

Modification Form for Permit BIO-UWO-0017

Permit Holder: Dale Laird

Approved Personnel

(Please stroke out any personnel to be removed)

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 Robert Lorentz
 Michael Stewart
 Cindy Shao
 Silvia Penuela
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 Jared Churko

Additional Personnel

(Please list additional personnel here)

John KELLY
Eric Huarey

Please stroke out any approved Biological Agent(s) to be removed

Write additional Biological Agent(s) for approval below. Give the full name

Approved Microorganisms

E. coli DH5 alpha, JM109

Approved Primary and Established Cells

Human (primary), skin biopsies, Rodent (primary), transgenic alpha mutant mouse, Human (established), HELA, 293T, 293 Hek, Tumour cell lines, Rodent (established), Keratinocytes, NRK BICR, MIRK, N2A,

MCF 10A cell line (from ATCC)

Approved Use of Human Source Material

Human blood (whole), Human tissues (unpreserved), Human tissues (preserved), ODDD Patients and relatives

Approved Genetic Modifications (Plasmids/Vectors)

JM109, T-EASY, PcDNA3, pEGFP, retrovirus AP-2, SV 40 Large T antigen, HEK, E1A oncogenes, HeLa, pGFP-V-RS

GMV-R-GECO1 plasmid 32444 (Level 2)

Approved Use
of Animals

mice

Approved Biological
Toxin(s)

Cholera Toxin (level 2)

Approved Gene
Therapy

Approved Plants and
Insects

** PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOLOGICAL AGENTS.*

*** PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOLOGICAL AGENTS USED AND HOW THEY WILL BE STORED, USED AND DISPOSED OF..*

As the Principal Investigator, I have ensured 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.shs.uwo.ca/workplace/newposition.htm>

Signature of Permit Holder: _____



Current Classification: 2 Containment Level for Added Biohazards: _____

Date of Last Biohazardous Agents Registry Form: Mar 27, 2009

Date of Last Modification (if applicable): Apr 16, 2010

BioSafety Officer(s): _____

Chair, Biohazards Subcommittee: _____ Date: _____

Subject: description

From: Cindy Shao <Cindy.Shao@schulich.uwo.ca>

Date: Tue, 21 Feb 2012 14:34:19 -0500

To: Jennifer Stanley <jstanle2@uwo.ca>

1. MCF10A cells is derived from human , mammary gland, epithelial cells. Using mammary epithelial growth medium from Clonetics for culture.
2. Cholera toxin, is used for 100ng/ml in MCF10A cell line culture medium to keep this cells character
3. The new plasmid will be used for transfection into rat or mouse culture cell lines to see protein expression and test its functions.

If you have any questions, please let me know.

Thank you

Cindy



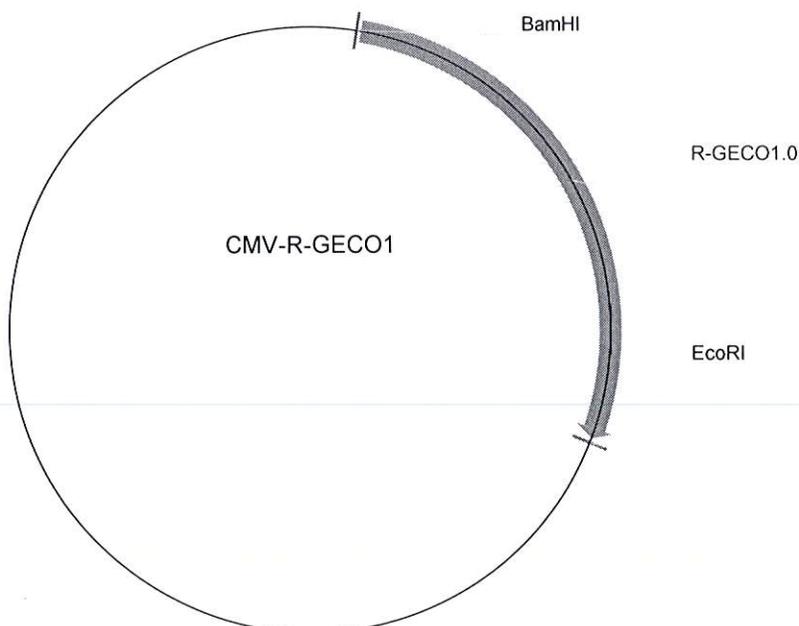
[Browse](#) > [Robert Campbell](#) > [Zhao et al](#) > CMV-R-GECO1

Plasmid 32444: CMV-R-GECO1

Gene/insert name: R-GECO1.0
Alt name: red intensimetric genetically encoded Ca²⁺-indicators for optical imaging
Insert size: 1254
Species: synthetic construct
GenBank ID: JN258411
Mutation: Substitutions relative to the mApple-derived analogue of GCaMP3:
T47A/L60P/E61V/S63V/E64S/R81G/K83R/Y134C/M158L/N164aD/V228A/
S290P/I366F/K380N/S404G/N414D/E430V
Vector backbone: Customized Vector
([Search Vector Database](#))
Vector type: Mammalian Expression
Backbone size w/o insert: 3200
Promoter: CMV
Cloning site 5': BamHI
Site destroyed during cloning: No
Cloning site 3': EcoRI
Site destroyed during cloning: No
5' sequencing primer: TAATACGACTCACTATAGGG [List of Sequencing Primers](#)
3' sequencing primer: TAGAAGGCACAGTCGAGG
Bacterial resistance: Ampicillin
Growth strain: DH10B
Growth temperature (°C): 37
High or low copy: High Copy
Sequence: [View sequences \(3\)](#)
Principal Investigator: Robert Campbell
Terms and Licenses: [MTA](#)

Comments: Note: Could not make stable cell line using this vector.

Addgene has sequenced a portion of this plasmid for verification. Click [here](#) for the sequencing result.



Article: [An Expanded Palette of Genetically Encoded Ca²⁺ Indicators](#), Zhao et al (Science. 2011 Sep 8. PubMed)

Please acknowledge the principal investigator and cite this article if you use this plasmid in a publication. Also, please include the text "Addgene plasmid 32444" in your Materials and Methods section.



An Expanded Palette of Genetically Encoded Ca²⁺ Indicators

Yongxin Zhao, *et al.*

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Supporting Online Material

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Materials and Methods

Fig. S1

References

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An Expanded Palette of Genetically Encoded Ca²⁺ Indicators

Yongxin Zhao,¹ Satoko Araki,² Jiahui Wu,¹ Takayuki Teramoto,³ Yu-Fen Chang,² Masahiro Nakano,² Ahmed S. Abdelfattah,¹ Manabi Fujiwara,³ Takeshi Ishihara,³ Takeharu Nagai,^{2,4} Robert E. Campbell^{1*}

Engineered fluorescent protein (FP) chimeras that modulate their fluorescence in response to changes in calcium ion (Ca²⁺) concentration are powerful tools for visualizing intracellular signaling activity. However, despite a decade of availability, the palette of single FP-based Ca²⁺ indicators has remained limited to a single green hue. We have expanded this palette by developing blue, improved green, and red intensometric indicators, as well as an emission ratiometric indicator with an 11,000% ratio change. This series enables improved single-color Ca²⁺ imaging in neurons and transgenic *Caenorhabditis elegans*. In HeLa cells, Ca²⁺ was imaged in three subcellular compartments, and, in conjunction with a cyan FP–yellow FP–based indicator, Ca²⁺ and adenosine 5′-triphosphate were simultaneously imaged. This palette of indicators paints the way to a colorful new era of Ca²⁺ imaging.

Fluorescent indicators for the quantification of intracellular Ca²⁺ have been indispensable tools of cell biology for three decades (1), yet the need for new indicators has continued to grow as advances in molecular biology and microscopy instrumentation reveal the limitations of each previous generation. Accordingly, there has been a push toward Ca²⁺ indicators that are longer wavelength, ratiometric, and higher signal-to-noise, and—since the advent

of green fluorescent protein (GFP)—genetically encodable. A ratiometric indicator excites or emits at distinct wavelengths in the Ca²⁺-free and Ca²⁺-bound states and provides the advantages of being quantitative and less susceptible to imaging artifacts. Two important classes of genetically encoded Ca²⁺ indicators are the Förster resonance energy transfer (FRET)-based cameleon type (2), and the single GFP type, such as GCaMP (3) and flash-pericam (4), that are dim in the absence of Ca²⁺ and bright when bound to Ca²⁺. GCaMPs are composed of a circularly permuted (cp) GFP fused to the calmodulin (CaM)-binding region of chicken myosin light chain kinase (M13) at its N terminus and a vertebrate CaM at its C terminus. Binding of Ca²⁺ causes the M13 and CaM domains to interact and the interface between CaM and the fluorescent protein (FP) to reorganize, which leads to an increase in fluores-

cence due to water-mediated interactions between the chromophore and R377 (5) of CaM (6, 7).

