,4 and 5) in comparison to the one non-angiogenic donor (MSC lot 2). In the one non-angiogenic donor the most notable growth factors secreted that had lower signal in the 3 angiogenic donors were: angiopoietin-2 (Ang-2), epidermal growth factor (EGF) and urinary type plasminogen activator (uPA). Although these results are only representative of 4 hMSC donors, the preliminary data potentially indicates different secretomes in angiogenic and non-angiogenic donors (Figure 19 and 20). This will need to be tested with larger donor numbers but some interesting candidates have been identified from this work.

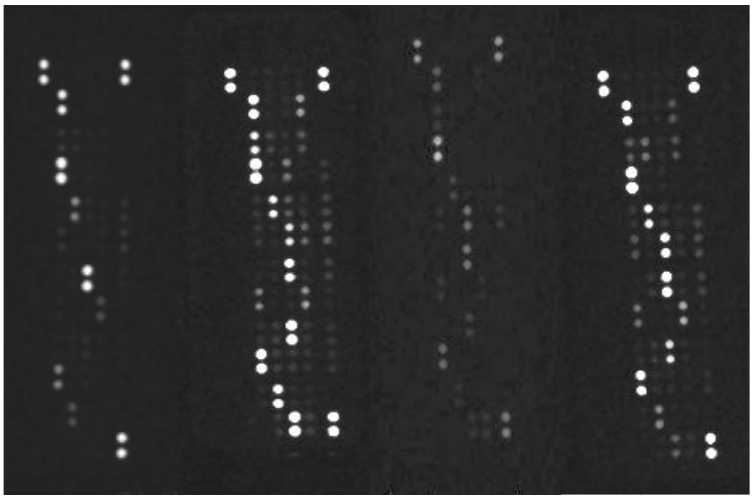


Figure 19: hMSC secretion profiles. Analysis of 24 hour hMSC conditioned media from 4 donor sets on an angiogenesis specific array. Photographs on a developed membrane illustrate a similar secretion profile across the angiogenic MSC donor lots (Lot 3-5) but show a noticeable difference when compared to the secretion profile of the MSC donor lot (Lot 2) that did not induce angiogenesis in vivo.

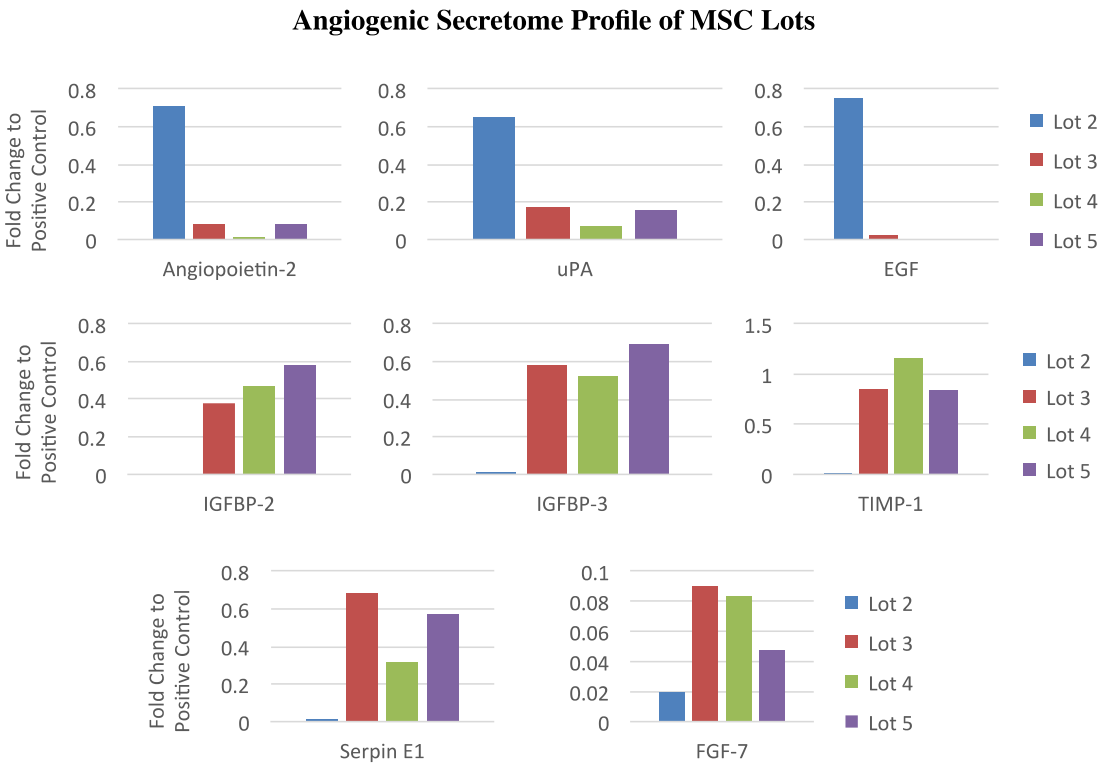


Figure 20: Angiogenic secretome of 4 MSC lots tested in an in vivo matrigel assay. *Semi quantitative analysis of the angiogenesis arrays* showing distinct differences in secretion profile for the following growth factors; FGF-7, IGFBP2, IGFBP3, Serpin-E1, TIMP1, EGF, uPA, VEGF-C and Ang-2. The data is expressed as average spot pixel density as a fold change to the internal positive control. *Abbreviations: FGF-7, fibroblast growth factor 7; IGFBP2, insulin growth factor binding protein 2, IGFBP3, insulin growth factor binding protein 3, TIMP-1, tissue inhibitor of metalloproteinases-1; EGF, epidermal growth factor; uPA, urokinase plasminogen activator, VEGF-C, vascular endothelial growth factor-C, Ang-2, angiopoietin-2.*

DISCUSSION

The initial intent of MSC therapy was to use their differentiation capacity to repair and regenerate injured tissues. However, studies exploring hMSC MOA have shown that these cells exert their beneficial effects primarily through the paracrine section of growth factors and cytokines (Caplan et al., 2011; Kwon et al., 2014). This has widened substantially the potential clinical indications of this therapy. The focus of this thesis has been the translational pathway to the clinic using MSCs as a treatment for patients with 'no option for revascularization' critical limb ischaemia. The therapeutic paradigm of using hMSCs to treat CLI is based on the consideration that they can deliver trophic support to ischaemic tissue by modulating inflammation, recruiting host tissue progenitor cells and by stimulating neo angiogenesis. While the benefit of this cell population is most likely derived from a series of these different pathways we believe that the angiogenesis pathway is critically involved and thus have focused on this in our early attempts to define a potency assay. Data from our group and the literature suggest that the administration of MSC in different animal models with ischemic injury results in increased tissue perfusion by increasing angiogenesis within the tissue injury site (Kinnaird et al., 2004; Bortolotti et al, 2015; Iwase et al, 2013). As our therapeutic rationale is focused on angiogenesis, our overall goal was to manufacture a MSC product that can effectively mediate an angiogenic response in vivo. In order to do this, it is first essential to establish a subset of well-standardized assays for these angiogenic pathways in order to generate comparative data and furthermore correlate in vitro surrogate measures of the cells bioactivity with an in vivo response.

As part of our product development in vitro and in vivo assays were specifically designed in order to provide quantitative information regarding the relevant biological activity/potency of our CT product. Our strategy was built in three separate stages. We first carried out a set of in vitro assays of angiogenesis to look at our product at a cellular level. We then sought to develop an in vivo assay where the cell product itself would be analysed, therefore we looked into the tissue level to observe the bioactivity of our cells in an animal model. Finally, we then completed a series of assays explored at the molecular level to see if there were any targets that could be used to further refine our understanding of the cellular mechanism of action.

To begin the functional characterization assays of the hMSC product we started with an assay focused at the cellular level. As the presumed MOA of hMSC is the stimulation of angiogenesis through paracrine secretions we opted to use the matrigel tube formation assay. The matrigel tube assay is a simple quantitative assay that is based on the ability of HUVEC to form 2D capillary like tubular structures when cultured on a basement membrane extract such as

matrigel. This in vitro assay represents a simple, rapid assay that can be used to study activators of angiogenesis. In the presence of angiogenic agents, HUVEC will migrate towards each other and align to form tube like structures that resemble a capillary network. Despite this being a well established assay of in vitro angiogenesis, we were unable to reproduce results from 2 separate protocols which implied that significant standardization was required before it could be used to assess the angiogenic potential of hMSCs. Much consideration was given to all aspects of this assay prior to its qualification for use in this study. To begin, the seeding density of HUVECs was optimized to 25,000 cells/well (Figure 7). The seeding density is critical to producing reliable and reproducible results. We found that 25,000 HUVECs per well was optimal for not producing any spontaneous angiogenesis in the negative control. Seeding more cells than 25,000 per well resulted in high baseline levels of tubulogenesis in the negative control which often resulted in difficulties in detecting differences between the positive and negative controls. Furthermore, once the optimal cell density was chosen, the dynamics of tubule formation was followed over 24 hours to assess the optimal time point for maximal tubule formation (Figure 7). We found after a series of assays that the tubules were fully formed at 18 hours and this represented the optimal time point to assess tubule formation (Figure 7). In addition to cell density and appropriate time point selection, another important factor that needed to be considered was the batch of matrigel used. As we wanted to use this assay to assess the stimulators of angiogenesis we opted for the use of growth factor reduced (GFR) matrigel. Despite the use of growth factor reduced matrigel, often we observed variances in tubule formation activity based on different preparations of GFR matrigel even from the same supplier. To account for this variability, pre-screening of the matrigel was performed before use in the assay and matrigel from the same lot was used for each assay for all the in vitro and in vivo tests performed. Once the optimal conditions of the assay were determined a series of tests were performed to further qualify the assay (Figure 8). To ensure assay qualification three known angiogenic agents were tested. FGF-2, PlGF and Il-8, all of which are known angiogenic stimulants, were tested.These factors individually stimulated angiogenesis in a consistent and reproducible manner over a series of 4 independent assays (Figure 8). To further progress our assay development, the matrigel plug assay, an in vivo system to assess angiogenesis was optimized. In this assay matrigel is injected subcutaneously into the dorsum of a mouse and, in the presence of appropriate angiogenic stimulation, neo-angiogenesis can be measured and quantified (Figure 9B). Therefore, as we wanted to correlate in vitro and in vivo results we carried out this assay using the same concentrations of FGF-2 and PlGF that were used in the in vitro assays (Figure 9A). After 7 days, strong angiogenesis was observed in both FGF-2 and PlGF in comparison to the control (Figure 9C). Although rigorous standardization of these assays was required, this data suggested that we had an effective set of appropriate assays and

therefore provided us with confidence that we could use them to assess the angiogenic potential of our hMSCs.

hMSC isolated from individual donors may have variability in angiogenic potency due to heterogeneity. This would be particularly important in the context of autologous transplantation where disease-induced dysfunction may be an additional important variable. Furthermore, as each manufacturing batch from donor cells represents a new lot, this makes lot-to-lot variability a valid consideration. Taking these points into consideration, we hypothesized that each lot of donor cells could act differently at stimulating angiogenesis. To test this hypothesis, we screened multiple hMSC donors in our in vitro angiogenesis assay. As hMSC angiogenic effects are proposed to be caused by secreted growth factors and cytokines we generated CM. Prior to the pivotal assay initiation, a pilot study to test the compatibility of MEM with the HUVECs utilized in the assay was performed (Figure 10). As HUVECs are very sensitive to the media in which they are grown in we wanted to test if the media that would be used to generate the CM would support tubulogenesis. To test this, a growth factor bullet kit of known angiogenic factors was added to the media and was assayed for angiogenesis against the negative control (MEM) and positive control. We found that our MEM media was capable of stimulating angiogenesis greater than the negative control albeit less than the positive control EGM (Figure 10). After making this observation, we next proceeded to generate conditioned media from distinct hMSC donors. We assayed the conditioned media from 12 hMSC donors and, as anticipated, found varying degrees of tubule formation across different donors' cells (Figure 11). Five hMSC donors were selected based on their in vitro assay performance and their angiogenenic potential was assessed in vivo (Figure 13-18). Matrigel was mixed with $3x10^5$ hMSC and injected subcutaneously into a BALB/c nude mouse. This assay was used to correlate our in vitro findings with in vivo response (Figure 13-18). Increased blood vessel formation and length density was observed in MSCs from which CM was found to be pro-angiogenic in vitro (Figure 13, 15, 16 and 17). However, one donor that was associated with a poor outcome in our in vitro test and did not show an increase in blood vessel number in vivo and overall performed poorly (Figure 14). These data suggest that the results produced using our in vitro assay could be used as a predictor of an associated angiogenic response in an in vivo model.

