Modification Form for Permit BIO-RRI-0006

Permit Holder: Robert Hegele

Approved Personnel
(Please stroke out any personnel to be removed)

Adam McIntyre

Rebecca Martins

Matthew Lanktree

Piya Lahiry

Chris Johansen

Additional Personnel
(Please list additional personnel here)

Jian Wang

Henian Cao

Matthew Ban

Joseph Dube

	Please stroke out any approved Biohazards to be removed below	**Write additional Biohazards for approval below. Give the full name - do not abbreviate.**
Approved Microorganisms	ME DH10B, ME DH5-alpha, ME DH5A T1	
Approved Primary and Established Cells	[Rodent] (established): 3T3-L1, NIH/3T3. [Non-human Primate] (established): COS-7 [Human] (established: HEK293, HEP62 [Human Primary]: Fibroblast- (GM05659, GM08398, , GM03348 GM03513, Fibroblast,	
Approved Use of Human Source Material	Blood, serum/plasma	Brain tissue (from Strong lab BIO-RRI-0035)
Approved Genetic Modifications (Plasmids/Vectors)	SV 40 Large T antigen (expressed in COS7 cells), pCDNA3 plasmid, pcDNA 3.1 Echo Expression Vector Kit. [ADD]: pCMV6, pcDNA3.1, pENTR 11, GFP-ICK-wt plasmid, ICK ORF Clone ID: IOH38087	
Approved Use of Animals		
Approved Biological Toxin(s)		

Adipose tissue (from Gros Lab)

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE STORED, USED AND DISPOSED OF..*

As the principal investigator, I have ensured that all of the personnel named on the form have been trained. I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca.

Signature of Permit Holder: Robert Hegele 2010.09.28 13:31:31 -04'00'

Current Classification: 2 **Containment Level for Added Biohazards:** _____

Date of Last Biohazardous Agents Registry Form: May 15, 2009

Date of Last Modification (if applicable): Apr 12, 2010

BioSafety Officer(s): Ronald Mosdale Sept. 29, 2010

Chair, Biohazards Subcommittee: _____ **Date:** _____

Western

robarts RESEARCH

October 7, 2010

To: Mr. Ron Noseworthy & RRI/UWO Biosafety Committee

RE: Use of human source materials from clinical samples from patients not from known risk groups.

Includes:

1. Collaborative research project with Dr. Michael Strong to sequence candidate genes in DNA from fresh, frozen human brain samples (normal controls, familial and sporadic ALS).
2. Collaborative research project with Dr. Rob Gros to sequence candidate genes in DNA and/or assay for gene expression in RNA from fresh or frozen human adipose tissue samples.

Specific biosafety procedures.

1. All staff will generally follow UWO procedure: Laboratory procedures to be followed when handling unfixed human blood, tissues or body fluids (HBBF). Universal level 2 precautions will be observed.
2. Brain tissue and adipose tissue has been added to Hegele BIO-RRI-0006 permit.
3. Tissue transport to Hegele lab: 1) Brain tissue will be transported from Dr. Strong's laboratory on the third floor of Robarts in 50 ml. conical tubes inside a sealed zip lock bag inside a freezer box. Box will be labelled with biohazard warning label and stored at -80 in Hegele freezer #3 in room 4295. Brain samples will be approximately 1 cm^3 each and will include normal, familial and sporadic ALS samples. 2) Adipose tissue will be transported from Dr. Gros's laboratory on the fourth floor of Robarts in 50 ml. conical tubes either frozen or at 4° then -80 in RNAlater solution. Box will be labelled with biohazard warning label and stored at -80 in Hegele freezer #3 in room 4295.
4. The 50 ml tubes will be transported to Hegele Level 2 Culture Suite (Room 4292) and thawed inside a Microzone HEPA filtered hood. Samples will be removed from the 50 ml conical tube and either homogenized in a Dounce homogenizer in Gentra PureGene lysis buffer solution using puncture proof gloves to hold the homogenizer or minced and manually processed in a plastic vessel in lysis solution. After homogenization or manual disruption, the solution will be transferred to plastic 15 ml conical tubes and DNA or RNA will be extracted as per the instructions on the kit. Instructions are attached for both DNA and RNA extraction.
5. All centrifugation will be carried out in a Beckman GS-6R centrifuge in 15 ml conical tubes inside a biosafety containment rotor.
6. Solid phase tissue waste (typically a pellet after lysis and proteinase treatment) will be discarded into the autoclave waste stream. All surfaces and apparatus will be

100 Perth Drive, Box 5015, London, ON N6A 5K8 Canada
TEL: 519-931-5271; FAX: 519-931-5218 www.robarts.ca

decontaminated with a fresh 1:10 bleach:water solution. We will autoclave the Dounce homogenizer between homogenizations. After use, all plastic pipette tips, plastic vessels and tubes will be placed in a fresh 1:10 bleach:water solution with a 30 minute residence time, then bleach solution will be decanted -- after a water rinse, tips and tubes will be placed in the autoclave waste stream.

7. The resultant DNA will undergo DNA sequence analysis for candidate genes and RNA will be used for gene expression assays. DNA aliquots are stored in either a 4 degree fridge or at -80 for subsequent use and RNA will be stored at -80 for subsequent use.

Robert A. Hegele MD, FRCPC, FACP, FAHA, FCAHS
Jacob J. Wolfe Distinguished Medical Research Chair in Human Gene Function
Edith Schulich Vinet Canada Research Chair in Human Genetics
Martha G. Blackburn Chair in Cardiovascular Research
Scientist, Robarts Research Institute
Distinguished Professor of Medicine and Biochemistry
The University of Western Ontario
hegele@robarts.ca

Third Edition April 2010

Gentra® Puregene® Handbook

For purification of archive-quality DNA from

- human whole blood
- bone marrow
- buffy coat
- buccal cells
- body fluids
- cultured cells
- tissue
- mouse tail
- yeast
- bacteria

QIAGEN

Sample & Assay Technologies

Protocol: DNA Purification from Tissue Using the Gentra Puregene Tissue Kit

This protocol is for purification of genomic DNA from 5–10 mg or 50–100 mg fresh or frozen solid tissue using the Gentra Puregene Tissue Kit.

Important point before starting

In some steps of the procedure, one of two choices can be made. Choose ▪ if processing 5–10 mg tissue; choose ▲ if processing 50–100 mg tissue.

Things to do before starting

Preheat water baths to 37°C for use in step 3, 55°C for use in step 2b, and 65°C for use in steps 2a and 15 of the procedure.

Procedure

1. **Dissect tissue sample quickly and freeze in liquid nitrogen.**

   Grind ▪ 5–10 mg or ▲ 50–100 mg frozen or fresh tissue in liquid nitrogen with a mortar and pestle. Work quickly and keep tissue on ice at all times, including when tissue is being weighed.

2. **Dispense ▪ 300 µl or ▲ 3 ml Cell Lysis Solution into a ▪ 1.5 ml or ▲ 15 ml grinder tube on ice, and add the ground tissue from the previous step. Complete cell lysis by following step 2a or 2b below:**

2a. **Heat at 65°C for 15 min to 1 h.**

2b. **If maximum yield is required, add ▪ 1.5 µl or ▲ 15 µl Puregene Proteinase K, mix by inverting 25 times, and incubate at 55°C for 3 h or until tissue has completely lysed. Invert tube periodically during the incubation.**

   The sample can be incubated at 55°C overnight for maximum yields.

3. **Add ▪ 1.5 µl or ▲ 15 µl RNase A Solution, and mix the sample by inverting 25 times. Incubate at 37°C for 15–60 min.**

4. **Incubate for ▪ 1 min or ▲ 3 min on ice to quickly cool the sample.**

5. **Add ▪ 100 µl or ▲ 1 ml Protein Precipitation Solution, and vortex vigorously for 20 s at high speed.**

6. **Centrifuge for ▪ 3 min at 13,000–16,000 x *g* or ▲ 10 min at 2000 x *g*.**

   The precipitated proteins should form a tight pellet. If the protein pellet is not tight, incubate on ice for 5 min and repeat the centrifugation.

7. **Pipet ▣ 300 µl or ▲ 3 ml isopropanol into a clean ▣ 1.5 ml microcentrifuge tube or ▲ 15 ml centrifuge tube and add the supernatant from the previous step by pouring carefully.**

   Be sure the protein pellet is not dislodged during pouring.

   Note: If the DNA yield is expected to be low (<1 µg) add ▣ 0.5 µl Glycogen Solution (cat. no. 158930).

8. **Mix by inverting gently 50 times.**
9. **Centrifuge for ▣ 1 min at 13,000–16,000 x *g* or ▲ 3 min at 2000 x *g*.**
10. **Carefully discard the supernatant, and drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube.**
11. **Add ▣ 300 µl or ▲ 3 ml of 70% ethanol and invert several times to wash the DNA pellet.**
12. **Centrifuge for ▣ 1 min at 13,000–16,000 x *g* or ▲ 1 min at 2000 x *g*.**
13. **Carefully discard the supernatant. Drain the tube on a clean piece of absorbent paper, taking care that the pellet remains in the tube. Allow to air dry for ▣ 5 min or ▲ 5–10 min.**

    The pellet might be loose and easily dislodged. Avoid over-drying the DNA pellet, as the DNA will be difficult to dissolve.

14. **Add ▣ 200 µl or ▲ 400 µl DNA Hydration Solution and vortex for 5 s at medium speed to mix.**
15. **Incubate at 65°C for 1 h to dissolve the DNA.**
16. **Incubate at room temperature overnight with gentle shaking. Ensure tube cap is tightly closed to avoid leakage. Samples can then be centrifuged briefly and transferred to a storage tube.**

Modification Form for Permit BIO-RRI-0006

Permit Holder: Robert Hegele

Approved Personnel
(Please stroke out any personnel to be removed)

Adam McIntyre

Rebecca Martins

Matthew Lanktree

Piya Lahiry

Chris Johansen

Additional Personnel
(Please list additional personnel here)

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. *
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Approved GMO	SV 40 Large T antigen (expressed in COS7 cells), pCDNA3 plasmid, pcDNA 3.1 Echo Expression Vector Kit. [ADD]: pCMV6, pcDNA3.1, pENTR 11	GFP-ICK wt plasmid: pcDNA-DEST53 Gateway vector ICK ORF clone ID:IOH3808/
Approved use of Animals		
Approved Toxin(s)		

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** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED.*

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Signature of Permit Holder:

Classification: 2

Date of Last Biohazardous Agents Registry Form: May 15, 2009

Date of Last Modification (if applicable): Nov 23, 2009

BioSafety Officer(s): JStanley April 9/10 Ronald Mosseroth

Chair, Biohazards Subcommittee:

Western

robarts RESEARCH

March 25, 2010

RE: Modification of Biosafety permit BIO-RRI-006
Permit holder: Dr. Robert A. Hegele

I wish to modify my permit to add

1) GFP-ICK-wt plasmid: pcDNA-DEST53 Gateway vector (Invitrogen, Catalog no. 12288-015) + ICK ORF clone (ID: IOH38087) - N-terminal GFP cassette - Antibiotic resistance: Ampicillin and Neomycin

2) GFP-CAT plasmid (control): pcDNA/GW-53/CAT Gateway vector (Invitrogen, included with the previous vector in order) - N-terminal GFP cassette - Antibiotic resistance: Ampicillin, Neomycin, and Chloramphenicol

These plasmids were used for functional characterization of a genomic variant discovered in a human subject. This work was published in 2009 (copy of paper attached):

Lahiry P, Wang J, Robinson J, Turowec J, Litchfield D, Lanktree M, Gloor G, Puffenberger EG, Strauss KA, Martens M, Ramsay DA, Rupar CA, Siu V and Hegele RA. A Multiplex Human Syndrome Implicates a Key Role for Intestinal Cell Kinase in Development of the Central Nervous, Skeletal, and Endocrine Systems. The American Journal of Human Genetics 2009, Feb;84(2):134-47. PMID: 19185282.

I have had a request from a research group in Australia and have a biological material MTA in place and I wish to process the export documentation.

Sincerely,

Robert A. Hegele MD, FRCPC, FACP, FAHA, FCAHS
Jacob J. Wolfe Distinguished Medical Research Chair in Human Gene Function
Edith Schulich Vinet Canada Research Chair in Human Genetics
Martha G. Blackburn Chair in Cardiovascular Research
Scientist, Robarts Research Institute
Distinguished Professor of Medicine and Biochemistry
The University of Western Ontario
hegele@robarts.ca

Australian Government
Department of Agriculture, Fisheries and Forestry
Australian Quarantine and Inspection Service

Quarantine Act 1908 Section 13(2AA)

Phone: (02) 6272 4578
Fax: (02) 6249 1798
File Ref: 08/12955

Permit to Import Quarantine Material

Permit: IP08017148 **Valid From:** 1 Oct 2008 **Valid To:** 1 Oct 2010

Importer	Exporter
Various Contacts Queensland University of Technology Institute of Health and Biomedical Innovation 60 Musk Avenue KELVIN GROVE QLD 4059 Attn: Prof Judith Clements	Various Suppliers Exporters Various Addresses In All countries

You are authorised to import the following material under the listed conditions

Note: This permit covers AQIS quarantine requirement only.

Imports may be subject to quarantine inspection on arrival to determine compliance with the listed permit conditions and freedom from contamination. Imports not in compliance or not appropriately identified or packaged and labelled in accordance with the import conditions they represent may be subject to seizure, treatment, re-export or destruction at the importer's expense.
Additionally, all foods imported into Australia must comply with the provisions of the *Imported Food Control Act 1992*, and may be inspected and/or analysed against the requirements of the Australia New Zealand Food Standards Code.
All imports containing or derived from Genetically Modified material must comply with the *Gene Technology Act 2000.*

It is the importer's responsibility to identify, and to ensure it has complied with, all requirements of any other regulatory organisations and advisory bodies prior to and after importation including The Australian Customs Service, The Department of Health and Ageing, Therapeutic Goods Administration, Australian Pesticides and Veterinary Medicines Authority, Department of the Environment, Water, Heritage and the Arts, Food Standards Australia New Zealand and any state agencies such as Departments of Agriculture and Health and Environmental Protection authorities. Importers should note that this list is not exhaustive.

Import conditions are subject to change at the discretion of the Director of Quarantine. This permit may be revoked without notice.

Notification of the import must be provided to AQIS for all imported goods other than goods imported as accompanied baggage or goods imported via the mail and not prescribed under the *Customs Act 1901*. Notification must be consistent with *Quarantine Regulations 2000* (examples include a Quarantine Entry or a Quarantine declaration).

Commodity Name	Condition Number(s)	Country	End Use
Antibodies (purified & ised against synthetic terial or against antigens derived from multicellular organisms)	PC0992 AND PC0701	All countries	In-vitro
Genetic Material (Purified plasmid and cosmid vectors including phages, bacteriophages and recombinant viral factors, not coding for virulence factors)	PC0992	All countries	In-vitro

This permit is granted subject to the condition that fees determined under Section 66E are paid

Stamp:

Delegate of Director of Quarantine
Printed Name Sally Grimes **Date** 1 Oct 2008

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Commodity Name	Condition Number(s)	Country	End Use
Genetic Material (Purified DNA/cDNA or RNA from multicellular organisms)	PC0992	All countries	In-vitro
Genetic Material (Yeast artificial chromosomes, plasmid or cosmid vectors in E.coli, Bacillus subtilis or Saccharomyces spp containing DNA and/or cDNA from these species or derived from multicellular organisms)	PC0992	All countries	In-vitro
Genetic Material (Purified recombinant bacterial proteins and lipids)	PC0992	All countries	In-vitro
Laboratory reagent (Purified animal proteins, hormones, albumins (including bovine serum albumin), enzymes and lipids (excluding antibodies))	PC0992 AND PC0701	All countries	In-vitro
Laboratory reagents (Cell culture supernatant fluid)	PC0992 AND PC0701	All countries	In-vitro
Cell lines (Human, rodent, rabbit, insect, amphibian, reptile, non-salmon finfish cell lines and hybridomas of these species)	PC0992	All countries	In-vitro
Cell lines (Established primate cell lines)	PC1329 AND PC0992	All countries	In-vitro
Laboratory reagent (Purified or recombinant proteins and lipids including peptides, hormones, growth factors, and enzymes (excluding antibodies))	PC0992 AND PC0701	All countries	In-vitro
Sera, blood, fluid or tissue samples (Sourced from all species (excluding salmonid fish, non-human primates, avians, ovines, caprines, bovines, cervines, equines or porcines))	PC0992 AND PC0701	All countries	In-vitro

Delegate of Director of Quarantine
Printed Name Sally Grimes **Date** 1 Oct 2008

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Condition	Condition Text

PC0701 PACKAGING REQUIREMENTS

1. The products must be imported in quantities of no greater than 20 mL or 20 g for each individually packaged unit.

PC0992 1. A valid copy of this Import Permit (or a method of identifying the Import Permit such as the Import Permit number) and all required documentation must accompany each consignment. Alternatively, necessary documentation will need to be presented to AQIS at the time of clearance. In order to facilitate clearance, airfreight or mail shipments should have all documentation securely attached to the outside of the package, and clearly marked "Attention Quarantine". Documentation may include Import Permit (or Import Permit number), and invoice.

DOCUMENTATION REQUIREMENTS

2. Each consignment must be clearly identified and linked to the relevant item(s) on the Import Permit. Identifying documentation must be available to the quarantine officer at the time of clearance. This documentation may include:

a) an accompanying invoice or airway bill; or

b) the physical labelling of the goods; or

c) an overseas supplier's declaration describing the goods.

3. If the product description on the Import Permit varies from the identifying documentation provided for clearance, the importer is responsible for providing evidence to the quarantine officer that the Import Permit covers the products in the consignment.

4. Providing all documentation is in order at the time of clearance, the consignment can be released from quarantine.

POST ENTRY / END USE CONDITIONS

5. This Import Permit allows for the importation of goods for in vitro laboratory studies (or in vivo use in laboratory organisms only), unless approved by AQIS for specific in vivo use in non-laboratory organisms.

6. This Import Permit does not permit the use of the samples for microbiological cultures or viral isolation, without prior written approval from AQIS.

7. Laboratory organisms include those defined in the following list and must be contained under laboratory or animal house conditions (or equivalent): guinea pigs, hamsters, mice, rabbits, rats, rodents or micro-organisms. Work in all other animals and plants is not permitted.

8. For in vivo use in non-laboratory organisms (eg. chickens, sheep, cattle, etc.) or plants a separate application for in vivo use must be lodged with, and approved by AQIS. This also applies if the product is to be used in veterinary vaccine or veterinary therapeutic manufacture.

9. This Import Permit does not permit the direct or indirect exposure of the imported materials or derivatives to non-laboratory organisms or plants.

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Condition	Condition Text

10. It is the importer's responsibility to ensure that the goods are labelled "In vitro use or in vivo use in laboratory organisms only" or equivalent on the smallest packaged unit prior to distribution.

11. It is the importer's responsibility to ensure compliance with all international (eg IATA) and domestic requirements concerning the safe handling, transport and labelling of biological material.

12. It is the end user's responsibility to ensure that all laboratory products are used in accordance with the current AS/NZS 2243 Safety in Laboratory standards and Office of Gene Technology Regulator (OGTR) requirements.

PC1329 DOCUMENTATION REQUIREMENTS

2. Each consignment must be accompanied by a supplier's declaration, stating:

a) The cell line has shown no signs of contamination including cytopathic effects, or adventitious microbial contamination (including viral contamination); and

b) (i) the cell line is greater than 2 years old; or

(ii) the cell line is less than 2 years old and was derived from animals with no history or clinical signs of infectious disease,

End of Condition Text

Delegate of Director of Quarantine
Printed Name Sally Grimes **Date** 1 Oct 2008

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2009 Biohazard Agents Registry, Dr. Robert Hegele 22-Oct-09

Name	CAT. NO.	SUPPLIER	MSDS	Comment	Use
3T3-L1	CL-173	ATCC	Y	Murine fibroblast	Mutant LMNA is transfected into these cells and immunofluorescence studies and Western analysis performed
NIH/3T3	CRL-1658	ATCC	Y	Murine	
COS-7: note SV40 promoter	CRL-1651	ATCC	Y	Green monkey (Ceropithecus aethiops)	
HEK293: note Adeno E1A promoter	CRL-1573	ATCC	Y	Human	
HepG2	CRL-11997	ATCC	Y	Human	
Fibroblast, finite primary cell line human	GM05659	Coriell Cell Repository	Y	Human: unaffected	Cells are cultured, morphology and growth curves evaluated and immunofluorescence studies, Western analysis, DNA and RNA isolated and microarray and high throughput sequencing analysis performed.
Fibroblast, finite primary cell line human	GM08398	Coriell Cell Repository	Y	Human: unaffected	
Fibroblast, finite primary cell line human	GM03348	Coriell Cell Repository	Y	Human: unaffected	
Fibroblast, finite primary cell line human	AG03513	Coriell Cell Repository	Y	Human: HGPS proband	
Fibroblast, finite primary cell line human	AG04456	Coriell Cell Repository	Y	Human: unaffected	
Fibroblast, finite primary cell line human	AG16409	Coriell Cell Repository	Y	Human: unaffected	
Fibroblast, finite primary cell line human	30950	Dr. T.C. Rupar	na	Human	Cells are cultured, DNA and RNA extracted and microarray and high throughtput sequencing analysis performed
Fibroblast, finite primary cell line human	40916	Dr. T.C. Rupar	na	Human	
Fibroblast, finite primary cell line human	20750	Dr. T.C. Rupar	na	Human	
Fibroblast, finite primary cell line human	70280	Dr. T.C. Rupar	na	Human	
pCMV6	PS100001	Origene	y		Transfection vectors used in the lab
pcDNA3.1	350492	Invitrogen	Y		
pENTR 11 dual selection vector	A10562	Invitrogen	Y		
Subcloning Efficiency™ DH5a™ Competent Cells	18265-017	Invitrogen	Y	E. Coli	Cells used to as vehicles for transfection of genetic material for overexpression studies and phenotypic characterization
Electromax DH10B competent cells	18290-015	Invitrogen		E. Coli	
ME DH10B competent cells	18297-010	Invitrogen		E. Coli	
ME DH5-alpha competent cells	18258-012	Invitrogen	Y	E. Coli	
ME DH5A T1 page resist comp cells	12034-013	Invitrogen		E. Coli	
GFP-ICK-wt plasmid: pcDNA-DEST53 Gateway vector	12288-015	Invitrogen		E. Coli	
ICK ORF clone	ID: IOH38087	Invitrogen		E. Coli	
Fibroblast, finite primary cell line human	HGADFN167	Progeria Research Foundation	Y	Human: HGPS proband	same use as Coriell Cell Repository

University of Western Ontario
Permit Summary

Permit Holder	Hegele, Robert A
Permit #	BIO-RRI-0006
Department	Robarts Research
Phone	5106612111 **Ext.** 25271
Email	hegele@robarts.ca
Approval Date	May 15, 2009 **Expiration Date** May 14, 2012
BioSafety Officer's Signature	J Stanley Robert Hegele

Organism	Cell Types	Human Source Material	GMO	Animals Used	Toxin
ME DH10B, ME DH5-alpha, ME DH5A T1	[Rodent] (established): 3T3-L1, NIH/3T3. [Non-human Primate] (established): COS-7 [Human] (established: HEK293, HEP62 [Human Primary]: Fibroblast- (GM05659, GM08398, , GM03348 GM03513, Fibroblast, finite primary cell line-human .	Blood, serum/plasma	SV 40 Large T antigen (expressed in COS7 cells), pCDNA3 plasmid, pcDNA 3.1 Echo Expression Vector Kit. [ADD]: pCMV6, pcDNA3.1, pENTR 11		

Building	Room	Room Area	Lab Phone	Ext.	Level
Robarts	4212				2
Robarts	4286				2
Robarts	4288				2
Robarts	4292				2
Robarts	4294				1

University of Western Ontario
Permit Summary

Permit Holder	Hegele, Robert A
Permit #	BIO-RRI-0006
Department	Robarts Research
Phone	5106612111 **Ext.** 25271
Email	hegele@robarts.ca
Approval Date	May 15, 2009 **Expiration Date** May 14, 2012
BioSafety Officer's Signature	J Stanley Ronald Mastrangelo

Permit Conditions

1 INTERNAL PERMIT HOLDER RESPONSIBILITIES

Comply with UWO BioSafety Safety Policies and Standard Operating Procedures. Ensure that the Health Canada Biosafety Guidelines, relevant regulations and safe laboratory practices are followed.