Although directed protein evolution has provided many improved FPs, it has not proven particularly effective for the production of improved GCaMPs. Directed evolution of FPs is typically guided by digital fluorescence imaging of large libraries of gene variants expressed in *Escherichia coli* colonies. In this manner, rare clones that harbor a mutation that confers a desirable trait, such as improved brightness or altered hue, can be identified in libraries of >10⁵ variants. Lacking an analogous screen for GCaMP Ca²⁺ response, researchers have identified improved variants by manual testing (7, 8) and a medium-throughput cell-based assay (9).

To accelerate the development of improved and hue-shifted GCaMP-type indicators, we developed a colony-based screen for Ca²⁺-dependent fluorescent changes (Fig. 1) [supporting online material (SOM) text]. The premise of this screening system is that Ca²⁺ indicators targeted to the *E. coli* periplasm can be shifted toward the Ca²⁺-free or Ca²⁺-bound states by experimental manipulation of the environmental Ca²⁺ concentration. Accordingly, screening of large libraries of genetic variants of GCaMP-type indicators can be achieved by digital fluorescence imaging, at both high- and low-Ca²⁺ conditions, of plates containing hundreds of *E. coli* colonies each. We used this screening system to undertake a process of directed evolution that explored the sequence space accessible from the most optimized single FP Ca²⁺ indicator, GCaMP3 (9). Initially, we created a large library by error-prone polymerase chain reaction (PCR) and screened ~2 × 10⁵ colonies to identify the offspring with the largest Ca²⁺-dependent changes in green fluorescence (10). After this and subsequent iterative rounds of library creation and screening

¹Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada. ²Research Institute for Electronic Science, Hokkaido University, Kita 20, Nishi 10 Kita-ku, Sapporo 001-0020, Japan. ³Department of Biology, Faculty of Sciences, Kyushu University, 6-10-1, Hakozaki, Higashi-ku, Fukuoka, 812-8581, Japan. ⁴PRESTO, Japan Science and Technology Agency, 5 Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan.

*To whom correspondence should be addressed. E-mail: robert.e.campbell@ualberta.ca

(fig. S1), we arrived at a set of three improved variants (figs. S2 and S3 and table S1). We designated these green fluorescent genetically encoded Ca^{2+} indicators for optical imaging as G-GECO1 (Fig. 2, A to C), G-GECO1.1, and G-GECO1.2. Parallel efforts to evolve flash-pericam (4) did not produce variants with properties that rivaled the G-GECOs. The G-GECOs share a Ca^{2+} -dependent increase in fluorescence (2300 to 2600%) that is approximately double that of GCaMP3 (table S2), which demonstrates the effectiveness of the molecular evolution strategy. The primary differences between the three variants are the apparent dissociation constants (K_d) for Ca^{2+} (750 nM, 620 nM, and 1150 nM, respectively; 540 nM for GCaMP3) and the fact that G-GECO1 exhibits the least fluorescence when excited at ~400 nm and may be preferred when used in conjunction with violet-excitable probes.

Our success in developing the G-GECOs led us to explore the evolution of GECOs with al-

tered fluorescent hues. We attempted to construct a blue fluorescent GECO (B-GECO) by introducing the GFP Y66H substitution (in which His replaces Tyr⁶⁶) to produce the histidine-derived chromophore of blue FP (BFP), into G-GECO1.1. To create an initial library of variants, we randomized three amino acids (V63, R377, and K380) with side chains in close proximity to the chromophore (6). Screening of this >18,000-member library led to the identification of a first-generation B-GECO with 400% signal change. Additional rounds of evolution ultimately produced B-GECO1 (table S1 and figs. S2, S4A, and S5A), which has a 700% signal change (Fig. 2, B and C) and a K_d of 480 nM (table S2) and is relatively pH-insensitive compared with G-GECO1 (22% versus 92% quenched at pH 6) (fig. S6).

Red-shifted fluorescent indicators are preferable to blue-shifted ones because of the lower phototoxicity and greater tissue penetration as-

sociated with longer wavelength light. To construct a potential red fluorescent GECO (R-GECO), we first replaced the cpGFP of G-GECO1.1 with an analogous cp version ($N_{\text{term}}-146$ to 231-GGTGGG-1 to 145- C_{term}) of the mApple red FP (11). The first-generation library was created by error-prone PCR and randomization of the first and last residues of cpmApple (63 and 302 by GECO numbering) (fig. S2). From this library, we identified R-GECO0.1, which exhibited a modest response to Ca^{2+} (60% increase). Additional rounds of directed evolution afforded major improvements and resulted in the production of R-GECO1 (table S1 and figs. S2, S4B, and S5B), which exceeds GCaMP3 in terms of its Ca^{2+} response. R-GECO1 has a 1600% intensity change (Fig. 2, B and C), a K_d of 480 nM, and absorption and emission maxima that are ~80 nm red-shifted relative to the G-GECO series (table S2).

Late in the directed evolution process, we used a PCR-based method (12) to recombine the mutations of advanced intermediates and final products of the B-GECO, G-GECO, and flash-pericam series. This recombination produced our most improved B- and G-GECOs, but also yielded some unexpected variants. For example, some of the brightest blue fluorescent variants had a tyrosine-derived chromophore and exhibited a Ca^{2+} -dependent change in the ratio of blue (~450 nm) to green (~510 nm) fluorescence when excited at ~400 nm (Fig. 2D). We speculate that, in the Ca^{2+} -free state, the chromophore of this blue-green emission ratiometric GECO (GEM-GECO) undergoes excited-state proton transfer (ESPT) and emits from the lower-energy (green fluorescent) anionic form. In the Ca^{2+} -bound state, ESPT is blocked, and the chromophore emits from the higher-energy (blue fluorescent) neutral form. Additional library creation and screening led to GEM-GECO1 (table S1 and figs. S2 and S4C), which exhibits an 11,000% ratio change and a K_d of 340 nM (table S2).

Yet another unexpected discovery in the recombination library was a green excitation ratiometric GECO (GEX-GECO), which exhibits a large Ca^{2+} -dependent change in the ratio between 400-nm and 488-nm excitation (fig. S5C). This indicator is analogous to the previously reported ratiometric-pericam that undergoes a ~1000% ratio change (4). Further directed

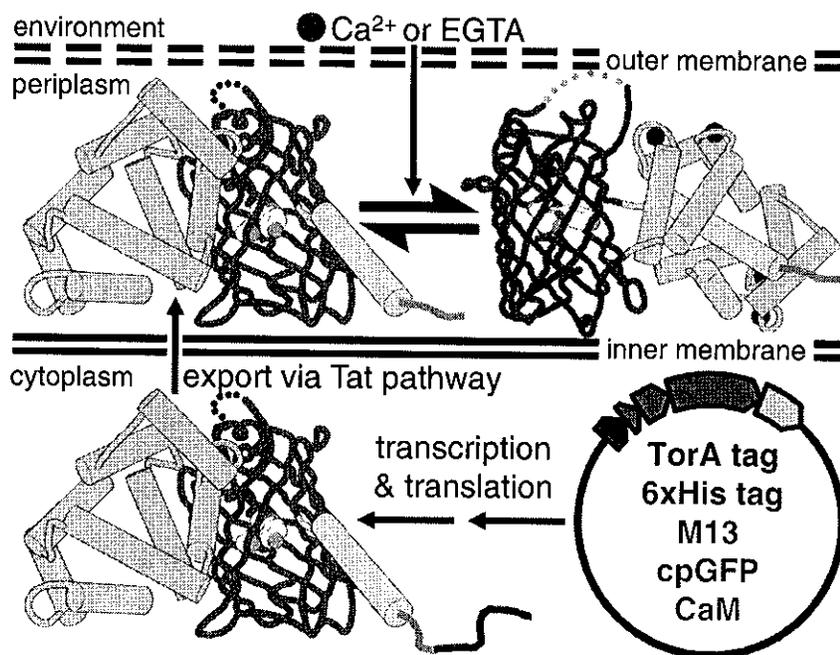


Fig. 1. Schematic of the system for image-based screening of *E. coli* colonies. The GCaMP variant, as represented by GCaMP2 (PDB ID 3EVU and 3EVR) (6), has a TorA periplasmic export tag (17).