As VEGF is one of the central growth factors involved in angiogenesis (Ferrara, 2001) we measured it in the hMSC CM. We found higher levels of VEGF secretion in 4 of our donors that stimulated angiogenesis in vitro and in vivo when compared to the 1 donor with poor angiogenic potential (Figure 18). In addition, we observed that increasing VEGF levels may be associated with a positive angiogenic outcome in our in vivo assay (Figure 18B and 18C). One of our donors (MSC lot 2; Figure 14) that had a low level of VEGF secretion also had a lower

number of vessels, shorter length densities than control (Figure 14) and other hMSC populations tested. Although we cannot definitively say, based on this, that VEGF is central to hMSC mediated angiogenic response, this data suggests that increasing VEGF levels may be associated with a positive angiogenic reponse in vivo. Further screening of MSC donors would be required to confirm this association. Nonetheless we will consider VEGF as a potential target for a potency measurement.

As MSC therapy has a paracrine MOA, we tested the capacity of the cells to produce and secrete relevant proteins. As we have tested cells from donors with angiogenic (Figure 13, 15, 16 and 17) and weakly angiogenic (Figure 14) phenotypes in both our in vitro and in vivo assays we wanted to use the biological data to screen to see if we could discover targets that may be useful in the future in defining the biological activity of the hMSC product. Protein secretion in 24-hour CM from 4 donors illustrate that fibroblast growth factor (FGF-7), insulin growth factor binding protein-2 (IGFBP2), insulin growth factor binding protein-3 (IGFBP3), Serpin-E1 and tissue inhibitor of metalloproteinases-1 (TIMP-1) were consistently expressed only in the angiogenic donors whereas epidermal growth factor (EGF), urinokinase plasminogen activator (uPA) and angiopoietin-2 (Ang-2) were only present in high amounts in the one non-angiogenic donor (Figure 20). Although these results provide important insights into the secretion profiles of hMSCs and in potential targets that may be useful for in further defining the biological activity of the product, the screening of more donors would be necessary in order to further confirm these results. Furthermore, if the factors do represent pro angiogenic factors by which hMSC drive vascularization, the importance of these factors could only be confirmed by knocking out or blocking the activity of these factors by using antibodies or inhibitors and then by testing them in in vivo or in vitro models. Carefully designed experiments could then provide information to help establish if each secreted factor alone, or in combination with the other factors, is needed for the desired cellular bioactivity.

Studies have shown that cryopreservation can exert functional changes with hMSCs and therefore diminish their intended biological effect (Pollock et al., 2015; Francois et al., 2011; Chinnadurai et al., 2016). Therefore, the second question we attempted to answer from the same in vivo study was the ability that cryopreserved hMSC possesses to produce blood vessels in vivo. In this study we have shown that MSCs derived after freezing and storage are capable of inducing angiogenesis in an in vivo model (Figure 13-17). This data is of great importance to our ongoing clinical trial where autologous patient samples are being plated, cultured expanded, cryopreserved and re-administered as frozen product to the patient. In that context, one limitation of the data presented in this chapter is that healthy donors were used. A similar series of tests as those described above would be useful for testing on CLI patients cells to see if the

same desired responses are observed as with that of the healthy donors as has been suggested in one publication (Gremmels et al., 2014).

Although the data described herein is fundamentally related to increasing our knowledge regarding biological characterization of hMSCs, it represents the beginning of a process for the development of an assay, or series of assays, that can be used as surrogate measures of angiogenesis and thus predict the angiogenic potency of the MSC product.

REFERENCES

AU, P., HURSH, D. A., LIM, A., MOOS, M. C., JR. , OH, S. S., SCHNEIDER, B. S. & WITTEN, C. M. 2012. FDA oversight of cell therapy clinical trials. *Sci Transl Med,* 4**,** 149fs31.

BAILEY, A. M., MENDICINO, M. & AU, P. 2014. An FDA perspective on preclinical development of cell-based regenerative medicine products. *Nat Biotechnol,* 32**,** 721-3.

BORTOLOTTI, F., UKOVICH, L., RAZBAN, V., MARTINELLI, V., RUOZI, G., PELOS, B., DORE, F., GIACCA, M. & ZACCHIGNA, S. 2015. In vivo therapeutic potential of mesenchymal stromal cells depends on the source and the isolation procedure. *Stem Cell Reports,* 4**,** 332-9.

BRAVERY, C. A., CARMEN, J., FONG, T., OPREA, W., HOOGENDOORN, K. H., WODA, J., BURGER, S. R., ROWLEY, J. A., BONYHADI, M. L. & VAN'T HOF, W. 2013. Potency assay development for cellular therapy products: an ISCT review of the requirements and experiences in the industry. *Cytotherapy,* 15**,** 9-19.

CAPLAN, A. I. & CORREA, D. 2011. The MSC: an injury drugstore. *Cell Stem Cell,* 9**,** 11-5.

CHINNADURAI, R., COPLAND, I. B., GARCIA, M. A., PETERSEN, C. T., LEWIS, C. N., WALLER, E. K., KIRK, A. D. & GALIPEAU, J. 2016. Cryopreserved Mesenchymal Stromal Cells Are Susceptible to T-Cell Mediated Apoptosis Which Is Partly Rescued by IFNgamma Licensing. *Stem Cells.*

DOCKERY, P. & FRAHER, J. 2007. The quantification of vascular beds: a stereological approach. *Exp Mol Pathol,* 82**,** 110-20.

EUROPEAN MEDICINES AGENCY, COMMITTEE FOR ADVANCED THERAPIES. 2007. Reflection paper on stem cell based medicinal products. Retrieved from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/02/WC500101692.pdf

EUROPEAN PARLIAMENT AND THE COUNCIL OF EUROPEAN UNION. 2007.

Regulation no 1394/2007 of the European parliament and the council of the 13 November 2007 on advanced therapy medicinal products amending Directive 2001/83/EC and regulation EC NO 726/2004. Retrieved from: http://ec.europa.eu/health/files/eudralex/vol-1/reg_2007_1394/reg_2007_1394_en.pdf

FRANCOIS, M., COPLAND, I. B., YUAN, S., ROMIEU-MOUREZ, R., WALLER, E. K. & GALIPEAU, J. 2012. Cryopreserved mesenchymal stromal cells display impaired immunosuppressive properties as a result of heat-shock response and impaired interferon-gamma licensing. *Cytotherapy,* 14**,** 147-52.

GARCIA, Y., BREEN, A., BURUGAPALLI, K., DOCKERY, P. & PANDIT, A. 2007. Stereological methods to assess tissue response for tissue-engineered scaffolds. *Biomaterials,* 28**,** 175-86.

IWASE, T., NAGAYA, N., FUJII, T., ITOH, T., MURAKAMI, S., MATSUMOTO, T., KANGAWA, K. & KITAMURA, S. 2005. Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia. *Cardiovasc Res,* 66**,** 543-51.

KINNAIRD, T., STABILE, E., BURNETT, M. S., SHOU, M., LEE, C. W., BARR, S., FUCHS, S. & EPSTEIN, S. E. 2004. Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation,* 109**,** 1543-9.

KWON, H. M., HUR, S. M., PARK, K. Y., KIM, C. K., KIM, Y. M., KIM, H. S., SHIN, H. C., WON, M. H., HA, K. S., KWON, Y. G., LEE, D. H. & KIM, Y. M. 2014. Multiple paracrine factors secreted by mesenchymal stem cells contribute to angiogenesis. *Vascul Pharmacol,* 63**,** 19-28.

PAS 93:2011. 2011. Characterization of human cells for clinical applications guide. Retrieved from: http://shop.bsigroup.com/en/forms/pass/pas-93/

POLLOCK, K., SUMSTAD, D., KADIDLO, D., MCKENNA, D. H. & HUBEL, A. 2015. Clinical mesenchymal stromal cell products undergo functional changes in response to freezing. *Cytotherapy,* 17**,** 38-45.

PORAT, Y., ABRAHAM, E., KARNIELI, O., NAHUM, S., WODA, J. & ZYLBERBERG, C. 2015. Critical elements in the development of cell therapy potency assays for ischemic conditions. *Cytotherapy,* 17**,** 817-31.

PRITCHETT, T. and LITTLE, L. 2011. “Hard cell” potency testing for cellular therapy products.*BioProcess International* 10(3)1-12.

U.S DEPARTMENT OF HEALTH AND HUMAN SERVICES, FOOD AND DRUG ADMINISTRATION.2011. Guidance for Industry, Potency Tests for Cellular and Gene Therapy Products. Retrieved from:

http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/UCM243392.pdf

SPALTRO, G., STRAINO, S., GAMBINI, E., BASSETTI, B., PERSICO, L., ZOLI, S., ZANOBINI, M., CAPOGROSSI, M. C., SPIRITO, R., QUARTI, C. & POMPILIO, G. 2015. Characterization of the Pall Celeris system as a point-of-care device for therapeutic angiogenesis. *Cytotherapy*, 17**,** 1302-13.

WATT, S. M., GULLO, F., VAN DER GARDE, M., MARKESON, D., CAMICIA, R., KHOO, C. P. & ZWAGINGA, J. J. 2013. The angiogenic properties of mesenchymal stem/stromal cells and their therapeutic potential. *Br Med Bull*, 108**,** 25-53.

Chapter 5: Thesis Summary

The focus of this thesis has been the translational pathway to the clinic using MSCs as a treatment for patients CLI.

In **Chapter 1** we describe the regulatory process surrounding the proposed testing of stem cell products in Ireland. Furthermore, we provide a key insight based on our own experiences of what first time academic investigators should consider prior to submitting their IMP application to support the human testing of their stem cell therapy product.

In **Chapter 2** we describe the preclinical toxicology study which was completed in order to characterize the safety profile of our IMP. In this study immunodeficient mice received $3x10^5$ GMP grade hMSCs via the intramuscular route and were monitored for 3 months for any toxicological effects. The administration of $3x10^5$ GMP grade hMSCs was not associated with any abnormal clinical signs, haematological or biochemical changes in comparison to the control. Furthermore, histological analysis did not uncover any tumours, ectopic tissue formation, or major inflammatory response associated with our GMP MSCs. Based on the results in this study we could conclude that the administration of $3x10^5$ GMP grade hMSCs via the intramuscular route was not associated with any adverse effects. This data was included in an investigator brochure that was sent to the HPRA in August 2013 to support our IMP application.

On the 25th of October 2013 a written response letter to our IMP application was sent to us by the HPRA requesting us to complete an "adequate and meaningful biodistribution study" to further support our application. Furthermore, they requested information regarding the analytical method employed in the biodistribution study along with information describing the methods of sensitivity and limits of detection.

In **Chapter 3**, we describe the development of an accurate, reproducible and quantitative, qPCR-based method of tracking the biodistributive fate of human cells in xenogenic models. The assay is a primer probe based PCR assay using custom-made primers to detect and quantify the hAlu sequences in a heterogeneous sample of human DNA and rodent DNA from whole organ gDNA extracts. The assay measures the amount of human gDNA by amplifying a hAlu repeat sequence, enabling the detection of 1 human cell equivalent in $1.5x10^6$ heterogeneous cells. Using this assay we investigated the distribution of $3x10^5$ IM injected GMP grade hMSCs in BALB/c nude mice. The gDNA was extracted from isolated organs and hAlu sequences were quantified by qPCR analysis. No hAlu sequences were detected in the brain, heart, lungs,

kidneys, spleen, or liver of mice that received human MSCs. However, hAlu was detected in the calf and thigh muscles of BALB/C nude mice after 3 months Between 5.32-11.79% of the hDNA persisted at the time of sacrifice. hDNA, indicating potential hMSC localization, was detected three months after cell administration in immune compromised animals only at the administration site and not in distal tissues. In conclusion, this assay is reproducible, inexpensive and effective at detecting hDNA to the level required for regulatory submission.

The data from this study was resubmitted to the HPRA along with our response letter. Based on the toxicology data presented in **Chapter 2** in combination with the biodistribution data in **Chapter 3,** the HPRA granted us permission to initiate a Phase 1b clinical trial in 'no option for revascularization' CLI patients.

From the previous two chapters we can see that there was a number of studies needed to complete the successful clinical trial application. However, despite trial approval we recognized that it is still important to carry on the lab work to future characterize our product. Characterization of the cell and its active substance is a fundamental part of the cell products development (PAS:93, 2011). A comprehensive understanding of the CT products properties and active substance is important in order to control its quality and therefore its safety and its efficacy (PAS:93,2011).

In **Chapter 4**, as part of our product development, we describe a series of assays that we used to measure the bioactivity of our MSC product. As we believe angiogenesis is the biological process underlying the therapeutic activity of our MSC we first began the optimization and standardization of robust in vitro and in vivo assays. After the optimization of our assays, we proceeded to use these assays to assess the functional biological effect of multiple MSC donors. In our in vitro tubule assay we observed MSC donor-to-donor variability at stimulating an angiogenic response. To correlate our in vitro response with an in vivo response, 5 hMSC donors cell lots were selected, based on their performance in the in vitro assay, and were tested in a matrigel plug mouse model. Increased blood vessel formation was observed in our in vivo assay in 4 donors that performed postively in our in vitro assay. These donors were classified as "angiogenic" donors. One donor that performed poorly in our in vitro pre-screen assay also performed poorly in vivo as evidenced by its inability to stimulate blood vessel formation greater than the negative control. This donor and was classified as a "non angiogenic" donor. Furthermore, VEGF ELISA revealed high levels of VEGF in the CM of the angiogenic donors and low levels of VEGF in non angiogenic donors. Furthermore we observed a potential association between VEGF levels in the MSC CM and MSC mediated blood vessel formation in vivo. An angiogenesis array was used in order to screen for targets that may be useful in the future in defining the bioactivity of our MSCs. The data revealed similar secretion profiles

across the angiogenic donors screened but differences in the one non-angiogenic donor. Although the data presented in this chapter has limitations with regard donor numbers, no definite conclusions can be formulated. However, what this data has shown is that our cryopreserved MSC product has the ability to stimulate blood vessel formation in vivo. Furthermore this data has increased our knowledge regarding the biological characterization of our MSC product.