1.1 Receive adequate biosafety training from the institution. Permit Holders are responsible for the provision of specific training and instruction in biohaza agent handling that is necessary for the safe use of this material in their own laboratories. Supervisors must ensure that workers understand the healt safety hazards of the work or task (due diligence).

1.2 Ensure that the UWO Biosafety Manual is available to all lab personnel under the permit.

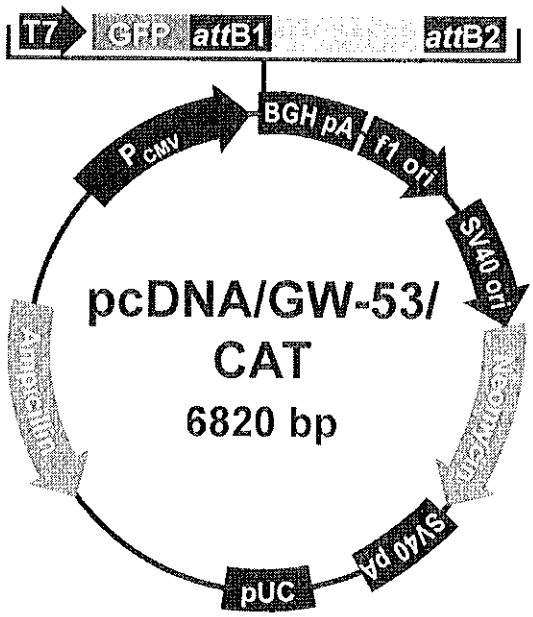
1.3 Report incidents of loss or theft of any biohazardous material immediately to the Biosafety Coordinator;

2 WORKER RESPONSIBILITIES
Be familiar with the UWO Biosafety Manual, attend all required safety training sessions and obey all safety regulations required by the UWO Biosafety Committee.

2.1 Report to the Permit Holder any incident involving known or suspected exposure, personal contamination or a spill involving a biohazardous agent.

I accept the above responsibilities as a Internal Permit Holder and I am accountable for following UWO BioSafety Guidelines and Procedures Manual for Contain Level 1 and 2 Laboratories.

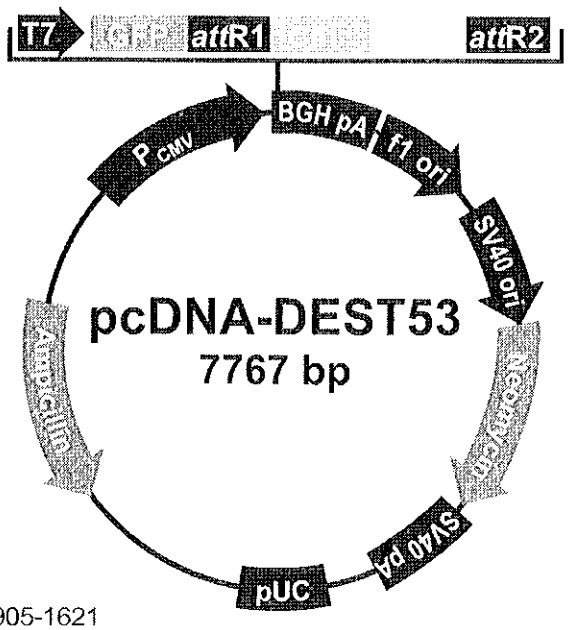
Permit Holder Name J. R. Hegele Signed _____ Date Sept. 9, 09



Comments for pcDNA/GW-53/CAT
6820 nucleotides

CMV promoter: bases 232-819
T7 promoter: bases 863-882
Cycle 3 GFP (N-terminal): bases 905-1621
*att*B1 recombination site: bases 1643-1667
CAT ORF: bases 1697-2353
*att*B2 recombination site: bases 2355-2379
BGH polyadenylation region: bases 2414-2641
f1 origin: bases 2687-3115
SV40 early promoter and origin: bases 3142-3450
Neomycin resistance ORF: bases 3525-4319
SV40 early polyadenylation region: bases 4493-4623
pUC origin: bases 5006-5679
Ampicillin resistance ORF (*bla*): bases 5824-6684 (c)
bla promoter: bases 6685-6783 (c)
(c) = complementary strand

Invitrogen™
life technologies



Comments for pcDNA-DEST53
7767 nucleotides

CMV promoter: bases 232-819
T7 promoter: bases 863-882
Cycle 3 GFP (N-terminal): bases 905-1621
*att*R1 recombination site: bases 1643-1767
Chloramphenicol resistance gene: bases 1876-2535
*ccd*B gene: bases 2856-3161
*att*R2 recombination site: bases 3202-3326
BGH polyadenylation region: bases 3361-3588
f1 origin: bases 3634-4062
SV40 early promoter and origin: bases 4089-4397
Neomycin resistance ORF: bases 4472-5266
SV40 early polyadenylation region: bases 5440-5570
pUC origin: bases 5953-6626
Ampicillin resistance ORF (*bla*): bases 6771-7631 (c)
bla promoter: bases 7632-7730 (c)

(c) = complementary strand

Invitrogen™
life technologies

MATERIAL SAFETY DATA SHEET

Revised 8/26/03
Replaces (None)
Printed 8/26/03

PCDNA-DEST53 (GATEWAY VECTOR)
INVITROGEN CORPORATION
MSDS ID: 12288

1. PRODUCT AND COMPANY INFORMATION

INVITROGEN CORPORATION
1600 FARADAY AVE.
CARLSBAD, CA 92008
760/603-7200

GIBCO PRODUCTS
INVITROGEN CORPORATION
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2270 INDUSTRIAL ST.
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CANADA L7P 1A1
905/335-2255

EMERGENCY NUMBER (SPILLS, EXPOSURES): 301/431-8585 (24 HOUR)
800/451-8346 (24 HOUR)
NON-EMERGENCY INFORMATION: 800/955-6288

Product Name: PCDNA-DEST53 (GATEWAY VECTOR)
Stock Number: 12288

NOTE: If this product is a kit or is supplied with more than one material, please refer to the MSDS for each component for hazard information.

Product Use:
These products are for laboratory research use only and are not intended for human or animal diagnostics, therpeutic, or other clinical uses.

Synonyms:
Not available.

2. COMPOSITION, INFORMATION ON INGREDIENTS

The following list shows components of this product classified as hazardous based on physical properties and health effects:

Component	CAS No.	Percent
EDTA	60-00-4	1 - 5
TRIZMA BASE	MIXTURE	60 - 100

3. HAZARDS IDENTIFICATION

********** EMERGENCY OVERVIEW **********
Warning!
Irritant.
Harmful if swallowed.
Harmful if absorbed.
Harmful by inhalation.
May cause allergic skin reaction.
Possible reproductive system hazard based on animal data.
****

Potential Health Effects:
Eye:
Can cause moderate irritation, tearing and reddening, but not likely to permanently injure eye tissue.

Skin:
Can cause moderate skin irritation, defatting, and dermatitis. Not likely to cause permanent damage.
May cause allergic skin reaction.
Upon prolonged or repeated exposure, harmful if absorbed through the skin.
May cause minor systemic damage.

Inhalation:
Can cause moderate respiratory irritation, dizziness, weakness, fatigue, nausea and headache.
Harmful! Can cause systemic damage (see "Target Organs).

Ingestion:
Mildly irritating to mouth, throat, and stomach. Can cause abdominal discomfort.
Harmful if swallowed. May cause systemic poisoning.

Chronic:
No data on cancer.
Contains a substance that is a possible reproductive system hazard based on animal studies at doses that could be encountered in the workplace.

4. FIRST AID MEASURES

Eye:
Immediately flush eyes with plenty of water for at least 20 minutes retracting eyelids often. Tilt the head to prevent chemical from transferring to the uncontaminated eye. Get immediate medical attention and monitor the eye daily as advised by your physician.

Skin:
Wash with soap and water. Remove contaminated clothing, launder

4. FIRST AID MEASURES (CONT.)

immediately, and discard contaminated leather goods. Get medical attention immediately.

Inhalation:
Remove to fresh air. If breathing is difficult, have a trained individual administer oxygen. If not breathing, give artificial respiration and have a trained individual administer oxygen. Get medical attention immediately.

Ingestion:
Severely irritating. Do not induce vomiting. Seek medical attention immediately. Drink 2 glasses of water or milk to dilute.

Note To Physician:
Treat symptomatically.

5. FIRE FIGHTING MEASURES

Flashpoint Deg C:
Not available.

Upper Flammable Limit %:
Not available.

Lower Flammable Limit %:
Not available.

Autoignition Temperature Deg C:
Not available.

Extinguishing Media:
Can cause moderate irritation, tearing and reddening, but not likely to permanently injure eye tissue.
Use water spray/fog for cooling.

Firefighting Techniques/Equipment:
Do not enter fire area without proper protection including self-contained breathing apparatus and full protective equipment. Fight fire from a safe distance and a protected location due to the potential of hazardous vapors and decomposition products.

Hazardous Combustion Products:
Includes carbon dioxide, carbon monoxide, dense smoke.

6. ACCIDENTAL RELEASE MEASURES

Accidental releases may be subject to special reporting requirements and other regulatory mandates. Refer to Section 8 for personal protection equipment recommendations.

6. ACCIDENTAL RELEASE MEASURES (CONT.)

Spill Cleanup:
Exposure to the spilled material may be irritating or harmful. Follow personal protective equipment recommendations found in Section VIII of this MSDS. Additional precautions may be necessary based on special circumstances created by the spill including; the material spilled, the quantity of the spill, the area in which the spill occurred. Also consider the expertise of employees in the area responding to the spill.
Ventilate the contaminated area.
Prevent the spread of any spill to minimize harm to human health and the environment if safe to do so. Wear complete and proper personal protective equipment following the recommendation of Section VIII at a minimum. Dike with suitable absorbent material like granulated clay. Gather and store in a sealed container pending a waste disposal evaluation.

7. HANDLING AND STORAGE

Storage of some materials is regulated by federal, state, and/or local laws.

Storage Pressure:
Ambient

Handling Procedures:
Harmful or irritating material. Avoid contacting and avoid breathing the material. Use only in a well ventilated area.
Keep closed or covered when not in use.

Storage Procedures:
Store in a cool dry ventilated location. Isolate from incompatible materials and conditions. Keep container(s) closed.
Suitable for most general chemical storage areas.

8. EXPOSURE CONTROLS, PERSONAL PROTECTION

Exposure Limits:

Component	OSHA PEL (ppm)	AGCIH TWA (ppm)
EDTA	Not established.	Not established.
TRIZMA BASE	Not established.	Not established.

Engineering Controls:
Local exhaust ventilation or other engineering controls are normally required when handling or using this product to avoid overexposure.

Personal Protective Equipment:

8. EXPOSURE CONTROLS, PERSONAL PROTECTION (CONT.)

Eye:
An eye wash station must be available where this product is used. Wear chemically resistant safety glasses with side shields when handling this product. Wear additional eye protection such as chemical splash goggles and/or face shield when the possibility exists for eye contact with splashing or spraying liquid, or airborne material. Do not wear contact lenses. Have an eye wash station available.

Skin:
Avoid skin contact by wearing chemically resistant gloves, an apron and other protective equipment depending upon conditions of use. Inspect gloves for chemical break-through and replace at regular intervals. Clean protective equipment regularly. Wash hands and other exposed areas with mild soap and water before eating, drinking, and when leaving work. Have a safety shower available.

Respiratory:
NIOSH approved air purifying respirator with dust/mist filter.
A respiratory protection program that meets OSHA's 29 CFR 1910.134 and ANSI Z88.2 requirements must be followed whenever workplace conditions warrant a respirator's use.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance/physical state:	Liquid solution / suspension
Odor:	No odor.
	Not established.
	Not established.
	Not established.
	Not established.
	Not established.
	Not established.
Specific Gravity/Density:	Not established.
Octanol/water Partition Coeff:	Not established.
Volatiles:	Not established.
Evaporation Rate:	Not established.
Viscosity:	Not established.

10. STABILITY AND REACTIVITY

Stability:
Stable under normal conditions.

Conditions to Avoid:
Strong oxidizing agents. High temperatures. Strong alkalies. Copper alloys. Aluminum alloys.

10. STABILITY AND REACTIVITY (CONT.)

Hazardous Decomposition Products:
Carbon monoxide. Carbon dioxide. Nitrogen oxides.

Hazardous Polymerization:
Hazardous polymerization will not occur.

11. TOXICOLOGICAL INFORMATION

Acute Toxicity:

Dermal/Skin:
Not determined.

Inhalation/Respiratory:
Not determined.

Oral/Ingestion:
TRIZMA BASE: 5900 MG/KG

Target Organs: Kidneys. Bone marrow.

Carcinogenicity:

NTP:
Not tested.

IARC:
Not listed.

OSHA:
Not regulated.

Other Toxicological Information

12. Ecological Information

Ecotoxicological Information: No ecological information available.

Environmental Fate (Degradation, Transformation, and Persistence):
Bioconcentration is not expected to occur.
Biodegrades slowly.

13. DISPOSAL CONSIDERATIONS

Regulatory Information:
Not applicable.

Disposal Method:
Clean up and dispose of waste in accordance with all federal, state, and local environmental regulations.
Dispose of by incineration following Federal, State, Local, or Provincial regulations.

14. TRANSPORT INFORMATION

Proper Shipping Name: Not Determined.
Subsidiary Hazards:

15. REGULATORY INFORMATION

UNITED STATES:

TSCA:
This product is solely for research and development purposes only and may not be used, processed or distributed for a commercial purpose. It may only be handled by technically qualified individuals.

Prop 65 Listed Chemicals: PROP 65 PERCENT
No Prop 65 Chemicals.

No 313 Chemicals

CANADA:

DSL/NDSL:
Not determined.

COMPONENT	WHMIS Classification
EDTA	D2A
TRIZMA BASE	D2B

EUROPEAN UNION:

PRODUCT RISK PHRASES:
None assigned.

PRODUCT SAFETY PHRASES:
Not applicable.

15. REGULATORY INFORMATION (CONT.)

PRODUCT CLASSIFICATION:
XI

Component	EINECS Number
EDTA	200-449-4
TRIZMA BASE	Not established.

16. OTHER INFORMATION

HMIS Rating 0-4:
FIRE: Not determined.
HEALTH: Not determined.
REACTIVITY: Not determined.

Abbreviations
N/A - Data is not applicable or not available
SARA - Superfund and Reauthorization Act
HMIS - Hazard Material Information System
WHMIS - Workplace Hazard Materials Information System
NTP - National Toxicology Program
OSHA - Occupational Health and Safety Administration
IARC - International Agency for Research on Cancer
PROP 65 - California Safe Drinking Water and Toxic Enforcement Act of 1986
EINECS - European Inventory of Existing Commercial Chemical Substances

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* The ORFCard has links to several external public sites for your convenience.
These links may be occasionally unavailable

Ultimate™ ORFCard for Clone ID IOH38087

Gene Information

Clone ID:	IOH38087
Organism:	*Homo sapiens*
Matching Nucleotide Accession:	NM_014920.3|Alignment
Related Accession(s):	AL031178|Alignment || AL162581|Alignment || AB023153|Alignment || AF152469|Alignment || AF225919|Alignment || AI699136|Alignment || AJ420557|Alignment || AK074892|Alignment || BC035807|Alignment || BX647493|Alignment
Gene Name:	intestinal cell (MAK-like) kinase
Gene Definition:	Homo sapiens, intestinal cell (MAK-like) kinase (ICK), transcript variant 1, mRNA
Gene Symbol:	ICK
Summary:	Eukaryotic protein kinases are enzymes that belong to a very extensive family of proteins which share a conserved catalytic core common with both serine/threonine and tyrosine protein kinases. This gene encodes an intestinal serine/threonine kinase harboring a dual phosphorylation site found in mitogen-activating protein (MAP) kinases. The protein localizes to the intestinal crypt region and is thought to be important in intestinal epithelial cell proliferation and differentiation. Alternative splicing has been observed at this locus and two variants, encoding the same isoform, have been identified.
Expression:	Sage Tag Expression || Virtual Northern || Digital Expression Profile
Transcript Variant 1:	This variant (1) represents the shorter transcript.
Transcript Variant 2:	This variant (2) has an additional exon in the 5' UTR, as compared to variant 1. Variants 1 and 2 encode the same isoform.
mRNA Record:	NM_014920|Alignment || NM_016513|Alignment
GO Category: biological process	development (GO:0007275) protein amino acid phosphorylation (GO:0006468) protein kinase cascade (GO:0007243) signal transduction (GO:0007165)
GO Category: molecular function	ATP binding (GO:0005524) magnesium ion binding (GO:0000287) protein serine/threonine kinase activity (GO:0004674) transferase activity (GO:0016740)
References:	GRIF: 22858 | PUBMED: ICK

ORF Information

ORF length (bp):	1899
Sequence:	Nucleotide || Peptide || Translation || Quality Scores || Quality Scores with Sequence

Clone Information

Collection Name:	Ultimate ORF Clones
Collection Type:	ORF Gateway™ Entry
Vector Name:	pENTR(tm)221
Vector Antibiotic:	Kanamycin
Host Name:	E.coli

Protein

Protein Accession:	CAI20261|Alignment || CAI19518|Alignment || BAA76780|Alignment || AAG43364|Alignment || AAF37278|Alignment || AAH35807|Alignment || Q9UPZ9|Alignment
Protein Record:	NP_055735|Alignment || NP_057597|Alignment
Physical Properties:	(aa) || 0.0 (MW) || 0.0 (pI)
Protease Digestion:	Trypsin | Lys-C | Arg-C | Asp-N | V8-bicarb | V8-phosph | Chymotrypsin | CNBr
Predicted Secondary Structure:	View Secondary Structure
Protein Model Search:	Swiss-Model BLAST
Product:	intestinal cell kinase

SNP Information

SNP:	All rs in gene region | rs in coding region only | rs with heterozygosity only
SNP Map to:	Protein

Genomic Link

LocusLink ID:	22858
Unigene ID:	Hs.417022
Genome Alignment:	Map to Human Genome using BLAT || Map to Ensembl Genome Browser

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ARTICLE

A Multiplex Human Syndrome Implicates a Key Role for Intestinal Cell Kinase in Development of Central Nervous, Skeletal, and Endocrine Systems

Piya Lahiry,[1,2] Jian Wang,[1] John F. Robinson,[1] Jacob P. Turowec,[2] David W. Litchfield,[2] Matthew B. Lanktree,[1,2] Gregory B. Gloor,[2] Erik G. Puffenberger,[3] Kevin A. Strauss,[3] Mildred B. Martens,[4] David A. Ramsay,[4] C. Anthony Rupar,[2,5,6] Victoria Siu,[2,5,6] and Robert A. Hegele[1,2,*]

Six infants in an Old Order Amish pedigree were observed to be affected with endocrine-cerebro-osteodysplasia (ECO). ECO is a previously unidentified neonatal lethal recessive disorder with multiple anomalies involving the endocrine, cerebral, and skeletal systems. Autozygosity mapping and sequencing identified a previously unknown missense mutation, R272Q, in *ICK*, encoding intestinal cell kinase (ICK). Our results established that R272 is conserved across species and among ethnicities, and three-dimensional analysis of the protein structure suggests protein instability due to the R272Q mutation. We also demonstrate that the R272Q mutant fails to localize at the nucleus and has diminished kinase activity. These findings suggest that ICK plays a key role in the development of multiple organ systems.

Introduction

Protein kinases belong to one of the largest and most functionally diverse gene families in eukaryotes, constituting approximately 1.7% of all human genes.[1] By phosphorylating substrates, kinases can direct activity, localization, and overall function of numerous target proteins. Kinases are particularly important for signal transduction and coordination of complex cellular functions, as seen with the mitogen-activated protein (MAP) kinases and cyclin-dependent kinases (CDKs), which play a central role in regulating mammalian cell proliferation and division.[2] Altered kinase activity or abnormal substrate phosphorylation has been implicated in monogenic diseases—"kinasopathies"—including endocrine disorders,[3] cancers,[4] immunodeficiencies,[5] and cardiovascular diseases.[6]

Inherited skeletal dysplasias or osteochondrodysplasias are characterized by abnormal development, growth, and maintenance of the skeleton.[7] Manifestations of skeletal dysplasias range from clinically undetectable to severe deformities and lethality.[7] A group of lethal autosomal-recessive skeletal dysplasias are the short rib-polydactyly (SRP) syndromes,[8] which include SRP type II or Majewski Syndrome (MIM: 263520).[9]

Herein we report a new syndrome, to our knowledge, comprising osteodysplasia, cerebral anomalies, and endocrine gland hypoplasia. A pedigree from an Old Order Amish community was identified as having six affected infants with this previously unreported multisystem, neonatal lethal condition, designated here as the endocrine-cerebro-osteodysplasia (ECO) syndrome. Because the Old Order Amish population reportedly have a high degree of consanguinity,[10] this pedigree was ideal for autozygosity mapping of the putative molecular defect.[11,12]

We delineate the clinical features of ECO and report the first mutation, R272Q (c.1305G→A), within *ICK*, encoding intestinal cell kinase (ICK). Biochemical and immunocytochemical studies indicate function and localization deficits, implicating ICK as a key player in development of the central nervous, skeletal, and endocrine systems.

Material and Methods

Patients and Biological Materials

Two families from an Old Order Amish community were referred for genetic assessment and counseling, and all six affected infants were examined by one of the authors (V.S.). Photos, blood, and tissue samples were provided for research purposes, with ethics approval (from the Office of Research Ethics at the University of Western Ontario) and informed consent from participating parents. Peripheral blood and skin biopsy for DNA extraction was collected from five affected children, three parents, and two unaffected siblings and used for autozygosity mapping. The genealogy of the families affected with the disease was prepared through interviews and local Amish community records. DNA from umbilical cord blood and buccal swabs was extracted from 257 ECO-unaffected individuals born into the Old Order Amish community for determining the mutant-allele frequency in the community. Autopsies were performed on three patients (IV-1, IV-2, and IV-8 from pedigree) after 33, 29, and 23 weeks of gestation, respectively.