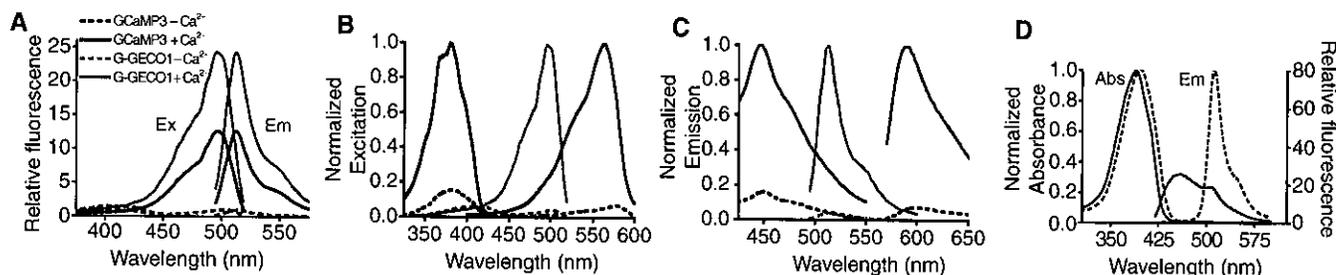


Fig. 2. Spectral profiles of GECOs. (A) Fluorescence excitation (Ex) and emission spectra (Em) normalized to the Ca^{2+} -free state. (B) Normalized excitation spectra of Ca^{2+} -free (dashed line) and Ca^{2+} -bound (solid line) B-GECO1 (blue), G-GECO1 (green), and R-GECO1 (red). (C) Emission spectra represented as in (B). (D) Absorbance (Abs) and emission spectra for Ca^{2+} -free (dashed line) and Ca^{2+} -bound (solid line) GEM-GECO1.

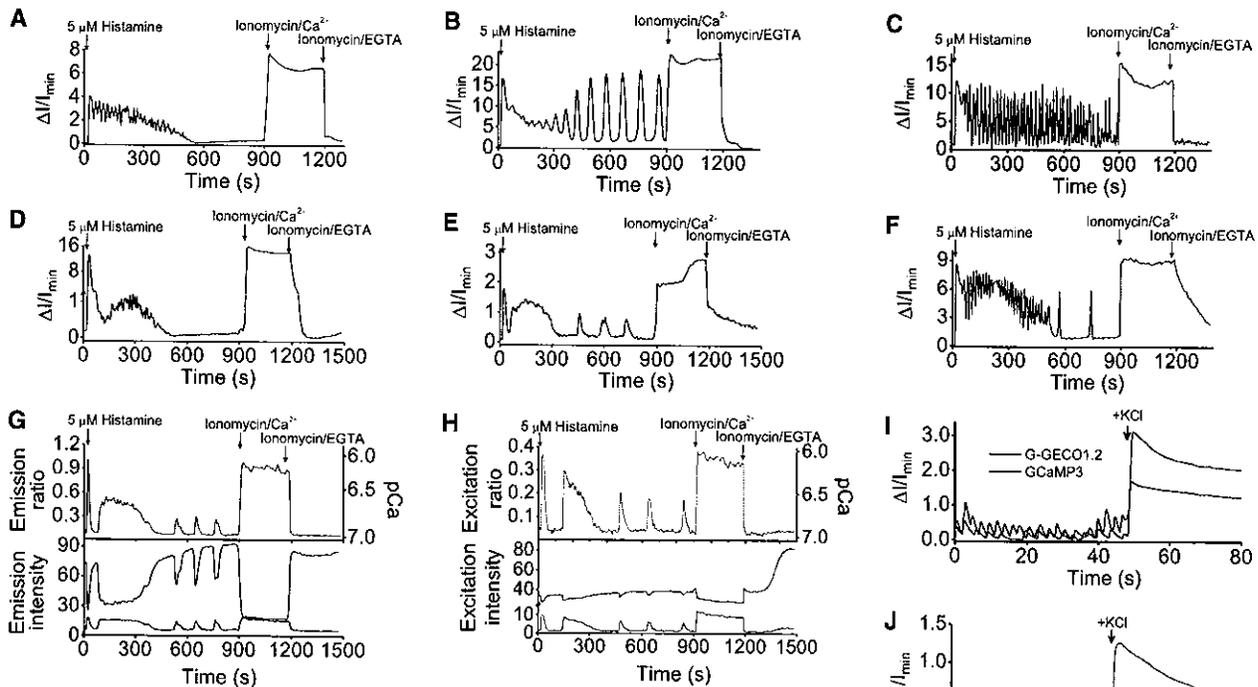


Fig. 3. Single-color imaging with GECOs. (A to H) Intensity versus time traces for transfected HeLa cells. (A) GCaMP3, (B) G-GECO1, (C) G-GECO1.1, (D) G-GECO1.2, (E) B-GECO1, (F) R-GECO1, (G) GEM-GECO1, and (H) GEX-GECO1. Ionomycin/Ca²⁺, ionomycin/EGTA, and the initial histamine-induced spike are generally consistent for a given variant (table S4), but the histamine-induced oscillations are highly variable between cells. Accordingly, lower-amplitude oscillations [e.g., (A) and (D)] do not necessarily indicate poorer indicator performance. (I) Imaging of spontaneous Ca²⁺ oscillations in neurons. (J) R-GECO1 imaged under conditions similar to those in (I). (K) Ratiometric imaging of *C. elegans* with GEM-GECO1 expressed in the AWA neuron. (L) Bright-field and fluorescence images of the worm imaged in (K). Scale bar, 10 μm. Filter specifications are in table S5.

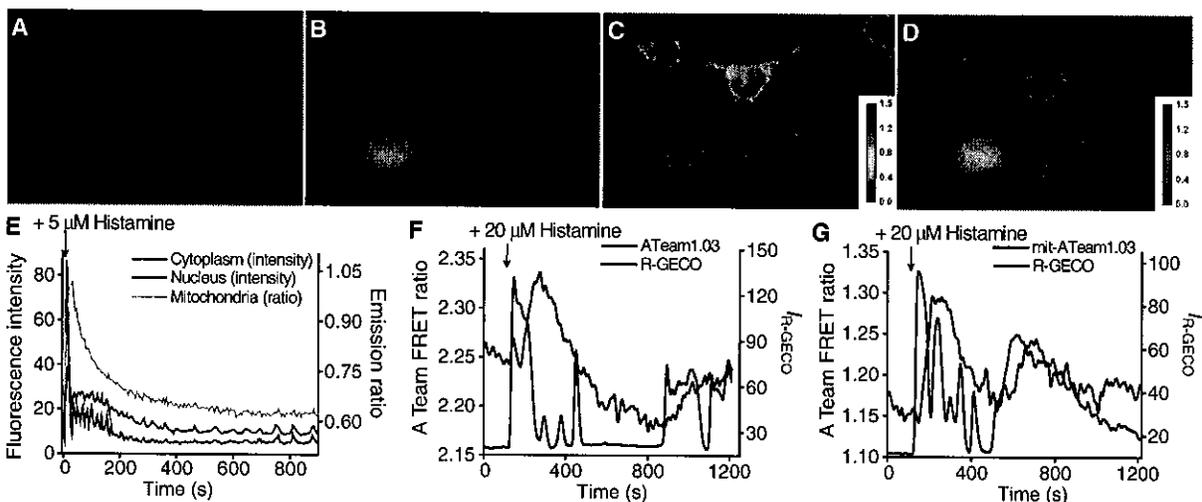


Fig. 4. Multicolor imaging with GECOs. (A to C) HeLa cells transfected with nucleus-localized R-GECO1, cytoplasmic G-GECO1, and mitochondria-localized GEM-GECO1. (A) Red fluorescence. (B) Green fluorescence with cyan (~470 nm) excitation. (C) Pseudocolored ratio of blue to green fluorescence with UV (~380-nm) excitation. (D) Merge of images (A) to (C), with GEM-GECO1 ratio in magenta. Images are from movie S1. (E) Intensity or ratio versus time traces for each channel represented in (A) to (C). Filter specifications are in table S5. (F) Imaging of cytoplasmic Ca²⁺ and ATP. (G) Imaging of cytoplasmic Ca²⁺ and mitochondrial ATP.

evolution produced GEX-GECO1 (table S1 and figs. S2 and S4D), which exhibits a 2600% change in excitation ratio, a K_d of 318 nM, and the most rapid approach to equilibrium (k_{obs}) of any of the GECOs (table S3 and fig. S7).

To explore the utility of our best GECOs, we performed a series of imaging experiments to investigate their performance relative to previously reported indicators and to determine whether they could be used for multicolor Ca^{2+} imaging in single cells. Initially, we expressed individual GECOs in HeLa cells and imaged their intensity (or ratio) during treatment with histamine and in situ dynamic range calibration (table S4 and Fig. 3, A to H). The performance of the GECOs in cells closely paralleled their performance in vitro. We next explored the use of the G- and R-GECO1 in dissociated rat hippocampal neurons (Fig. 3, I and J). Consistent with our HeLa experiments, G-GECO1 proved superior to, and R-GECO1 comparable to, GCaMP3 for imaging of spontaneous Ca^{2+} oscillations. To achieve imaging of neuronal activity in a whole animal, we turned to GEM-GECO1 because of its large ratiometric change. Exposure of a transgenic *Caenorhabditis elegans*, with GEM-GECO1 expressed in the AWA sensory neuron, to a perfused solution of diacetyl produced a 44% ratio change (Fig. 3, K and L), a substantial improvement over the 18% ratio change obtained with YC3.60 under similar conditions (13). This improvement was obtained with a cyan FP–yellow FP (CFP–YFP) filter set that provided diminished autofluorescence signal but is not ideally matched to the GEM-GECO1 spectrum.