Impact of the Work:

- Development of a novel, sensitive and reproducible qPCR assay that allows for the detection of human cells in mice. The PCR protocol allows for the detection of 1 human cell equivalent in $1.5x10^6$ murine cells. This detection limit is superior to all previously published protocols.

- The data from **Chapter 2** and **Chapter 3** of this thesis was included in an investigational medicinal product application to support the clinical testing of hMSC in 'no option for revascularization' CLI patients. Based on this data the HPRA granted Prof O'Brien permission to initiate a Phase 1b clinical trial in this patient population. The trial is currently ongoing and 3 patients have been admistered hMSCs.

Publication Outputs:

Review article co-author

- O'Brien T, Creane M, Windebank, AJ, Tersic A, Dietz AB. **Translating stem cell research to the clinic: a primer on translational considerations for your first stem cell protocol.** *Stem Cell Research and Therapy.* 2015.

Research article first author

Cytotherapy under revision;

- Creane M, Howard L, O'Brien T. **Biodistribution and Rentention of Locally Administered Human Mesenchymal Stromal Cells: Quantitative PCR-Based Detection of Human DNA in Murine Organs**.

Manuscript in preparation

For submission in Toxicologic Pathology;

- Creane M et al. **A Three Month Toxicology Study of Human Bone Marrow Derived Mesenchymal Stromal Cells Administered Once by The Intramuscular Route.**

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Appendices & Supporting Documents

APPENDIX 1: STANDARD OPERATING PROCEDURE

Standard Operating Procedure	SOP-RS-3	Supersedes: SOP-RS-2.	
MSC Biodistribution Analysis			
Reviewed By: Reviewed Date:		New Review Date	

1. Purpose

1.1. Preclinical biodistribution studies tracking the distribution of human mesenchymal stem cells (hMSCs) after administration are an Irish Medicines Board (IMB) regulatory requirement prior to a clinical trial initiation. This SOP describes a method to quantitatively detect the presence/absence of human genomic DNA in tissues of mice that have received intramuscular injections of hMSCs. The identification of human genomic DNA is not intended to indicate the viability of the cell, but only the presence or absence of human DNA and the original cellular equivalent containing that quantity of human DNA.

Quantitative polymerase chain reaction (qPCR) is a method that uses fluorescent markers to monitor the production of amplification products during each cycle of the PCR reaction. The accumulation of the fluorescent signal is measured at the exponential phase of the reaction to enable rapid and precise quantitation of the PCR product of interest. Here the presence of human DNA within murine tissues will be detected using qPCR, which is specific for the human Alu sequence.

This procedure uses qPCR to detect human DNA by amplifying the human Alu sequence in whole organ DNA extracts.

2. Scope

2.1. This procedure details how to isolate DNA from rodent tissues, quantify the amount of DNA and perform qPCR to detect the presence/absence of human DNA in murine tissues.

3. Responsibilities

3.1. Responsibility of the operators to ensure that this procedure is followed correctly.

3.2. Operators must record any deviations or unexpected events that occur during execution of this procedure.

3.3. Responsibility of technical approver to review the data upon completion of this protocol.

4. Training Requirements

4.1. All operators executing this procedure shall be trained to the level they understand the procedures outlined in this SOP.

5. Health and Safety

5.1. Operators must be familiar with practices for safe working procedures with genetically modified organsims NCBES 03-03-02 and waste disposal of GMO waste NCBES 03-11-02

6. Cross Reference and Related Documents

6.1. NCBES 03-01-02	Training of personnel with GMO work
6.2. NCBES 03-03-02	Safe working procedures for use if genetically modified organisms
6.3. NCBES 03-11-02	Disposal of solid GMO/GMM waste

7. Nomenclature

QC	Quality control
SOP	Standard operating procedure
g	Grams
mg	Milligrams
ml	Milliliters
µl	Microliters
qPCR	Quantitative polymerase chain reaction
hMSCs	Human mesenchymal stem cells
DNA	Deoxyribonucleic Acid
Standard Curve	A graph that is created using known dilutions of human genomic DNA and is used for quantification.
Alu	Dimeric sequences derived from the 7SL RNA gene that are approximately 300 base pairs long. Alu insertational elements are one of the most abundant SINES (short interspersed elements) in the human genome.
IMB	Irish Medicines Board
GMO	Genetically modified organism

8. Materials and Equipment

Materials	Manufacturer	Catalogue Number
Serological pipettes: 5 ml 10ml 25ml	 Sarstedt Sarstedt Sarstedt	 86.1253.001 86.1254.001 86.1685.001
50ml Centrifuge tubes	Sarstedt	62.547.254
Micropipette tips 10 µl	TipOne	S1120-3810
Micropipette tips 20 µl	TipOne	S112-1810
Micropipette tips 200 µl	TipOne	S1120-8810
Micropipette tips 1000 µl	TipOne	S1122-1830
Cell strainer, 100 µm yellow	Fisherbrand	22363549
10 ml syringe	BD Emerald	307736
Forward primer TGGTGGCTCTCTCCTGTAAT	Biosciences	Custom manufactured
Probe TGAGGCAGGAGAATCGCTTGAACC FAM-MGB	Biosciences	Custom manufactured
Reverse primer GATCTCGGCTCACTGCAAC	Biosciences	Custom manufactured
PCR Water	Bioline	BIO-37080
Ethanol 200 Proof	Sigma Aldrich	E7023-500ml
Microtube 1.5ml	Sarstedt	72.960.001
MicroAmp Fast Optical 96-Well Reaction Plate with Barcode (0.1ml)	Applied Biosystems	4346906
ISOLATE II Genomic DNA Kit	Bioline	BIO-52067
MicroAMP Optical Adhesive Film	Applied Biosystems	4311971
Human DNA (200ng/µl)	Bioline	BIO-35025
Proteinase K 20mg/ml	Bioline	BIO37084
Quant-iT PicoGreen dsDNA Assay Kit	Invitrogen	P7589
96F Non Treated Black Microwell SI	Thermo Scientific/NUNC	37105
PCR Water	Bioline	BIO-37080
FG TaqMan Gene Expression	Applied Biosystems	4369016

Mastermix		
Tissue Culture Dish VENTS NUNCLON D SI	Thermo Scientific/NUNC	50288

Equipment	Calibration Certificate Number
Stuart Scientific Shaker/Incubator SI20H	Temperature tracker NUIG documentation
Tube rack	Not applicable
Refrigerators and Freezers 4 degree C -20 degree C -80 degree C	 Not applicable Not applicable Not applicable
Vortex	Not applicable
Pipette Aid	Not applicable
Eppendorf Microcentrifuge 54159	04089
Pipettes: Set 1 Gilson P10 Gilson P20 Gilson P200 Transferpette P1000	 P13018503 P13018608 P13018505 P13018518
Pipettes: Set 2 Gilson P2 Gilson P200 Gilson P1000	 P13018589 P13018609 P13018610
Perkin Elmer VICTOR^3 1420 Multilabel Counter	
Eppendorf Holder	Not applicable
StepOne Plus Real Time PCR Machine	NUIG documentation
Fisher Scientific MH-214 Microbalance	04088

9. Procedure

9.1. Record all equipment/materials used throughout this protocol in Forms 5-8.

9.2. Anytime a disposable plastic filter tip or syringe plunger comes in contact with a tissue sample, use it with that one and only tissue sample and dispose of it immediately upon use to avoid cross contamination between samples.

9.3. Complete tissue digestion

9.3.1. Wear gloves for all procedures to avoid contamination of samples with hDNA

9.3.2. Remove buffer GL from the Isolate II kit and leave at room temperature.

9.3.3. Remove the proteinase K (20mg/ml) from the -20 degree freezer and store it in a 4 degree C refrigerator until required for addition to the tissue lysate.

9.3.4. Pre-heat the oven/incubator to 55 +/- 5 degrees C along with the temperature tracker.

9.3.5. Remove tissues from -80 degree C freezer and place in bucket of ice such that the lid/cap is above the ice level to avoid contamination.

9.3.6. Remove the 25mm Nunclon tissue culture dish from the sterile packaging. Record the lot number in Form 5.

9.3.7. Label the Nunclon dish base or lid with the organ name and animal number.

9.3.8. Transfer labeled 25mm dishes to the bench adjacent to the microbalance. Place the base or lid of one dish in the center of the balance.

9.3.9. Close microbalance doors and tare to 0.0000 g.

9.3.10. Remove labeled dish from the balance and place on the bench top. With a P1000 pipette and clean tip, remove the tissue sample from the cryovial and place it in the 25mm dish.

9.3.11. Place the dish containing the tissue in the balance and record the weight of the tissue in g on Form 1 rounding to the nearest 2 decimal places.

9.3.12. Remove the 25mm dish from the balance, leaving it on the bench top at room temperature.

9.3.13. Repeat steps 9.3.9 through 9.3.12 for each individual tissue.

9.3.14. On Form 1 with the use of Annex 1, convert the weight of the tissue from g to mg by multiplying by 1,000.

9.3.15. Using the chart in Annex 1, determine the amount of buffer GL and proteinase K that is required to digest each tissue. Record these volumes adjacent to the respective tissue in Form 1.

9.3.16. Label the lid and side of a sterile 50ml Sarstedt tube with the tissue name and animal ID number, creating a tube for each individual tissue.

9.3.17. Unwrap a sterile 100 µm Fisherbrand cell filter and place it directly in the open 50ml tube, using a fresh filter for each tissue.

9.3.18. Using the plunger from a sterile BD Emerald 10ml syringe press the tissue onto the sterile cell filter.

9.3.19. Add the appropriate amount of buffer GL (according to Annex 1) to the top of the cell filter using a P1000 and/or P200 pipette and P1000/P200 tip, moistening the entire cell filter surface. Continue to press the moistened tissue through the filter.

9.3.20. Scrape the underside of the cell filter with the plunger to remove homogenized tissue. Rinse any adherent tissue from the plunger with the buffer GL flow-through using a P1000 pipette and tip.

9.3.21. Any small tissue clumps remaining in the top of the cell filter are scraped into the GL buffer flow through with a P1000 pipette and P1000 pipette tip.

9.3.22. Add the appropriate volume of proteinase K (according to Annex 1) to the homogenized tissue in buffer GL using a P200 pipette and P200 pipette tip.

9.3.23. Scrape any adherent tissue from the side of the 50ml tube into the GL buffer with a clean P1000 tip.

9.3.24. Place the homogenized tissue in a 50ml tube heat-resistant rack and leave it on the bench top.

9.3.25. Repeat steps 9.3.17 through 9.3.24 for each remaining tissue.

9.3.26. Vortex the 50ml tubes vigorously at maximum speed for 15 seconds

9.3.27. Place the 50ml tubes in a rack and transport them to the pre-heated shaker/incubator.

9.3.28. Place the samples along with a temperature monitor inside the incubator and rock at a frequency of 70 strokes per minute for 12-20 hours. Record the start time of the incubation on Form 1.

9.3.29. Place label on outside of incubator reading ‘Do Not Open – Study in Progress’.

9.4. Genomic DNA Isolation from Digested Tissues

9.4.1. Pre-heat a small incubator to 70 degrees C.

9.4.2. Pre-heat the elution buffer to 70 degrees C within the small incubator in 9.4.1.

9.4.3. Remove the digested tissue from the shaker/incubator from step 9.3.28. Record incubation end time on Form 2.

9.4.4. To each tissue digest, directly add the appropriate volume of G3 lysis buffer as determined in Annex 1, recording this volume in Form 2.

9.4.4.1. When using a new kit, G3 is created by combining G1 and G2 according to the manufacturer’s instructions.

9.4.5. Vortex vigorously at maximum speed for 15 seconds.

9.4.6. Incubate the tissue digest at 70 degrees C for 10 minutes.

9.4.7. Remove the 50ml tube from the incubator.

9.4.8. Add the appropriate volume of ethanol as dictated in Annex 1, recording the volume added in document 2.

9.4.9. Vortex vigorously at maximum speed for 15 seconds.

9.4.10. Using a 5,10 or 25 ml serological pipette as appropriate, measure the total volume of the contents of the tube and record in Form 2.

9.4.11. Calculate the volume of lysate containing 25mg of tissue.

9.4.11.1. Divide the total volume of tissue lysate (in ml) by tissue weight (in mg)

9.4.11.2. Multiply the product of 9.4.11.1 by 25 (mg) to determine the lysate volume (ml) equivalent to 25 mg of tissue.

9.4.11.3. Record this value in Form 2.

9.4.11.4. At this point the tissue lysates can be stored at -20 degrees C until DNA isolation. Record the freezer number and shelf number where the samples are stored in Form 2.