Histology

Routine tissue samples including liver, kidney, and central nervous system (CNS) from each of the three autopsy patients were collected

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for histopathological examination, formaldehyde fixed, paraffin embedded, sectioned, and then stained with hemolysin and eosin. Neuropathological examination was performed on formalin-fixed brain and spinal cord of each of the three autopsy patients.

Genotyping

DNA from five affected infants and five unaffected parents and siblings were genotyped for single-nucleotide polymorphisms (SNPs) with GeneChip Mapping 500K Array Set (Affymetrix, Santa Clara, CA, USA) at the London Regional Genomics Centre. 250 ng of double-stranded genomic DNA was digested with either *Nsp* or *Sty*, followed by adaptor ligation and PCR amplification with generic primers. PCR products were then purified, fragmented with *DNaseI*, labeled with terminal deoxytransferase, and finally hybridized to the Mapping 250K Nsp or Sty GeneChips. SNP genotypes were determined with the B-RLMM algorithm implemented in Affymetrix GTYPE software.

Autozygosity Mapping

Autozygosity (homozygosity) mapping was performed with Agilent GT v2.0 (Agilent Technologies, Santa Clara, CA, USA), which scans the genome for regions that are identical by descent. SNP allele frequencies from controls of European descent were used for estimating the two-point logarithm of the odds (LOD) scores for each SNP. Location scores, which are the summation of two-point LOD scores for a block of homozygous SNPs, were then calculated for providing a relative measure of likelihood that the region harbors the disease gene.[13]

Mutation Analysis

Eleven of the 36 positional candidate genes in the linked region on chromosome 6p were screened by genomic DNA sequencing in an affected individual, a parent, an unaffected sibling, and a non-Amish control. Candidate genes from genomic DNA were PCR amplified with primer pairs designed for all coding exons and intron-exon boundaries. All amplicons were then sequenced with the sequencing platform of the London Regional Genomics Centre Sequencing Facility.

Cosegregation of the exon 7 *ICK* mutant with disease in the pedigree was demonstrated with direct sequence analysis of all available family members. The target sequence (552 base pairs [bp]) was amplified with primers 5′ CTC ATT CCA TAC AGT GCC ACA and 3′ GAA TTA CAT GCC AAT TTT CAA AG, followed by electrophoresis purification on a 1.5% agarose gel and analysis on a ABI 3730 DNA Sequencer (Applied Biosystems, Mississauga, ON, Canada). A total of 13 family members, including the affected individuals, were studied.

SNaPshot and TaqMan assays were used for identifying allele frequency of the *ICK* variation in exon 7, c.1305G→A (GI: 156671211), within 257 Old Order Amish controls and 2855 ethnically diverse and healthy non-Amish controls, respectively. For SNaPshot, which is a rapid allele-specific genotyping method, the purified 552 bp amplicon (with the above primers) was subjected to ddNTP extension (SnaPShot, Applied Biosystems) with primer 5′ CAG TGG GAT CCC AAG AAA C and analyzed by ABI 3730 DNA Sequencer. TaqMan quantitative real-time PCR assays were performed with an ABI 7900 sequence detection system (Applied Biosystems) for providing allele discrimination with PCR primers (forward primer: 5′ GCT CCT GAG AGA CAT GCT TCA; reverse primer: 5′ AAG AAA ATG GAA GAA AAC CTG ACT AGC T) and two allele-specific TaqMan probes synthesized for detecting the *ICK* variation (allele G: 5′ VIC-CCC AAG AAA CGA CCA AC and mutant allele A: 5′ FAM-CCA AGA AAC AAC CAA C).

In Silico Analysis

Conservation of the ICK protein across species was determined with ClustalW, which is a multiple-sequence-alignment computer program, by initially creating a phylogenetic tree of the query sequence.[14] Impact of the amino acid mutation (R→Q at residue 272) on ICK protein structure, function, and pathological implication was predicted with four online tools, namely PMUT,[15] PolyPhen,[16] SNPs3D,[17] and SIFT.[18]

The crystal structure of human CDK2 in complex with isopentenyladenine (PDB ID: 2EXM), solved by Schulze-Gahmen et al.[19], was used as a basis for modeling the ICK with and without the R→Q mutation at residue 272. For mimicking ICK, 2EXM was substituted at A183P and I186V. The resulting structure was visualized in the program PyMOL (v0.99, DeLano Scientific, San Francisco, CA, USA).[20] With the Rosetta Design program,[21] used for approximating the change in potential energy (in kilocalories) of the ICK structure with the R→Q mutation, side chains of nearby contacting amino acids were allowed to vary in conformation. Change in energy values (in kilocalories) was replicated in Eris server, which is a protein-stability prediction server that calculates the change in protein stability caused by mutations.[22] Eris server has the added feature of allowing backbone motion of the amino acids, which is crucial for protein-stability estimation of small-to-large mutations.

Plasmids and Cell Culture

The Ultimate ORF Clone of human *ICK* cDNA (clone ID: IOH38087) was provided in the Gateway entry vector, pENTR221, containing a kanamycin-resistance cassette (Invitrogen, Carlsbad, CA, USA). The R→Q mutation was introduced into the wild-type *ICK* clone within pENT221 in vitro with the GeneTailor Site-Directed Mutagenesis System (Invitrogen). With Clonase II (Invitrogen) for aiding homologous recombination, the wild-type and mutant *ICK* cDNA was cloned directionally into the Gateway destination vector, pcDNA-DEST53, containing an N-terminal green fluorescent protein (GFP) tag and neomycin-resistance cassette. All clones were sequence verified. The plasmid pcDNA/GW-53/CAT, which contained an N-terminal GFP tag, neomycin-resistance cassette, and chloramphenicol-acetyltransferase (CAT) cassette, was provided as a vector control. HEK293 cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum.

Immunocytochemistry

For assessing nuclear localization of *ICK* constructs, HEK293 cells were grown on coverslips in six-well 35 mm dishes to 60%–70% confluency, followed by transfection with either wild-type (WT), R272Q mutant ICK-expression plasmid, or control vector containing a CAT cassette (4 μg DNA) by a calcium-phosphate-based method. 48 hr after transfection, cells were washed two times with PBS, fixed with 4% paraformaldehyde, and stained with Hoechst dye (2.5 μg/ml in PBS) (Sigma-Aldrich, Oakville, ON, Canada) on ice for 20 min. Cells were then washed three times with PBS and mounted on glass slides with PermaFluor Aqueous Mounting Medium (Fisher, Markham, ON, Canada). Images were captured with FITC and UV filter sets and 40× objective with a Leica (Deerfield, IL, USA) DMI6000B inverted fluorescence microscope, followed by image acquisition with the Leica Application Suite (LAS v. 2.8.1).

Protein Quantification

HEK293 cells were grown in 225 cm^2 flasks until 60%–70% confluency was reached, followed by transfection with GFP-tagged

expression constructs of either WT, R272Q mutant, or control vector containing a CAT cassette (96 μg DNA) by a calcium-phosphate-based method. 48 hr after transfection, cells were harvested in ice-cold PBS and lysed in lysis buffer (20 mmol/L Tris-HCl [pH = 7.4], 50 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 25 mmol/L NaF supplemented with phosphatase and protease inhibitors). The lysate was cleared by centrifugation. Cell lysates were precleared with immobilized protein-G beads (Fisher) for 3 hr at 4°C and then incubated with anti-GFP (3 μg) for 2 hr at 4°C, followed by incubation with immobilized protein-G beads (Fisher) overnight at 4°C. The beads were washed extensively with lysis buffer and then divided for immunoblotting and kinase assay.

GFP-immunoprecipitated beads of all three constructs were boiled in the SDS loading buffer for 5 min. Proteins were then resolved by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Invitrogen). The membranes were blocked in TBS containing 0.1% Tween-20 and 5% fat-free dry milk for 1 hr and then incubated with anti-ICK (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The membrane was then incubated with horseradish peroxidase-conjugated anti-goat secondary antibodies (1: 50,000, Santa Cruz Biotechnology) for 1 hr, followed by ICK protein visualization with enhanced chemiluminescence-detection Luminol reagent according to the manufacturer's instructions (Santa Cruz Biotechnology).

Kinase Assays

For assessing kinase activity, GFP-immunoprecipitated WT ICK, R272Q mutant ICK, and vector control were washed extensively in kinase assay buffer (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$, supplemented with 5 mM DTT, protease inhibitors and phosphatase inhibitors). The samples were then incubated with 5 μCi [γ-^{32}P] ATP, 100 μM ATP, and 5 μg purified myelin basic protein (MBP) (Millipore, Billerica, MA, USA) at 30°C for 15 min in 50 μl kinase assay buffer. As a positive control, active MAPK 2 (Millipore) was incubated with 5 μCi [γ-^{32}P] ATP, 100 μM ATP, and 5 μg MBP at 30°C for 10 min in 25 μl kinase assay buffer. Proteins in the reaction were separated by 15% SDS-PAGE. The gels were dried, and ^{32}P was detected by autoradiography.

Statistical Analysis

Immunofluorescence localization data were analyzed with Pearson's chi-square test with SAS v9.1 (SAS Institute, Cary, NC, USA).

Results

Clinical and Pathological Features of ECO

Three affected individuals from two Old Order Amish families were originally reported as having a Majewski-hydrolethalus phenotype in 2004 (S. Bakker and V. Siu, 2004, Am. Soc. Hum. Genet., abstract). Three further infants were subsequently born, and the phenotype was further characterized (Figure 1A and Table 1).

The infants have had a high birth weight (90th percentile)[23] in four out of six times that this was measured. Some of the excess weight may be attributed to the excess fluid associated with severe hydrocephalus. In all of the pregnancies in which antenatal ultrasound was performed, multiple anomalies including ventriculomegaly, cleft lip, and shortened limbs were observed in the second trimester. Polyhydramnios occurred in one pregnancy. Spontaneous onset of labor occurred in two of the pregnancies at 33–34 weeks gestation. One infant (IV-1) showed no spontaneous respirations at birth, was intubated for a day, then extubated and taken home for palliative care. He died on day 3. The second infant (IV-7) had severe hydrocephalus, requiring drainage of 350 ml of cerebrospinal fluid from the ventricles to enable vaginal delivery. The baby died during birth. The other pregnancies were induced between 23 and 29 weeks gestation because of the severity of the malformations. Integrated prenatal screening was undertaken in one pregnancy and was screen positive for increased risk of Trisomy-21, with a low unconjugated estriol of 0.18 MoM and a low alpha-fetoprotein of 0.48 MoM. Adrenal hypoplasia found in this infant (IV-8) may potentially account for the low prenatal estriol levels.

The face shows a wide nasal bridge and flattened nasal tip with median cleft lip in four of the six cases and a small premaxilla with bilateral cleft lip and palate in the remaining two cases. There is swelling of the tissue derived from the maxillary prominences. The eyes are small and sunken, with a cystic component observed in two cases. As expected, the eyelids were fused in the three infants born between 23 and 25 weeks gestation, but they were also fused in the infant born at 29 weeks (eyelids normally remain fused until approximately 26 weeks gestation)[23]. Ears show varying degrees of dysplasia. The lower lip is deficient laterally, and the chin is small. One infant (IV-2) had two congenital supernumerary teeth at 29 weeks gestation.

The upper limbs are markedly shortened, with a characteristic bowing of the forearms, ulnar deviation of the hands, and postaxial polydactyly. X-rays reveal angulation of the diaphyses. Palmar creases are abnormal. Usually the index fingers have only one interphalangeal crease, whereas the other digits lack at least one, if not both, interphalangeal creases. There is severe brachydactyly of the digits on the hands, as well as syndactyly involving various combinations of digits two to six. The hips are abducted, with the thighs held at 90 degrees to the lower legs. There is a very wide gap between the halluces and the second toes, reminiscent of atelosteogenesis type 2. On X-rays, the proximal metaphyses of the femur and tibia are widened. Overall, the radiologic findings are most consistent with a diagnosis of Majewski syndrome.

Chest tends to be broad, with widely spaced nipples only in patient IV-1. No congenital heart defect was found in any of the three autopsies. The adrenal glands were hypoplastic in two cases and absent in one case.

Four genotypic males have all had varying degrees of abnormal differentiation of the external genitalia. Patient IV-7, born at 34 weeks, had apparent sex reversal, with unfused urogenital folds and microphallus. Position of the urethral orifice was not determined, and no autopsy was done for assessing for testes. Patients IV-1, IV-2, and

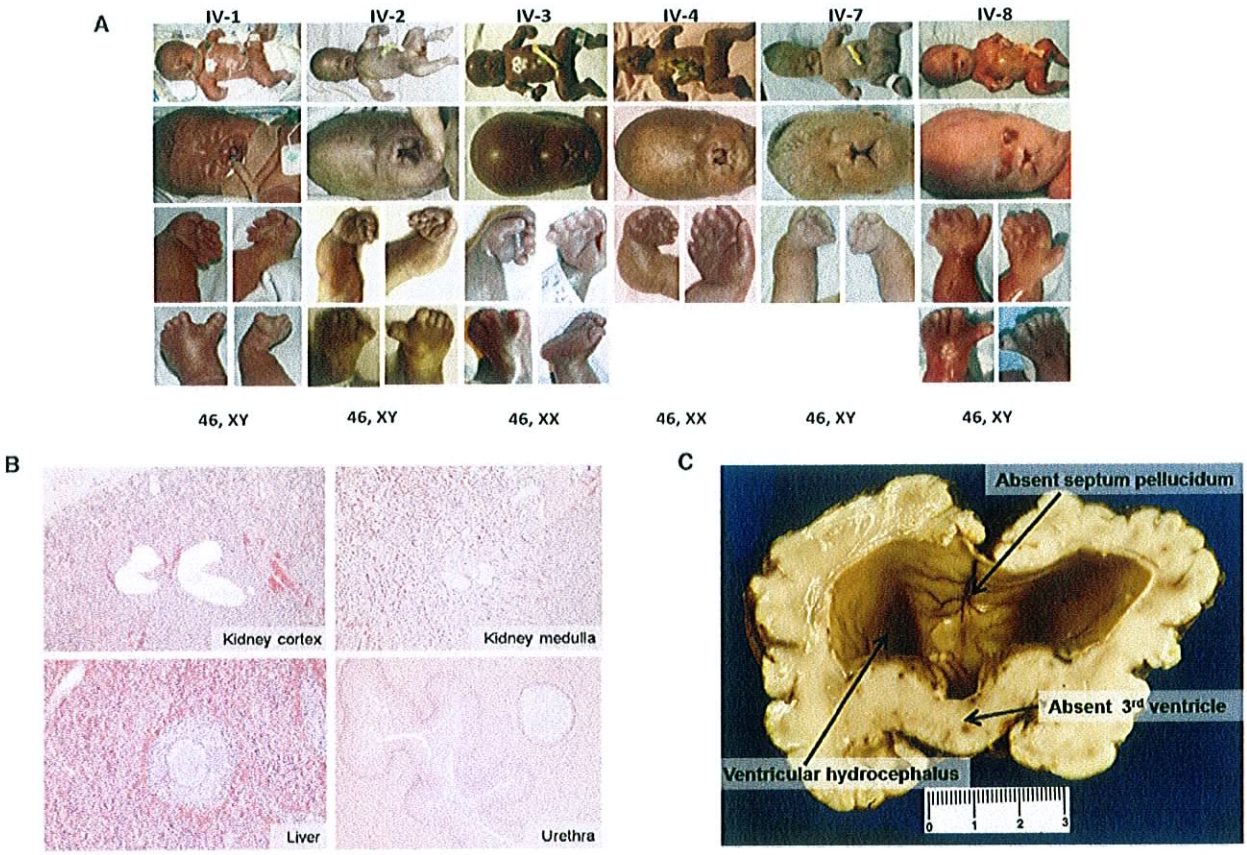

Figure 1. Clinical and Pathological Findings in ECO Patients

(A) Serial photographs of all six affected individuals (left to right from pedigree) showing full body with polydactyly and micromelia, craniofacial abnormalities including cleft lip and palate, bowed forearms, wide-gapped hallux (not shown for IV-4 and IV-7), and karyotype (top to bottom).

(B) Kidney cortex from patient IV-1 (top left) and medulla from patient IV-2 (top right) show cystically dilated tubules (hematoxylin and eosin staining [H&E], 40× magnification). Liver sections from the 29-week-gestated patient IV-2 (bottom left) illustrate the persistence of circular ductal plates (100× magnification). Squamous metaplasia is seen in the intraprostatic urethra from patient IV-1 (bottom right) (H&E, 40× magnification).

(C) Representative gross coronal view of the brain of patient IV-1, presenting with absent septum pellucidum, secondary to ventricular hydrocephalus, and fused thalami due to a lack of the third ventricle.

IV-8 had microphallus, hypoplastic scrotum with no rugae, prominent scrotal raphe, and bilateral cryptorchidism. Although the cryptorchidism may not be significant in the context of prematurity (two were delivered at 24 and 28 weeks), IV-1 was delivered at 33 weeks gestation and had first-degree hypospadias. The urethral opening was present at the tip of the microphallus in the other two infants. The two genotypic females had unfused urogenital folds. Born at 24 weeks gestation, IV-3 had unusually prominent labia majora, whereas IV-4 (25 weeks gestation) had normal external female genitalia.

Tissue specimens examined from autopsies of patients IV-1, IV-2, and IV-8 (Figure 1B) showed cystically dilated tubules to a variable extent in both the medulla and the cortex of the kidney, as well as persistence of circular ductal plates in the liver. In addition, patient IV-8 had squamous metaplasia of the urethra and periurethral glands in the prostate.

Neuropathology findings were extensive, with the main features being evidence of holoprosencephaly, hypoplastic or absent corpus callosum, agenesis of the pituitary, and cerebral cortex malformations. Patients IV-1 (Figure 1C) and IV-8 had hydrocephalus in the form of ventricular dilatation, absence of the third ventricle, dysmorphic septum pellucidum, and malformed diencephalic elements. It is conceivable that the apparent overgrowth of the medial diencephalic structures led to the obliteration of the third ventricle, initiating secondary hydrocephalus, followed by the rupture or absence of the septum pellucidum. In patient IV-2, there is a development of a semilobar holoprosencephaly, which indicates an earlier initiation of the genetic defect resulting in hypertrophic diencephalic elements.

Overall, affected individuals had ventricular hydrocephalus, midline cleft lip and palate, abnormal bone development manifesting as micromelia, bowing of the long bones, postaxial polydactyly, hypoplastic adrenal and pituitary

Table 1. Clinical and Pathological Description[a]

Clinical Features	Affected Individuals (year of birth)					
	IV-1 (2004)	IV-2 (2005)	IV-3 (2005)	IV-4 (2006)	IV-7 (2002)	IV-8 (2003)
Age at delivery (weeks)	33	29	24	25	34	23
Sex	male	male	female	female	male	male
Karyotype	46, XY	46, XY	46, XX	46, XX	46, XY	46, XY
Height[40]	40 cm (3rd percentile)	37.3 cm (10th percentile)	29 cm (75th percentile)	32.5 cm (10th percentile)		33.1 cm (90th percentile)
Weight[40]	2250 grams (90th percentile)	1400 grams (90th percentile)	675 grams (25th percentile)	897 grams (90th percentile)	1871 grams (10th percentile)	667 grams (90th percentile)
Head circumference[40]	36.5 cm (>97th percentile)	31.7 cm (>97th percentile)	24 cm (20th percentile)	28 cm (3rd percentile)		23.3 cm (90th percentile)
Autopsy	+	+	−	−	−	+
Oral						
Cleft palate	midline	midline	notch in alveolar ridge	midline	midline	midline
Cleft lip	bilateral	median	median	bilateral	median	median
Presence of premaxilla	+	−	−	+, tiny	−	−
Prominent upper lip region	−	+	−	+	−	−
Hypoplastic/absent epiglottis	+	−				+
Hypoplastic/absent larynx	+	−				+
Facial						
Midface hypoplasia	+	+	+	+	+	+
Hypoplastic eyes	+	+	−	+	present; right: hypoplastic, left: cystic	cystic
Retinal dysplasia	−	−				+, cataracts
Deep-set eyes	+	+	+	+		+
Fused eyelids		+	+	+		+
Hypotelorism			+			
Flat and wide nasal bridge	+	+	+	+	+	+
Dysplastic and low-set ears	+	+		dysplastic	+	+
Micrognathia	+	+	+	+	+	+
Excess skin below chin	+	+	−	−	+	+
Teeth	−	two in lower jaw	−	−	−	−
Skeletal						
Dolicocephalic	−	+	−	−	+	+
Prominent xyphoid	−	+	−	−	−	−
Polydactyly (postaxial)	4 limbs	4 limbs	3 limbs	3 limbs	3 limbs	4 limbs
Syndactyly	+	+	+	+	+	+
Brachydactyly	+	+	+	+	+	+
Single transverse palmar crease	unilateral	bilateral			unilateral	
Ulnar deviation of hands	+	+	+	+	+	+
Bowing of forearms (radius and ulna)	+	+	+	+	+	+
Bowing of lower legs (fibula and tibia)	+	−	−	−	−	+
Hitch-hikers' thumbs	−	−	−	−	−	unilateral
Abducted hips	+	+	+	+	+	+
Wide gap between first and second toe	+	+	unilateral-right	+	+	+
Talipes equinovaris	−	−	−	−	−	−
Chest width	broad with wide-spaced nipples	−	−	−	narrow	−

Table 1. *Continued*

Clinical Features	Affected Individuals (year of birth)					
	IV-1 (2004)	IV-2 (2005)	IV-3 (2005)	IV-4 (2006)	IV-7 (2002)	IV-8 (2003)
Micromelia	+	+	+	+	+	+
[b]Radiography						
Abnormal long bones (radius, ulna, tibia, fibula)	short diaphysis					
Short and incurved ulnae	+					
Short and ovoid tibiae	+					
Abnormal humerus	short diaphysis					
Abnormal femur	short and ovoid					
Abnormal Ilium	+					
Abnormal/ hypoplastic acetabular roof	+					
Central nervous system						
Evidence of holoprosencephaly	failure of separation of diencephalic elements	semilobar, monoventricular, fusion of basal ganglia and thalami				diencephalic agenesis
Corpus callosum	+	agenesis				hypoplastic
Absence of septum pellucidum	+					+
Hydrocephalus (ventriculomegaly)	communicating					+
Dysmorphic cerebral aqueduct	+	stenosis				
Olfactory bulbs	+	absent				
Cerebral cortex malformation	frontal and occipital regions	focal polymicrogyria				rudimentary sulcation
Brainstem malformation	+	+				small brainstem
Cerebellar abnormalities	hemorrhagic, dysmorphic peduncles					small cerebellum
Hippocampus agenesis	+	+				
Leptomeningeal glioneruronal heterotopia	+					
Spinal cord malformation	+	–				
Endocrine system						
Pituitary gland	absent	not identified				
Adrenal glands	hypoplastic	absent				hypoplastic
Other						
Pulmonary hypoplasia						+
Gastrointestinal anomalies	tube-like stomach	small stomach				–
Squamous metaplasia of bladder	+	–				–
External genitalia	microphallus, hypoplastic scrotum	microphallus, hypoplastic scrotum	normal	normal	sex reversal; hypoplastic labia majora	present, microphallus
Cryptorchidism	–	bilateral	–	–	–	–
Polyhydramnios	–	–	–	–	present	–

Note: Blank cells indicate that information was unavailable.