The palette of GECOs transforms Ca^{2+} imaging from a monochromatic to a multichromatic endeavor. With appropriate targeting and selection of GECO hues, it should be possible to visualize correlated changes in Ca^{2+} in different organelles of a single cell. To explore this possibility, we imaged HeLa cells cotransfected with plasmids encoding targeted versions of R-GECO1, G-GECO1, and either B-GECO1 or GEM-GECO1 (Fig. 4, A to E). As mitochondrial autofluorescence interferes with the emission of B-GECO1, but not with the ratiometric emission of GEM-GECO1, we prefer the latter three-color combination. To determine whether R-GECO1 could be used to image Ca^{2+} dynamics in conjunction with a CFP–YFP FRET-based indicator, we co-expressed R-GECO1 and ATeam1.03, a FRET-based adenosine 5'-triphosphate (ATP) indicator (14), in HeLa cells. As recently observed with a synthetic Ca^{2+} indicator (15), histamine treatment resulted in transient increases in cytoplasmic (Fig. 4F) and mitochondrial (Fig. 4G) ATP concentrations, albeit with a lag relative to cytoplasmic Ca^{2+} .

In summary, we have engineered a palette of GECOs and have demonstrated that they open the door to Ca^{2+} imaging experiments that were previously impractical. Specifically, these indicators enable imaging of multiple Ca^{2+} indicators in single cells; imaging of neuronal activity

in *C. elegans* with improved sensitivity; and, in the case of R-GECO1, multiparameter imaging with CFP–YFP FRET-based biosensors. In addition, GEM-GECO1 and R-GECO1 should facilitate imaging of neuronal activity after optogenetic channel activation, because they are excited at wavelengths spectrally distinct from the action spectra of certain channels (16).

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5. Single-letter abbreviations for amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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10. Materials and methods are available as supporting material on Science Online.
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15. M. Nakano, H. Imamura, T. Nagai, H. Noji, *ACS Chem. Biol.* **6**, 709 (2011).
16. F. Zhang *et al.*, *Nat. Protoc.* **5**, 439 (2010).
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Acknowledgments: We thank L. L. Looger for GCaMP3 cDNA (Addgene plasmid 22692) and H. Imamura for AT1.03 and mitAT1.03 plasmids. Technical advice or support was provided by J. H. Weiner, Q. Tran, K. Saito, T. Matsuda, N. Sato, N. Yonezawa, the Nikon imaging Center at Hokkaido University, and the University of Alberta Molecular Biology Service Unit. Sequences have been deposited in GenBank (accession nos. JN258409 to JN258415). Plasmid requests will be handled through Addgene and covered under the Uniform Biological Material Transfer Agreement. This work was supported by Alberta Innovates (Y.Z.); Japan society for the Promotion of Science (JSPS), Canadian Institutes of Health Research, and Natural Sciences and Engineering Research Council of Canada (R.E.C.); Ministry of Education, Culture, Sports, Science, and Technology of Japan Grants in Aid for Scientific Research (KAKENHI) 20115003 and 22150003 (T.I.); and PRESTO from the Japan Science and Technology Agency and FIRST from JSPS (T.N.). R.E.C. holds a Tier II Canada Research Chair in Bioanalytical Chemistry.

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1208592/DC1
Materials and Methods
SOM Text
Figs. S1 to S9
Tables S1 to S6
Movie S1
References (18–24)

18 May 2011; accepted 17 August 2011
Published online 8 September 2011;
10.1126/science.1208592

Unfolded Proteins Are Ire1-Activating Ligands That Directly Induce the Unfolded Protein Response

Brooke M. Gardner¹ and Peter Walter^{1,2*}

The unfolded protein response (UPR) detects the accumulation of unfolded proteins in the endoplasmic reticulum (ER) and adjusts the protein-folding capacity to the needs of the cell. Under conditions of ER stress, the transmembrane protein Ire1 oligomerizes to activate its cytoplasmic kinase and ribonuclease domains. It is unclear what feature of ER stress Ire1 detects. We found that the core ER-luminal domain (cLD) of yeast Ire1 binds to unfolded proteins in yeast cells and to peptides primarily composed of basic and hydrophobic residues in vitro. Mutation of amino acid side chains exposed in a putative peptide-binding groove of Ire1 cLD impaired peptide binding. Peptide binding caused Ire1 cLD oligomerization in vitro, suggesting that direct binding to unfolded proteins activates the UPR.

Secretory and transmembrane proteins fold within the endoplasmic reticulum (ER). Contingent on proper folding, they are either exported to the Golgi apparatus or degraded. An

accumulation of unfolded proteins activates the unfolded protein response (UPR), a transcriptional program that restores ER protein-folding homeostasis (1). The transmembrane protein Ire1 induces the UPR by oligomerizing. Oligomerization activates Ire1's cytoplasmic kinase and ribonuclease (RNase) domains and initiates a nonconventional mRNA splicing reaction of *HAC1* mRNA. Spliced *HAC1* mRNA is translated to produce the transcription factor Hac1, which up-regulates protein-folding

¹Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA 94158, USA.
²Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, CA 94158, USA.

*To whom correspondence should be addressed. E-mail: peter@walterlab.ucsf.edu

* *Cholera Toxin*

Cell Biology

ATCC® Number: **CRL-10317™** Price:**\$279.00**

Designations: **MCF 10A**
 Depositors: Michigan Cancer Foundation
 Biosafety Level: 1
 Shipped: frozen
 Medium & Serum: See Propagation
 Growth Properties: adherent
 Organism: *Homo sapiens*
 Morphology: epithelial

Source: **Organ:** mammary gland; breast
Disease: fibrocystic disease
Cell Type: epithelial
 In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Permits/Forms:
 Isolation: **Isolation date:** August 22, 1984
 Applications: transfection host
 Tumorigenic: No

Amelogenin: X
 CSF1PO: 10,12
 D13S317: 8,9
 D16S539: 11,12
 DNA Profile (STR): D5S818: 10,13
 D7S820: 10,11
 THO1: 8,9.3
 TPOX: 9,11
 vWA: 15,17

Isoenzymes: AK-1, 1 [23084]
 ES-D, 1 [23084]
 G6PD, B [23084]
 GLO-I, 1-2 [23084]
 PGM1, 1-2 [23084]
 PGM3, 1 [23084]

Age: 36 years
 Gender: female
 Ethnicity: Caucasian

Related Links▶[NCBI Entrez Search](#)[Make a Deposit](#)[Frequently Asked Questions](#)[Material Transfer Agreement](#)[Technical Support](#)[Related Cell Culture Products](#)**Login Required▶**[Product Information Sheet](#)**BioProducts**[Cell, microbial and molecular genomics products for the life](#)

- [sciences](#)

BioServices[Bio-materials management; basic repository to complex partnership-](#)

- [level services](#)

BioStandards[Biological Reference Material and Consensus Standards for the life science](#)

- [community](#)

The MCF 10A cell line is a non-tumorigenic epithelial cell line.

The line was produced by long term culture in serum free medium with low Ca⁺⁺ concentration.

MCF 10A was derived from adherent cells in the population. Cells derived from a floating population are available (see MCF 10F, ATCC [CRL-10318](#)).

The cells are positive for epithelial sialomucins, cytokeratins and milk fat globule antigen.

They exhibit three dimensional growth in collagen, and form domes in confluent cultures.

Comments:

Thus far, the cells have shown no signs of terminal differentiation or senescence.

The line is responsive to insulin, glucocorticoids, cholera enterotoxin, and epidermal growth factor (EGF).

By electron microscopy the cells display characteristics of luminal ductal cells but not of myoepithelial cells.

They also express breast specific antigens as detected by positive reaction with MFA-Breast and MC-5 monoclonal antibodies.

The calcium content of the medium exerts a strong effect on the morphology of the cells.

ATCC complete growth medium: The base medium for this cell line (MEBM) along with the additives can be obtained from Lonza/Clonetics Corporation as a kit: MEGM, Kit Catalog No. CC-3150. ATCC does not use the GA-1000 (gentamycin-amphotericin B mix) provided with kit. To make the complete growth medium, you will need to add the following components to the kit (sold separately):

Propagation:



- 100 ng/ml cholera toxin

Note: Do not filter complete medium

Temperature: 37.0°C

Atmosphere: air, 95%; carbon dioxide (CO₂), 5%

Protocol: Remove medium and rinse monolayer with PBS (ATCC Cat# 30-2200). Add 3.0 ml 0.05% trypsin, 0.53 mM EDTA and incubate at 37C for 15 minutes. To neutralize trypsin, add 3 ml solution of 0.1% soybean trypsin inhibitor. Centrifuge cell suspension at 125 xg for 5 to 10 minutes. Resuspend cell pellet in complete culture medium. Add appropriate aliquots of cell suspension to new culture vessels.