9.5. Isolation of Genomic DNA

9.5.1. Remove the tissue lysate tubes from the -20 degrees C and let them thaw over ice.

9.5.2. Label ISOLATE II spin column lid with sample name and animal number. Place it in a provided collection tube. Create one column/tube combination for each tissue sample.

9.5.3. Add the calculated lysate volume equivalent to 25mg of tissue (from Form 2) to each spin column and centrifuge in the Eppendorf microcentrifuge 54159 at 13000 rpm for 1 min.

9.5.4. Freeze the remaining tissue digest from step 9.5.1 at -20 degrees C. Record freezer number and shelf number where samples stored in Form 2.

9.5.5. Discard the flow through and recombine the spin column and collection tube.

9.5.6. Add 500 µl of GW1 wash buffer to each collection tube and spin at 13000 rpm for 1 min.

9.5.7. Discard the flow through and replace the spin column into the collection tube.

9.5.8. Add 600 µl of GW2 wash buffer and spin at 13000 rpm for 1 min.

9.5.8.1. When using a new kit, ethanol needs to be added to GW2 before use according to the manufacture's instructions.

9.5.9. Discard the flow through and spin for 1 minute at 13000 rpm to remove excess ethanol.

9.5.10. Discard flow tube and place the spin column in a labeled 1.5ml Eppendorf tube.

9.5.11. Elute sample DNA by adding 50 µl of preheated elution buffer G (9.4.2) to the membrane followed by 3 min incubation at room temperature.

9.5.12. Centrifuge the spin column at 13000rpm for 1 min. Repeat step 9.5.11 on the same spin column with a fresh 50 µl elution buffer, combining this flow through with the flow through of step 9.3.9 to make 100 µl of eluted DNA total.

9.5.13. Freeze DNA at -20 degrees C until use or store on ice if proceeding to pico green analysis.

9.6. Pico Green Analysis of DNA Concentration

9.6.1. Remove the Quant-iT Pico green dsDNA assay kit from the -20 degree C freezer and allow reagents to thaw on the bench top.

9.6.2. If the DNA samples from 9.5.13 are frozen, place them in an ice bucket and allow them to thaw. Vortex the samples thoroughly before proceeding.

9.6.3. Dilute the 20x TE stock by removing 1ml of stock (with a P1000 pipette and pipette tip) and placing it in a 50ml Sarstedt tube. Add 19ml of deionized water, replace the cap, vortex and invert the solution several times to mix.

9.6.4. Dilute the Pico Green solution as described in Table 1 relative to the number of wells to be used in the assay. Once made, shield it from light by placing the reagent in a dark drawer or wrapping the tube in aluminum foil. The Pico Green solution must be made up fresh for each assay.

Number of Sample Wells	Volume of Pico Green (µl)	Volume of 1x TE (ml)
35 wells	20 µl	3.8 ml
75 wells	40 µl	7.6 ml
110 wells	60 µl	11.4 ml

Table 1 : Preparation of Pico green Solution

9.6.5. Dilute the hDNA standard stock (contained in the Quant-iT PicoGreen dsDNA assay kit) 50 fold (example: 20 µl DNA stock to 980 µl 1x TE from 9.6.3) in a 1.5 ml Eppendorf tube. Replace the cap and vortex briefly to mix the solution.

9.6.6. From the DNA standard solution prepare the 8 DNA standards according to table 2 below. Use P20, P200 and P1000 pipettes and pipette tips as appropriate, creating each solution in a clean 1.5 ml Eppendorf tube

9.6.6.1. Use the DNA stock created in 9.6.5 combined with the TE diluted in 9.6.3 to create each standard. Use new pipette tips when preparing each standard in order to avoid contamination.

DNA Stock (µl)	1x TE (µl)	Final DNA Concentration (ng DNA per well)
400	0	200
200	200	100
100	300	50
40	360	20
20	380	10
10	390	5
4	396	2
0	400	0

Table 2: Preparation of hDNA standards for the Pico Green assay

9.6.7. Label a fresh 1.5 ml Eppendorf tube for each digested tissue sample with the organ name and animal ID number.

9.6.8. Dilute each right thigh, right calf, brain, heart and spleen DNA sample (1:200) individually in the tube from 9.6.8 by combining 2 µl of sample with 398 µl of TE from step 9.4.3.

9.6.9. Dilute each lung, liver, kidney DNA sample (1:500) individually in the tube from 9.6.8 by combining 2 µl of sample with 998 µl of TE from step 9.4.3.

9.6.10. Place 100 µl of diluted standard (9.6.6) or sample (9.6.8) into each of three wells of a 96F non treated black microwell plate according to the plate diagram in Annex 2.

9.6.11. Add 100 µl of Pico Green solution (9.6.4) to each standard and sample. Once the appropriate volume has been added per well then discard the excess Pico Green stock solution.

9.6.12. Incubate the plate at room temperature sheltered from light for 3 minutes.

9.6.13. Place the 96 well plate into the plate reader and click on the Perkin Elmer software.

9.6.14. As presented in Figure 1, select the protocol Fluorescein 485/535nm, 0.1 seconds ensuring the plate reader reads from the top of the well. Highlight the appropriate wells to be measured (labeled in annex 2) and click save.

9.6.15. Initiate the plate reader to take measurements.

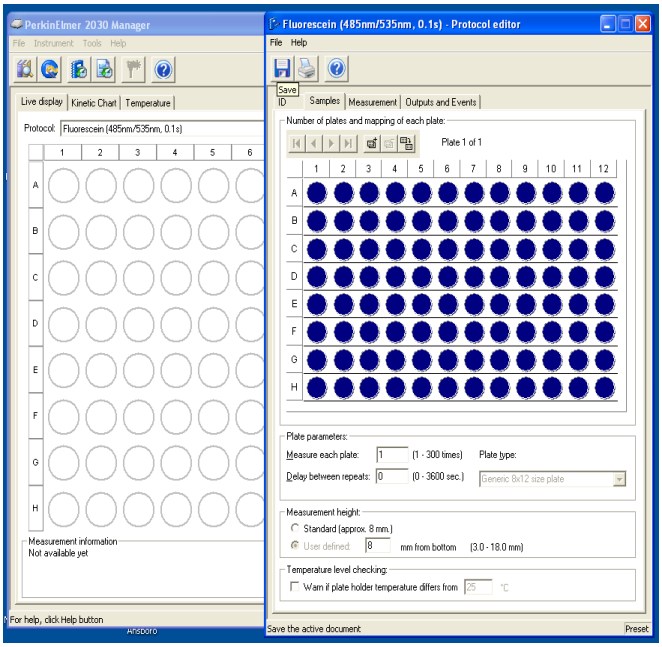

Figure 1: Perkin Elmer 2033 Manager. Set program to Fluroscein 485/535nm, 0.1 sec and highlight the appropriate wells as shown.

9.6.16. Export the resultant data in a Microsoft Excel sheet.

9.6.17. Using Excel, calculate the concentration of the DNA sample

9.6.17.1. Average the triplicate values for each standard in the standard curve.

9.6.17.2. Subtract the average value of the 0 ng blank in the standard curve from each sample and standard.

9.6.17.3. Plot the standard curve in an XY Scatter graph such that the Nanograms of DNA are along the X-axis and the OD is along the Y-axis.

9.6.17.4. Draw a line of best fit and determine the line equation. Ensure the R^2 value is greater than 0.97.

9.6.17.5. Average the triplicate values for each digested tissue DNA sample. Ensure the sample values fit within the standard curve. If not, repeat the entire assay.

9.6.17.6. Using the line equation, calculate the DNA content (ng) within each digested tissue sample well.

9.6.17.7. Multiply the value in 9.6.16.6 by the dilution factor (200 or 500 as appropriate in sections 9.6.8 and 9.6.9) and divide by 200 (µl) to determine the DNA concentration in ng/µl.

9.7. qPCR Analysis

9.7.1. Remove from the freezer all DNA samples, DNA standards and the primer/probe mix. Allow them to thaw in an ice bucket. Remove from the refrigerator the Taqman master mix and store it on ice on the bench top.

9.7.2. Immediately before the qPCR assay, create a solution of primers/probe suspended in Taqman mastermix. Store the solution on ice for no longer than 2 hours.

9.7.2.1. For the number of assay well plus 2 additional wells, pipette 10 µl of Taqman mastermix into a clean Eppendorf tube (ex: for 10 samples, prepare a mix for 12 wells by pipetting 120 µl of mastermix) followed by 1 µl of primer/probe solution (ex: for 12 wells, add 12 µl of primer/probe mix to the 120 µl of mastermix).

9.7.2.2. Record the volumes used in Form 3.

9.7.3. Pipette 11 µl of the solution from step 9.7.2 into each standard or sample well of a 96 well qPCR plate

9.7.4. Into each standard well (columns 1 and 2, rows A-G), pipette 8 µl of PCR water and 1 µl of the appropriate standard.

9.7.4.1. To create qPCR standards of hDNA, combine the following as illustrated in table 3 in a fresh 1.5 ml Eppendorf tube. Store the standards at -20 degrees C, thawing on an ice bucket before use.

9.7.4.2. To create a combined murine muscle DNA/hDNA standard, add 1 µl of the 20 ng/well human DNA standard (as prepared in table 3 below) with a volume of murine muscle (uninjected, control) genomic DNA equivalent to 400 ng DNA. Finally add PCR water to make up a total volume of 9 µl. This can be added directly to the PCR plate in duplicate, then supplemented with 11 µl of the master mix created in step 9.7.2.1.

Standard (ng/well)	hDNA	PCR-quality water
20	1 µl of Bioline stock DNA	9 µl
2	1 µl of Bioline stock DNA	99 µl
0.2	1 µl of Bioline stock DNA	999 µl
0.02	1 µl of the 0.2 ng/well stock	9 µl
0.002	1 µl of the 0.2ng/well stock	99 µl
0	0 µl	1 ml

Table 3: Preparation of hDNA standards for the qPCR reaction

9.7.5. Into each sample well, pipette 400 ng of organ DNA according to the calculations in step 9.6.16. Record this volume in Form 3.

9.7.6. Into each sample well, pipette the appropriate volume of PCR water according to the volume determined in Form 3.

9.7.7. Gently tap the 96 well plate on the bench top to ensure sample is retained at the bottom of the well.

9.7.8. Remove the adhesive backing from one piece of Microamp optical adhesive film and adhere the film to the top of the plate, avoiding fingerprints.

9.8. qPCR Analysis

9.8.1. Open the Step One Software v2.3.

9.8.2. As demonstrated in figure 2, under the New Experiment menu, choose Advanced Setup and highlight the Experimental Properties menu.

9.8.2.1. In Experiment Name, record the date of the qPCR assay, the animal group and sex and the animal numbers included on each PCR plate.

9.8.2.2. Identify the instrument employed as the Step One Plus Instrument (96 wells).

9.8.2.3. Choose Comparative CT as the type of experiment.

9.8.2.4. Highlight TaqMAn Reagent and Standard (2 hour run) as the assay type.

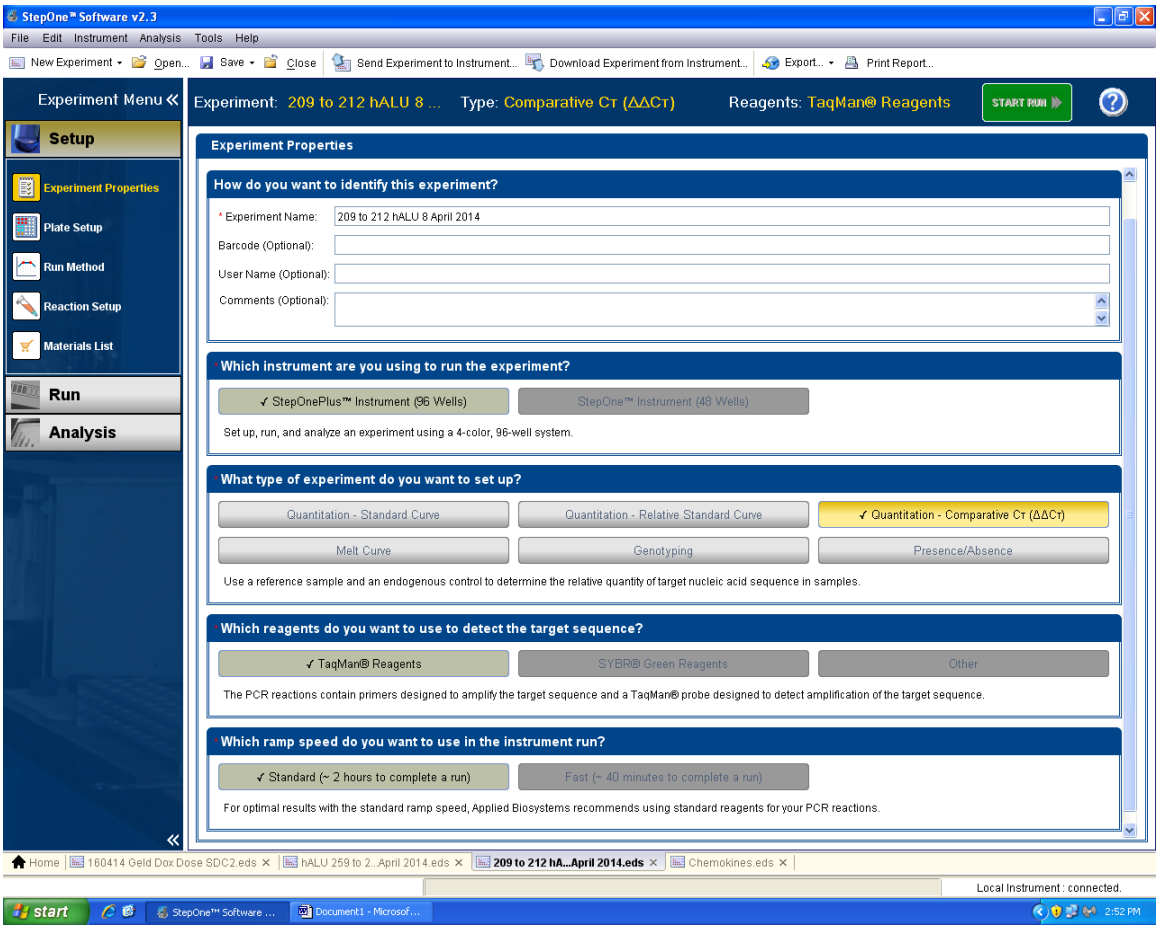

Figure 2: Determining experimental properties by choosing the options best reflecting the experimental setup.