[a] Information gathered from attending-physician reports.

[b] Report from International Skeletal Dysplasia Registry (Cedars-Sinai Medical Center) for Patient IV-1.

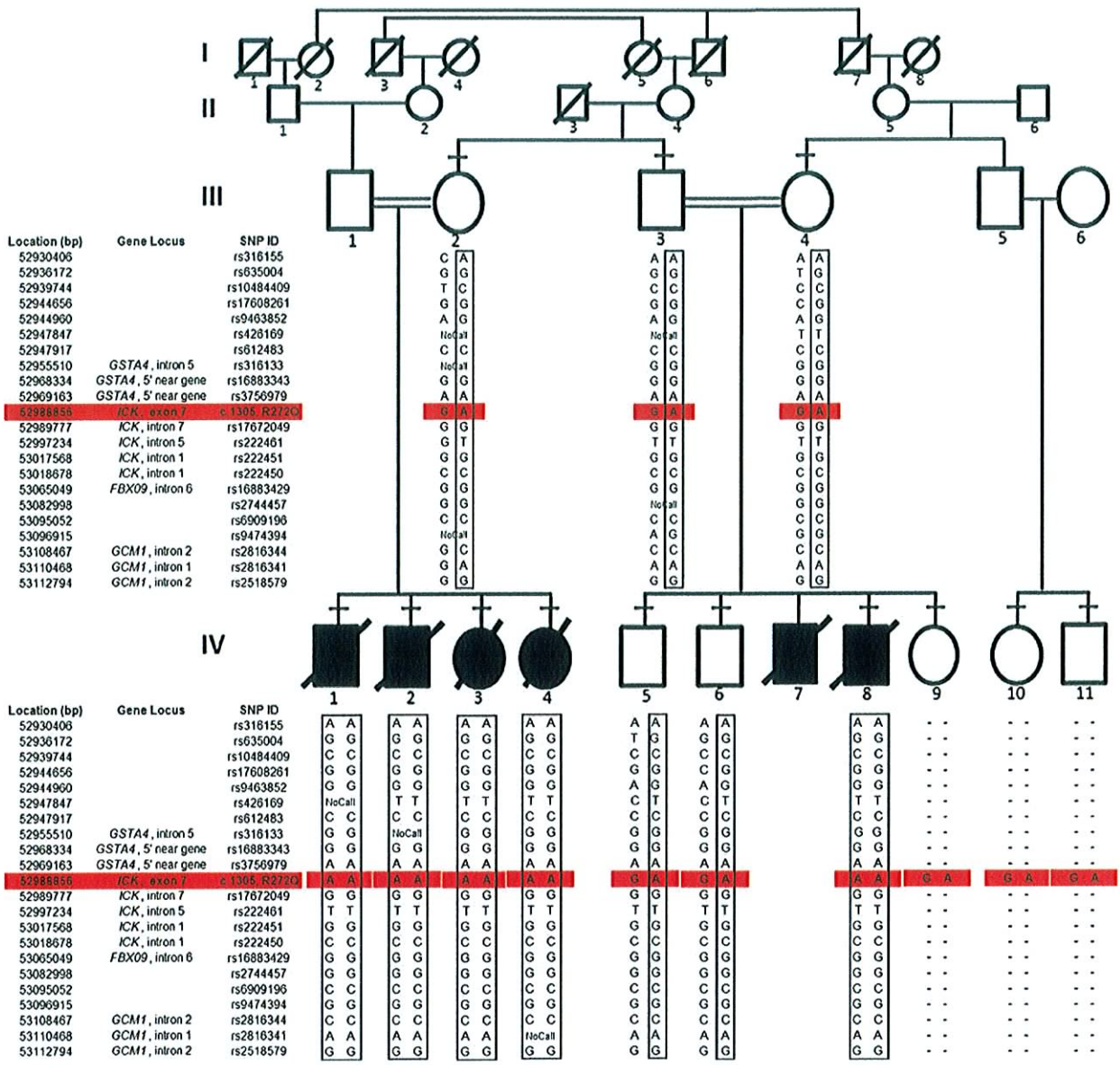

Figure 2. SNP-Based Genotyping of ECO-Affected Pedigree Identified a Homozygous Region in Chromosome 6p

ECO in a consanguineous Old Order Amish pedigree. The inheritance of the disease follows an autosomal-recessive mode of transmission. Affected individuals are shown as blackened squares (male) and circles (female). Diagonal lines across symbols indicate deceased individuals. A consanguineous marriage is shown by a double line between two individuals. Confirmation of autozygosity-based linkage was performed by observing the SNP-based genotyping within the candidate region on 6p12.2–p12. A portion of the homozygous region within patients is shown in boxes. The *ICK* variation at the coding sequence 1305 (G→A, accession number: NM_016513) that causes a R→Q change at residue 272 in the protein was analyzed (see Figure 3C for more details) and highlighted in red. Horizontal dashes above symbols indicate individuals who underwent DNA analysis, and hyphens indicate nongenotyped markers.

glands, and ambiguous genitalia. Many of the malformations observed involve a defect of apoptosis, especially the cleft lip and palate, syndactyly, prolonged persistence of fusion of the eyelids, and unfused urogenital folds.

Autozygosity Mapping of a Candidate Locus on Chromosome 6p

Pedigree analysis (Figure 2) of affected individuals revealed that the syndrome followed an autosomal-recessive inheritance pattern, with two parental consanguineous matings (III-1 with III-2 and III-3 with III-4). Genome-wide autozygosity mapping performed on five ECO-affected infants and three of the four parents from the pedigree unambiguously mapped ECO to 6p12.2–p12 with a location score of 462, the summation of two-point LOD data,[13,24] which spans over 1.8 Mb of the chromosome (Figure 3A). This region in chromosome 6p is bound by SNPs rs2397061 and rs627217 and is comprised of 397 genotyped

contiguous SNPs (Figure 3B). Autozygosity-based linkage was confirmed by haplotype analysis of the SNP genotypes within the candidate region at chromosome 6p (Figure 2).

Identification of the Causative Mutation, R272Q, in *ICK*

The candidate interval harbored a total of 36 known and hypothetical genes. We prioritized sequencing of candidate genes on the basis of the following criteria: (1) gene involvement in other single-gene disorders, (2) the role and tissue specificity of the encoded protein, and (3) the number of exons per gene, for feasibility reasons. On the basis of this priority list, exons of 11 candidate genes were directly sequenced from the genomic DNA of one of each of the following: affected individuals, unaffected siblings, parents, and non-Amish controls. This sequencing led to the discovery of numerous DNA sequence variations (Table S1 available online). In an autosomal-recessive mode of inheritance, the disease causing the variation would be due to a homozygous genotype observed only in the affected individual, and the parent would be an obligate carrier of the causative allele. The only such variation that also affected the gene and/or its gene product was in *ICK*. The *ICK* gene, composed of 12 exons, had a nonsynonymous nucleotide change c.1305G→A in exon 7 (Figure 3C) in the DNA of the affected individual. This nucleotide change results in an amino acid change from arginine to glutamine at residue 272 (R272Q), which lies within the nuclear-localization-signal domain of ICK (Figure 3D). DNA sequencing further demonstrated that this alteration was homozygous in all five affected individuals, whereas the phenotypically unaffected parents and siblings were heterozygotes, consistent with the predicted autosomal-recessive pattern of inheritance. Complete linkage of ECO to the R272Q mutation (at chromosome 6p) within the pedigree was observed, with a two-point linkage LOD score of 3.61 (at recombination fraction = 0).

In Silico Analysis Demonstrates R272 to Be Conserved and Required for Protein Stability

The ICK protein belongs to the CDK subfamily, which is within the CMGC serine-threonine protein kinase family.[1] R272 in human ICK is conserved in the CMGC group of kinases, which includes the CDK, MAP kinase, glycogen synthase kinase 3 (GSK3), and CDC-like kinase (CLK) families. ICK homologs were identified across phylogeny, ranging from *Homo sapiens* to *Candida albicans*. Using ClustalW protein sequence alignment, we observed R272 to be conserved in all 84 known orthologous ICK homologs (a representative set is shown in Figure 4A). The conservation of R272 suggests that mutations in this amino acid are not well tolerated, given that it may be involved in crucial aspects of structure and function. A previous study of ICK that mutagenized several residues, including R272, suggested that the arginine is required to create a functionally and structurally stable conformation by forming ionic bonds with glutamic acid at residue 189.[25] There is presently no three-dimensional (3D) model of ICK because it has not yet been crystallized. However, CDK2 and ICK share very high amino acid homology within the critical functional domain of interest. For homology-based modeling of ICK in PyMol,[20] the 3D region harboring the nuclear-localization signal of CDK2 was substituted at only 2 of the 35 residues (residues 183 and 186). As visualized in Figure 4B, when arginine is mutated to glutamine, the bond with glutamic acid is disrupted such that the glutamic acid rotates its side chain so that it is no longer buried in the protein but becomes surface exposed, suggesting a cause for protein instability. Instability was confirmed with thermodynamic values acquired from Rosetta[21] and Eris,[22] two discrete protein-structure programs. Both Rosetta and Eris, which predict protein-packing energy changes, predicted that the R272Q mutant protein was less stable (by 5.1 and 3.4 kCal/mol, respectively) compared to the wild-type protein. In addition, four different bioinformatic programs predicted a deleterious effect of R272Q mutation on the ICK protein ("pathological," "probably damaging," "affected protein function," and "deleterious" from PMUT, PolyPhen, SIFT, and SNPs3D, respectively).

Mutation Screening in Amish and Non-Amish Populations Suggests R272Q to Be a Private Mutation

The frequency of the A allele in *ICK* at exon 7, c.1305 G→A, was assessed within members in the Old Order Amish community as well as individuals from other ethnic groups. Genotype analysis of 257 healthy Old Order Amish controls demonstrated no AA homozygote but identified five heterozygote GA carriers outside the ECO-affected family. The A allele frequency in the Old Order Amish community was 0.97%; thus, ~1 in 10,000 Old Order Amish subjects would be predicted to have the AA genotype. TaqMan-based genotyping of the *ICK* mutation in an additional 2855 individuals from six ethnic groups demonstrated the complete absence of the A allele, indicating that this mutation was specific to the Amish.

Nuclear Localization Affected by ICK R272Q Mutation

GFP-tagged ICK wild-type overexpressed in HEK293 cells localized predominately to the nucleus, whereas the GFP-tagged ICK R272Q mutant localized predominantly to the cytoplasm (Figure 5A). Therefore, this single R272Q point mutation was sufficient to cause a loss of nuclear localization. Statistical evidence in a bar graph is provided in Figure S1.

Loss of Kinase Activity of ICK with R272Q Mutation

We studied kinase activity in the presence of the R272Q mutation. GFP-labeled ICK wild-type and R272Q mutant were overexpressed in HEK293 cells prior to isolation via immunoprecipitation, and subsequent kinase-activity assays were performed for phosphotransferase activity. Incorporation of ^{32}P into the exogenous substrate, MBP, was catalyzed by ICK wild-type, but no such incorporation was detected in the presence of the ICK R272Q mutant (Figure 5B). The lower panel for Figure 5B indicates that

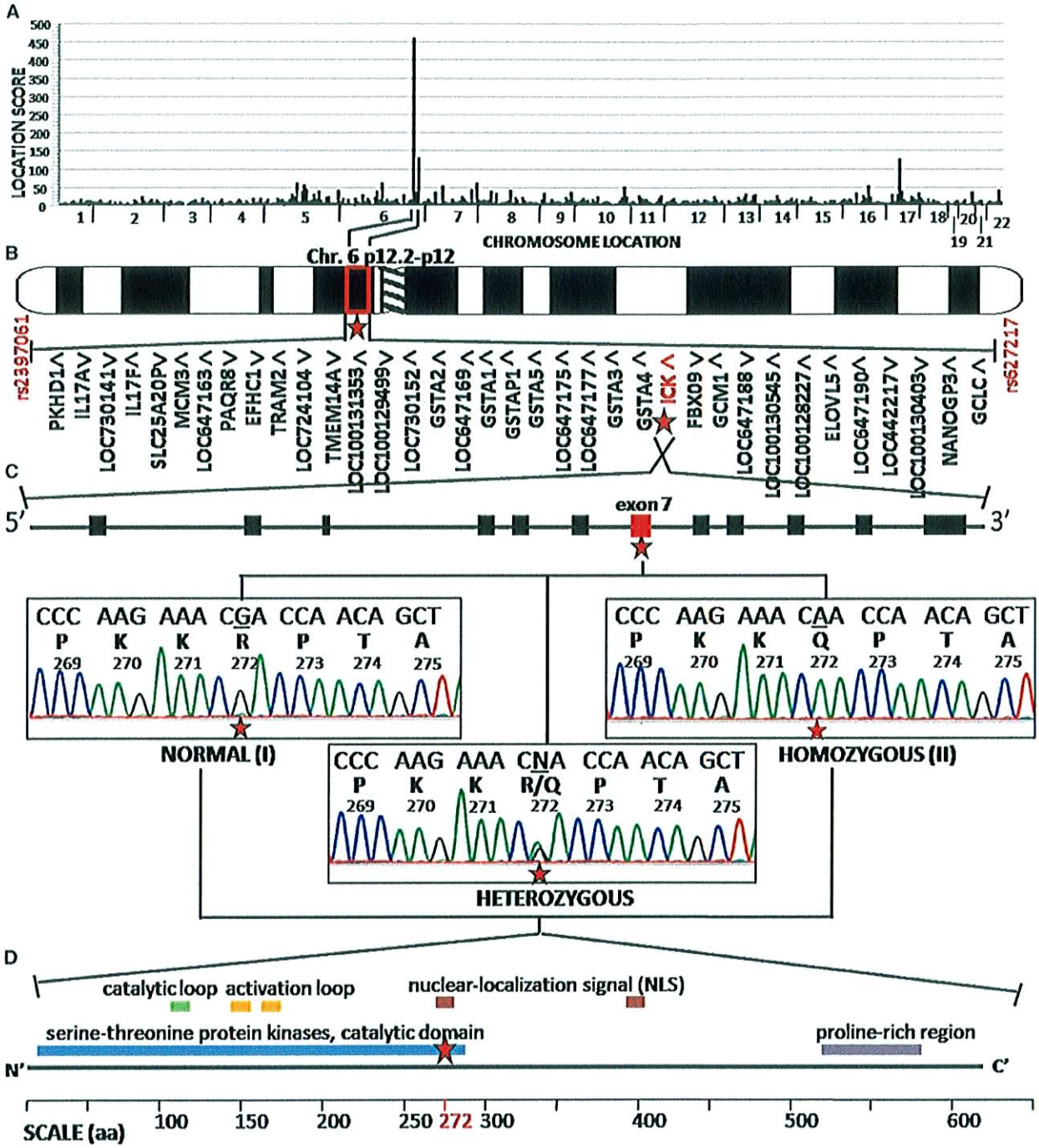

Figure 3. Autozygosity Mapping of ECO-Affected Pedigree Identified an Amino Acid Change, R272Q, in ICK

(A) Autozygosity mapping with SNP genotypes from 500,000 SNP microarrays across the chromosomes (x axis) yielded SNP haplotype location scores (y axis), with the highest peak on chromosome 6. Location score is the summation of the point LOD scores representing the likelihood of observing contiguous homozygosity in all five affected individuals of each SNP within the region of homozygosity.[13]

(B) Expanded view of the candidate locus shows the peak to be on chromosome 6p12.2–p12 (indicated by a star), which is defined by SNPs rs2397061 and rs627217 and consists of 36 candidate genes, including *ICK* (indicated in red with a star), distributed over 1.8 Mb of the genome. Transcriptional direction is indicated by arrowheads.

(C) The genomic structure of ICK gene consists of 12 coding exons with a nonsynonymous nucleotide change in exon 7 (indicated in red with a star) that alters the amino acid of arginine to glutamine at residue 272 (R272Q). DNA sequence analysis of ICK exon 7 from genomic DNA of a normal (I, top left tracing) individual, an affected (II, top right tracing) individual, and a R272Q heterozygote (bottom middle tracing). For each tracing, a normal nucleotide sequence is shown in the top line of letters, with single-letter amino acid codes and codon numbers beneath. The position of the mutated nucleotides is indicated by the star.

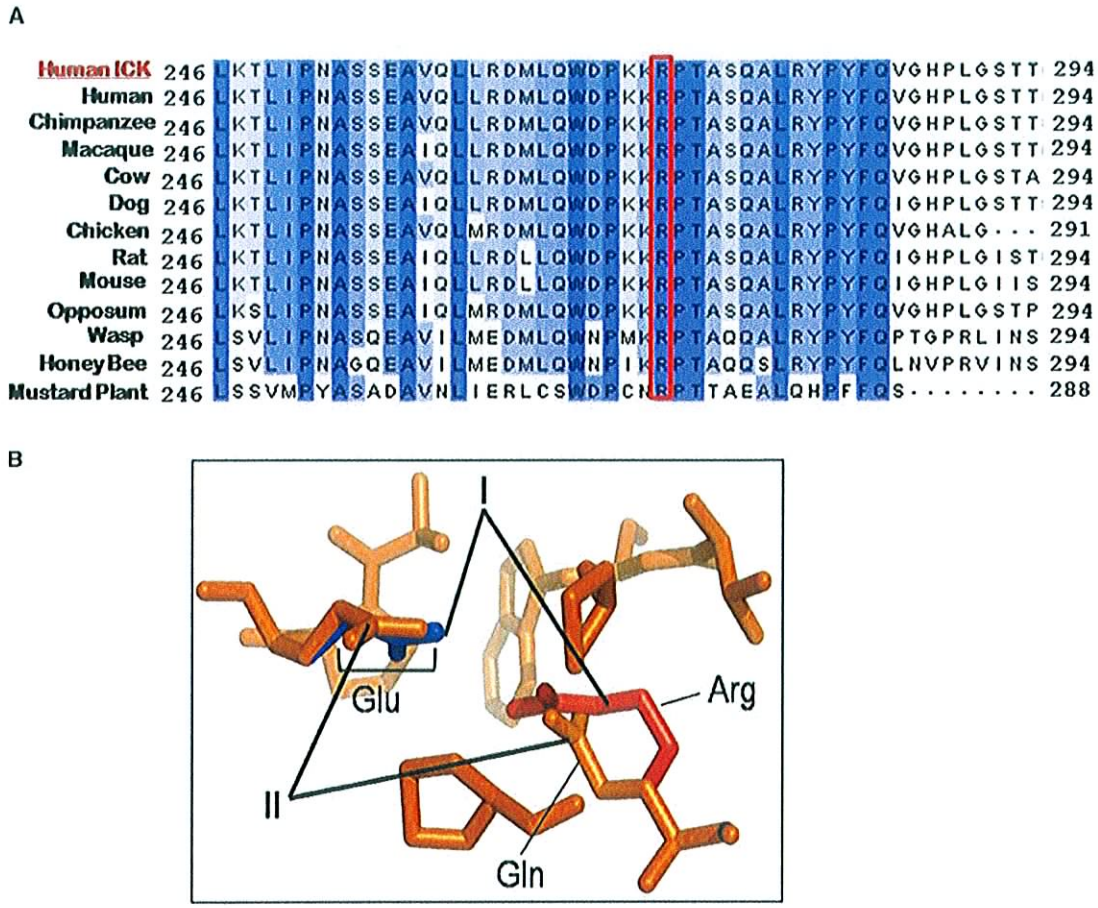

Figure 4. ICK Protein Analysis Demonstrates that R272 Is Highly Conserved and that the R272Q Mutation Alters Protein Structure

(A) Multiple alignments demonstrate that R272 residue is highly conserved across a representative set of species-specific ICK homologs. A ClustalW analysis of the ICK region encompassing the mutation site at residue 272 (highlighted in red) in aligned homologs with multiple divergent sequences is shown. The residues shaded in blue indicate amino acids that are similar between homologs.

(B) A magnified look at the 3D region surrounding residue 272 in ICK according to PyMOL modeling, such that the normal (I) protein is superimposed on the R272Q mutant (II) protein. This modeling predicts that the arginine (Arg, in red) and glutamic acid (Glu, in blue) form an ionic pair because of close proximity, in normal or wild-type protein (indicated by I). By comparison, the R272Q mutant (indicated by II) is a basic polar to neutral polar substitution and predicts a change in structure such that the glutamine (Gln, in orange) and glutamic acid (Glu, in orange) can no longer ion pair, leaving the glutamic acid exposed to the surface of the protein rather than buried within the 3D structure.

equal amounts of ICK protein were assayed for kinase activity. Bands for the ICK constructs were observed specifically, providing solid evidence for specificity of the ICK antibody. Overall, the assay for ^{32}P incorporation suggests that the R272Q mutation significantly impairs kinase activity.

Discussion

Rare congenital disorders provide new information on the biological pathways in human organogenesis.[26] We report in an Old Order Amish community a previously unidentified and phenotypically distinct syndrome, ECO, whose clinical manifestations occur early in development and include dysplasias of skeletal, cerebral, and endocrine systems resulting in neonatal mortality. In affected patients, we identified a homozygous missense mutation, R272Q, in the kinase ICK. R272 was highly conserved across species and kinase family members. Moreover, the protein carrying the mutation was predicted to change the conformation of the structure, as visualized in PyMol, and to cause protein instability, as predicted by the

(D) The domain structure of ICK protein consists of the protein serine-threonine kinase catalytic domain, a catalytic loop, two activation loops, two nuclear-localization-signal sites, and a proline-rich region, from the N-terminal to C-terminal end. The amino acid (aa) 272 (indicated in red) lies within the nuclear-localization signal (indicated with a star).