Subculturing:

Subcultivation Ratio: A subcultivation ratio of 1:3 to 1:4 is recommended

Medium Renewal: Every 2 to 3 days

Preservation:

Freeze medium: Complete growth medium supplemented with 7.5% (v/v) DMSO

Storage temperature: liquid nitrogen vapor phase

Related Products:

derived from same individual: ATCC [CRL-10318](#)

derived from same individual: ATCC [CRL-10780](#)

derived from same individual: ATCC [CRL-10781](#)

21968: Soule H, McGrath CM. Immortal human mammary epithelial cell lines. US Patent 5,026,637 dated Jun 25 1991
22025: Pauley RJ, et al. Immortal human mammary epithelial cell sublines. US Patent 5,206,165 dated Apr 27 1993

22248: Soule HD, McGrath CM. A simplified method for passage and long-term growth of human mammary epithelial cells. In Vitro Cell. Dev. Biol. 22: 6-12, 1986. PubMed:

References:

2418007

*
✱

23084: Soule HD, et al. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res. 50: 6075-6086, 1990. PubMed: 1975513

23085: Tait L, et al. Ultrastructural and immunocytochemical characterization of an immortalized human breast epithelial cell line, MCF-10. Cancer Res. 50: 6087-6094, 1990. PubMed: 1697506

[Return to Top](#)

Material Safety Data Sheet

Version 4.3
Revision Date 12/01/2011
Print Date 02/16/2012

1. PRODUCT AND COMPANY IDENTIFICATION

Product name : **Cholera Toxin Vibrio cholerae**

Product Number : C8052
Brand : Sigma
Product Use : For laboratory research purposes.

Supplier : Sigma-Aldrich Canada, Ltd
2149 Winston Park Drive
OAKVILLE ON L6H 6J8
CANADA

Manufacturer : Sigma-Aldrich Corporation
3050 Spruce St.
St. Louis, Missouri 63103
USA

Telephone : +1 9058299500
Fax : +1 9058299292
Emergency Phone # (For both supplier and manufacturer) : 1-800-424-9300

Preparation Information : Sigma-Aldrich Corporation
Product Safety - Americas Region
1-800-521-8956

2. HAZARDS IDENTIFICATION

Emergency Overview

Target Organs

Bowel

WHMIS Classification

D2B Toxic Material Causing Other Toxic Effects Moderate skin irritant
Moderate eye irritant

GHS Classification

Acute toxicity, Oral (Category 4)
Acute toxicity, Dermal (Category 4)
Skin irritation (Category 3)
Acute aquatic toxicity (Category 3)
Chronic aquatic toxicity (Category 3)

GHS Label elements, including precautionary statements

Pictogram



Signal word Warning

Hazard statement(s)

H302 + H312 Harmful if swallowed or in contact with skin.
H316 Causes mild skin irritation.
H412 Harmful to aquatic life with long lasting effects.

Precautionary statement(s)

P273 Avoid release to the environment.
P280 Wear protective gloves/ protective clothing.

HMIS Classification

Health hazard: 2
Chronic Health Hazard: *

Flammability: 0
Physical hazards: 0

Potential Health Effects

Inhalation May be harmful if inhaled. Causes respiratory tract irritation.
Skin Harmful if absorbed through skin. Causes skin irritation.
Eyes Causes eye irritation.
Ingestion Harmful if swallowed.

3. COMPOSITION/INFORMATION ON INGREDIENTS

Synonyms : Cholera enterotoxin
Cholergen

CAS-No.	EC-No.	Index-No.	Concentration
Tris (hydroxymethyl) aminomethane			
77-86-1	201-064-4	-	>= 5.82 - <= 5.94 %
2-Amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride			
1185-53-1	214-684-5	-	>= 31.3 - <= 31.9 %
Edetate disodium dihydrate			
6381-92-6	205-358-3	-	>= 1.83 - <= 1.87 %
Sodium chloride			
7647-14-5	231-598-3	-	>= 57.6 - <= 58.8 %
Exotoxin, vibrio cholerae			
9012-63-9	-	-	>= 0.5 - <= 2.5 %
Sodium azide			
26628-22-8	247-852-1	011-004-00-7	>= 0.96 - <= 0.98 %

4. FIRST AID MEASURES

General advice

Consult a physician. Show this safety data sheet to the doctor in attendance. Move out of dangerous area.

If inhaled

If breathed in, move person into fresh air. If not breathing, give artificial respiration. Consult a physician.

In case of skin contact

Wash off with soap and plenty of water. Consult a physician.

In case of eye contact

Flush eyes with water as a precaution.

If swallowed

Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.

5. FIREFIGHTING MEASURES

Conditions of flammability

Not flammable or combustible.

Suitable extinguishing media

Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

Special protective equipment for firefighters

Wear self contained breathing apparatus for fire fighting if necessary.

Hazardous combustion products

Hazardous decomposition products formed under fire conditions. - Nature of decomposition products not known.
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx), Hydrogen chloride gas, Sodium oxides

Explosion data - sensitivity to mechanical impact

no data available

Explosion data - sensitivity to static discharge

no data available

6. ACCIDENTAL RELEASE MEASURES**Personal precautions**

Use personal protective equipment. Avoid dust formation. Avoid breathing vapors, mist or gas. Ensure adequate ventilation. Avoid breathing dust.

Environmental precautions

Prevent further leakage or spillage if safe to do so. Do not let product enter drains. Discharge into the environment must be avoided.

Methods and materials for containment and cleaning up

Pick up and arrange disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE**Precautions for safe handling**

Avoid contact with skin and eyes. Avoid formation of dust and aerosols. Provide appropriate exhaust ventilation at places where dust is formed.

Conditions for safe storage

Keep container tightly closed in a dry and well-ventilated place.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Contains no substances with occupational exposure limit values.

Personal protective equipment**Respiratory protection**

For nuisance exposures use type P95 (US) or type P1 (EU EN 143) particle respirator. For higher level protection use type OV/AG/P99 (US) or type ABEK-P2 (EU EN 143) respirator cartridges. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection

Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands.

Eye protection

Safety glasses with side-shields conforming to EN166 Use equipment for eye protection tested and approved under appropriate government standards such as NIOSH (US) or EN 166(EU).

Skin and body protection

Complete suit protecting against chemicals, The type of protective equipment must be selected according to the concentration and amount of the dangerous substance at the specific workplace.

Hygiene measures

Handle in accordance with good industrial hygiene and safety practice. Wash hands before breaks and at the end of workday.

Specific engineering controls

Use mechanical exhaust or laboratory fumehood to avoid exposure.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	solid
Colour	no data available

Safety data

pH	no data available
Melting point/freezing point	no data available
Boiling point	no data available
Flash point	no data available
Ignition temperature	no data available
Autoignition temperature	no data available
Lower explosion limit	no data available
Upper explosion limit	no data available
Vapour pressure	no data available
Density	no data available
Water solubility	no data available
Partition coefficient: n-octanol/water	no data available
Relative vapour density	no data available
Odour	no data available
Odour Threshold	no data available
Evaporation rate	no data available

10. STABILITY AND REACTIVITY

Chemical stability

Stable under recommended storage conditions.

Possibility of hazardous reactions

no data available

Conditions to avoid

no data available

Materials to avoid

Dimethyl sulfate, Acid chlorides, Halogenated hydrocarbon, Metals, Acids

Hazardous decomposition products

Hazardous decomposition products formed under fire conditions. - Nature of decomposition products not known.
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx), Hydrogen chloride gas, Sodium oxides
Other decomposition products - no data available

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Oral LD50

no data available

Inhalation LC50

no data available

Dermal LD50

no data available

Other information on acute toxicity

no data available

Skin corrosion/irritation

no data available

Serious eye damage/eye irritation

Eyes: no data available

Respiratory or skin sensitization

no data available

Germ cell mutagenicity

no data available

Carcinogenicity

IARC: No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.

Reproductive toxicity

no data available

Teratogenicity

no data available

Specific target organ toxicity - single exposure (Globally Harmonized System)

no data available

Specific target organ toxicity - repeated exposure (Globally Harmonized System)

no data available

Aspiration hazard

no data available

Potential health effects

Inhalation	May be harmful if inhaled. Causes respiratory tract irritation.
Ingestion	Harmful if swallowed.
Skin	Harmful if absorbed through skin. Causes skin irritation.
Eyes	Causes eye irritation.

Signs and Symptoms of Exposure

Laboratory experiments in animals have shown sodium azide to produce a profound hypotensive effect, demyelination of myelinated nerve fibers in the central nervous system, testicular damage, blindness, attacks of rigidity, and hepatic and cerebral effects.