9.8.3. As demonstrated in figure 3, click on the plate setup menu on the left column of the home page.

9.8.3.1. Define the target as Human Alu and the reporter as FAM and the quencher as NFQ-MGB.

9.8.3.2. Define the sample names for each standard in the standard curve (ex: 200 ng, 20 ng, 2 ng, 0.2 ng etc) and for each organ (ex: right thigh, heart, brain etc).

9.8.3.3. Under the assign targets menu, highlight each standard and sample well according to the plate setup in annex 3 and assign the Human ALU target to every well by clicking on the adjacent box.

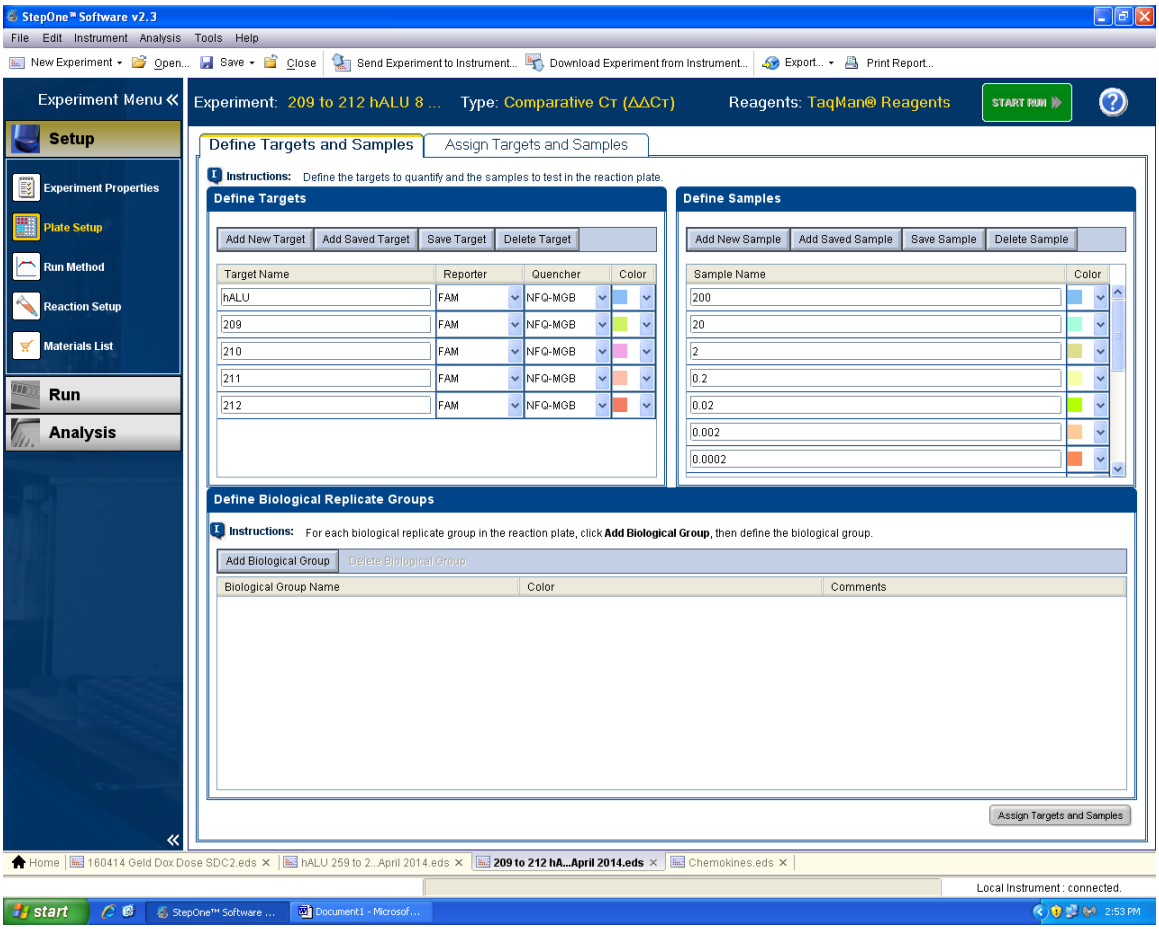

Figure 3: Plate Setup Menu. The contents of each well as well as the target of the qPCR are individually identified within this menu.

9.8.3.4. In the plate diagram created in 9.8.3.3, highlight each sample and standard well and assign the individual sample name to the well, as shown in figure 4.

9.8.3.5. Identify the passive reference dye as Rox in the lower left corner menu.

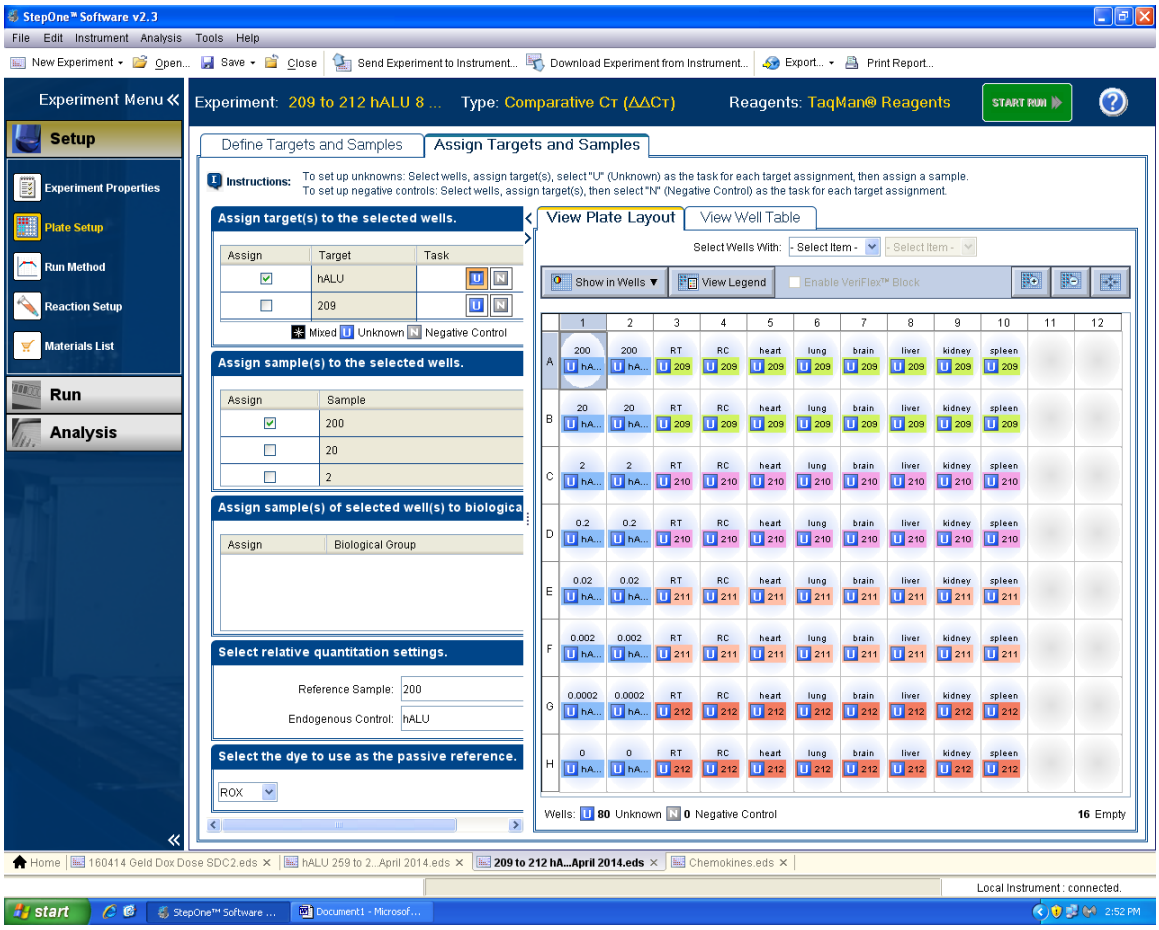

Figure 4: Plate Setup Menu. The contents of each well as well as the target of the qPCR are individually identified within this menu.

9.8.4. Click on Run Method on the left menu

9.8.4.1. At the top of the run diagram, change the reaction volume to 20 µl.

9.8.4.2. Ensure the run diagram matches the screen shot exactly as demonstrated.

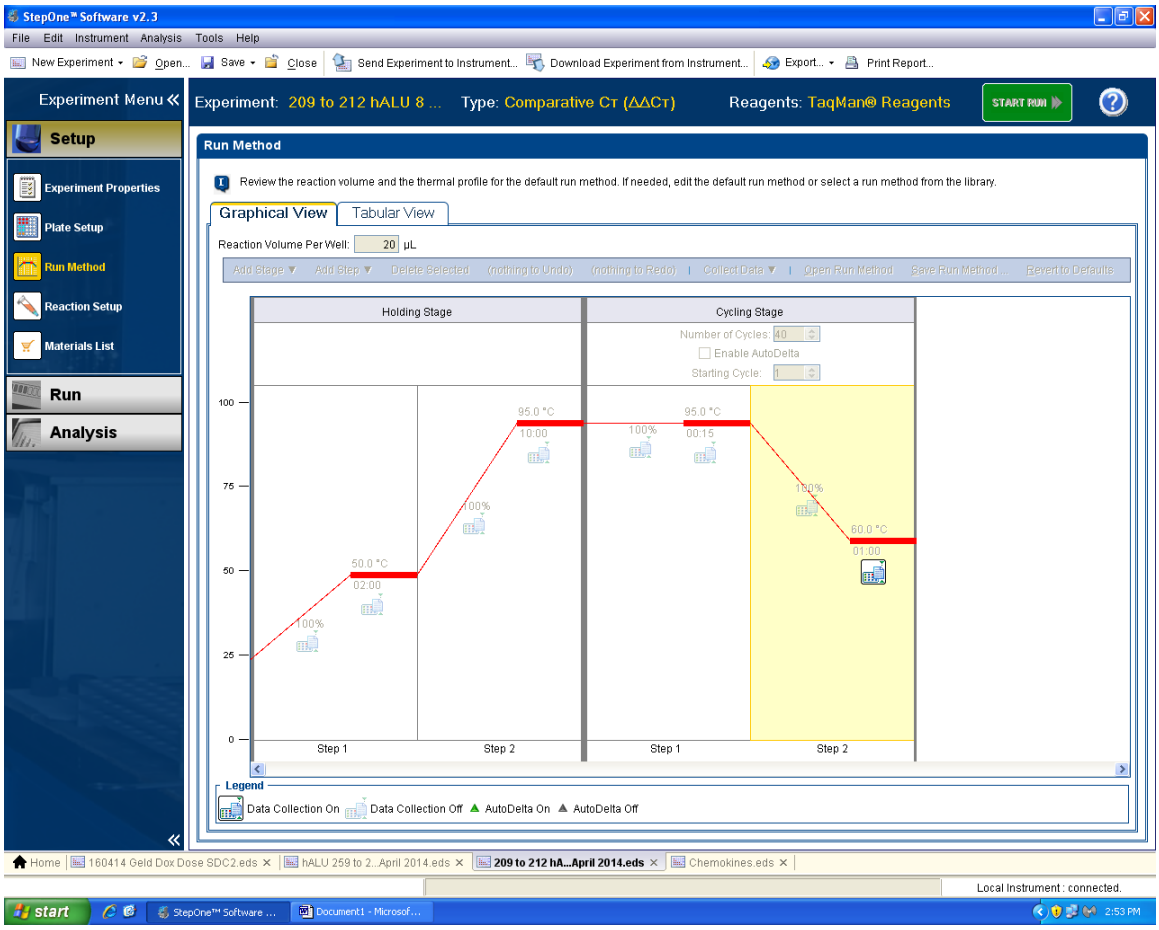

Figure 5: Run Method. Graphical view of the reaction temperatures of the heating, cycling and melt curve stages.

