GM Microorganism Risk Assessment

Full Assessment, Old Format

PI Responsible: Downes, Prof Peter
Division: Molecular Physiology
Assessor: Nick Leslie
Extn: 86263
Approver: Ian Scragg
Approval Date: 15/2/2008
Review Date: 15/2/2009

Serial Number: USOGMM1107 **Version:** 2
SS Code No:
Created: 24/1/2007, ,
Modified: 16/12/2008, 4:30:38 PM, lgrayson
Assessment Complete: ◉ Yes
Assessment Reviewed by BSA: ◉ Yes
Permission Granted by HoD: ◉ Yes

Final Classification of Project: **Class 2**

Title: **Expression and RNA based knockdown of components of intracellular signal transduction pathways in mammalian cells using retroviral vectors**

Lab: Floor 2
Bldg: Sir James Black Centre

1: Brief Description of Project

We aim to use retroviruses to express, or knock down the expression of, intracellular signalling molecules, including protein and lipid kinases (e.g. AMPK, PI3K, PKB, InsR), lipid and protein phosphatases (e.g. PTEN, SHIP2) and associated proteins (e.g. adaptor, scaffolding proteins, substrates, amino acid transporters). Viruses will be used to infect commonly used tissue culture cell lines (eg HEK293, HeLa, L6, 3T3-L1, U87MG, 1321N1, MCF10A and MDCK cells) or embryonic stem cells, hepatocytes, fibroblast and myoblast cells derived from rodents. The effects that this has on signal transduction pathways will be determined.

Viral vectors and virus
Viral vectors will either be expression vectors, containing cDNAs encoding signalling molecules for transcription and translation, or be 'knock-down' vectors, encoding specific short hairpin RNA molecules for RNA interference. Examples of the vectors that will be used include:

Lentiviral/Retroviral expression vectors
pHR-SIN-CSGW is derived from human immunodeficiency virus 1 (HIV) genome but with the viral genes deleted. This vector contains the viral long terminal repeat (LTR) but with the U3 region deleted in the 3' LTR to prevent viral enhancer and promoter transfer into target cells, thus rendering it a so-called 'self inactivated vector'. The vector also contains the Rev Response Element (RRE) to enhance expression, packaging signals, the HIV central polypurine tract (cPPT) to increase viral titre and a WPRE* (Woodchuck hepatitis virus post-transcriptional regulatory element) to enhance mRNA stability. Expression of the inserted gene comes from a SFFV (spleen focus forming virus) promoter. Derivatives of this plasmid with altered restriction sites for cDNA insertion will be used.

*SACGM recently issued an information note detailing concerns over the safety of viral vectors containing the WPRE sequence. WPRE is capable of expressing part of the X protein from WHV. A number of publications have suggested that truncated hepadna virus X-proteins may have oncogenic properties (Tu et al 2001 Cancer research 61, 7803; Sirma et al 1999, Oncogene 18, 4848). This has implications for human health & safety and the environment. SACGM recommends that containment level 2 be used for work with all lentiviral vectors or viral vectors containing wild-type WPRE, or truncated forms of WPRE, unless the X-protein promoter and start codon have been truncated.

pLenti6/V5-D-TOPO (Invitrogen) is a similar self inactivating Lentiviral vector for the expression of encoded proteins from a CMV promoter in infected cells.

The retroviral vector pWZL hygro (Serrano et al, 1996, Cell, 88, p593), based upon Murine Moloney sarcoma virus, contains long terminal repeats, the viral gag gene and an inserted hygromycin resistance cassette, within a bacterial plasmid vector. To produce infective virus particles, the virus requires pol and env sequences provided by co-transfection or a packaging cell line.

pQCXI vectors (Clontech) - These "retro-X" vectors are self-inactivating retroviral vectors designed to express a target gene alongside a selectable or fluorescent marker gene. They are based upon the Murine Maloney Leukemia Virus. These are of similar design to the pWZL vectors, except that they lack the viral gag gene, requiring this function in trans from the packaging cell line.

Lentiviral/Retroviral RNAi vectors
pLKO.1 (Addgene) is replication incompetent self inactivating lentiviral vector for siRNA expression, encoding a puromycin resistance gene.

pMKO.1puro (Addgene) is a modification of the pQCXIN vector (Clontech) in which the puromycin resistance gene was introduced and the human U6 promoter to drive the expression of inserted cDNAs.

The Origene retroviral silencing plasmid pRS, contains murine retroviral long terminal repeats (LTR), puromycin resistant gene and a U6 small nuclear RNA gene promoter to effectively express the inserted hairpin DNA and to achieve RNA interference upon introduction into a mammalian cell.

Two strategies will be employed to generate viral particles:

1. Vectors are co-transfected into human embryonic kidney (HEK) 293T cells with an HIV packaging plasmid, CMVR8.91, encoding the gag/pol, tat and rev genes but where the virulence genes vif, vpu and nef are deleted and a third plasmid, VSV-G, expressing envelope protein. The transfected cells therefore contain viral genes on split plasmids. These plasmids are in wide use and details of the plasmids and the demonstration that they do not give rise to replication competent viruses are available in Zufferey et al. Nat. Biotechnol. 15:871-875, 1997 and Zufferey et al, J. Virol. 72:9873-9880, 1998. There is evidence that such replication defective viruses are not pathogenic when tested in mice and a minimum of 3 recombination events are necessary to produce replication competent, recombinant virus. The use of VSV-G envelope will give rise to pantropic virus able to infect the broadest range of mammalian and non-mammalian cells.