Synergistic effects

no data available

Additional Information

RTECS: Not available

12. ECOLOGICAL INFORMATION

Toxicity

no data available

Persistence and degradability

no data available

Bioaccumulative potential

no data available

Mobility in soil

no data available

PBT and vPvB assessment

no data available

Other adverse effects

An environmental hazard cannot be excluded in the event of unprofessional handling or disposal.

Harmful to aquatic life with long lasting effects.

13. DISPOSAL CONSIDERATIONS

Product

Offer surplus and non-recyclable solutions to a licensed disposal company. Contact a licensed professional waste disposal service to dispose of this material.

Contaminated packaging

Dispose of as unused product.

14. TRANSPORT INFORMATION

DOT (US)

Not dangerous goods

IMDG

Not dangerous goods

IATA

Not dangerous goods

15. REGULATORY INFORMATION

WHMIS Classification

D2B	Toxic Material Causing Other Toxic Effects	Moderate skin irritant
		Moderate eye irritant

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations and the MSDS contains all the information required by the Controlled Products Regulations.

16. OTHER INFORMATION

Text of H-code(s) and R-phrases mentioned in Section 3

Further information

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----- Original Message -----

Subject:Re: Fwd: Fwd: New MTA for Dale Laird Lab (Addgene Order 80610) -
Biosafety Modification

Date:Fri, 03 Feb 2012 10:57:05 -0500

From:Cindy Shao <Cindy.Shao@schulich.uwo.ca>

To:Jennifer Stanley <jstanle2@uwo.ca>

CC:Dale Laird <Dale.Laird@schulich.uwo.ca>

Q1: LD₅₀ is 250 (ug/kg)

Q2: 100ng/ml in 500ml so we use 50ug/ml at one time

Q3: 1mg in store

Q4: no

Thank you

Cindy

>>> Jennifer Stanley <jstanle2@uwo.ca> 2/3/2012 10:47 AM >>>
HI there -

Thanks! We'll go up and check the mail.

Thanks for adding the cholera. It was on your permit in 2005 but your approval has since expired.

Please tell us the following info about the cholera use:

- What is the LD₅₀ (specify species) of the toxin? **
- How much of the toxin is handled at one time*? **
 - How much of the toxin is stored*? **
- Will any biological toxins be used in live animals?

Thanks!
Jennifer



TOXIN USE RISK ASSESSMENT

Name of Toxin:	Cholera toxin
Proposed Use Dose:	50 µg
Proposed Storage Dose:	1000 µg
LD₅₀ (species):	250 µg

Calculation:	
	$250 \mu\text{g/kg} \quad \times \quad 50 \text{ kg/person}$
	Dose per person based on LD ₅₀ in µg = 12500
	LD₅₀ per person with safety factor of 10 based on LD₅₀ in µg = 1250

Comments/Recommendations:

Modification Form for Permit BIO-UWO-0017

Permit Holder: Dale Laird

Approved Personnel

(Please stroke out any personnel to be removed)

Jamie Simek
Cindy Pan
Ruchi Bhalla
Jared Churko
Silvia Peneula (Peneula)
Isabelle Plante
Xiang-Qun Gong
Cindy Shao
Jason Martin
Robert Lorentz
Jack Lee
~~Jennifer Siu~~

Additional Personnel

(Please list additional personnel here)

MICHAEL STEWART

Approved Microorganisms

Please stroke out any approved Biohazards to be removed below

E. coli DH5 alpha, JM109

Write additional Biohazards for approval below. *

Approved Primary and Established Cells

Human (primary), skin biopsies, Rodent (primary), transgenic alpha mutant mouse, Human (established), HELA, 293T, 293 Hek, Tumour cell lines, Rodent (established), Keratinocytes, NRK BiCR, MIRK, N2A,

Established cell lines:
B16-F0, B16-F10, B16-BL6
(mouse skin melanoma)

Approved Use of Human Source Material

Human blood (whole), Human tissues (unpreserved), Human tissues (preserved), ODDD Patients and relatives

Approved Genetic Modifications (Plasmids/Vectors)

JM109, T-EASY, PcDNA3, pEGFP, retrovirus AP-2, SV 40 Large T antigen, HEK, E1A oncogenes, HeLa, pGFP-V-RS

Approved Use of Animals

mice

Approved Biological
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 STORED, USED AND DISPOSED OF..

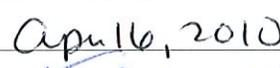
As the principal investigator, I have ensured that all of the personnel named on the form have been trained. I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). 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: 

Current Classification: 2 Containment Level for Added Biohazards: 1

Date of Last Biohazardous Agents Registry Form: Mar 27, 2009

Date of Last Modification (if applicable): Dec 21, 2009

BioSafety Officer(s):   April 16, 2010

Chair, Biohazards Subcommittee:  Date: _____

Modification Form for Permit BIO-UWO-0017

Permit holder: Dale Laird

Additional Biohazards for approval:

Established cell lines:

B16-F0, B16-F10, B16-BL6 (mouse skin melanoma). The first two cell lines were obtained from ATCC (see data sheets enclosed) and are Biosafety level 1. The B16-BL6 cell line was originally derived from B16-F10 (see paper attached by Poste, et al.) and it was kindly provided to us by Dr. Moulay Alaoui-Jamali of the Lady Davis Institute at McGill University.

These cell lines will be used for cell culture in vitro, shRNA silencing of Pannexins, protein and RNA extractions, and other cell biology and biochemistry work currently performed with other cell lines in our lab. They will be handled and disposed according to the current biosafety regulations.



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Customers in Europe, Australia, Canada, China, Hong Kong, India, Israel, Japan, Korea, Macau, Mexico, New Zealand, Singapore, and Taiwan, R.O.C. must contact a [local distributor](#) for pricing information and to place an order for ATCC cultures and products.

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Cell Biology

ATCC® Number: CRL-6322™
Price: \$331.00

Designations: B16-F0

Related Links ▶
Biosafety Level: 1

[NCBI Entrez Search](#)
Shipped: frozen

[Make a Deposit](#)
Medium & Serum: [See Propagation](#)
[Frequently Asked Questions](#)
Growth Properties: adherent

[Material Transfer Agreement](#)
Organism: *Mus musculus* (mouse)

[Technical Support](#)
Morphology: Spindle shaped

[Related Cell Culture Products](#)
Source: **Organ:** skin
Strain: C57BL/6J
Disease: melanoma

Permits/Forms: In addition to the [MTA](#) mentioned above, other [ATCC and/or regulatory permits](#) may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please [click here](#) for information regarding the specific requirements for shipment to your location.

Applications: transfection host ([Roche FuGENE® Transfection Reagents](#)
[Nucleofection technology from Lonza](#))

Tumorigenic: Yes

Propagation: **ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.

Atmosphere: air, 95%; carbon dioxide (CO₂), 5%

Temperature: 37.0°C

Subculturing: **Protocol:**

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
 Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:4 to 1:10 is recommended

Medium Renewal: Every 2 to 3 days



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Cell Biology

ATCC® Number: CRL-6475™ [Order this Item](#)
Price: \$269.00

Designations: B16-F10

Related Links ▶
Biosafety Level: 1

[NCBI Entrez Search](#)
Shipped: frozen

[Cell Micrograph](#)
Medium & Serum: [See Propagation](#)
[Make a Deposit](#)
Growth Properties: adherent

[Frequently Asked Questions](#)
Organism: *Mus musculus* (mouse)

[Material Transfer Agreement](#)
Morphology: melanocyte

Source: Organ: skin

Strain: C57BL/6J

Disease: melanoma

Permits/Forms: In addition to the [MTA](#) mentioned above, other [ATCC and/or regulatory permits](#) may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please [click here](#) for information regarding the specific requirements for shipment to your location.

Applications: transfection host ([technology from amaxa](#))

Propagation: **ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.

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4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:10 is recommended

Medium Renewal: Every 2 to 3 days

Cell Line Designation: B16-F0
ATCC[®] Catalog No. CRL-6322[™]

Table of Contents:

- Cell Line Description
- Biosafety Level
- Use Restrictions
- Handling Procedure for Frozen Cells
- Handling Procedure for Flask Cultures
- Subculturing Procedure
- Medium Renewal Procedure
- Complete Growth Medium
- Cryoprotectant Medium
- Replacement Policy

Cell Line Description

Organism: *Mus musculus* (mouse)

Strain: C57BL/6J

Tissue: melanoma; skin

Tumorigenic: yes, in syngeneic mice

Growth properties: adherent

Morphology: spindle shaped

Depositors: Naval Biosciences Laboratory

Comments: Confirmed as a murine cell line by ATCC.

Tests for microbial contamination were negative.

DISCLAIMER: This cell line was deposited by the Naval Biosciences Laboratory. Researchers should be aware that one purpose of the NBL bank was to produce and distribute early passage cultures of cells from various clinical materials. Many of these lines, unlike most others available and published in the ATCC catalog, are primary and mixed. Those from tumors, for example, may consist of mixtures of stromal and cancer cells in which the former cell types predominate.