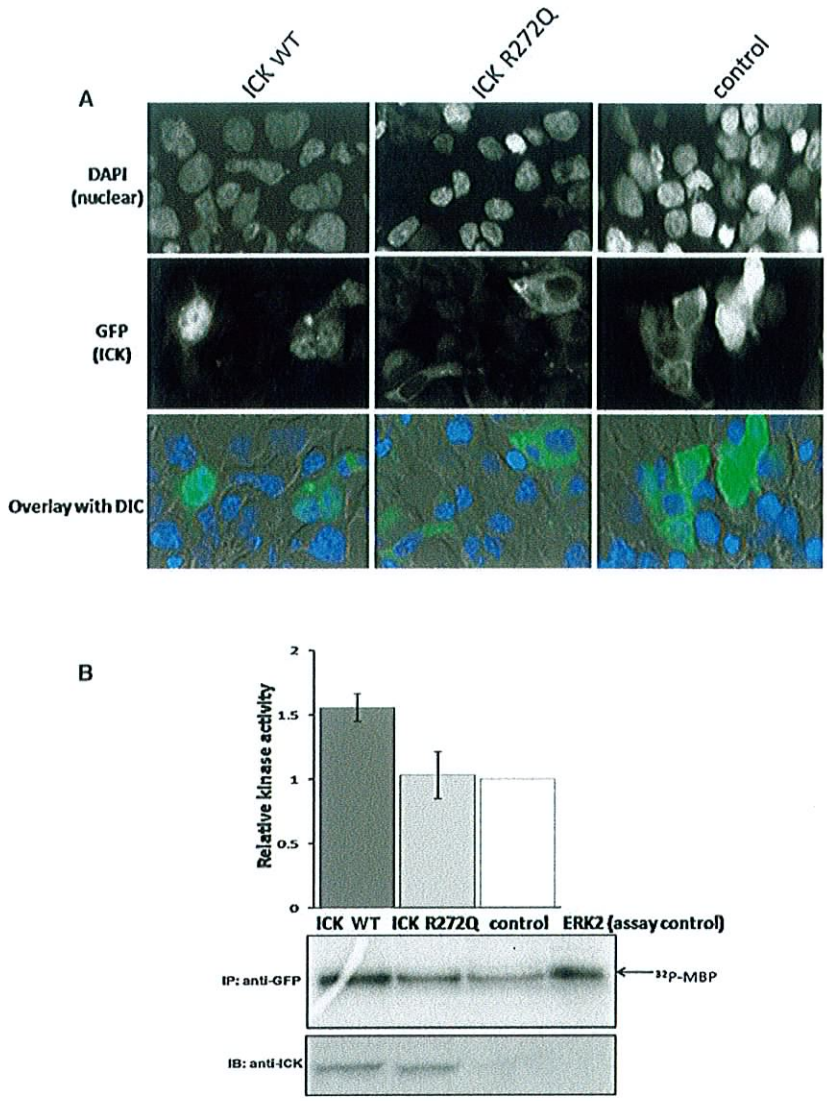

Figure 5. Altered Subcellular Localization and Protein Activity of the R272Q ICK Mutation

(A) Subcellular localization of wild-type and mutant ICK is mainly nuclear and cytoplasmic, respectively. HEK293 cells were transfected for 48 hr with wild-type ICK (ICK WT), mutant ICK (ICK R272Q), or vector control tagged with GFP and analyzed by immunofluorescence microscopy at 40× magnification. Transfected cells were identified by GFP fluorescence (green), and nuclei were stained with DAPI (blue). The signals obtained for DAPI and GFP are shown separately (rows 1 and 2), and an overlay of both fluorescence signals with differential interference contrast (DIC) is shown in row 3. We counted 502, 501, and 501 transfected cells per construct of wild-type ICK, mutant ICK, and control, respectively. The difference in subcellular localization is statistically significant (chi-square p value = 3.9×10^{-97}), such that the wild-type and the mutant ICK protein localizes in the nucleus of 71.9% and 9.78% of the transfected cells, respectively. In addition, blinded counting was performed in a small subset of the whole, and the overall results of the much larger cell numbers were representative of this blinded sample.

(B) In vitro kinase assays were performed with MBP as substrate with anti-GFP immunoprecipates obtained from HEK293 transfected cells and active-purified ERK2 as a positive control (top panel). HEK293 cells were transfected for 48 hr with GFP-tagged ICK WT, ICK R272Q, or vector control followed by anti-GFP immunoprecipitation. Bar graphs indicate means ± standard deviations from three sets of experiments, showing the relative kinase activities normalized to vector control quantitated by densitometry. The autoradiograph shows results of one experiment demonstrating incorporation of ^{32}P into MBP. The upper blot shows results of a representative experiment demonstrating the levels of MBP phosphorylation. The lower blot, developed with anti-ICK, demonstrates equal amounts of ICK WT and ICK R272Q.

change in energy values in the programs Rosetta Design and Eris. The R272Q mutation impairs nuclear localization and kinase activity. The *ICK* R272Q mutation underlying the ECO syndrome implicates intestinal cell kinase as central to the development of several organ systems in humans.

Although ECO is, to our knowledge, a distinct new syndrome, it does share some clinical features with previously reported syndromes, such as Majewski syndrome and hydrolethalus. Majewski syndrome, described in 1971, maps to chromosome 4 and is a lethal form of neonatal dwarfism characterized by short ribs, micromelia, polysyndactyly, median cleft lip, polycystic kidneys, ambiguous genitalia, and hypoplastic epiglottis, larynx, and lungs.[27] Hydrolethalus, described in 1981, maps to chromosome 11q24.2 and is a neonatal lethal autosomal-recessive syndrome characterized by multiple congenital anomalies including hydramnios, hydrocephalus, a "keyhole" defect of the occipital bone, low-set ears, and midline malformations, such as heart and brain defects, cleft lip or palate, an abnormally shaped nose or jaw, incomplete lung development, and abnormal genitalia.[28] The causative genes for both Majewski syndrome and hydrolethalus have yet to be identified,[29] but neither locus overlaps with 6p12.2–p12 harboring *ICK*. A transitional clinical condition, pseudotrisomy 13 (MIM 264480), consists of characteristics found in both Majewski syndrome and hydrolethalus, including holoprosencephaly, hydrocephalus, polydactyly, heart defects, and facial anomalies consistent with the trisomy 13

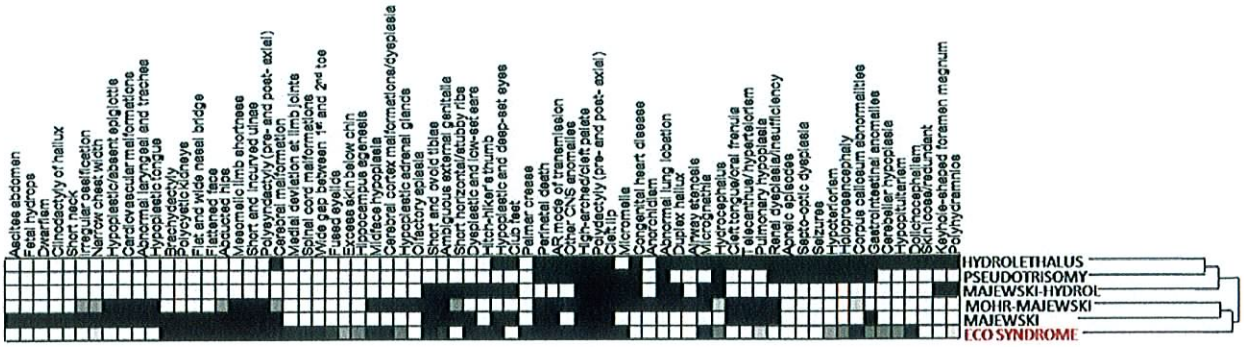

Figure 6. Schematic of Overlapping Features between Six Disorders, including ECO
The grid shows organ-system involvement, with darkened cells indicating the presence of the subphenotype for each disease. Prevalence of disorder is in a white-to-black gradient system in which a frequency of 0% is white, 50% is gray, and 100% is black. The reordering of the disorders was the result of hierarchical cluster analysis that clustered the disease on the basis of similarities in organ-system involvement. The diseases included are (from top to bottom) Hydrolethalus, Pseudotrisomy 13, Majewski Hydrolethalus, Mohr-Majewski, Majewski syndrome, and ECO (highlighted in red).

condition without any chromosomal defects.[30] Another clinical condition that bears some similarity to Majewski syndrome and hydrolethalus was reported in 1992, uniting two distinct genetic conditions such that the two conditions are suspected to be causally related.[31] "Majewski hydrolethalus" was characterized by short limbs and ribs and abnormal tibiae consistent with Majewski syndrome as well as hydraminos, hydrocephalus, and keyhole-shaped occipital bone consistent with hydrolethalus syndrome.[32] Again, the causative gene is unknown. Another distinct yet related condition with an unknown causative gene is the autosomal-recessive Mohr-Majewski syndrome (MIM: 258860).[33]

Hierarchical cluster analysis reordered these six related diseases on the basis of clinical features such that ECO had the closest clinical description to the Majewski and Mohr-Majewski syndromes (Figure 6). However, Majewski and Mohr-Majewski have the presence of short ribs and cardiac defects, respectively, which are features that are not observed in ECO-affected infants. Despite the absence of overlap of loci from mapping studies, DNA sequencing of *ICK* in individuals with Majewski and hydrolethalus syndromes might help to establish whether these disorders are allelic or whether ECO truly represents a distinct and unique disorder.

ICK was initially cloned from the human intestinal crypt with degenerate primers specific for MAP kinases.[34,35] The designation ICK appears to be a misnomer, given that ICK is ubiquitously expressed in adult human tissues.[34,35] Alternatively, this protein has sometimes been called MAK-related kinase (MRK) in previous reports.[35,36] ICK has a variant, ICKb (accession number AAH35807); however, ICKb is 337 amino acids shorter with an alternative C terminus, such that ICKb is predominantly in the cytoplasm.[25] ICK shares 38% to 40% identity to the catalytic domains of CDKs, which are involved in cell-cycle transition, and MAP kinases, regulators of cell-cycle entry.[25] ICK is activated by dual phophorylation of the TDY motif (Y-159 and T-157), such that it autophosphorylates at Y-159 and can be phosphorylated by human kinase cell-cycle-related kinase (CCRK), while being deactivated by protein phosphatase 5 (PP5).[37] It may be speculated that because the residue 272 mutation of ICK prevents nuclear localization, it hinders phosphorylation of the ICK by the nuclear-residing CCRK, which is ultimately required for kinase activity of ICK.

A previous in vitro study of *ICK* coincidentally mutagenized residue 272 from arginine to alanine (R272A). Like R272Q, this non-naturally occurring R272A mutant showed impaired nuclear localization and kinase activity.[25] As determined by in vitro studies, ICK can phosphorylate exogenous substrates, such as myelin basic protein[25,36] and *Scythe*, an antiapoptotic protein required during mammalian development.[38] Scythe (BAT3) is a nuclear protein that is implicated in apoptosis.[39] *Scythe* knockout mice display brain heteropia (abnormal migration), hydrocephalus, and dilated and hypoplastic kidneys as well as lung abnormalities, leading to perinatal death.[39] In addition to endocrine hypoplasia, syndactyly, cleft lip, and cleft palate, ECO-affected infants also develop phenotypes observed with Scythe deficiency. Because ICK is involved in phosphorylation and activation of Scythe, it is presumable that an ICK mutation would include defects observed with Scythe inactivation. Overall, ICK seems to be involved in cell-cycle regulation and apoptosis during mammalian development. Other evidence that ICK has an effect on embryological development is the observed presence of *ICK* mRNA in placental tissue,[35] although specific experiments will need to be performed for confirmation. Previous work[36] has suggested that ICK has a role in development of the myocardium, but because ECO syndrome is a multiorgan disorder, this kinase must have a role in development of other tissues and organ systems. Interestingly, no cardiac anomalies have been observed in ECO syndrome patients.

Thus, we have characterized a previously unreported human syndrome—ECO—in which mutated ICK is

functionally impaired. This single point mutation at residue 272 not only affects structure, but also function, such that it can no longer localize properly, which has detrimental downstream effects and ultimately leads to abnormal fetal development. These findings suggest that ICK plays a key role in the development of multiple organ systems.

Supplemental Data

Supplemental Data include one table and one figure and can be found with this article online at http://www.ajhg.org/.

Acknowledgments

We acknowledge the generous cooperation of the families participating in this study. We acknowledge the excellent technical assistance of Rebecca Provost, Rachel Rollings, Caroline O'Neil, and Roger Dewar. R.A.H. is a Career Investigator of the Heart and Stroke Foundation of Ontario and holds the Edith Schulich Vinet Canada Research Chair (Tier I) in Human Genetics and the Jacob J. Wolfe Distinguished Medical Research Chair. P.L. was supported by the Canadian Institutes of Health Research (CIHR) Scriver Family MD/PhD studentship award and the HSFO Vascular Training Program. This work was supported by a team grant from the CIHR (CTP-79853, MOP-37854), the Heart and Stroke Foundation of Ontario, Genome Canada through the Ontario Genomics Institute, and grants from the Children's Health Research Institute, the Lawson Health Research Institute, and the Garrod Association.

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Web Resources

The URLs for data presented herein are as follows:

ClustalW alignments, http://www.clustal.org
Eris Server, http://troll.med.unc.edu/eris/login.php
London Regional Genomics Centre, http://www.lrgc.ca
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim
PMUT, http://mmb2.pcb.ub.es:8080/PMut
PolyPhen, http://coot.embl.de/PolyPhen/
PyMol, http://pymol.sourceforge.net
Rosetta Design, http://rosettadesign.med.unc.edu
SIFT, http://blocks.fhcrc.org/sift/SIFT.html
SNPs3D, http://www.SNPs3D.org

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Modification Form for Permit BIO-RRI-0006

Permit Holder: Robert Hegele

Approved Personnel
(Please stroke out any personnel to be removed)

Additional Personnel
(Please list additional personnel here)

J Wang, H Cao, R Martins, R Hassell
M Ban, A McIntyre, B Kennedy, J Robinson,
P lahiry, C Johansen, M Lanktree

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. *
Approved Microorganisms	ME DH10B, ME DH5-alpha, ME DH5A T1	
Approved Cells	[Rodent] (established): 3T3-L1, NIH/3T3. [Non-human Primate] (established): COS-7 [Human] (established: HEK293, HEP62 [Human Primary]: Fibroblast- (GM05659, GM08398, , GM03348 GM03513, Fibroblast,	Fibroblast, finite primary cell line human AG16409 Coriell Cell Repository Fibroblast, finite primary cell line human HGADFN167 Progeria Research Foundation
Approved Use of Human Source Material	Blood, serum/plasma	
Approved GMO	SV 40 Large T antigen (expressed in COS7 cells), pCDNA3 plasmid, pcDNA 3.1 Echo Expression Vector Kit. [ADD]: pCMV6, pcDNA3.1, pENTR 11	
Approved use of Animals		
Approved Toxin(s)		

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED.*

As the principal investigator, I have ensured that all of the personnel named on the form have been trained. I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca.

Signature of Permit Holder:

Classification: 2

Date of Last Biohazardous Agents Registry Form: May 15, 2009

Date of Last Modification (if applicable): October 22, 2009

BioSafety Officer(s): Nov 20/09

Chair, Biohazards Subcommittee:

2009 Biohazard Agents Registry, Dr. Robert Hegele

22-Oct-09

Name	CAT. NO.	SUPPLIER	MSDS	Comment	Use
3T3-L1	CL-173	ATCC	Y	Murine fibroblast	Mutant LMNA is transfected into these cells and immunofluorescence studies and Western analysis performed
NIH/3T3	CRL-1658	ATCC	Y	Murine	
COS-7: note SV40 promoter	CRL-1651	ATCC	Y	Green monkey (Ceropithecus aethiops)	
HEK293: note Adeno E1A promoter	CRL-1573	ATCC	Y	Human	
HepG2	CRL-11997	ATCC	Y	Human	
Fibroblast, finite primary cell line human	GM05659	Coriell Cell Repository	Y	Human: unaffected	Cells are cultured, morphology and growth curves evaluated and immunofluorescence studies, Western analysis, DNA and RNA isolated and microarray and high throughput sequencing analysis performed.
Fibroblast, finite primary cell line human	GM08398	Coriell Cell Repository	Y	Human: unaffected	
Fibroblast, finite primary cell line human	GM03348	Coriell Cell Repository	Y	Human: unaffected	
Fibroblast, finite primary cell line human	AG03513	Coriell Cell Repository	Y	Human: HGPS proband	
Fibroblast, finite primary cell line human	AG04456	Coriell Cell Repository	Y	Human: unaffected	
Fibroblast, finite primary cell line human	AG16409	Coriell Cell Repository	Y	Human: unaffected	
Fibroblast, finite primary cell line human	30950	Dr. T.C. Rupar	na	Human	Cells are cultured, DNA and RNA extracted and microarray and high throughtput sequencing analysis performed
Fibroblast, finite primary cell line human	40916	Dr. T.C. Rupar	na	Human	
Fibroblast, finite primary cell line human	20750	Dr. T.C. Rupar	na	Human	
Fibroblast, finite primary cell line human	70280	Dr. T.C. Rupar	na	Human	
pCMV6	PS100001	Origene	y		Transfection vectors used in the lab
pcDNA3.1	350492	Invitrogen	Y		
pENTR 11 dual selection vector	A10562	Invitrogen	Y		
Subcloning Efficiency™ DH5a™ Competent Cells	18265-017	Invitrogen	Y	E. Coli	Cells used to as vehicles for transfection of genetic material for overexpression studies and phenotypic characterization
Electromax DH10B competent cells	18290-015	Invitrogen		E. Coli	
ME DH10B competent cells	18297-010	Invitrogen		E. Coli	
ME DH5-alpha competent cells	18258-012	Invitrogen	Y	E. Coli	
ME DH5A T1 page resist comp cells	12034-013	Invitrogen		E. Coli	
Fibroblast, finite primary cell line human	HGADFN167	Progeria Research Foundation	Y	Human: HGPS proband	same use as Coriell Cell Repository

Catalog ID: **AG16409**

Product (Source): CELL CULTURE

ADD TO CART

- Overview
- Characterizations
- Phenotypic Data
- Publications
- External Links
- Images
- Products

Overview

Collection NIA Aging Cell Culture Repository
Subcollection Apparently Healthy Collection
Sample Description APPARENTLY HEALTHY NON-FETAL TISSUE
Biopsy Source Unspecified
Cell Type Fibroblast
Tissue Type Skin
Transformant Untransformed
Species Homo sapiens
Common Name Human
Age 12 YR
Sex Male
Race Caucasian
Family 1936
Family Member 1
Relation to Proband proband
Clinically Affected No
Confirmation Clinical summary/Case history
ISCN 46,XY
Remarks The donor was clinically normal having suffered a cervical spine injury at age 5. He was ventilator-dependent. He died of brain death with cardiorespiratory arrest at age 12. The culture was initiated on 7/12/2000 using explants of minced skin tissue taken post-mortem. The cell morphology is fibroblast-like. The karyotype is 46,XY with 4% of the cells examined showing random chromosome loss and 2% showing random chromosomal aberrations.

Catalog ID AG16409
Product Cell Culture
Pricing Commercial Pricing: $155.00
Academic and not-for-profit pricing: $85.00
NIA Grantees: $0.00
How to Order Online Ordering
Assurance Form (Must have current form on file)
Statement of Research Intent Form (Information can be entered electronically when order is placed. Do not fax form to Customer Service)

Characterizations

Sample Description APPARENTLY HEALTHY NON-FETAL TISSUE
PDL at Freeze 4
Passage Frozen 2

IDENTIFICATION OF SPECIES OF ORIGIN Species of Origin Confirmed by Nucleoside Phosphorylase,Glucose-6-Phosphate Dehydrogenase, and Lactate Dehydrogenase Isoenzyme Electrophoresis

Phenotypic Data

Remark The donor was clinically normal having suffered a cervical spine injury at age 5. He was ventilator-dependent. He died of brain death with cardiorespiratory arrest at age 12. The culture was initiated on 7/12/2000 using explants of minced skin tissue taken post-mortem. The cell morphology is fibroblast-like. The karyotype is 46,XY with 4% of the cells examined showing random chromosome loss and 2% showing random chromosomal aberrations.

Publications

Data are not available

External Links

dbSNP dbSNP ID: 1113

Images

Data are not available

Protocols

PDL at Freeze	4
Passage Frozen	2
Split Ratio	1:4
Temperature	37 C
Percent CO2	5%
Medium	Eagle's Minimum Essential Medium with Earle's salts and non-essential amino acids
Serum	10% fetal bovine serum (not inactivated
Substrate	None specified
Subcultivation Method	trypsin-EDTA

Public Health Agency of Canada / Agence de la santé publique du Canada

Name and/or Organization: **University of Western Ontario**
Robarts Research Institute
Attn: Dr. Robert Hegele

Address: P.O. Box 5015
100 Perth Drive, Rm 4-25
London, ON
N6A 5K8

The following biological material does not require a Public Health Agency of Canada import permit under the HPIR*:

Human fibroblast cell line from healthy donor (AG16409), as provided by Coriell Institute for Medical Research, 403 Haddon Avenue, Camden, NJ, USA 08103.

Marianne Heisz
Chief, Importation and Regulatory Affairs

JULY 16, 2009
Date

NOTICE

***HPIR (HUMAN PATHOGENS IMPORTATION REGULATIONS)**

- We are in receipt of your application for an importation permit for biological materials. The **HPIR** apply **only** to the importation of infectious substances which cause human disease and their subsequent distribution or transfer. Other materials, which are deemed by the importer to be non-infectious for humans, **do not** require a permit under these regulations. It should be noted that the importation of biological materials may also be subject to other federal, provincial and municipal laws.
- For animal or plant pathogens one **must** apply to The Canadian Food Inspection Agency (CFIA) for a permit to import. If this material is of animal or plant origin it may also require a permit from the CFIA. Please contact the CFIA for their consideration. CFIA contact numbers are as follows:
  (613) 221-7068 for information concerning animal pathogens/material
  (613) 225-2342 [ext. 4334] for information concerning plant pathogens/material
- Importation of this material may also be subject to the requirements of the *New Substances Notification Regulations (Organisms)* of the *Canadian Environmental Protection Act, 1999*, administered by Environment Canada and Health Canada. Please contact the New Substances Information Line at 1-800-567-1999 or nsn-infoline@ec.gc.ca.
- You may be required to provide the Canada Border Services Agency (CBSA) customs officers with a declaration that the imported material is non-infectious and non-hazardous.

Should you require further information, please contact:
Office of Laboratory Security
Centre for Emergency Preparedness and Response
(613) 957-1779

Canada

The Progeria Research Foundation
Cell and Tissue Bank

Dear Dr. Robinson,

October 19th, 2009

Blackburn Cardiovascular Genetics Laboratory
Robarts Research Institute
Room 4-28, PO box 5015, 100 Perth Drive
London, On, Canada, N6A 5K8

Please find enclosed one flask each of the following cell lines which you requested from the PRF Cell and Tissue Bank for your research.

Cell Line #	Passage #	Clinically Affected?	Relation to Proband	Age at Donation	Exon 11 mutation Yes or no C→T
HGADFN167	4	yes	proband	8 yrs. 5mos	yes

Please place cells at 37°C and 5% CO_2 for 24 hours upon receipt and then change the culture medium and split (if necessary) as directed. Please see the attached sheet containing specific culture conditions. If you have any further questions do not hesitate to contact me at leslie_gordon@brown.edu or Lorraine Fast (Laboratory Technician) at 401-444-7564 or lfast1@lifespan.org.

Sincerely,

Leslie B. Gordon, MD, PhD
Principal Investigator, The Progeria Research Foundation Cell and Tissue Bank

Email: progeria@netzero.net Website: www.progeriaresearch.org
P. O. Box 3453, Peabody, MA 01961-3453 Tel: (978) 535-2594 Fax: (978) 535-5849

Culture Conditions:
DMEM (Gibco 11960-044), + 2mM L-Glutamine, Pen/Strep and 15% FBS.

Split Conditions:
0.25% Trypsin/ EDTA - 1ml/T25 flask, evenly coating cells. Incubate 2-3 minutes. Gently tip flask to dislodge. Pool cells in culture medium and replate or freeze down.