9.8.5. Click on Reaction Setup in the left hand menu and ensure the final volume of the reaction is 20 µl (figure 6).

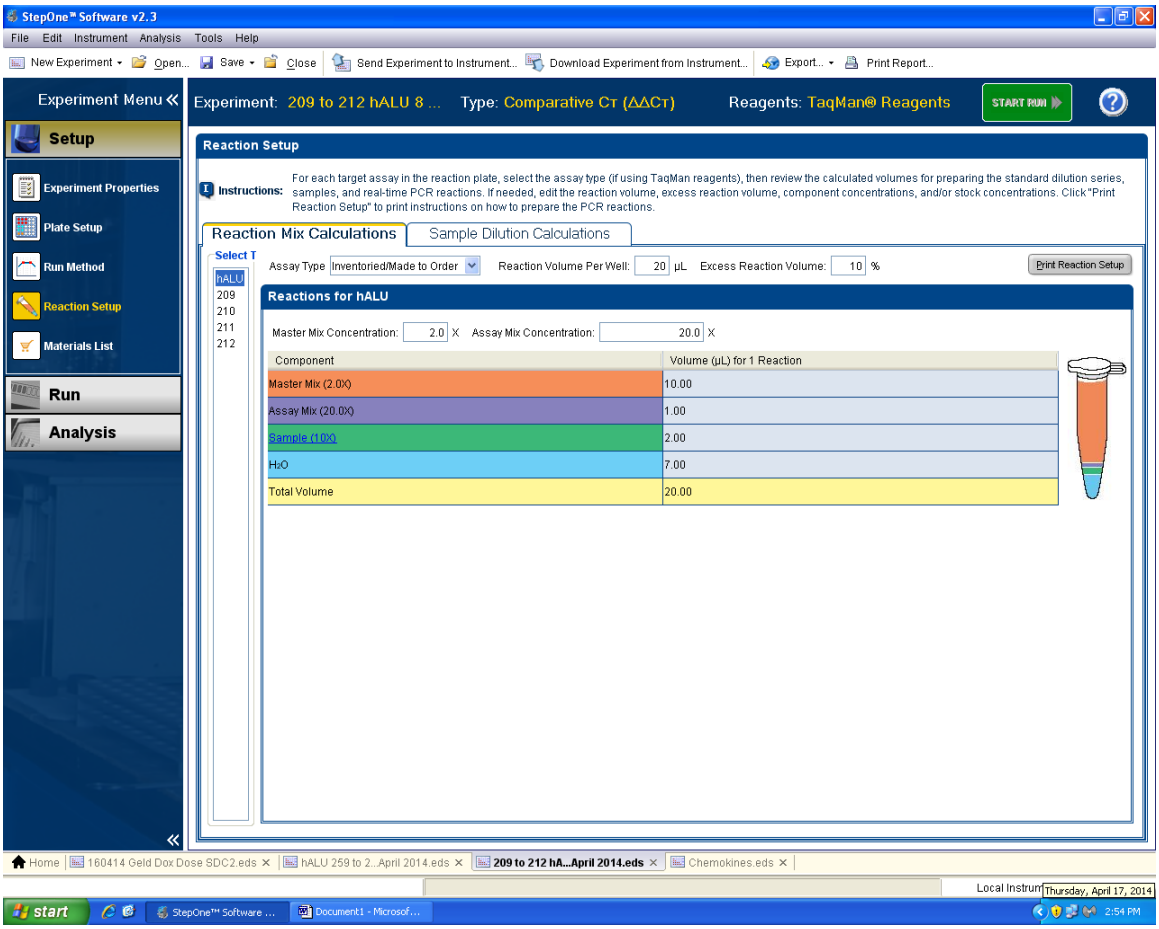

Figure 6: Reaction Setup menu where the final volume of the reaction must be recorded.

9.8.6. On the upper menu, click on Save. Keep the folder name as the name of the assay created in step 9.8.2.1. If required change the destination folder. Click Save.

9.8.7. Load the plate into the Step One machine ensuring the A1 well is in the upper left corner. Close the door, holding it in place until the machine raises the plate.

9.8.8. Click the Start Run (green) button on the upper right hand side of the screen.

9.8.9. Allow the software to run for approximately 2 hours.

9.8.10. Upon completion, click Download Experiment from the Instrument and save the resultant information in the same file as in step 9.8.6.

9.8.11. In the Export menu check every box in step one to download all relevant data.

9.8.11.1. Choose and appropriate file name and destination and click Start Export.

9.8.12. Remove the resultant excel sheet from the computer for analysis.

9.9. **Data Analysis**

9.9.1. Using Excel, average the replicate data for each standard curve and sample value.

9.9.2. Plot the standard curve values on a XY Scatter graph such that the Predicted Number of human gemomes (per well) is on the logarithmic X-axis and the average Ct value on the Y-axis.

9.9.2.1. Regularly when plotting the Standard Curve, the upper standard of 200 ng/well is saturated and should be eliminated from the assay. Similarly, the lowest standard of 0.0002 ng/well is indistinguishable from the 0 ng/well standard and should be eliminated.

9.9.2.2. To determine the predicted number of human genomes per well based on DNA weight for each point on the standard curve, consult the table below.

DNA Standard (ng/well)	Equivalent Number of Human Genomes Based on DNA (ng)
20	3,030
2	303
0.2	30
0.02	3.0
0.002	0.3
0	0

Table 4: **QPCR standard curve standards for ex vivo quantification of human cells**

9.9.3. Draw the best fit line and determine the line equation.

9.9.4. Using the line equation and the organ DNA Ct values, calculate the number of human gemomes detected in the PCR well.

9.9.5. Calculate the total DNA isolated in each 25 mg tissue sample by multiplying the volume of elution buffer (9.5.12) used to extract the DNA from the column (usually 100 µl) by the DNA concentration of the extracted DNA (ng/µl) determined in 9.6.16.7.

9.9.5.1. Convert the quantity of DNA in 25 mg of digested tissue from ng to µg by dividing by 1,000.

9.9.6. Calculate the ng DNA isolated per organ by multiplying the total DNA in 25 mg of tissue (9.9.4), divided by 25, by the weight of the tissue (9.3.14) in mg.

9.9.6.1. To determine the weight of the organ in mg, multiply the weight of the organ in grams by 1,000.

9.9.7. Account for the dilution of this sample by dividing the value in 9.9.6 by 0.1.

9.9.8. Calculate the number of human gemomes present in the organ by multiplying the value in 9.9.7 by the value determined in 9.9.4.

Form 1: Tissue Digestion Buffer Composition

Date: _____ Animal ID Number: _____

Start time of incubation _____

Tissue	Tissue Weight (g)	Tissue Weight (mg)	GL Buffer (ml)	Proteinase K (µl)
Brain				
Lung				
Liver				
Heart				
Kidney				
Spleen				
Right Thigh				
Right Calf				

Recorded By: _____

Approved By: _____

Form 2: Tissue Digestion Buffer Composition

Date: _____ Animal ID Number: _____

End time of incubation _____

-20 Degree C Freezer number _____

-20 Degree C Freezer shelf number _____

Recorded By: _____

Tissue	Volume G3 (ml)	Volume Ethanol (ml)	Total Volume Digested Tissue (ml)	Volume of 25 mg Tissue (ml)
Brain				
Lung				
Liver				
Heart				
Kidney				
Spleen				
Right Thigh				
Right Calf				

Approved By: _____

Form 3: Dilution of DNA sample for qPCR

Date: _____ Animal ID Number: _____

Recorded By: _____

Approved By: _____

Tissue	Stock DNA Concentration (ng/µl)	Volume Containing 400 ng DNA (µl)	Volume of Water to Add to Reach 9 µl total	Initial Upon Addition To Well
Brain				
Lung				
Liver				
Heart				
Kidney				
Spleen				
Right Thigh				
Right Calf				

Form 4 : Verification of the qPCR setup

Date: _____ Animal ID Number (s): _____

Experimental Name : _____ Notebook number/page of resultant data_____

Setup	Verification of StepOne Plus Settings (Yes/No)
Experimental properties	
Plate Setup	
Run Method	
Reaction Setup	
Save	
Start Run	

Recorded By: _____

Approved By: _____

Form 5: Record of Lot Numbers for Assay Materials: Tissue Digest

Date: _____ Animal ID Number (s): _____

Materials	Manufacturer	Lot number	Initials
Serological pipettes: 5 ml 10ml 25ml	Sarstedt		
50ml Centrifuge tubes	Sarstedt		
Micropipette tips 10 µl	TipOne		
Micropipette tips 20 µl	TipOne		
Micropipette tips 200 µl	TipOne		
Micropipette tips 1000 µl	TipOne		
Cell strainer, 100 µm yellow	Fisherbrand		
10 ml syringe	BD Emerald		
50ml Centrifuge tubes	Sarstedt		
Ethanol 200 proof	Sigma Aldrich		
Proteinase K 20mg/ml	Bioline		
Isolate II genomic DNA kit	Bioline		
Tissue Culture Dish VENTS NUNCLON D SI	Thermo Scientific/NUNC		

Form 6: Record of Lot Numbers for Assay Materials: DNA Purification

Date: _____ Animal ID Number (s): _____

Materials	Manufacturer	Lot number	Initials
Serological pipettes: 5 ml 10ml 25ml	Sarstedt		
50ml Centrifuge tubes	Sarstedt		
Micropipette tips 10 µl	TipOne		
Micropipette tips 20 µl	TipOne		
Micropipette tips 200 µl	TipOne		
Micropipette tips 1000 µl	TipOne		
Ethanol 200 Proof	Sigma Aldrich		
Microtube 1.5ml	Sarstedt		
ISOLATE II Genomic DNA Kit	Bioline		

Form 7: Record of Lot Numbers for Assay Materials: Pico Green

Date: _____ Animal ID Number (s): _____

Materials	Manufacturer	Lot number	Initials
Serological pipettes: 5 ml 10ml 25ml	Sarstedt		
50ml Centrifuge tubes	Sarstedt		
Micropipette tips 10 µl	TipOne		
Micropipette tips 20 µl	TipOne		
Micropipette tips 200 µl	TipOne		
Micropipette tips 1000 µl	TipOne		
Microtube 1.5ml	Sarstedt		
Quant-iT PicoGreen dsDNA Assay Kit	Invitrogen		
96F Non Treated Black microwell SI	Thermo Scientific/NUNC		

Form 8: Record of Lot Numbers for Assay Materials: qPCR

Date: _____ Animal ID Number (s): _____

Materials	Manufacturer	Lot number	Initials
Micropipette tips 10 µl	TipOne		
Micropipette tips 20 µl	TipOne		
Micropipette tips 200 µl	TipOne		
Micropipette tips 1000 µl	TipOne		
Taqman primer/probe solution	Bioline		
PCR Water	Bioline		
Microtube 1.5ml	Sarstedt		
MicroAmp Fast Optical 96-Well Reaction Plate with Barcode (0.1ml)	Applied Biosystems		
MicroAMP Optical Adhesive Film	Applied Biosystems		
Human DNA standard	Bioline		
PCR Water	Bioline		
Taqman mastermix	Applied Biosystems		

Annex 1: DNA Isolation Calculations

Tissue Weight (g)	Tissue Weight (mg)	GL buffer (ml)	Proteinase K (µl)	G3 Buffer (ml)	Ethanol (ml)
0.10	100.00	0.72	100.00	0.80	0.84
0.11	110.00	0.79	110.00	0.88	0.92
0.12	120.00	0.86	120.00	0.96	1.01
0.13	130.00	0.94	130.00	1.04	1.09
0.14	140.00	1.01	140.00	1.12	1.18
0.15	150.00	1.08	150.00	1.20	1.26
0.16	160.00	1.15	160.00	1.28	1.34
0.17	170.00	1.22	170.00	1.36	1.43
0.18	180.00	1.30	180.00	1.44	1.51
0.19	190.00	1.37	190.00	1.52	1.60
0.20	200.00	1.44	200.00	1.60	1.68
0.21	210.00	1.51	210.00	1.68	1.76
0.22	220.00	1.58	220.00	1.76	1.85
0.23	230.00	1.66	230.00	1.84	1.93
0.24	240.00	1.73	240.00	1.92	2.02
0.25	250.00	1.80	250.00	2.00	2.10
0.26	260.00	1.87	260.00	2.08	2.18
0.27	270.00	1.94	270.00	2.16	2.27
0.28	280.00	2.02	280.00	2.24	2.35
0.29	290.00	2.09	290.00	2.32	2.44
0.30	300.00	2.16	300.00	2.40	2.52
0.31	310.00	2.23	310.00	2.48	2.60
0.32	320.00	2.30	320.00	2.56	2.69
0.33	330.00	2.38	330.00	2.64	2.77
0.34	340.00	2.45	340.00	2.72	2.86
0.35	350.00	2.52	350.00	2.80	2.94
0.36	360.00	2.59	360.00	2.88	3.02
0.37	370.00	2.66	370.00	2.96	3.11
0.38	380.00	2.74	380.00	3.04	3.19