2. Phoenix helper-free retrovirus producer lines will be employed to generate retroviruses generated by Gary P. Nolans' lab, Stanford University Medical Centre, USA (http://www.stanford.edu/group/nolan/retroviral_systems/phx.html). Phoenix is a second-generation retrovirus producer line for the generation of helper free ecotropic and amphotropic retroviruses. The lines are based on the 293T cell line, a human embryonic kidney line transformed with adenovirus E1a and carrying a temperature sensitive T antigen co-selected with neomycin. The lines were created by placing into 293T cells constructs capable of producing gag-pol, and envelope protein for ecotropic and amphotropic viruses. Using this approach, both the gag-pol and envelope constructs non-moloney promoters were used to minimise recombination potential. Different promoters for gag-pol and envelope are also used to minimise their inter-recombination potential. Two cell lines were created, Phoenix-Eco and Phoenix-Ampho, both of which have been extensively tested for helper virus production and established as being helper-virus free. Whenever possible, when experiments can be performed using rodent cells, ecotropic viruses will be produced that will not infect human cells.

After transfection, media is harvested from the transfected cells 48, 72 and 96 hours later. Viral particles are then purified by filtering through a 0.45mM membrane and then used to infect target cells. Viral particles produced may be amphotropic or pantropic and may be used to infect both human and mouse cell lines and primary cells. All plasmid stocks will be propagated in standard E. Coli host cell lines such as XL1 Blue and prepared according to standard protocols. No special precautions are required for general laboratory handling.

Inserts

The inserts to be used will consist of kinases, phosphatases or related proteins that interact or are affected by these enzymes. In addition, viruses will be used to express short interfering RNAs (siRNAs) which down-regulate the expression of their cellular target mRNAs. These siRNAs will be used to target the same proteins listed above. As signalling pathways regulate cell growth and control transformation, it is possible, therefore, that changing the expression of genes from this pathway could be harmful. As signalling pathways also affect the immune system, it is also possible that expression of these proteins could therefore produce adverse inflammatory effects and immune suppression. Insertion of the above inserts is not expected to alter the tissue tropism, or increase the infectivity or pathogenicity of the recipient vector. Scope for recombination with wild type virus is limited, and due to the packaging limits of this retrovirus, if any such recombinants did arise they would be inviable.

2: Hazards to Human Health

A. Identification of potential harmful properties, and consideration of their severity and likelihood of occurrence.

It is theoretically possible that recombination between the retroviral vector and the retroviral components (transfected or stably integrated) in the packaging cells could produce replication competent retroviruses (RCRs). However, the proposed vectors are in wide use and details of the plasmids and the demonstration that they do not give rise to replication competent viruses are available in Zufferey et al. Nat. Biotechnol. 15:871-875, 1997 and Zufferey et al, J. Virol. 72:9873-9880, 1998. There is evidence that such replication defective viruses are not pathogenic when tested in mice and a minimum of 3 recombination events are necessary to produce replication competent, recombinant virus. The formation of RCRs will be tested for as described [http://www.stewartlab.net/Protocols/Making_Virus_1.htm].

The effects of expression or knockdown of signalling components due to accidental contamination are unknown for many signalling molecules. It is possible that cell growth or immune function could be affected by such contamination. It is known that some of the signalling pathways under study regulate cell growth, survival and proliferation. Although retroviral particles and infected cultured cells would not survive outside culture medium, thorough measures must be taken to avoid personal contamination.

It is also possible that accidental personal contamination with the non-replicative virus would lead to chromosomal insertion of the virus in some cells of the worker. This apparently random insertion could inactivate or activate genes close to its point of insertion, with unknown consequences. However, the chances of such insertion occurring, and at such a pathologically significant site seem very small. Similar risks are associated with many delivery systems.

The risk of replicative viral infection with these viruses seem remote. As described, these systems have been developed to minimise this risk, which is also indicated by the development of these viruses for gene human therapy (see eg Braybrooke et al, 2005, Clin. Cancer Res. 11, 1512 ; Barquinero et al 2004 Gene Ther. 11 S3-9).

Infection of the worker is most likely to occur as a result of injection via sharps contaminated with the viral vector. This risk is minimised by prohibiting the use of sharps unless it is absolutely essential.

B. Assign a provisional Containment Level.

○ Class 1/Level 1 Containment ◉ Class 2/Level 2 Containment ○ Class 3/Level 3 Containment

C. Nature of Work

Brief description of nature of work (include maximum culture volumes)

Standard tissue culture techniques will be applied.
Culture volumes below one litre.

Additional containment measures for work of this nature

3: Hazards to the Environment

The prepared viruses are capable of infecting human and other animal cells. However, since the viruses are non-replicative, and extensive containment measures are in place to rule out the escape of viable virus, the hazard to the environment beyond the laboratory space is negligible

4: If risks identified in Part 3 are not effectively zero specify additional containment measures require to reduce all risks to effectively zero.