Freezing Conditions:
10% DMSO in culture media

Email: progeria@netzero.net Website: www.progeriaresearch.org
P. O. Box 3453, Peabody, MA 01961-3453 Tel: (978) 535-2594 Fax: (978) 535-5849

Public Health Agency of Canada / Agence de la santé publique du Canada

Name and/or Organization: **University of Western Ontario**
Robarts Research Institute
Attn: Dr. Robert Hegele

Address: P.O. Box 5015, Rm 4-25
100 Perth Drive
London, ON
N6A 5K8

The following biological material does not require a Public Health Agency of Canada import permit under the HPIR*:

Human fibroblast cell line from donor with Hutchinson-Gilford Progeria Syndrome (HGADFN167), as provided by The Progena Research Foundation Cell Bank, 532 Lowell Street, Peabody, MA, USA, 01960.

Marianne Heisz
Chief, Importation and Regulatory Affairs

JULY 16, 2009
Date

NOTICE

***HPIR (HUMAN PATHOGENS IMPORTATION REGULATIONS)**

- We are in receipt of your application for an importation permit for biological materials. The **HPIR** apply **only** to the importation of infectious substances which cause human disease and their subsequent distribution or transfer. Other materials, which are deemed by the importer to be non-infectious for humans, **do not** require a permit under these regulations. It should be noted that the importation of biological materials may also be subject to other federal, provincial and municipal laws.
- For animal or plant pathogens one **must** apply to The Canadian Food Inspection Agency (CFIA) for a permit to import. If this material is of animal or plant origin it may also require a permit from the CFIA. Please contact the CFIA for their consideration. CFIA contact numbers are as follows:
  (613) 221-7068 for information concerning animal pathogens/material
  (613) 225-2342 [ext. 4334] for information concerning plant pathogens/material
- Importation of this material may also be subject to the requirements of the *New Substances Notification Regulations (Organisms)* of the *Canadian Environmental Protection Act, 1999*, administered by Environment Canada and Health Canada. Please contact the New Substances Information Line at 1-800-567-1999 or nsn-infoline@ec.gc.ca.
- You may be required to provide the Canada Border Services Agency (CBSA) customs officers with a declaration that the imported material is non-infectious and non-hazardous.

Should you require further information, please contact:
Office of Laboratory Security
Centre for Emergency Preparedness and Response
(613) 957-1779

Canada

The Progeria Research Foundation, Inc

DECLARATION STATEMENT

The contents of this package are as follows:

Cultured Human dermal fibroblasts specimen in medium containing 15% fetal bovine serum (certified free of infectious agents) in a sealed collection tube.

These samples are considered to be non-infectious and are for research purposes only.

These samples are being shipped from Dr. Leslie Gordon and Dr. Douglas Hixson for studies funded by The Progeria Research Foundation. Samples are packed in approved blood mailers and are perishable. Samples ***are not*** known to be infectious. **Please do not delay**.

If you have any questions or concerns, please contact Dr. Leslie Gordon, Principal Investigator, at the following phone number: (508) 889-6655
Sincerely,

Leslie B. Gordon, MD, PhD

P. O. Box 3453, Peabody, MA 01961-3453
Tel: (978) 535-2594, Fax: (978) 535-5849,
Email: info@progeriaresearch.org

www.progeriaresearch.org

THE UNIVERSITY OF WESTERN ONTARIO
BIOHAZARDOUS AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: March 27, 2009
Biosafety Website: www.uwo.ca/humanresources/biosafety/

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biohazardous agents is described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans or involving plants, fungi, or insects that require Public Health Agency of Canada (PHAC) or Canadian Food Inspection Agency (CFIA) permits.

This form must ~~also~~ be updated at least every 3 years or when there are changes to the biohazards being used.

Containment Levels will be established in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Public Health Agency of Canada (PHAC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Completed forms are to be returned to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190) for distribution to the Biohazard Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135 or biosafety@uwo.ca. If there are changes to the information on this form (excluding grant title and funding agencies), contact Occupational Health and Safety for a modification form. See website: www.uwo.ca/humanresources/biosafety

PRINCIPAL INVESTIGATOR	Dr. Robert Hegele
SIGNATURE	
DEPARTMENT	Robarts Research Institute, Vascular Biology Research Group
ADDRESS	100 Perth Dr. Room 4-06
PHONE NUMBER	519-931-5271 Lab 519-931-5777 ext. 34412
EMERGENCY PHONE NUMBER(S)	519-931-5271
EMAIL	hegele@robarts.ca

Location of experimental work to be carried out: Building(s) RRI Room(s) 4288, 4292, 4286

*For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to the University of Western Ontario Biosafety Officer (See Section 12.0, Approvals).

FUNDING AGENCY/AGENCIES: HSFO, CIHR
GRANT TITLE(S):

PLEASE ATTACH A BRIEF DESCRIPTION OF YOUR WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED. PROJECTS SUBMITTED WITHOUT A SUMMARY WILL NOT BE REVIEWED.

Names of all personnel working under Principal Investigators supervision in this location:

Jian Wang
Henian Cao
Brooke Kennedy
John Robinson
Alena Hassell
Rebecca Provost
Matthew Ban
Chris Johansen
Piya Lahiry
Matthew Lanktree

* DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED*

1.0 Microorganisms

1.1 Does your work involve the use of microorganisms or biological agents of plant or animal origin (including but not limited to viruses, prions, parasites, bacteria)? ☒YES ○ NO
If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? ○ YES ☒NO
If YES, please give the name of the species. _____
What is the origin of the microorganism(s)? _____
Please describe the risk (if any) of escape and how this will be mitigated:

_____
_____
_____

Please attach the CFIA permit.
Please describe any CFIA permit conditions:

_____
_____
_____

1.2 Please complete the table below:

Name of Biological agent(s)*	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
DH 10B	○ Yes ☒No	○ Yes ☒No	○ Yes ☒No	0.007-0.010	Invitrogen	☒1 ○2 ○3
DH 5α	○ Yes ☒No	○ Yes ☒No	○ Yes ☒No	0.007-0.010	"	☒1 ○2 ○3
	○ Yes ○ No	○ Yes ○ No	○ Yes ○ No			○1 ○2 ○3
	○ Yes ○ No	○ Yes ○ No	○ Yes ○ No			○1 ○2 ○3

*Please attach a Material Safety Data Sheet or equivalent from the supplier.

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ☒YES ○ NO
If no, please proceed to Section 3.0

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture in the table below

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	☒Yes ○ No	See Attached	Not applicable
Rodent	☒Yes ○ No	" "	"
Non-human primate	○ Yes ☒No		
Other (specify)	○ Yes ☒No		

* DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED*

2.3 Please indicate the type of established cells that will be grown in culture in the table below.

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Supplier / Source
Human	☒ Yes ○ No	See Attached	See Attached
Rodent	☒ Yes ○ No	" "	ATCC
Non-human primate	☒ Yes ○ No	COS-7	ATCC
Other (specify)	○ Yes ○ No		

*Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information. see www.atcc.org)

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required ○ 1 ☒ 2 ○ 3

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? ☒ YES ○ NO
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	**Source/Supplier /Company Name**	**Is Human Source Material Known to Be Infected With An Infectious Agent? YES/NO**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid	Research Subjects	○ Yes ☒ No		○ 1 ☒ 2 ○ 3
Human Blood (fraction) or other Body Fluid	Research Subjects	○ Yes ☒ No		○ 1 ☒ 2 ○ 3
Human Organs or Tissues (unpreserved)		○ Yes ○ No		○ 1 ○ 2 ○ 3
Human Organs or Tissues (preserved)		○ Yes ○ No		○ 1 ○ 2 ○ 3

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? ☒ YES ○ NO If no, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? ☒ YES, complete table below ○ NO

Bacteria Used for Cloning *	Plasmid(s) *	Source of Plasmid	Gene Transfected	Describe the change that results
DH5α	pcDNA 3.1 CAT	Bacteria (Invitrogen)	APOE	Morphological

* Please attach a Material Data Sheet or equivalent if available.

4.3 Will genetic modification(s) involving viral vectors be done? O YES, complete table below ☒NO

Virus Used for Transduction *	Vector(s) *	Source of Vector	Gene Transfected	Describe the change that results

* Please attach a Material Safety Data Sheet or equivalent.

4.4 Will genetic sequences from the following be involved?

- HIV O YES, please specify _____ ☒NO
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens O YES, specify _____ ☒NO
- SV 40 Large T antigen ☒YES O NO
- E1A oncogene ☒YES will be O NO
- Known oncogenes O YES, please specify _____ O NO
- Other human or animal pathogen and or their toxins O YES, please specify _____ ☒NO

4.5 Will virus be replication defective? ☒YES O NO

4.6 Will virus be infectious to humans or animals? O YES ☒NO

4.7 Will this be expected to increase the containment level required? O YES ☒NO

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials be conducted using the viral vector in 4.0? O YES ☒NO
If no, please proceed to Section 6.0 If YES attach a full description of the make-up of the virus.

5.2 Will virus be able to replicate in the host? O YES O NO

5.3 How will the virus be administered? _____

5.4 Please give the Health Care Facility where the clinical trial will be conducted:_____

5.5 Has human ethics approval been obtained? O YES, number: _____ O NO O PENDING

6.0 Animal Experiments

6.1 Will live animals be used? O YES ☒NO If no, please proceed to section 7.0

6.2 Name of animal species to be used_____

6.3 AUS protocol # _____

6.4 Will any of the agents listed be used in live animals O YES, specify: _____ O NO

7.0 Use of Animal species with Zoonotic Hazards

7.1 Will any of the following animals or their organs, tissues, lavages or other body fluids including blood be used?

- Pound source dogs O YES ☒ NO
- Pound source cats O YES ☒ NO
- Cattle, sheep or goats O YES ☒ NO
- Non-human primates O YES, please specify species _____ ☒ NO
- Wild caught animals O YES, please specify species & colony # _____ ☒ NO
- Birds O YES ☒ NO
- Others (wild or domestic) O YES, please specify _____ ☒ NO

8.0 Biological Toxins

8.1 Will toxins of biological origin be used? O YES ☒ NO If no, please proceed to Section 9.0

8.2 If YES, please name the toxin(s)_____
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

8.3 What is the LD_{50} (specify species) of the toxin_____

8.4 How much of the toxin is handled at one time*? _____

8.5 How much of the toxin is stored*? _____

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

9.0 Insects Requiring CFIA Permits

9.1 Do you use insects that require a permit from the CFIA? O YES ☒ NO
If no, please proceed to Section 10.0

9.2 If YES, please give the name of the species. _____

9.3 What is the origin of the insect? _____

9.4 What is the lifestage of the insect? _____

9.5 What is your intention? O Initiate and maintain colony, give location: _____
O "One-off" use, give location: _____

9.6 Please describe the risk (if any) of escape and how this will be mitigated:

_____
_____
_____

9.7 Please attach the CFIA permit.

9.8 Please describe any CFIA permit conditions

_____
_____
_____

* DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED*

10.0 Plants Requiring CFIA Permits

10.1 Do you use plants that require a permit from the CFIA? O YES ☒ NO
If no, please proceed to Section 11.0

10.2 If YES, please give the name of the species. _____

10.3 What is the origin of the plant? _____

10.4 What is the form of the plant (seed, seedling, plant, tree...)? _____

10.5 What is your intention? O Grow and maintain a crop O "One-time" use

10.6 Do you do any modifications to the plant? O YES O NO
If yes, please describe: _____
_____

10.7 Please describe the risk (if any) of loss of the material from the lab and how this will be mitigated:
_____
_____
_____

10.8 Is the CFIA permit attached? O YES O NO

10.9 Please describe any CFIA permit conditions:
_____
_____
_____

11.0 Import Requirements

11.1 Will any of the above agents be imported? ☒ YES, please give country of origin USA
If no, please proceed to Section 10.0 O NO

Letter attached Stating NOT Required

11.2 Has an Import Permit been obtained from HC for human pathogens? ☒ YES O NO

11.3 Has an import permit been obtained from CFIA for animal or plant pathogens? O YES ☒ NO

11.4 Has the import permit been sent to OHS? O YES, please provide permit # _____ O NO

12.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biohazardous agents in Sections 1.0 to 9.0 have been trained.

SIGNATURE _____ May 1/09

* DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED*

13.0 Containment Levels

13.1 For the work described in sections 1.0 to 9.0, please indicate the highest PHAC or CFIA Containment Level required. O 1 ☒ 2 O 3

13.2 Has the facility been certified by OHS for this level of containment?
☒ YES, permit # if on-campus B10-RR1-0006
O NO, please certify
O NOT REQUIRED for Level 1 containment

14.0 Procedures to be Followed

14.1 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca/

SIGNATURE [signature] Date: Apr. 24, 2009

15.0 Approvals

UWO Biohazard Subcommittee: SIGNATURE: [signature]
Date: 15 May 2009

Safety Officer for Institution where experiments will take place: SIGNATURE: [signature]
Date: May 07, 2009

Safety Officer for University of Western Ontario (if different from above): SIGNATURE: [signature]
Date: May 15/09

Approval Number: B10-RR1-0006 Expiry Date (3 years from Approval): May 14, 2012

Special Conditions of Approval:

* DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED*

2009 Biohazard Agents Registry, Dr. Robert Hegele

Name	CAT. NO.	SUPPLIER	MSDS	Comment	Use
3T3-L1	CL-173	ATCC	Y	Murine fibroblast	Mutant LMNA is transfected into
NIH/3T3	CRL-1658	ATCC	Y	Murine	cells and immunofluorescence st
COS-7: note SV40 promoter	CRL-1651	ATCC	Y	Green monkey (Ceropithecus aethiops)	and Western analysis performed
HEK293: note Adeno E1A promoter	CRL-1573	ATCC	Y	Human	
HepG2	CRL-11997	ATCC	Y	Human	
Fibroblast, finite primary cell line human	GM05659	Coriell Cell Repository	Y	Human: unaffected	Cells are cultured, morphology ar growth curves evaluated and immunofluorescence studies, We analysis, DNA and RNA isolated a microarray and high throughput sequencing analysis performed.
Fibroblast, finite primary cell line human	GM08398	Coriell Cell Repository	Y	Human: unaffected	
Fibroblast, finite primary cell line human	GM03348	Coriell Cell Repository	Y	Human: unaffected	
Fibroblast, finite primary cell line human	AG03513	Coriell Cell Repository	Y	Human: HGPS proband	
Fibroblast, finite primary cell line human	AG04456	Coriell Cell Repository	Y	Human: unaffected	
Fibroblast, finite primary cell line human	AG06234	Coriell Cell Repository	Y	Human: HGPS proband	
Fibroblast, finite primary cell line human	AG06297	Coriell Cell Repository	Y	Human: unaffected	
Fibroblast, finite primary cell line human	30950	Dr. T.C. Rupar	na	Human	Cells are cultured, DNA and RNA
Fibroblast, finite primary cell line human	40916	Dr. T.C. Rupar	na	Human	extracted and microarray and hig
Fibroblast, finite primary cell line human	20750	Dr. T.C. Rupar	na	Human	throughtput sequencing analysis
Fibroblast, finite primary cell line human	70280	Dr. T.C. Rupar	na	Human	performed
pCMV6	PS100001	Origene	y		Transfection vectors used in the l
pcDNA3.1	350492	Invitrogen	Y		
pENTR 11 dual selection vector	A10562	Invitrogen	Y		
Subcloning Efficiency™ DH5a™ Competent Cells	18265-017	Invitrogen	Y	E. Coli	Cells used to as vehicles for trans
Electromax DH10B competent cells	18290-015	Invitrogen		E. Coli	of genetic material for overexpre
ME DH10B competent cells	18297-010	Invitrogen		E. Coli	studies and phenotypic characte
ME DH5-alpha competent cells	18258-012	Invitrogen	Y	E. Coli	
ME DH5A T1 page resist comp cells	12034-013	Invitrogen		E. Coli	

In 2003, we proposed in HSFO #NA5320 to expand understanding of single-gene predisposition to cardiovascular disease (CVD) and since found causative genes and mutations for 3 forms of partial lipodystrophy, namely familial partial lipodystrophies types 2 and 3 (FPLD2, FPLD3) and acquired partial lipodystrophy (APL). Using "phenomics", defined as integrated multidisciplinary research to understand the complex consequences of genomic variation through systematic discovery and cataloguing of standardized phenotypes, we developed standardized, optimized quantitative non-invasive imaging procedures for carotid arteries and adipose stores, all key to this renewal application. The work was recognized by merit awards from several groups, including the American Heart Association in 2004 and the Genetics Society of Canada in 2006. ***We will build on the scientific momentum from the initial funding period with this renewal application.***

◦ **BACKGROUND AND SIGNIFICANCE:** The constellation of disturbed carbohydrate and insulin metabolism, with central obesity, dyslipidemia (elevated triglycerides [TG] with depressed HDL cholesterol), hypertension, and type 2 DM (T2DM) is called the 'metabolic syndrome' (MetS). Evaluation of patients with extreme monogenic forms of MetS will help us to understand common MetS, just as the study of patients with monogenic dyslipidemias improved understanding and treatment of those diseases. Some monogenic forms of MetS have been molecularly characterized – including those discovered in the Hegele lab - providing important insights and model systems for common MetS.

◦ **PROPOSED RESEARCH:** We propose to continue to expand our database of lipodystrophy kindreds, to significantly increase our characterization of biochemical, vascular and adipose phenotypes in monogenic MetS and then to assess these new markers in the general population. Specific aims are:

1) To extend our measurements of traditional and non-traditional serum biomarker phenotypes of subjects with FPLD2, FPLD3, APL, other lipodystrophies and familial hypercholesterolemia (FH, positive control for early CVD) and to contrast these according to molecular basis of the disease.
2) To measure baseline non-invasive ultrasound (US) vascular and magnetic resonance imaging (MRI) adipose phenotypes in subjects with FPLD2, FPLD3, APL, other forms of lipodystrophy and FH, and to contrast these according to molecular basis of the disease.
3) To serially measure non-invasive US vascular and MRI adipose phenotypes in subjects with FPLD2, FPLD3, APL, other forms of lipodystrophy and FH in order to evaluate disease progression, and to contrast these according to molecular basis of the disease.
4) To determine association between risk factors, intermediate phenotypes and atherosclerosis read-outs variables in subjects with FPLD2, FPLD3, APL, other forms of lipodystrophy and FH.

We expect to observe between-genotype differences in phenotypes such as: 1) lipoprotein, metabolic, cytokines, chemokines and other serum biomarkers; 2) carotid IMT at baseline and serial progression; 3) association of traditional and non-traditional risk factors with IMT; and 4) distribution of adipose stores in different forms of lipodystrophy. The presence of novel serum and imaging biomarkers identified in the studies of patients with monogenic MetS will subsequently be evaluated in individuals from the general population with MetS. We expect that some biomarkers that we will identify as being part of the monogenic MetS metabolic signature profile will be translated to the "garden variety" form of MetS.

◦ **RELEVANCE:** Our past record of finding determinants of CVD risk in Canadian families with monogenic MetS will continue with renewed funding of this project. Our success in finding MetS genetic determinants in pilot studies leads us to expect that this expanded set of samples will provide new insights. MetS is an important new prevalent CVD risk factor, with numerous metabolic abnormalities and a very strong relationship with vascular disease. Better characterization of early phenotypes in monogenic MetS – sensitive "phenomics" – may provide new clues to help solve the puzzle of the common metabolic syndrome. Any between-genotype differences in phenotype severity will help to plan future experiments assessing specific diagnosis and/or treatment.

Since 1989, the Hegele lab has identified: 1) the genomic basis of 10 human diseases; and 2) >10 human mutations disease-causing mutations in dyslipidemia and other metabolic disorders. Among other recognitions, this work received the 2004 CM Hogg Basic Science and Clinical Research Award from the American Heart Association and the 2006 WF Grant & PB Mocnik Award of Excellence from the Genetics Society of Canada. This new application proposes to take this discovery record in a new direction, with the ***objective*** to ***define genomic factors underlying elevated plasma triglyceride*** (TG).

- **BACKGROUND AND SIGNIFICANCE:** Elevated plasma TG contributes to increased risk of cardiovascular disease (CVD). Despite ~20 years of research, there has been little progress in defining the molecular basis of susceptibility to two genetic disorders with very high TG, namely hyperlipoproteinemia (HLP) types 5 and 3. Furthermore, the role of apo C-I in human TG metabolism remains poorly defined. The time is ripe for re-addressing these issues due to the convergence of several factors, including availability of sufficient numbers of affected subjects, specification of numerous candidate genes, access to robust genomic technologies and the fortuitous discovery of a natural human *APOC1* mutation in a well-characterized population.
- **PROPOSED RESEARCH:** We will advance the understanding of the genetic basis of human dyslipoproteinemia by identifying and classifying new human genomic mutations in candidate genes affecting lipoprotein metabolism. To this end, we will study patients with primary dyslipoproteinemias characterized by elevated plasma TG and we will take advantage of a newly discovered human mutation in *APOC1* as a "probe" to learn more about the normal and pathophysiological function of this protein.

Hypotheses:

1) Multiple rare mutations in multiple candidate genes in lipoprotein metabolism are present in a substantial proportion of subjects with HLP type 5.
2) Multiple rare mutations in multiple candidate genes in lipoprotein metabolism are present in a substantial proportion of subjects with HLP type 3 homozygotes for the *APOE* E3/E2 genotype.
3) The private *APOC1* T45S polymorphism of the Oji-Cree will be associated with metabolic phenotypes, analogous to phenotypes seen in induced-mutant mouse models.

- **Specific aim 1: Advancing the understanding of HLP type 5 through:** a) extending the mutational spectrum in causative genes through large-scale genomic DNA sequence analysis; b) identifying mutations in candidate HLP type 5 genes; c) using array-based detection methods to identify common regions of the genome with large-scale copy number variations (CNVs) that are shared between HLP type 5 subjects; and d) defining *in vitro* mechanism(s) of disease of selected HLP type 5 mutations.
- **Specific aim 2: Advancing the understanding of HLP type 3 through:** a) extending the mutational spectrum in causative genes through large-scale genomic DNA sequence analysis in HLP type 3 subjects who have the *APOE* E2/2 genotype; b) identifying mutations in candidate HLP type 3 genes; c) using array-based detection methods to identify common regions of the genome with large-scale copy number variations (CNVs) that are shared between HLP type 3 subjects; and d) defining *in vitro* mechanism(s) of disease of selected HLP type 3 mutations.
- **Specific aim 3: Advancing the understanding of the physiological and pathophysiological role of** apo C-I by taking advantage of the private *APOC1* T45S polymorphism of the Oji-Cree and intensively studying its association with metabolic phenotypes.
- **RELEVANCE.** Serum TG is an important emerging CVD risk factor, with numerous determinants. Better characterization of the genetic determinants of elevated TG starting with simpler familial forms such as HLP types 5 and 3 will provide new clues to help solve the puzzle of the common hypertriglyceridemia. The results will provide genomic data that will identify potentially important new metabolic pathways for elevated TG and generate hypotheses for future cell biology, biochemical and clinical studies. The *APOC1* studies in the Oji-Cree will help to clarify the normal and pathophysiological role of this protein in humans. Our past record of finding determinants of CVD risk in Canadian patients at risk for CHD will continue with this project.