0.39	390.00	2.81	390.00	3.12	3.28
0.40	400.00	2.88	400.00	3.20	3.36
0.41	410.00	2.95	410.00	3.28	3.44
0.42	420.00	3.02	420.00	3.36	3.53
0.43	430.00	3.10	430.00	3.44	3.61
0.44	440.00	3.17	440.00	3.52	3.70
0.45	450.00	3.24	450.00	3.60	3.78
0.46	460.00	3.31	460.00	3.68	3.86
0.47	470.00	3.38	470.00	3.76	3.95
0.48	480.00	3.46	480.00	3.84	4.03
0.49	490.00	3.53	490.00	3.92	4.12
0.50	500.00	3.60	500.00	4.00	4.20
0.51	510.00	3.67	510.00	4.08	4.28
0.52	520.00	3.74	520.00	4.16	4.37
0.53	530.00	3.82	530.00	4.24	4.45
0.54	540.00	3.89	540.00	4.32	4.54
0.55	550.00	3.96	550.00	4.40	4.62
0.56	560.00	4.03	560.00	4.48	4.70
0.57	570.00	4.10	570.00	4.56	4.79
0.58	580.00	4.18	580.00	4.64	4.87
0.59	590.00	4.25	590.00	4.72	4.96
0.60	600.00	4.32	600.00	4.80	5.04
0.61	610.00	4.39	610.00	4.88	5.12
0.62	620.00	4.46	620.00	4.96	5.21
0.63	630.00	4.54	630.00	5.04	5.29
0.64	640.00	4.61	640.00	5.12	5.38
0.65	650.00	4.68	650.00	5.20	5.46
0.66	660.00	4.75	660.00	5.28	5.54
0.67	670.00	4.82	670.00	5.36	5.63
0.68	680.00	4.90	680.00	5.44	5.71
0.69	690.00	4.97	690.00	5.52	5.80
0.70	700.00	5.04	700.00	5.60	5.88
0.71	710.00	5.11	710.00	5.68	5.96

0.72	720.00	5.18	720.00	5.76	6.05
0.73	730.00	5.26	730.00	5.84	6.13
0.74	740.00	5.33	740.00	5.92	6.22
0.75	750.00	5.40	750.00	6.00	6.30
0.76	760.00	5.47	760.00	6.08	6.38
0.77	770.00	5.54	770.00	6.16	6.47
0.78	780.00	5.62	780.00	6.24	6.55
0.79	790.00	5.69	790.00	6.32	6.64
0.80	800.00	5.76	800.00	6.40	6.72
0.81	810.00	5.83	810.00	6.48	6.80
0.82	820.00	5.90	820.00	6.56	6.89
0.83	830.00	5.98	830.00	6.64	6.97
0.84	840.00	6.05	840.00	6.72	7.06
0.85	850.00	6.12	850.00	6.80	7.14
0.86	860.00	6.19	860.00	6.88	7.22
0.87	870.00	6.26	870.00	6.96	7.31
0.88	880.00	6.34	880.00	7.04	7.39
0.89	890.00	6.41	890.00	7.12	7.48
0.90	900.00	6.48	900.00	7.20	7.56
0.91	910.00	6.55	910.00	7.28	7.64
0.92	920.00	6.62	920.00	7.36	7.73
0.93	930.00	6.70	930.00	7.44	7.81
0.94	940.00	6.77	940.00	7.52	7.90
0.95	950.00	6.84	950.00	7.60	7.98
0.96	960.00	6.91	960.00	7.68	8.06
0.97	970.00	6.98	970.00	7.76	8.15
0.98	980.00	7.06	980.00	7.84	8.23
0.99	990.00	7.13	990.00	7.92	8.32
1.00	1,000.00	7.20	1,000.00	8.00	8.40
1.01	1,010.00	7.27	1,010.00	8.08	8.48
1.02	1,020.00	7.34	1,020.00	8.16	8.57
1.03	1,030.00	7.42	1,030.00	8.24	8.65
1.04	1,040.00	7.49	1,040.00	8.32	8.74

1.05	1,050.00	7.56	1,050.00	8.40	8.82
1.06	1,060.00	7.63	1,060.00	8.48	8.90
1.07	1,070.00	7.70	1,070.00	8.56	8.99
1.08	1,080.00	7.78	1,080.00	8.64	9.07
1.09	1,090.00	7.85	1,090.00	8.72	9.16
1.10	1,100.00	7.92	1,100.00	8.80	9.24
1.11	1,110.00	7.99	1,110.00	8.88	9.32
1.12	1,120.00	8.06	1,120.00	8.96	9.41
1.13	1,130.00	8.14	1,130.00	9.04	9.49
1.14	1,140.00	8.21	1,140.00	9.12	9.58
1.15	1,150.00	8.28	1,150.00	9.20	9.66
1.16	1,160.00	8.35	1,160.00	9.28	9.74
1.17	1,170.00	8.42	1,170.00	9.36	9.83
1.18	1,180.00	8.50	1,180.00	9.44	9.91
1.19	1,190.00	8.57	1,190.00	9.52	10.00
1.20	1,200.00	8.64	1,200.00	9.60	10.08
1.21	1,210.00	8.71	1,210.00	9.68	10.16
1.22	1,220.00	8.78	1,220.00	9.76	10.25
1.23	1,230.00	8.86	1,230.00	9.84	10.33
1.24	1,240.00	8.93	1,240.00	9.92	10.42
1.25	1,250.00	9.00	1,250.00	10.00	10.50
1.26	1,260.00	9.07	1,260.00	10.08	10.58
1.27	1,270.00	9.14	1,270.00	10.16	10.67
1.28	1,280.00	9.22	1,280.00	10.24	10.75
1.29	1,290.00	9.29	1,290.00	10.32	10.84
1.30	1,300.00	9.36	1,300.00	10.40	10.92
1.31	1,310.00	9.43	1,310.00	10.48	11.00
1.32	1,320.00	9.50	1,320.00	10.56	11.09
1.33	1,330.00	9.58	1,330.00	10.64	11.17
1.34	1,340.00	9.65	1,340.00	10.72	11.26
1.35	1,350.00	9.72	1,350.00	10.80	11.34
1.36	1,360.00	9.79	1,360.00	10.88	11.42
1.37	1,370.00	9.86	1,370.00	10.96	11.51

1.38	1,380.00	9.94	1,380.00	11.04	11.59
1.39	1,390.00	10.01	1,390.00	11.12	11.68
1.40	1,400.00	10.08	1,400.00	11.20	11.76
1.41	1,410.00	10.15	1,410.00	11.28	11.84
1.42	1,420.00	10.22	1,420.00	11.36	11.93
1.43	1,430.00	10.30	1,430.00	11.44	12.01
1.44	1,440.00	10.37	1,440.00	11.52	12.10
1.45	1,450.00	10.44	1,450.00	11.60	12.18
1.46	1,460.00	10.51	1,460.00	11.68	12.26
1.47	1,470.00	10.58	1,470.00	11.76	12.35
1.48	1,480.00	10.66	1,480.00	11.84	12.43
1.49	1,490.00	10.73	1,490.00	11.92	12.52
1.50	1,500.00	10.80	1,500.00	12.00	12.60
1.51	1,510.00	10.87	1,510.00	12.08	12.68
1.52	1,520.00	10.94	1,520.00	12.16	12.77
1.53	1,530.00	11.02	1,530.00	12.24	12.85
1.54	1,540.00	11.09	1,540.00	12.32	12.94
1.55	1,550.00	11.16	1,550.00	12.40	13.02
1.56	1,560.00	11.23	1,560.00	12.48	13.10
1.57	1,570.00	11.30	1,570.00	12.56	13.19
1.58	1,580.00	11.38	1,580.00	12.64	13.27
1.59	1,590.00	11.45	1,590.00	12.72	13.36
1.60	1,600.00	11.52	1,600.00	12.80	13.44
1.61	1,610.00	11.59	1,610.00	12.88	13.52
1.62	1,620.00	11.66	1,620.00	12.96	13.61
1.63	1,630.00	11.74	1,630.00	13.04	13.69
1.64	1,640.00	11.81	1,640.00	13.12	13.78
1.65	1,650.00	11.88	1,650.00	13.20	13.86
1.66	1,660.00	11.95	1,660.00	13.28	13.94
1.67	1,670.00	12.02	1,670.00	13.36	14.03
1.68	1,680.00	12.10	1,680.00	13.44	14.11
1.69	1,690.00	12.17	1,690.00	13.52	14.20
1.70	1,700.00	12.24	1,700.00	13.60	14.28

1.71	1,710.00	12.31	1,710.00	13.68	14.36
1.72	1,720.00	12.38	1,720.00	13.76	14.45
1.73	1,730.00	12.46	1,730.00	13.84	14.53
1.74	1,740.00	12.53	1,740.00	13.92	14.62
1.75	1,750.00	12.60	1,750.00	14.00	14.70
1.76	1,760.00	12.67	1,760.00	14.08	14.78
1.77	1,770.00	12.74	1,770.00	14.16	14.87
1.78	1,780.00	12.82	1,780.00	14.24	14.95
1.79	1,790.00	12.89	1,790.00	14.32	15.04
1.80	1,800.00	12.96	1,800.00	14.40	15.12
1.81	1,810.00	13.03	1,810.00	14.48	15.20
1.82	1,820.00	13.10	1,820.00	14.56	15.29
1.83	1,830.00	13.18	1,830.00	14.64	15.37
1.84	1,840.00	13.25	1,840.00	14.72	15.46
1.85	1,850.00	13.32	1,850.00	14.80	15.54
1.86	1,860.00	13.39	1,860.00	14.88	15.62
1.87	1,870.00	13.46	1,870.00	14.96	15.71
1.88	1,880.00	13.54	1,880.00	15.04	15.79
1.89	1,890.00	13.61	1,890.00	15.12	15.88
1.90	1,900.00	13.68	1,900.00	15.20	15.96
1.91	1,910.00	13.75	1,910.00	15.28	16.04
1.92	1,920.00	13.82	1,920.00	15.36	16.13
1.93	1,930.00	13.90	1,930.00	15.44	16.21
1.94	1,940.00	13.97	1,940.00	15.52	16.30
1.95	1,950.00	14.04	1,950.00	15.60	16.38
1.96	1,960.00	14.11	1,960.00	15.68	16.46
1.97	1,970.00	14.18	1,970.00	15.76	16.55
1.98	1,980.00	14.26	1,980.00	15.84	16.63
1.99	1,990.00	14.33	1,990.00	15.92	16.72
2.00	2,000.00	14.40	2,000.00	16.00	16.80

Annex 2: Pico Green Plate Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	200 ng	200 ng	Right Thigh 1	Right Calf 1	Brain 1	Heart 1	Lung 1	Liver 1	Kidney 1	Spleen 1		
B	100 ng	100 ng	Right Thigh 1	Right Calf 1	Brain 1	Heart 1	Lung 1	Liver 1	Kidney 1	Spleen 1		
C	50 ng	50 ng	Right Thigh 2	Right Calf 2	Brain 2	Heart 2	Lung 2	Liver 2	Kidney 2	Spleen 2		
D	25 ng	25 ng	Right Thigh 2	Right Calf 2	Brain 2	Heart 2	Lung 2	Liver 2	Kidney 2	Spleen 2		
E	12.5 ng	12.5 ng	Right Thigh 3	Right Calf 3	Brain 3	Heart 3	Lung 3	Liver 3	Kidney 3	Spleen 3		
F	6 ng	6 ng	Right Thigh 3	Right Calf 3	Brain 3	Heart 3	Lung 3	Liver 3	Kidney 3	Spleen 3		
G	3 ng	3 ng	Right Thigh 4	Right Calf 4	Brain 4	Heart 4	Lung 4	Liver 4	Kidney 4	Spleen 4		
H	0 ng	0 ng	Right Thigh 4	Right Calf 4	Brain 4	Heart 4	Lung 4	Liver 4	Kidney 4	Spleen 4		

Note: Within each tissue DNA sample, the number (1-4) represents replicates of each biologic sample. For example, A4-A12 and B4-B12 are technical replicates from the same animal (animal 1).

Annex 3: qPCR Assay Plate Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	200 ng Standard	200 ng Standard	Right thigh 1	Right calf 1	Heart 1	Lung 1	Brain 1	Liver 1	Kidney 1	Spleen 1		
B	20 ng Standard	20 ng Standard	Right thigh 1	Right calf 1	Heart 1	Lung 1	Brain 1	Liver 1	Kidney 1	Spleen 1		
C	2 ng Standard	2 ng Standard	Right thigh 2	Right calf 2	Heart 2	Lung 2	Brain 2	Liver 2	Kidney 2	Spleen 2		
D	0.2 ng Standard	0.2 ng Standard	Right thigh 2	Right calf 2	Heart 2	Lung 2	Brain 2	Liver 2	Kidney 2	Spleen 2		
E	0.02 ng Standard	0.02 ng Standard	Right thigh 3	Right calf 3	Heart 3	Lung 3	Brain 3	Liver 3	Kidney 3	Spleen 3		
F	0.002 ng Standard	0.002 ng Standard	Right thigh 3	Right calf 3	Heart 3	Lung 3	Brain 3	Liver 3	Kidney 3	Spleen 3		
G	0.0002 ng Standard	0.0002 ng Standard	Right thigh 4	Right calf 4	Heart 4	Lung 4	Brain 4	Liver 4	Kidney 4	Spleen 4		
H	0 ng Standard	0 ng Standard	Right thigh 4	Right calf 4	Heart 4	Lung 4	Brain 4	Liver 4	Kidney 4	Spleen 4		

Note: Within each tissue DNA sample, the number (1-4) represents replicates of each biologic sample. For example, A4-A12 and B4-B12 are technical replicates from the same animal (animal 1).