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

Use Restrictions

~~These cells are distributed for research purposes only.~~ ATCC recommends that individuals contemplating commercial use of any cell line first contact the originating investigator to negotiate an agreement. Third party distribution of this cell line is discouraged, since this practice has resulted in the unintentional spreading of cell lines contaminated with inappropriate animal cells or microbes.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at -70°C . Storage at -70°C will result in loss of viability.

SAFETY PRECAUTION: ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. It is important to note that some vials leak when submerged in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.

1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).
2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. *All of the operations from this point on should be carried out under strict aseptic conditions.*
3. Transfer the vial contents to a centrifuge tube containing 9.0 ml complete culture medium and spin at approximately $125 \times g$ for 5 to 7 minutes. Discard supernatant.
4. Resuspend the cell pellet with the recommended complete medium and dispense into a 25 cm^2 culture flask. It is important to avoid excessive alkalinity of the medium during recovery of the cells. *It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).*
5. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended if using the medium described on this product sheet.

Handling Procedure for Flask Cultures

The flask was seeded with cells, grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the



Product Information Sheet for CRL-6322

Lot number: 58254426

Designation: B16-F0 Description: Melanoma
Total Cells/mL: 8.5×10^5
Expected Viability: 85.1% to 96.3%
Ampule Passage No.: Unknown
Population Doubling (PDL): N/A
Dilute Ampule Content: 1:10 (T-25) or 1:15 (T-75)
Volume/Ampule: 1 mL
Date Frozen: 12/18/08

A T-25 setup at a dilution of 1:10, using culture medium as described in the product information sheet, reaches approximately 60% to 70% confluence in 2 days.

- If the cells are still attached, aseptically remove all but 5 to 10 ml of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO₂ in air atmosphere until they are ready to be subcultured.
- If the cells are not attached, aseptically remove the entire contents of the flask and centrifuge at 125 x g for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 ml of this medium and add to 25 cm² flask. Incubate at 37°C in a 5% CO₂ in air atmosphere until cells are ready to be subcultured.

Subculturing Procedure

Volumes used in this protocol are for 75 cm² flasks; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

- Remove and discard culture medium.
- Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.
- Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).

Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

- Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
- Add appropriate aliquots of the cell suspension to new culture vessels.
Subculture ratio: 1:6 to 1:10
- Incubate cultures at 37°C.

Note: For more information on enzymatic dissociation and subculturing of cell lines consult Chapter 13 in **Culture Of Animal Cells: A Manual Of Basic Technique** by R. Ian Freshney, 5th edition, published by Wiley-Liss, N.Y., 2005.

Medium Renewal

Every 2 to 3 days

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium:

- fetal bovine serum to a final concentration of 10%

This medium is formulated for use with a 5% CO₂ in air atmosphere. (Standard DMEM formulations contain 3.7 g/L

sodium bicarbonate and a 10% CO₂ in air atmosphere is then recommended).

ATCC tested fetal bovine serum is available as ATCC[®] Catalog No. 30-2020 (500ml) and ATCC[®] Catalog No. 30-2021 (100ml).

Cryoprotectant Medium

Complete culture medium described above supplemented with 5% (v/v) DMSO. Cell culture tested DMSO is available as ATCC[®] Catalog No. 4-X.

Additional Information

Additional product and technical information can be obtained from the catalog references and the ATCC Web site at www.atcc.org, or by e-mail at tech@atcc.org.

References

(Additional references may be available in the catalog description at www.atcc.org)

Fidler IJ. **Biological behavior of malignant melanoma cells correlated to their survival in vivo.** *Cancer Res.* 35: 218-224, 1975 PubMed: 75074100

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Fleming, D.O., Richardson, J. H., Tulis, J.J. and Vesley, D., (1995) **Laboratory Safety: Principles and Practice.** Second edition, ASM press, Washington, DC.

ATCC Warranty

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Disclaimers

This product is intended for laboratory research purposes only. It is not intended for use in humans.

While ATCC uses reasonable efforts to include accurate and up-to-date information on this product sheet, ATCC makes no warranties or representations as to its accuracy. Citations



Product Information Sheet for CRL-6475

Accession number: 58078645

Designation: B16-F10 Description: Melanoma

Total Cells/mL: 7.3×10^5

Expected Viability: 66.7% to 75.9%

Ampule Passage No.: Unknown

Population Doubling (PDL): N/A

Dilute Ampule Content: 1:10 (T-25) or 1:15 (T-75)

Volume/Ampule: 1 mL

Date Frozen: 10/01/08

A T-25 setup at a dilution of 1:10, using culture medium as described in the product information sheet, reaches approximately 60% to 70% confluence in 2 days.

B16-
first → BL6 cells
report

In Vitro Selection of Murine B16 Melanoma Variants with Enhanced Tissue-invasive Properties

G. Poste,^{1, 2} J. Doll,¹ I. R. Hart,³ and I. J. Fidler³

Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, New York 14263 [G. P., J. D.], and Cancer Biology Program, National Cancer Institute, Frederick Cancer Research Center, Frederick, Maryland 21701 [I. R. H., I. J. F.]

ABSTRACT

New assay methods have been devised to quantitate tumor cell invasion of tissues of differing histological complexity maintained as organ cultures *in vitro* (chorioallantoic membrane of chicken, mouse urinary bladder, and canine blood vessel). In addition to quantitating tumor cell invasion, these methods also allow recovery of invasive cells for comparison with noninvasive cells. These methods have been used to select variant sublines from murine B16-F1 and B16-F10 melanoma lines that display significantly greater tissue-invasive abilities than the parent lines. B16 variant sublines selected *in vitro* for increased invasiveness through the bladder wall or vein also show a significant increase in their ability to form spontaneous and experimental metastases *in vivo*. In contrast, cells from the same parent cell line selected for increased invasiveness through the chorioallantoic membrane do not show significant alterations in metastatic behavior. We conclude that invasive variants can be isolated from the parent B16 tumor by several *in vitro* methods and that the level of expression of the invasive phenotype *in vivo* may be determined by the severity of the selection procedure *in vitro*.

INTRODUCTION

Invasion of lymphatics and blood vessels by malignant cells provides a major pathway for the dissemination of neoplastic cells within the body (for reviews, see Refs. 7, 8, and 27). In addition, following their arrest in the capillary beds of different organs, circulating tumor cells invade the wall of these vessels and escape into the extravascular tissue(s) where they establish metastases. Despite its obvious importance in the metastatic process, relatively little is known about the mechanism(s) of tumor invasion. This deficiency reflects the formidable technical problems encountered in studying invasion *in vivo* and the lack of quantitative methods for studying invasion *in vitro* and for recovering invasive cells once invasion has taken place.

A substantial body of evidence has been assembled in the last few years which indicates that primary malignant neoplasms are not homogeneous entities of cells with uniform properties but instead contain subpopulations of tumor cells with widely differing metastatic abilities (for reviews, see Refs. 7, 8, and 27). This phenotypic heterogeneity dictates that examination of heterogeneous unselected tumor cell populations may offer little insight into the cellular properties responsible for invasion and/or metastasis if only very few cells within

the population express these behavioral traits. A more productive approach to the experimental analysis of the cellular properties needed for successful invasion is to isolate invasive tumor cell subpopulations and compare them with poorly invasive or noninvasive tumor cells derived from the same parent cell population. In this report, we describe methods for the *in vitro* isolation of a series of murine B16 melanoma cell variants with enhanced invasive properties and an initial characterization of their behavior *in vivo*.

MATERIALS AND METHODS

Cells. The origin and properties of the B16-F1 (low potential for lung colonization) and B16-F10 (high potential for lung colonization) sublines of the B16 melanoma have been described in detail previously (5, 12). Cultures of mouse embryo fibroblasts were prepared by trypsinization of 14- to 16-day-old C57BL/6 mouse fetuses as described elsewhere (29). Homogeneous peritoneal macrophage cultures were obtained from C57BL/6 mice inoculated i.p. with sodium thioglycollate (Baltimore Biological Laboratories, Cockeysville, Md.) as described previously (29). All cells were incubated in plastic flasks or Petri dishes in CMEM⁴ (12, 29). The components of CMEM were obtained from Flow Laboratories, Inc., Rockville, Md., and the Grand Island Biological Co., Grand Island, N. Y. Cell cultures were incubated at 37° in a humidified atmosphere containing 5% CO₂ in air. All cultures were free of *Mycoplasma* species and pathogenic murine viruses (12), and they remained so throughout the experiments. In certain experiments, cell cultures (except macrophages) were labeled with [¹²⁵I]dUrd as detailed in Ref. 5. Briefly, actively growing nonconfluent monolayer cell cultures were incubated for 24 hr in CMEM containing [¹²⁵I]dUrd (0.2 μCi/ml; specific activity, 200 μCi/mmol; New England Nuclear, Boston, Mass.). This method labels more than 95% of the cells and does not alter their metastatic behavior *in vivo* (5).