2. A complete but succinct summary of the proposed research on this page.
Présentez sur cette page un résumé complet du projet de recherche.

Background: The importance of well-defined CVD phenotypes was highlighted by recent coining of the term 'phenomics', defined as integrated multidisciplinary research to understand the complex consequences of genomic variation. Sensitive new phenomic tools may reveal new phenotypic markers, sometimes called 'early' or 'intermediate' phenotypes. Because well-established 'traditional' risk factors do not account for all CVD events, 'non-traditional' risk factors have been proposed, including insulin resistance (IR). Evaluation of monogenic IR syndromes might help understand common IR, just as the study of monogenic forms of dyslipidemia, such as familial hypercholesterolemia (FH), improved understanding of these diseases. Some monogenic forms of IR have been molecularly characterized, providing interesting and sometimes important insights. One of these is Dunnigan-type familial partial lipodystrophy (FPLD), which results from mutation in one of two genes: *LMNA* or *PPARG*. FPLD is associated with early atherosclerosis and exists in Canadian families.

PROPOSED RESEARCH

Study objectives

1) To measure traditional and non-traditional biochemical phenotypes of subjects with FPLD-*LMNA*, FPLD-*PPARG*, and FH (positive control monogenic disease with atherosclerosis), and to contrast them according to molecular basis of the disease.
2) To measure baseline non-invasive ultrasound (US) and magnetic resonance imaging (MRI) carotid phenotypes of FPLD-*LMNA*, FPLD-*PPARG* and FH subjects, and to contrast these according to molecular basis of the disease.
3) Over three years, to serially measure non-invasive US and MRI vascular structure phenotypes of FPLD-*LMNA*, FPLD-*PPARG*, and FH subjects in order to evaluate disease progression, and to contrast these according to molecular basis of the disease.
4) To determine associations between risk factors, intermediate phenotypes, and atherosclerosis read-outs variables in FPLD-*LMNA*, FPLD-*PPARG*, and FH subjects.

Experimental strategy to test for genotype-phenotype associations:

Subjects: Patients with FPLD-*LMNA* (N=60), FPLD-*PPARG* (N=20), FH (N=30) and matched controls (N=60)
Genotype: DNA sequence determination of *LMNA*, *PPARG* or *LDLR* as appropriate
Phenotypes: 1) Lipoprotein profile: cholesterol (total, HDL & LDL), TG, apo B & A1, Lp(a). 2) metabolic variables: insulin, glucose, glycosylated hemoglobin (HbA1C), CRP, C-peptide, free fatty acids, adiponectin, leptin, resistin, ASP; 3) Coagulation and inflammatory variables: FBG, PAI-1, TNF-α, IL-6; 4) Carotid US: IMT, TPA, TPV; 5) Carotid MRI: carotid wall volume, plaque composition; 6) Total body MRI: fat redistribution in selected individuals (3 per group)
Hypotheses: We will observe between-genotype differences in: 1) lipoprotein, metabolic, coagulation and inflammatory markers corrected for age, sex and body mass; 2) carotid US and carotid MRI variables; 3) carotid US and MRI plaque progression; 4) association of traditional and non-traditional risk factors with non-invasive vascular read-outs; 5) distribution of adipose stores in FPLD-*LMNA* versus FPLD-*PPARG*.

Management plan: Years 1 and 2: Baseline DNA, biochemical and imaging analysis in 130 subjects; Years 2 and 3: 1-year and 2-year follow-up US and MRI for 130 subjects.

Relevance: Monogenic illnesses have provided key insights into CVD risk factors such as hypertension and dyslipidemia. IR is an important newer risk factor, with a very strong relationship with vascular disease. Better characterization of subjects with monogenic IR using sensitive methods – 'phenomics' – may provide clues to help solve the puzzle of the common metabolic syndrome.

Executive Summary

The vision of this program is to establish an integrated facility providing Ontario scientists with access to all aspects of disease gene discovery, including collection of family material, full genome scanning, fine genetic and physical mapping, identification of disease genes, mutational spectrum analysis and diagnostic testing applications. By having team members working towards the common goal of disease gene identification and by integrating the team into the genomics community in Ontario, The Facility for Advanced Genetic Analysis will be unique in the world.

The collaborating institutions include the University of Ottawa (Ottawa Hospital Research Institute), The Hospital for Sick Children, The Toronto Hospital and The John P. Robarts Research Institute.

A variety of commercial applications have been identified for the output of this project, including products in the area of pharmacogenomics, gene therapy and diagnostic screening tests.

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. DO NOT EXCEED THE SPACE PROVIDED.

The overall goal of this proposal is to image inflammation in atherosclerosis by developing and testing novel activatable MR imaging agents that can sense myeloperoxidase (MPO) activity in atheromata. Emerging evidence suggests that activated macrophages secrete various enzymes that mediate inflammation in atherosclerosis, and that MPO in particular may be indicative of high-risk ("vulnerable") lesions. A recent clinical trial has further shown that elevated MPO levels strongly predict adverse cardiovascular outcomes in patients with chest pain. It is therefore hypothesized that imaging of local MPO activity will be useful in identifying vulnerable atherosclerotic lesions.

We have recently described two novel approaches for imaging peroxidases using either low molecular weight amplifiable paramagnetic substrates (Mol Imaging 2002;1:16-23) or superparamagnetic enzyme pseudosubstrates (Nanolett 2004;4:119-122). The latter method is based on magnetic relaxation switching (MRSW) a phenomenon that occurs when magnetic nanoparticles are brought into close contact to each other (R2 increase of 4-6 fold with negligible R1 change). These activatable, "smart" agents harness enzyme mediated amplification strategies and can be used to quantitatively measure enzyme activity and inhibition by MR imaging. The current proposal represents an effort to integrate investigators with different expertises to develop, implement and validate the larger field of *enzyme imaging* in atherosclerosis. Together, the team will translate basic results into new methodologies for *in vivo* MR imaging. The ultimate goal of this research is to develop clinically useful imaging tools for the molecular assessment of atherosclerosis in vivo, which are currently limited.

The overall objective of the Hegele lab is to discover the genetic basis of atherosclerosis susceptibility. This is being achieved through two complementary human genetic strategies: 1) studies of monogenic (Mendelian) forms of atherosclerosis in Canadian probands and families; and 2) studies of complex traits related to atherosclerosis in Canadian communities and subpopulations. Recently, the lab's focus has shifted to also include studies on the genetic basis of the metabolic syndrome (MetS), type 2 diabetes (T2DM) and obesity. Programs, objectives and operating grant data are summarized in Table 1.

Table 1: Research programs for the Hegele lab under HSFO CI award #4380

Program name	Specific objective	Funding source(s)
Monogenic human disease models of atherosclerosis	1) Discover gene defects	CIHR MET-13939 (2000-03)
	2) Assess for monogenic MetS	CGDN (2005-2009)
	b) genetics of the metabolic dyslipidemias	
	c) genetics of monogenic premature aging	
	3) Functional studies of LPLD mutations	CIHR MET-43939 (2000-03)
	4) Carotenoidemias and atherosclerosis	CIHR MET-53030 (2000-03)
	5) Human molecular genetics of dyslipidemia	CIHR MET-13017 (2000-05)
	6) Phenomics of atherosclerosis	HSFO NA5320 (2003-06)
	7) Object. CDM and complications	CDA-16200 (2003-06)
	8) Extending mutation spectrum of known human disease genes	CIHR MET-53030 (2003-05)
Complex human traits related to atherosclerosis	Find genetic determinants of atherosclerosis endpoints and intermediate traits in ethnic groups, including Canadian aboriginal people	HSFO CI award #4380 (2006-05) CIHR ERN-43685 (2000-05) HHRI CRT-43825 (2000-05)

A) Monogenic human disease models of atherosclerosis and intermediate phenotypes. He continues to HSFO is that several of these monogenic diseases are associated with atherosclerosis and serve as natural model systems of atherosclerosis. Characterizing the mutant gene in these monogenic diseases reveals new pathogenic pathways, establishes the importance in humans of a molecular pathway, and permits molecular stratification of mutant gene carriers. This will be accomplished by: 1) study of the human molecular genetics of LPLD; 2) *in vitro* structure-function studies of new LPLD mutations; 3) studies of other haemopathies such as Hutchinson-Gilford progeria syndrome and Werner syndrome; 4) study of the human molecular genetics of selected dyslipidemias such as familial hypobetalipoproteinemia (FHBL); 5) study of the phenomics of atherosclerosis by evaluation of extended families with monogenic forms of MetS and dyslipidemia; 6) continuation of studies of Object. to identify those susceptible to vascular complications; 7) extending the mutation spectrum in human disease genes such as apolipoproteinemia Tangier Disease, MODY, familial combined hyperlipidemia, familial combined hypobetalipoproteinemia and congenital cataracts; and 8) [illegible]

B) Complex human traits related to atherosclerosis. 1) Genetic determinants of atherosclerosis endpoints and intermediate traits in Canadian ethnic groups. The inheritance of CHD and its [illegible] is complex. Even for the simple genetic models, we have used the comparative [illegible] association and linkage analysis. We study homogeneous subgroups, such as MetS [illegible] Important [illegible] essential hypertension, familial combined hyperlipidemia, Chinese Canadians, Aboriginal Canadians, South Asian Canadians and [illegible] with premature atherosclerosis. [illegible] We address [illegible] phenotypes, endpoints [illegible] susceptibility, progression and regression of atherosclerosis. [illegible] approach to incorporate the recently developed [illegible] genotyping technologies. [illegible] genotyping arrays, [illegible] Pooled DNA genotyping, [illegible] [illegible] specific candidate genes and [illegible] whole genome scans.

Relevance. [illegible] genetic basis of atherosclerosis [illegible] MetS and CHD. [illegible] genetic determinants of [illegible] and [illegible] studies of MetS and CHD. [illegible] therapeutic agents [illegible] [illegible] medications and therapeutic [illegible] [illegible] [illegible] [illegible] [illegible]

Summary of Research Proposal

The metabolic syndrome (MetS) affects 25% of Canadian adults and is a potent risk factor fo atherosclerosis. Our strategy to understand the common MetS and dyslipidemia is to study rare human monogenic forms, such as familial partial lipodystrophy (FPLD) and familial hypercholesterolemi (FH). We will build on the ongoing record of discovery of causative genes for familial partia lipodystrophy types 2 (FPLD2; MIM 151660) and 3 (FPLD3; MIM 604367) and of human disease mutations causing hyperlipidemia, obesity and diabetes funded by CIHR application #MOP13430.

OBJECTIVE: *To discover and define additional genetic determinants of obesity and plasma lipoproteins by studying rare monogenic human MetS in Canadian families and communities.*

Goals: 1) Advancing the understanding of FPLD by: a) extending the mutation spectrum in FPLD and FPLD3; b) identifying mutations in new FPLD genes; c) defining *in vitro* mechanism(s) of disease of selected mutations; d) extending the FPLD phenotype; defining phenotypic-genotype correlations. **2) Advancing the understanding of FH by:** a) extending the mutation spectrum in FH; b) identifying mutations in new FH genes; c) extending the FH phenotype; defining phenotypic-genotype correlations.

Experimental details: 1a) Extending the mutational spectrum in FPLD2 and FPLD3. Conventional direct sequencing of genomic DNA (gDNA) from 83 FPLD probands showed that 51 had no mutation in genes for FPLD2 [*LMNA* encoding lamin A/C] or FPLD3 [*PPARG* encoding peroxisome proliferator activated receptor-γ]. The candidate gene approach showed that two patients had novel rare missense mutations in *EMD* encoding emerin. These families will be expanded and evidence for causation will be obtained from co-segregation with phenotype, absence from normal controls and *in vitro* functional assessment. We will determine whether rare *EMD* missense mutations represent a new molecular form of FPLD – "FPLD4". The remaining 49 subjects with no mutation will have these genes re-screened using a new method to detect deletions called MLPA. Some probands with normal MLPA testing of candidate genes may have mutations in new FPLD genes. **1b) Identifying mutations in new FPLD genes.** We will use a candidate gene approach focusing on direct sequence analysis of gDNA. Candidates include genes involved in nuclear envelope biology, such as *LMNB1, LMNB2, LBR, LAP, NARF, SENP2, MAN1* and *ZMPSTE24/FACE1*, and genes with a functional link to lipodystrophy, such as *RXRA, SREBP1, AGPAT2* and *BSCL2*. Also, some families may be sufficiently informative to perform linkage analysis. **1c) *In vitro* studies for selected FPLD mutations.** *LMNA* T496fs is the first splicing mutation in FPLD2. We will express the *LMNA* mutant in model cell lines – liver, adipocyte and fibroblast – and will assess effects on cellular phenotype morphologically, including assessment of fat content and assessment of 3-D nuclear structure, co-immunoprecipitation with other nuclear lamin constituents, such as emerin and with mRNA expression profiling. *PPARG* Y355X will be studied *in vitro* collaboratively with Dr. Todd Leff. **1d) Extending the FPLD phenotype.** We will: i) extend novel FPLD3 kindreds with γ1 promoter -14A>G and E1 fs ΔAATG; ii) perform lipidomic, proteomic and transcriptomic comparison of FPLD2 and FPLD3; iii) define the phenotype of pre-symptomatic FPLD children. **2a) Extending the mutational spectrum in FH.** Conventional direct sequencing of gDNA from 109 FH probands showed that 62 had no mutation in the HCHOLAD1 gene [*LDLR*]. We will re-screen these genes from gDNA of affected subjects using MLPA. We will sequence from gDNA of candidate genes including *APOB, NARC1/PCSK9* and *ARH*. **2b) New FH genes.** Some FH families have no mutation in a known gene and may be informative for linkage analysis. **2c) Extending the FH phenotype.** We will: i) extend novel FH families and compare phenotypes between molecular forms.

Relevance. Demonstrating mutant *LMNA* in FPLD introduced a new mechanism – abnormal nuclear envelope structure and function – to the study of MetS and its metabolic complications. Disease mutations can serve as probes for new pathways or mechanisms. The planned studies will also help determine whether haploinsufficiency is an important disease mechanism in FPLD and whether simple replacement with normal gene product is feasible. Mutations in new will specify new pathways, disease mechanisms and therapeutic drug targets.

HEGELE, Robert A. SUMMARY OF RESEARCH PROPOSAL
"Genetic determinants of blood pressure"

We have submitted a duplicate of this proposal to HSFC, in which we propose to study Canadian genetic isolates to find genetic determinants of blood pressure (BP) primarily by testing for associations with candidate genes, whose products are involved in vascular biology. This approach has proven to be successful with 15 publications since 1997 on the topic of genetic determinants of BP.

OBJECTIVE

To discover new genetic determinants of BP and related traits in genetically isolated Canadian populations

HYPOTHESIS

Genomic variation within selected candidate genes is associated with variation in BP in Alberta Hutterites (AH), Ontario Oji-Cree (OC) and Keewatin Inuit (KI)

SPECIFIC AIMS

1. To evaluate reported candidate genes that contribute to variation in BP
2. To evaluate new candidate gene SNPs already discovered in our lab
3. To characterize new candidate gene SNPs, which can then be evaluated

Component 1: Candidate gene association studies in will be carried out in adults from our three samples, including evaluation of SNPs discovered in the Hegele lab (*CTSS* -256*G/A*; *LMNA* 1908C/T) and evaluation of previously reported SNPs (namely, *AGT* -6G/A, M/T174 & T/M235; *AGTR1* 1166A/C; *CYP11B2* -344C/T, *B3ADR* Y/R64, *B2ADR* R/G16 & Q/E27). Genotype X gender interactions will also be assessed. candidate gene associations will be evaluated in a sample of adolescent OC.

Component 2: Defining promoter sequence variants in *DAX1*. We showed that variation in the *DAX1* coding sequence is associated with hypotension. We will test the hypothesis that common variation in the *DAX1* promoter is associated with BP variation

Component 3: Additional candidate gene association studies. Other candidate genes for BP variation containing SNPs for which *in vitro* loss of function has been demonstrated include the *POMC*, *MTHFR*, *ET1*, *GRL* and *ANP* genes, and these are present priorities for evaluation in our study samples.

Relevance. Characterizing the genomic basis of BP variation is a first step towards developing: 1) diagnostic tests to stratify patients for evidence-based clinical decision making and intervention, and 2) novel targets for classical pharmacologic and/or genetic interventions. Given the success in identifying genetic determinants of BP, there is merit in studying this set of samples to investigate new candidate genes.

Time course, personnel and management plan: *Years 1 and 2:* Component 1 *Year 3:* Component 1 and 2. *Year 4:* Component 2 and 3; *Year 5:* Component 3. *Personnel.* Candidate genotyping, sequence analysis and transient gene expression and most statistical analyses will be performed by Dr. Jian Wang (Research Associate).

V SCIENTIFIC SUMMARY (Maximum one (1) page)

Structural and Functional Annotation of the Human Genome for Disease Study

In this application, we plan integrated and comprehensive computational and laboratory-based experiments to produce a more sophisticated and complete structural and functional annotation of human genomic variation, within four major, integrated themes. These themes are: Theme 1 (Scherer): To completely characterize the recently described phenomenon of large-scale copy variations (LCVs) in the human genome; Theme 2 (Hughes and Frey): To elucidate all gene coding sequences; Theme 3 (Blencowe and Frey) To characterize all splicing isoforms of all genes; and Theme 4 (Hegele): To evaluate the role of these newer genomic variations in mechanisms underlying selected human diseases, specifically atherosclerosis, diabetes and breast cancer. Since a complete understanding of genome biology is the ultimate goal of genomic investigation, analysis of patients will play a focal role in increasing our knowledge of function and regulation of the genes and types of variation involved. Our efforts to construct comprehensive new genomic datasets will enhance the characterization of clinical samples, in particular, for monogenic versions of complex diseases such as atherosclerosis, diabetes and breast cancer. Each of the four integrated and complementary themes will use high-throughput technologies within the OGI/Genome Resource Platform to assist in data gathering and will build additional capacity and new infrastructure. State-of-the-art technologies and advanced computational applications that we have developed in recent collaborations will be extended and applied to support each of our four Themes. Importantly, our activities will also be co-ordinated with the Canadian and International scientific community to enhance their application. Partnerships with the Sanger Institute, the European Bioinformatics Institute, the Human Epigenetics Consortium, Affymetrix, Agilent, Rosetta/Merck, and other USA and European genome scientists, have already been established to facilitate the work.

The annotated databases from this project will be made available to the international community, via links to existing genomic resources. They will serve as an international resource for biomedical researchers to address research inquiry into the spectrum of genomic variation in health and disease. The methods and algorithms used to generate the datasets we will also represent intellectual property. Furthermore, the databases will be maintained and curated in Canadian and European centres, in part from support from by private sector co-funds. The addition of the broad range of phenotype data linked to the unprecedented depth of genomic data will permit sophisticated modelling and hypothesis testing. The new markers of inter-individual genomic and early phenotype variation developed as a result of this project will provide new reagents for individual diagnosis and therapies.

A multi-disciplinary, multi-institutional GE^{3}LS team will address: (i) conceptions of health, disease, illness, normalcy & disability; (ii) socioeconomics of monogenic health services; (iii) informed choice to participate in monogenic disease research; (iv) professional & educational issues in the conduct of monogenic disease research.

Understanding the structure and organization of chromosomes, genes, transcripts and their corresponding variants is the first step towards systematic analysis of the normal function of genes and their regulation in an organism. Furthermore, annotation of the full range of human genomic variation provides a starting point for understanding inter-individual differences underlying various phenotypic conditions, including disease states. Canada's long-standing track record in genetic and genomic discovery, coupled with a comprehensive health care network, creates an unrivalled opportunity to make an international impact in the discovery of all types of human genetic variations and mechanisms of disease. Ultimately, our genomic discoveries will provide both fundamental and applied information contributing to the diagnosis and treatment of illness, spearheading improvements in health care for Canadians and for communities world-wide.

CIHR Team in Circumpolar Health Research:
Averting Emerging Chronic Diseases in Northern Populations:

1. Overview

Northern populations in the circumpolar region have begun to experience the emergence of chronic diseases such as cardiovascular diseases, diabetes, obesity and the metabolic syndrome which have occurred in other populations undergoing rapid social, cultural and economic transition.

Our Team Grant will create, develop and sustain an international, collaborative research program to monitor the burden of emerging chronic diseases among northern peoples; investigate genetic, behavioural and environmental risk factors that may be unique to these populations; and design and evaluate interventions in order to avert future epidemics. It formalizes an existing network across several circumpolar countries, linking academic research centres, regional health authorities, and indigenous peoples' organizations. It leverages funding from other sources and incorporates as integral components knowledge translation, research dissemination and training support. It aims to create a long-lasting legacy of enhanced capacity for robust health research in the North and for the North and improvement in the health of northern residents.

1.1 Importance and relevance

While there are many different pressing health concerns affecting northern populations, strategically the time to conduct intensive research into chronic diseases prevention is now, when the problem is still relatively limited in scope and early in its evolution. Northern populations are in the unique situation where rapid translation of research into policies, programs and practices can have a significant impact on improving health. Northern populations share many characteristics, including small size, remoteness and lack of human resources, and a team approach to research, especially one that adopts a circumpolar perspective, is essential. Our proposal is a direct response to the recommendations of the tri-council *Dialogue on Northern Research* workshop held in Whitehorse in 2004 [www.nserc.gc.ca/about/northern_summ_e.htm].

1.2 Overall Objectives

The proposed research program addresses the following broad scientific and public health questions. Specific objectives relating to individual projects are presented in Section 9.

- What is the current burden and distribution of chronic diseases among northern populations and what genetic, behavioural and environmental factors are responsible for their development?
- Why do northern communities differ in their risk of chronic diseases and in the impact of various health determinants?
- Why do northern peoples fare poorly compared to the general population of the larger nation-states with which they are associated?
- What can be done to reduce the burden and impact of prevalent and emerging chronic diseases affecting northern peoples?

The proposed Team, in executing its research plan, aims to cross traditional academic disciplines, while connecting multiple investigators, institutions, research sites and geographic regions. At the end of the 5-year Team Grant, the following short-term process outcomes or "deliverables" are expected.

3. Objectives of the Program Grant

Cellular Programs and Responses in Atherosclerosis: Linking Genotype to Phenotype

Our Central Theme is: *A key to understanding atherosclerosis is the elucidation of genetic determinants and ascertaining the impact of these determinants on metabolic outcomes and vascular cell responses - the phenomics of atherosclerosis.*

Objectives and Achievements from the Currently Funded HSFO Program

This application is the renewal of an HSFO Program Grant that has been tremendously successful. Our group established 4 important lines of investigation to examine cell responses in atherosclerosis, which has effectively linked our 5 laboratories to a common objective. Our discoveries of gene expression profiles directed us towards novel genes, pathways, and mechanisms of gene regulation that underlie vascular cell responses and function. Through strategic admixture of our complimentary research technologies, approaches and skill sets, the productivity emanating from the 4 objectives has been excellent. This degree of success would not have been possible without the HSFO Program Grant.