APPENDIX 2: GENERAL DISCUSSION, LESSONS LEARNED AND FUTURE PERSPECTIVE

In this section, I discuss several lessons in which we learned throughout the translational process and provide some insight into future developments in the field.

Good Regulatory Plan is Key to Successful IMP Application Assembly.

The development of a regulatory plan is key to successfully navigating the CT product through the translational process. The development of a plan early is important to ensure the efficient use of resources and to save time in fundamental areas at this stage. Additionally, each member should become aware of the cell therapy regulation for that country and become aware of the basic principle of GXPs, for example good manufacturing practices, good laboratory practices and good clinical practices. Early preparation prior to the pre IMP application meeting is critical to an effective plan to ensure productive interactions with the regulatory body. As preclinical safety studies require the testing of the intended medicinal product, it is recommended to have close interactions with the GMP teams prior to the study design. Efficient communications between the regulatory agency and the IMP application assembly team is crucial in the case of stem cell therapies due to the unique scientific challenges associated with each product. Therefore, good planning will ensure that there is sufficient data available to the regulatory body in order for them to provide some meaningful feedback.

Good Laboratory Practice

Preclinical safety studies are very important to the development process of the CT product. This declaration only becomes understandable when one considers the role in which they play in the acceptance of the IMP applications. The toxicology studies are used to identify any local or systemic toxicity as a result of the administered cell dose and the results generated from these studies are used to extrapolate to humans to ensure a safe dose is administered to the intended patient population.

As government funding is becoming more and more competitive to secure, academic institutions are turning to industry as additional, or alternative, funding resource. Due to the high costs, time commitment and organization required for appropriate documentation which is required for GLP compliance, academic institutions often avoid conducting research under GLP. While GLPs do not apply to proof of concept or efficacy studies GLP, it is a requirement for any safety studies intended to support an IMP application.

The overall purpose of GLP, is to provide guidance for the conduct of preclinical toxicology and biodistribution studies to assure the production of reliable and integrable data. Furthermore, GLP puts in place a documentation system that allows for the complete reconstruction of the study by independent groups after it has been completed thus ensuring study reproducibility and reduces the chance for error and fraud. Operational costs (proper validation and calibration of equipment) for GLP studies are on average 30% more expensive than non-GLP studies which makes it very difficult for academic institutes to fully implement such systems. While academic institutes do not dismiss that GLP studies should be carried out, they do recommend that regulatory agencies accept their data to support regulatory finding. While regulatory agencies fully state that toxicology studies must be carried out under GLP, they do accommodate academic institutions by accepting other studies such as efficacy and biodistribution studies that are conducted under 'the spirit of GLP'. This means that although the experiments were not certified GLP or monitored by a quality assurance unit, best laboratory practices were maintained throughout the experiment and extensive documenting was in place to ensure some degree of traceability.

Regulatory Body Expectations

The investigator will have a better understanding of what the regulatory expectations are after the Pre submission meeting. While the regulatory expectations will be determined based on the type of CT product being proposed for human testing, there is an assumed regulatory expectation when submitting the data for an IMP application.

The first thing that the regulatory body would like to see is that each study has a protocol. It is expected that each toxicology and biodistribution used to support an IMP application have their own unique study protocol to which it clearly states the sponsors name, study title and purpose of the study. While the overall purpose of the study protocol is to provide the individuals conducting the study (in house or contracted) with enough information to establish what the study objectives are, it is also in place to define the methods which must be used in order to reach these objectives. The key components of the protocol include the identification of the control and test articles, the test system, the experimental design, route of administration and frequency of dosing, frequency of tests and analyses, recording methods and detailed description of how the tests will be conducted. The experimental design and frequency of testing and data capture represent the most important parts of this document and are the most important for the regulatory body to see.

When submitting the IMP application, a statement describing the status of the study should be included. If the study was completed under GLP then a statement confirming GLP compliance must be provided. If the study was not conducted under GLP compliance, then a brief statement providing

justification of non-compliance must be included. Often investigators may have needed to perform a hybrid study where 30% of the study is non-GLP compliant but 70% of it was. For example, for specialized surgeries the investigator may have needed to conduct the animal surgeries in house due to the lack of expertise under GLP conditions. However, even though the surgeries where conducted under non-compliance, the clinical pathology and histopathological analysis was outsourced and conducted under GLP.

Whether the study is GLP or not the regulatory authority expects clear documentation from all the study related activities. This includes not only full documentation from protocol driven activities but also full documentation of any deviations from expected practices. Although the study protocol should be strictly adhered to, any deviation from expected practices as well as unanticipated events should be noted and clearly documented. Also, the actions taken after the deviations have occurred and to what impact these deviations had on the study outcome must be recorded.

The regulatory body will expect complete study reports for all studies used to support the safety and rationale for the clinical trial. The reports should provide full tabulation of the data. While data can be presented in summary form in the main body of the text it is important that individual animal data of every parameter evaluated in the study is presented in the appendix. Furthermore, it is recommended that summary or individual data be presented in the form of text tables. The use of text tables is the most effective method as it presents significant findings based on dose and sex and therefore enables the reader to quickly understand the data that impacts the interpretation of the CT product or any dose related toxicities associated with it.

Cell Bank

According to our data in **Chapter 4** of this thesis, angiogenesis can be achieved by administering hMSCs secreting one or more pro angiogenic growth factors. The purpose of the assays was to establish the angiogenic potential of our cell population. In view of that, these cells can achieve their angiogenic effects in these assays, cell banks could be potentially established in our lab containing cells that have been selected to have a desired level of potency. The selection of the cells of desired potency could be established based on the secretion of one or more proangiogenic factors in combination with positive performances in both in vitro and in vivo assays. As in vitro and in vivo assay are required, limited lots and duration of testing represents an issue for the testing autologous cells. Therefore, we suggest that the system would be more favourable to an allogeneic administration of cells. In this case the cells can be expanded, assayed to validate that the cells have desired potency and stored for use in an off the shelf product. Furthermore, these cell banks may not only be used for purpose of treatment of patients but also for research purposes. Further in vitro and in vivo assays

using cells of known potency would be further useful for testing in normal and diseased models to further elucidate the mechanism in which MSC modulate their angiogenic effects. Furthermore, the storage of cells known potency would enable comparison with subpotent cells to assess differences in biological response between cells. In addition, these cells could be screened for differences between the cells in order to understand why the different responses are occurring. We have already screened our MSC donors and highlighted some agents that we believe may be critical to MSCs angiogenic potential.

Heterogeneity in MSCs Angiogenic Bioactivity

The safety record of MSCs at large has been excellent. As of now there has been no clinical observations made between the administration of MSCs with delivery toxicity, malignancy, tumour formation or death (Lalu et al., 2012). Despite this impressive safety record there can be a high degree of variability associated with MSCs bioactivity.

One of the most interesting results of **Chapter 4** was the observation of donor to donor variation in our MSC population angiogenic potential. While it is well known that MSCs activity can be altered by supplements in which the cells are exposed, initial cell seeding density, cell culture conditions and passage number one of the most interesting hypothesis in the literature at the moment is that of Pacini et al. They suggest that the presence of a nestin and CD-31 positive mesodermal progenitor cell (MPC) in isolated MSC cultures is responsible for the differences observed in the angiogenic potential of the MSC populations. According to their hierarchical model MPCs are considered the true progenitor of MSCs and constitute approx. 1-3% of the bone marrow mononuclear cells. After isolation they remain in a quiescent state and display a fried egg morphology (Pacini et al 2014). When these cells are supplemented with fetal bovine serum mesengenic differentiation of the cell occurs and the MPC differentiate into "early MSC'. Early MSCs represent a nestin, CD-31 positive cell that is a slowly proliferative, but angiogenic. Under persistent mesengenic differentiation (i.e. increasing passage) the cells further differentiate into a 'late MSCs". Late MSCs are nestin negative, proliferate and display the ability to differentiate in bone cartilage and fat. Furthermore, they do not display angiogiogenic properties. Pacini et al therefore claims that the angiogenic potential of MSC is due to heterogeneity of MSC and MPC subpopulations with the donor lot. Furthermore, the interpopulation variability is not only affected by different donors but due to the isolation and culture techniques. The isolation techniques protocols may affect the number of MPC, early or late MSCs within the culture. In addition to these, MPC populations are resistant to trypsin and it is therefore hypothesized that they become lost during subsequent passages and this may further explain the reduced angiogenic potential with increasing passage number (Pacini et al.2014).

Importance of Biodistribution Studies

Biodistribtution represents one of the most important safety tests that can be used by investigators when building a risk profile for their CT (Sensebe, et al., 2013). Determining where the cells go and if they survive after administration has both efficacy and safety implications. For toxicology determining where the cells go is important so that investigator can start looking for toxic activities. Doing this represents a more thorough screening procedure. Non-persisting cells with weak biodisribution potential are less likely to be a long-term hazard. The examination of the disappearance of such cells may enable the investigator to provide a justification to reduce the duration of later studies. Furthermore, if negative qPCR signal is obtained from sampling of non-target organs at early time points it may be used to justify a less arduous tissue sectioning protocol at intermediate time points. The next point that we would like to highlight is that biodistribution may impact both positively and negatively preclinical and clinical potency of the CT product. Wragg *et al* demonstrated that after 4 weeks persistence of their CT product was only detected in the surrounding neurovascular bundles within the ischemic muscles whereas no cells were detected in normally perfused mice. Fischer *et al* shows that cell size and adhesion molecules play a role in pulmonary passage after IV infusion. They compared pulmonary passage of MSCS, bone MNCs, multipotent adult progenitor cells and neural Stem Cells (Fischer er al., 2009). They found that MSCs were 30 times less likely to pass through the lungs when compared to BMNCs and linked this outcome to cell size (Fischer et al., 2009). Furthermore, the found that inactivation of VCAM-1 counter ligand on the surface of the MSCs resulted in a significant increase in the MSCs ability to reach the circulatory system thus suggesting that VCAM was involved in MSC adhesive ability to the vascular endothelium (Fischer et al., 2009). Therefore, we would like to make the point that independent of us demonstrating the angiogenic potential of our hMSC lot as shown in **Chapter 4** we also have to pay attention to their cell size and possible changes in target tissue receptors when transplanting to diseased models as this would have a big effect on the response of the product.

Advice for New PhDs

On beginning your career in translational research it is imperative to always have the clinic in mind. At this moment in time, every grant application will require you to provide justification on how the proposed scientific work will translate into the issue of health. My suggestion is that with every experiment you design it is important consider why you are proposing that experiment and evaluate if the body of work generated form it will directly or in some other form contribute to helping patients in the future. I was lucky to have a mentor who is a clinician scientist with a laboratory as he thought me to think in ways in which new and current technology could be applicable to the clinical setting.

Finally, bridging the gap between discovery and clinical trials is an onerous task, and therefore requires a systematic approach. Researchers in the field must not become disheartened by the unanswered questions that surround cell therapies. It is rare that everything will go straightforward in translating from the bench to the bedside. The prospect of stem cell therapy offers great promise and only in understanding more about the *in vivo* science of stem cells, the greater the possibility that we can safely and lucratively realise our vision of clinically promoting tissue repair and regeneration.

REFERENCES

LALU, M. M., MCINTYRE, L., PUGLIESE, C., FERGUSSON, D., WINSTON, B. W., MARSHALL, J. C., GRANTON, J., STEWART, D. J. & CANADIAN CRITICAL CARE TRIALS, G. 2012. Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS One,* 7**,** e47559.

PACINI, S. & PETRINI, I. 2014. Are MSCs angiogenic cells? New insights on human nestin-positive bone marrow-derived multipotent cells. *Front Cell Dev Biol,* 2**,** 20.

SENSEBE, L. & FLEURY-CAPPELLESSO, S. 2013. Biodistribution of mesenchymal stem/stromal cells in a preclinical setting. *Stem Cells Int,* 2013**,** 678063.

WRAGG, A., MELLAD, J. A., BELTRAN, L. E., KONOPLYANNIKOV, M., SAN, H., BOOZER, S., DEANS, R. J., MATHUR, A., LEDERMAN, R. J., KOVACIC, J. C. & BOEHM, M. 2008. VEGFR1/CXCR4-positive progenitor cells modulate local inflammation and augment tissue perfusion by a SDF-1-dependent mechanism. *J Mol Med (Berl),* 86**,** 1221-32.