5: Waste Disposal

Retroviruses are heat labile.
Virkon is effective at 1% for 10 min (efficacy data available on manufacturer's web site) and will be used for surface disinfection.

Liquid waste is collected in a sealable, robust, autoclavable container, autoclaved then disposed of to drains. If using a microbiological safety cabinet store the container within the cabinet during use and seal before removal. Aspirator set-ups are not used.

Solid waste (including plastic pipettes and agar plates) is collected in a lined, biohazard labelled, autoclavable bin, autoclaved then disposed of as normal refuse.

Large glass pipettes are not used.

Sharps waste is collected in an autoclavable sharp-safe container, autoclaved then disposed of as clinical waste. If using a microbiological safety cabinet store the container within the cabinet during use and engage temporary closure before removal. The use of sharps must be avoided unless essential.

6: Validation and Monitoring

<u>Autoclave Testing and Maintenance</u>
During the first four years after installation an annual 12-point validation test, employing independent thermocouples, is used to demonstrate that the autoclave holds the specified temperature and pressure for the required period of time. Thereafter, autoclaves are serviced every 6 months by a reputable service provider and calibrated annually to ensure the validation criteria are met. During normal, daily operation indicator tape and, in the case of liquid waste, a temperature probe placed at the centre of the load, are used to ensure the required conditions are achieved. Servicing and testing is arranged and test reports are kept by the CLS Health & Safety Coordinator..

<u>Maintaining PPE</u>
Users are required to routinely check their PPE (e.g. lab coat, safety glasses) and keep it in good order. Defective PPE must be repaired or replaced immediately. Laboratory Managers are required to ensure the appropriate PPE is readily available and keep an inspection record for

non-standard PPE, e.g. that used in Liquid Nitrogen facilities.

Inspections, Audits and Continual Monitoring
Safety Inspections are carried out regularly to ensure health & safety policy & procedures are being followed and that the required risk assessments and training records are complete and up to date. Inspections are timetabled and inspection teams selected by the CLS Health & Safety Working Group. Inspection team members are selected from CLS Health & Safety personnel and senior management. Inspection reports aresubmitted to the CLS Health and Safety Management Committee for review. Audits performed by an external, independent body are arranged by the CLS Health & Safety Working Group when deemed necessary by the CLS Health & Safety Management Committee. Lab Managers and Biological Safety Advisors are required to continually monitor safety standards and compliance with Health & Safety Policy & Procedures, within their designated area, and report problems and non-compliance to the CLS Health and Safety Working Group.

MSC Testing and Maintenance (TC suites only)
MSCs are serviced and operator protection (KI) tested on an annual basis by a reputable service provider. A certificate of conformity to the required standard is displayed on each cabinet. The Health and Safety Information Officer is responsible for arranging the servicing schedule, ensuring fumigation/decontamination is carried out prior to testing and issuing and keeping a copy of the certificates of conformity. Users are required to perform a visual check on all alarms and indicators before each use and report any defects immediately to their Lab Manager. Note: If an MSC is moved to a new location, or equipment in a room containing a cabinet is significantly rearranged, to the extent where it may affect the airflows within the room, the cabinet must be KI tested before use to ensure operator protection has not been compromised.

Negative Pressure Testing (TC suites only)
Pressure differentials in TC suites are checked regularly to ensure the suite is at an air pressure negative to the immediate surroundings. Checks are arranged by the CLS Health & Safety Information Officer.

7: Storage and Transport

Samples must be doubly contained during transport within/between buildings & clearly labelled with a contact name & number, the nature of the sample & the biohazard symbol. Inner container/tube must be robust & leak-proof. Outer container must be robust, leak-proof & contain enough absorbent material to absorb the total volume of sample should the inner container leak.

Samples in liquid nitrogen cryo-stores must be contained in proper cryo-tubes & stored in the liquid nitrogen vapour phase to eliminate risk of tube explosion upon initial warming. Samples stored in fridges/freezers must be doubly contained. Fridges, freezers & cryogenic storage vessels must be secure, biohazard labelled & subject to a well maintained inventory system.

8: Emergency Plan

Required? ○ Yes ◉ No

If not required for Class 2 or 3 activities give reason:

Activity is small scale and risk is low.

9: Health Surveillance

Required? ○ Yes ◉ No

If yes, give details:

10: License required? (animal, fish, bee and plant pathogens only)

Required? ○ Yes ◉ No

11: Final Classification of project

○ Class 1 ◉ Class 2 ○ Class 3

12: Additional Information and Comments

13: References

Retroviral vectors
Braybrooke et al, 2005, Clin. Cancer Res. 11, 1512
Barquinero et al 2004 Gene Ther. 11 S3-9
Rigg et al 1996 Virology 218 p290
Serrano et al, 1996, Cell, 88, p593
Zufferey et al. 1997 Nat. Biotechnol. 15:871-875
Zufferey et al. 1998, J. Virol. 72:9873-9880
Stewart et al. 2003, RNA, 9, 493-501