Our objectives for 2001-2005 were:

1. To define differential gene expression profiles in smooth muscle cells (SMCs) and macrophages.
2. To determine how gene mutations and single nucleotide polymorphisms (SNPs) associated with dyslipidemia, insulin resistance and the metabolic syndrome influence vascular cells.
3. To elucidate inflammatory cascades in cardiovascular disease.
4. To understand the relationship between alterations in G protein-coupled receptor (GPCR) signaling and vascular cell responses.

Progress:

Over the past 4 years, our HSFO group peer-reviewed publication record has been excellent and each manuscript has benefited from the programmatic approach we have adopted. Of significance, 25 of these manuscripts constituted formal collaborations between group PIs, as documented by shared authorships. Here we provide selected examples of our productivity in which major discoveries were made;, these advances would not have occurred without this integrative program.

Discovery of novel pathways driving diverse SMC phenotypes. We were the first, and to date only, group to successfully clone non-transformed SMCs from the media of the normal human adult artery. In doing so, we discovered distinct SMC subtypes. We have capitalized on this discovery, and the unique differentiation capacity of one SMC subtype, by undertaking microarray analysis and functional studies of putative novel SMC regulators. Of the many noteworthy findings, we discovered that lipoprotein lipase was differentially expressed and that this enzyme imparted to a subpopulation of SMCs a strong predisposition to accumulate lipids and differentiate into foam cells. We also discovered that Pre B-cell colony-enhancing factor regulates histone deacetylation and drives SMCs to a quiescent state, which attenuates the aging process. These findings opened an entirely new paradigm for foam cell formation and a novel cascade regulating SMC function and viability, with important consequences for plaque stabilization and potential therapy. These discoveries arose from collaborations between Drs. Huff, Pickering, and Hegele and have been published in *Circ Res* (2001), *ATVB* (2004), and *Circ Res* (2005).

Influence of gene mutations and variants, associated with dyslipidemia and the metabolic syndrome on vascular cells: We successfully applied genomic technologies to be the first in the world to discover the genetic basis of human monogenic forms of atherosclerosis, including familial partial lipodystrophy (FPLD) types 2 and 3, due respectively to mutations in *LMNA*, encoding nuclear lamin A/C, and *PPARG*, encoding peroxisomal proliferator-activated receptor gamma. These discoveries quickly lead to identification of novel pre-symptomatic metabolic phenotypes of insulin resistance that are predictors of future disease risk, together with novel vascular phenotypes ascertained through *in vivo* carotid ultrasound in affected subjects with familial partial lipodystrophy (FPLD). In other studies, we discovered and characterized at the transcriptional level a functional promoter variant in *PCK1*, encoding phosphoenol-pyruvate carboxykinase-1, the key enzyme in gluconeogenesis, and then

proceeded to show disparate associations between this *PCK1* variant and two vascular phenotypes, "plaque volume" derived from 3-D ultrasound and conventional intima-media thickness (IMT), in aboriginal Canadians with type 2 diabetes. Furthermore, we elucidated superiority of "plaque volume" over IMT as a biomarker of atherosclerosis in aboriginal Canadians with diabetes. This has established our key analytical strategies as a validated experimental paradigm for defining genotype-phenotype correlations in atherosclerosis. These discoveries arose from collaborations between Drs. Hegele, Huff and Pickering that were published in *Circulation* (2001), *ATVB* (2003), *JCEM* (2004) and *Stroke* (2005).

Elucidation of novel signalling events in vascular regulation and inflammation. We have made several key advances pertaining to novel pathways through which G-protein-coupled receptors (GPCRs) regulate events central to vascular function. Major discoveries include identification of a nitric oxide-based mechanism by which aldosterone mediates acute vasoconstriction via GPCR signaling. We also established, using microarray analysis, that the phenomenon of blunted ß-adrenergic receptor signalling in SMCs derived from hypertensive animals extends beyond the failure of SMCs to relax, since it also manifests as a global suppression of growth-related gene expression. Our focus on uncoupling of GPCRs from G-protein interactions led to the surprising finding that the GPCR regulatory protein ß-arrestin interacted with RalGDS to reorganize the cell cytoskeleton. Furthermore, a GPCR polymorphism was discovered that is associated with constitutive binding of ß-arrestin to a specific GPCR (fMLP receptor) and to increased plasma C-reactive protein, highlighting a new linkage between GPCR signaling and vascular inflammation. These discoveries arose from collaborations between Drs Ferguson, Feldman, Pickering and Hegele that have been published in *Nature Cell Biology* (2002), *Circulation* (2003), and *Molecular Pharmacology* (2005) as well as a submission to *J Biol Chem*.

Proposal for Renewal of HSFO Program

Background: Atherosclerosis develops over time as a consequence of hemodynamic stress, abnormal metabolism, and local biological perturbations including monocyte and lymphocyte recruitment to the arterial intima, smooth muscle cell (SMC) migration and proliferation, matrix production, foam cell formation, and thrombus deposition. While several genes responsible for rare single-gene vascular disorders have been described, unravelling the genetic determinants of the complex pathogenesis of common atherosclerosis remains a significant challenge. Our group proposes to combine efforts across a spectrum of model systems and technologies in order to efficiently accumulate new insights into the genomic-phenomic interplay that underlies atherosclerosis.

Hypothesis: *Elucidation of genetic determinants and ascertainment of their impact on metabolic and vascular responses will reveal novel pathways involved in initiation and progression of atherosclerosis and will provide new insights into, and targets for, prevention and treatment.*

Our Experimental Approaches: Each investigator brings unique *in vitro* and *in vivo* models and approaches to understanding the pathologenesis of atherosclerosis. We will utilize a systems approach through which we will integrate discovery science with our hypothesis-driven research. This integrated HSFO Program Grant capitalizes on our now well-developed core resources, including DNA sequencing, high-density microarray analysis, confocal microscopy, metabolic phenotyping, molecular pathology, and vascular imaging. Together with the established expertise of the principal investigators, this approach will add substantial value to each existing component research program. Three integrated objectives have been identified.

Specific Objectives:

1. To link genotype to phenotype in vascular disease.
2. To elucidate novel cellular programs in smooth muscle cells and macrophages that underlie the productive remodeling of the atherosclerotic artery.
3. To define novel signalling pathways that dynamically regulate vascular cell function.

Objective 1: To link genotype to phenotype in vascular disease

Introduction: The genetic basis of atherosclerosis and its constituent risk factors, such as metabolic syndrome (MetS), is exceedingly complex. Over the current term of this Program Grant, we made

substantial strides towards elucidating genomic determinants of atherosclerosis, of its risk factors and of vascular cell behaviour. Our research direction is both to expand the repertoire of genetic/genomic players and then to link these with structural and functional phenotypes in humans, animal models and vascular cells. Our key experimental paradigms are: 1) gene discovery in model systems, including ethnic communities, vulnerable families, animals, organs, tissues and cells; 2) characterization of genotype-phenotype relationships, including intervention studies, in human monogenic disease states and in other model systems; 3) microanalyses of vulnerable plaque heterogeneity, linking morphology, cellular and molecular pathways, and the transcriptome.

One important context in which to examine these paradigms is the MetS, a phenotype found in 25% of Canadians that is a potent risk factor for atherosclerosis. Our strategy to understand MetS is to study rare human monogenic forms, such as FPLD and human genetic variants causing hyperlipidemia, hypertension, obesity, diabetes and vascular traits. Genes discovered using this paradigm provide the springboard for the application of our full spectrum of scientific expertise and core technologies.

Proposed research: In research supported by GIAs 1, 2, 3 and 5, Drs. Hegele, Pickering and Huff will advance understanding of atherosclerosis-related traits by: a) identifying mutations in new genes; b) extending the mutational spectrum in monogenic forms of disease in which the gene is already known; c) using functional assays to define disease mechanisms that are caused by specific human gene variants; and d) extending metabolic and vascular phenotypes in molecularly-characterized patients and disease families. Phenotyping methods include extensive analyses of a wide range of biochemical markers and metabolic indices together with the use of novel imaging modalities (such as 3D-ultrasound and MRI) to expedite whole patient phenotyping with a focus on vascular structure and fat distribution profiles. Additional phenotypes include *in vivo* plaque morphology, plaque compartmentalization using laser capture microscopy and expression analysis of aging genes.

Determinants of human atherosclerosis. We will apply our genomic analysis platforms, including expression arrays, 500K SNP DNA arrays for both large-scale copy number variation and association analyses, together with state-of-the-art bioinformatics methods, in order to identify new gene clusters: genes and/or variants that are associated with metabolic and/or vascular phenotypes. Gene variants so identified can be examined for association with human phenotypes in independent replication samples. Validated novel genes and variants associated with metabolic or vascular phenotypes at the population level can then be extensively studied using biochemical, cellular and molecular biological approaches in order to expand our understanding of new pathways. Conversely, candidate genes arising from unbiased experiments in model systems can be studied for association with MetS, dyslipidemia and accelerated atherosclerosis in families and populations. Examples of functionally characterized genes in which variants were or will be identified and studied for phenotype associations in various populations include *PBEF*, *WTAP*, *HSP47*, *fMLP*, *NPC1L1*, *AC6*, and *GPR30*. We will extend novel disease families and compare phenotypes between different molecular forms of the same gene product, which will allow us to define variability in the phenotype. We will combine this information with strategic use of functional experiments in animal and/or cell models. These approaches used together will elucidate novel pathways in pathogenesis of vascular disease. As we have repeatedly demonstrated, human disease mutations can serve as probes for new pathways or mechanisms.

Determinants of insulin-related phenotypes in atherosclerosis. Insulin resistance is associated with a specific pattern of hepatic gene expression that is associated with overproduction of atherogenic lipoproteins, partly as a consequence of the failure of insulin to activate specific intracellular signaling pathways. We discovered that, like insulin, naturally occurring molecules called flavonoids reverse this pattern of gene expression. Importantly, these flavonoids activate insulin signaling pathways independent of the insulin receptor, potentially via a novel GPCR. Experiments in cultured human hepatocytes and in diet-induced insulin-resistant LDL-receptor knockout mice are proposed to implicate specific components of the cell signaling pathways and to determine whether flavonoids attenuate insulin resistance and atherogenesis.

Determinants of cellular aging in atherosclerosis. Genotype-phenotype relationships will also be studied in the milieu of cellular aging and atherosclerotic plaque stability. Atherosclerotic plaques are exposed to stresses that can accelerate cellular aging (by oxidation and replication) which could, in

turn, have profound implications for plaque stability. We have discovered that the longevity of vascular SMCs can be extended by manipulating the expression of genes that generate or consume NAD. We will define the topographical expression profile of these and other longevity/survival genes in discrete regions of human atherosclerotic tissue dissected by laser capture microscopy. The gene expression fingerprint relevant to aging will be mapped within plaque subregions and according to morphological features such as fibrous cap thickness. These multi-level transcriptome-phenotype linkages will reveal novel molecular patterns intrinsic to plaque aging and stability.

Mutations in new genes, and elucidation of vital gene expression programs, will specify new pathways, disease mechanisms and therapeutic targets. We have a potent collaborative partnership involving all members of this application. Our ready access to leading edge genomic map data, genotypes, sequences, expression profiles and phenomics uniquely positions us to make ground-breaking, multi-faceted discoveries in an accelerated manner, resulting in increased understanding of atherosclerosis.

Objective 2: Elucidate novel cellular programs in smooth muscle cells and macrophages that underlie the productive remodeling of the atherosclerotic artery.

Introduction: Vascular SMCs are normally quiescent, with their primary function being to contract and relax in response to physiological stimuli. In vascular disease however SMCs acquire new attributes that enable them to proliferate, migrate, produce and degrade extracellular matrix (ECM). SMCs that adopt this more assertive synthetic phenotype are critical for repairing diseased arteries and stabilizing atherosclerotic plaques. However, synthetic SMCs often fail to prevent plaque rupture. One reason is that SMCs replicate as the aging process accelerates which creates susceptibility to apoptosis and enfeebles their ability to perform vital functions. We discovered novel genes that regulate the maintenance of SMCs within a maturation-competent, slowly aging state. Our group also discovered that hyperlipidemia profoundly affects SMCs, forcing them into a phenotype akin to macrophage foam cells, which in turn incapacitates the elaboration of extracellular matrix. Macrophage-derived foam cells themselves play a focal role at each stage of lesion development, yet the pathways regulating their survival and governing their ability to efflux cholesterol remain poorly understood.

Proposed Research: ***Molecular determinants of plaque cell viability.*** In research supported by GIAs 3, 4 and S-GIA 1, Drs. Huff, Pickering and Hegele will investigate novel and recently discovered regulatory pathways that direct the performance of SMCs and macrophages. One major advance is our discovery of Pre-B-Cell Colony Enhancing Factor (PBEF), an enzyme that drives the activity of sirtuins, which, through deacetylation of key nuclear proteins, act as guardians against cell senescence. We hypothesize that enhanced PBEF activity will attenuate SMC aging and will sustain their ability to productively remodel the artery wall. This function will be evaluated using virus-mediated gene transfer into human cells. We also propose that PBEF enhances macrophage survival, allowing them to retain their ability to efficiently efflux cholesterol via the reverse cholesterol transport pathway. Expression profiles from microarray analysis of SMCs and macrophages that have been activated by PBEF will be interrogated for genes that program survival and longevity. Comparing transcriptomes and functional responses within and between these 2 cell types will provide a unique integrative picture of novel and potentially powerful determinants of the SMC and macrophage contribution to plaque stability.

Molecular determinants of foam cell formation. A second major discovery is that cholesterol efflux from macrophages can be strikingly enhanced through selective activation of endogenous oxysterol production. Oxysterols activate the liver X receptor which drives the expression of cholesterol efflux transporters. To broaden the implications of this oxysterol-based reprogramming of gene expression, we will define the potential for oxysterol-induced cholesterol efflux in SMC-derived foam cells.

Cellular and extracellular matrix determinants of plaque composition. The assembly and stability of extracellular matrix (ECM) fibrils also determines productive arterial remodeling and plaque stability. However, as SMCs age and accumulate lipids, the assembly and stability of the ECM decline precipitously. We propose that impelling both PBEF-mediated cell survival and cholesterol efflux pathways will attenuate or even reverse this decline. We will test this hypothesis using our novel models of fibrillogenesis and foam cell formation induced by atherogenic human lipoproteins isolated from genetically-defined dyslipidemic patients (see objective 1). ECM synthesis and degradation by SMCs and macrophages, respectively, will be measured using our well established techniques. The impact of

enhanced oxysterol synthesis on atherogenesis and plaque composition will be assessed *in vivo* in LDL-receptor knockout mice. The proposed link to activation of LXR-mediated gene expression will be tested in detail using laser capture microscopy to dissect subpopulations of arterial cells.

Objective 3. Defining novel signaling pathways that dynamically regulate vascular cell function:

Introduction: G protein-coupled receptors (GPCRs) play a central role in regulating vascular SMC responses to circulating and locally released hormones. The regulation of vascular smooth muscle tone represents a balance between endothelial and vascular smooth muscle receptor-mediated mechanisms of regulation, the activation of GPCRs linked to vasodilation and vasoconstriction and GPCR-regulated signaling linked to proliferation. Moreover, the activity of the GPCRs regulating these pathways is modulated by intracellular mechanisms that define receptor G protein-coupling, desensitization and resensitization. We discovered that the steroid hormone aldosterone mediates acute effects on vascular smooth muscle tone by mediating contractile responses potentially through the activation of the orphan GPCR GPR-30. In addition, the activity of different GPCRs linked to vasodilation and vasoconstriction of vascular SMCs can be differentially regulated based on differences in: 1) their intracellular trafficking patterns between various endosomal compartments; 2) their association with different Rab GTPases and 3) their propensity to either resensitize or remain desensitized.

Proposed Research: Research supported by GIAs 6, 7 and 8, Drs. Feldman, Ferguson and Pickering will examine the impact of novel components of GPCR signaling on vascular SMC function.

Determinants of vascular tone. We will exploit our recent discovery that aldosterone acutely regulates vascular tone through GPCR signaling. We will test the hypothesis that aldosterone mediates its acute effects in SMCs via the activation of the orphan GPCR, GPR-30. The biochemical consequences in human SMCs challenged acutely with aldosterone will be examined following the targeted knockdown by short hairpin RNA or overexpression of either GPR-30 or the mineralocorticoid receptor, using viral vectors. Functional consequences will be evaluated in single cells by dynamic time-lapse imaging. The concept of aldosterone-mediated acute activation of GPCR signaling will be extended to endothelial cells and intact vessels in organ culture using our well developed model systems. The role of this aldosterone-induced signaling in the pathogenesis of hypertension will be elucidated *in vivo* using genetic (SHR) and acquired (Dahl salt-sensitive) rat models of hypertension and ultimately in humans with hypertension using gluteal biopsies and perfusion myography.

Cellular determinants of GPCR signaling in the vasculature. We will capitalize on our discovery that specific RabGTPases are critical for GPCR recycling. This has led us to hypothesize that Rab GTPases critially regulate human SMC contractile function by orchestrating the endocytosis, trafficking, recycling and resensitization of the β_2 adrenergic receptor and angiotensin-1 receptor. We will assess whether the resensitization of endogenous β_2AR and $AT_{1A}R$ in adult human arterial SMCs is altered by the adenoviral-induced expression of dominant-negative or constitutively active GFP-tagged Rab5, Rab4 and Rab11 proteins. Using confocal microscopy and biochemical determinations, we will determine whether these Rab GTPases regulate the endocytosis and trafficking of GPCRs to endosomes (Rab5) as well as the recycling of receptors back to the cell surface via rapid (Rab4) and slow (Rab11) recycling endosomes. Alterations in SMC-mediated vascular responsiveness will be assessed in organ cultures from rat aortic and mesenteric arteries transfected with adenoviral constructs encoding dominant negative and constitutively active Rab GTPases. The long-term goal of these experiments will be to develop mice in which Rab GTPases are over-expressed or knocked out, in a vascular smooth muscle-specific manner, and assess the impact on hypertension and predisposition to atherosclerosis.

Summary

This program will create significant synergies resulting in the establishment of coherent lines of investigation that link our laboratories to a central theme – the phenomics of atherosclerosis. Our proposed studies in humans, *ex vivo* tissues, animal models and cells will capitalize on state-of-the-art technologies to elucidate genetic determinants of atherosclerosis and their impact on metabolism and vascular function. Our results will allow us to identify novel pathways involved in the pathogenesis of this complex disease and provide an enhanced and improved rationale for the diagnosis and treatment of atherosclerosis.

Public Health Agency of Canada | Agence de santé publique du Canada

Your file / Votre référence

Our file / Notre référence

Date issued: October 4, 2006

Name and/or Organization: **Robarts Research Institute**
Attn: Dr. Robert A. Hegele

Address: 100 Perth Drive, Room 4-06
London, ON
N6A 5K8

The following biological material does not require a Public Health Agency of Canada import permit under the HPIR*:

Human fibroblast cell lines from healthy donor (AG04456) and from donor with Hutchinson-Gilford Progeria Syndrome (AG03513), as provided by Coriell Cell Repositories, NJ, USA.

Paul J. Payette, Ph.D.
Director, Office of Laboratory Security

October 4, 2006
Date

NOTICE

***HPIR (HUMAN PATHOGENS IMPORTATION REGULATIONS)**

- We are in receipt of your application for an importation permit for biological materials. The **HPIR** apply **only** to the importation of infectious substances which cause human disease and their subsequent distribution or transfer. Other materials, which are deemed by the importer to be non-infectious for humans, **do not** require a permit under these regulations. It should be noted that the importation of biological materials may also be subject to other federal, provincial and municipal laws.
- For animal or plant pathogens one **must** apply to The Canadian Food Inspection Agency (CFIA) for a permit to import. If this material is of animal or plant origin it may also require a permit from the CFIA. Please contact the CFIA for their consideration. CFIA contact numbers are as follows:
  (613) 221-7068 for information concerning animal pathogens/material
  (613) 225-2342 [ext. 4334] for information concerning plant pathogens/material
- Importation of this material may also be subject to the requirements of the *New Substances Notification Regulations (Organisms)* of the *Canadian Environmental Protection Act, 1999*, administered by Environment Canada and Health Canada. Please contact the New Substances Information Line at 1-800-567-1999 or nsn-infoline@ec.gc.ca.
- You may be required to provide the Canada Border Services Agency (CBSA) customs officers with a declaration that the imported material is non-infectious and non-hazardous.

Should you require further information, please contact:
Office of Laboratory Security
Centre for Emergency Preparedness and Response
(613) 957-1779

Canada

2009 Biohazard Agents Registry, Dr. Robert Hegele

Name	CAT. NO.	SUPPLIER	MSDS	Comment	Use
3T3-L1 rodent	CL-173	ATCC	Y	Murine fibroblast	Mutant LMNA is transfected into t
NIH/3T3	CRL-1658	ATCC	Y	Murine	cells and immunofluorescence stu
COS-7: ~~note SV40 promoter~~ non human Primate	CRL-1651	ATCC	Y	Green monkey (Ceropithecus aethiops)	and Western analysis performed
HEK293: ~~note Adeno E1A promoter~~	CRL-1573	ATCC	Y	Human	
HepG2 human	CRL-11997	ATCC	Y	Human	
Fibroblast, finite primary cell line human	GM05659	Coriell Cell Repository	Y	Human: unaffected	Cells are cultured, morphology an
Fibroblast, finite primary cell line human human	GM08398	Coriell Cell Repository	Y	Human: unaffected	growth curves evaluated and immunofluorescence studies, Wes
Fibroblast, finite primary cell line human	GM03348	Coriell Cell Repository	Y	Human: unaffected	analysis, DNA and RNA isolated an
Fibroblast, finite primary cell line human	AG03513	Coriell Cell Repository	Y	Human: HGPS proband	microarray and high throughput sequencing analysis performed.
Fibroblast, finite primary cell line human	AG04456	Coriell Cell Repository	Y	Human: unaffected	
Fibroblast, finite primary cell line human	AG06234	Coriell Cell Repository	Y	Human: HGPS proband	
Fibroblast, finite primary cell line human	AG06297	Coriell Cell Repository	Y	Human: unaffected	
Fibroblast, finite primary cell line human	30950	Dr. T.C. Rupar	na	Human	Cells are cultured, DNA and RNA
Fibroblast, finite primary cell line human	40916	Dr. T.C. Rupar	na	Human	extracted and microarray and higl
Fibroblast, finite primary cell line human	20750	Dr. T.C. Rupar	na	Human	throughtput sequencing analysis
Fibroblast, finite primary cell line human	70280	Dr. T.C. Rupar	na	Human	performed
pCMV6 list under GMO	PS100001	Origene	y		Transfection vectors used in the l
pcDNA3.1	350492	Invitrogen	Y		
pENTR 11 ~~dual selection vector~~	A10562	Invitrogen	Y		
~~Subcloning Efficiency~~™ DH5α™ ~~Competent Cells~~	18265-017	Invitrogen	Y	E. Coli	Cells used to as vehicles for transi
~~Electromax DH10B competent cells~~	18290-015	Invitrogen		E. Coli	of genetic material for overexpre:
~~ME DH10B competent cells~~	18297-010	Invitrogen		E. Coli	studies and phenotypic character
~~ME DH5-alpha competent cells~~	18258-012	Invitrogen	Y	E. Coli	
~~ME DH5A T1 page resist comp cells~~	12034-013	Invitrogen		E. Coli	

organisms