Assay of Tumor Cell Invasion in CAM and Selection of Invasive Cell Variants. Newly fertilized eggs were incubated at 38° in a humidified atmosphere (relative humidity, approximately 75%) in an egg incubator (Favorite Incubator; Leahy Mfg. Co., Higginsville, Mo.) until Day 8 after fertilization, when the CAM was dropped using the false air sac method as described elsewhere (28). Two days later, the CAM was harvested and dissected by a sterile technique into pieces approximately 0.625 inch square for eventual transfer to the

¹ Recipient of USPHS Research Grant CA 18260 and NIH Core Support Grant 17809 to Roswell Park Memorial Institute Cancer Cell Center.

² To whom requests for reprints should be addressed.

³ Recipient of National Cancer Institute Contract NO1-CO-75380 with Litton Bionetics Inc.

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⁴ The abbreviations used are: CMEM, Eagle's minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and 2-fold concentrated vitamin solution; [¹²⁵I]dUrd, [¹²⁵I]dodeoxyuridine; CAM, chorioallantoic membrane; o.d., outside diameter; i.d., internal diameter; MEM, Eagle's CMEM (see above) without serum; HBSS, Hanks' balanced salt solution.

penetrating the new CAM's were, in turn, harvested and passaged again through fresh CAM's up to 14 times. These cells will be described by the prefix CP (CAM-passage) followed by the number of times the cells had been passaged through CAM (e.g., B16-F1-CP10 = B16-F1 cells passaged 10 times through the CAM).

In most selection experiments, nutrient agar was used in the lower chamber. Normal CAM cells were unable to grow in agar, but B16 tumor cells could form colonies in agar because of their loss of anchorage dependence. In certain experiments, fluid culture medium (CMEM) or factors which are chemotactic for macrophages or leukocytes and/or certain tumor cells were placed in the bottom compartment to determine if penetration of radiolabeled tumor cells was affected by such chemotactic stimuli. Bacterial culture filtrates containing leukotactic factors were produced from cultures of *Escherichia coli* (WHO reference strain 16) as described by Ward *et al.* (41). Serum fractions containing leukotactic factors were produced by zymosan activation of human serum in the presence of 1 M ϵ -aminocaproic acid (40). Zymosan-activated sera were incubated with trypsin using the method described by Orr *et al.* (25) to generate material containing factors from the third and fifth components of complement which are chemotactic for some tumor cells (25). All of the above materials were added to MEM at a final concentration of 75 μ l/ml. In other experiments, the bottom chamber was filled with cell-free culture supernatant fluid harvested from the same tumor cell population added to the upper chamber.

Assay of Tumor Cell Invasion in Mouse Urinary Bladder and Selection of Invasive Cell Variants. Adult male C57BL/6 mice were killed by ether inhalation. The mice were submerged in a 5% Wescodyne solution (West Chemical Products, New York, N. Y.), immersed in 70% ethanol for 2 min, and placed into a laminar flow hood. The abdominal cavity was opened in a sterile fashion, and the urogenital system was fully exposed (Fig. 3). Urine within the urinary bladder was expressed by gentle finger pressure (sterile surgical gloves were worn during these procedures), after which the urethra was occluded by application of a hemostat. One testis was displaced from the scrotal sac, and the ductus deferens was identified and spread across an open pair of forceps, with care being taken not to rupture the duct. The ductus deferens was penetrated (Fig. 3) using a 27-gauge needle (Becton, Dickinson, and Company, Rutherford, N. J.) attached to a tuberculin syringe, and 0.3 to 0.4 ml of tumor cell suspension (0.9 to 1.2×10^6 cells) was injected slowly. The injected cells reached the urethra via the emergence of the ductus deferens in the prostrate, and retrograde urethral flow carried suspended tumor cells to the bladder which gradually became filled with cells (Fig. 4). A ligature of 2-0 chromic catgut (Detnatel, Queens Village, New York, N. Y.) was placed around the neck of the bladder distal to the entrance of the ureters and tied tightly (Fig. 4). The bladder was then excised from the abdominal cavity using hot scissors (flamed) in order to cauterize the cut tissue. The ligated isolated bladder was then vigorously rinsed in 4 changes of HBSS containing gentamicin sulfate (0.1 mg/ml; Schering Corporation, Kenilworth, N. J.). The excised bladders were then placed in sterile 60-mm plastic Petri dishes containing a preformed base of semisolid agar produced by pouring 5 ml of agar (see below) into dishes followed by chilling at 4° for 10 min to solidify the agar. The semisolid agar medium was prepared by

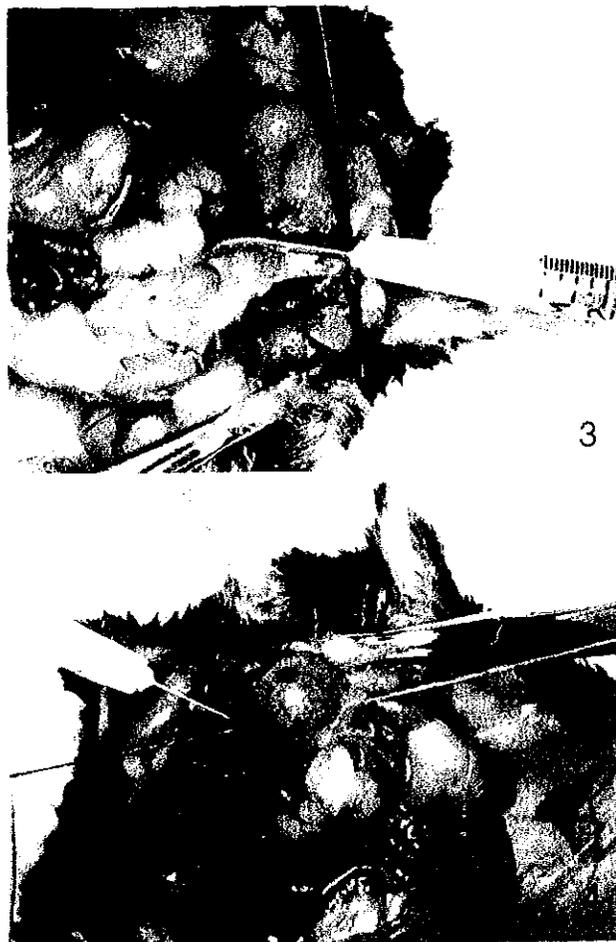


Fig. 3. Retrograde filling of murine urinary bladder with tumor cells via the vas deferens. The bladder has been emptied mechanically, and the ductus deferens is cannulated by a 27-gauge needle.

Fig. 4. Tumor cell suspension (0.3 to 0.4 ml) has been injected into the ductus deferens, and the urinary bladder is filled by retrograde flow. Hemostat occludes the penile urethra and ligature is 2-0 catgut.

mixing 20 ml 2 \times MEM, 5 ml fetal calf serum, 5 ml tryptose phosphate broth (all reagents from Grand Island Biological Co.), and 20 ml 1.25% agar (Difco Laboratories, Detroit, Mich.). The excised bladders were placed on the agar base, neck vertically up, and semisolid supporting agar, composed of 2 volumes of agar base to 1 volume of CMEM, was added to a point just below the ligature. Each bladder was restrained in the vertical position by stretching the free ends of the ligating catgut at 180° to one another and placing the Petri dish lid on the free ends. Thus, solidification of the agar maintained the bladders in the correct upright position. One bladder was placed in a single Petri dish, and between 5 and 10 bladders were used to obtain cells at each passage level (see below). Individual bladders were removed from the agar at daily intervals after incubation at 37° in a humidified atmosphere containing 5% CO₂ for up to 5 days. The agar situated around the site where the base of the bladder had rested was then pipetted up and added to 1 ml CMEM in a single well of a Costar 16-mm multiwell dish (Costar, Cambridge, Mass.). These dishes were incubated under the same conditions as described above, and the wells were examined daily for tumor colonies. When tumor

hollow fiber capillary bundles have been replaced by a 15-cm length of ultrahigh-molecular-weight polyethylene tube. The vein is fitted onto this tube before being placed into the chamber. Segments of dog femoral vein were perfused *in situ* with ice-cold 0.9% NaCl solution before removal from the animal. Collateral vessels were tied off or occluded by a diathermy needle before removing the main portion of vein. Veins were fitted onto the tube by inserting the tube into the lumen of the vessel for a sufficient distance to completely cover the tube. Two encircling catgut ties were then made at each end of the tube, and the excess vein was trimmed with a scalpel. The tube and vessel were then placed into the polycarbonate outer chamber and sealed with screw-fit end pieces and silicon rubber sealing O-rings. Both ends of the tube were then connected to 0.132 inch i.d. x 0.183 inch o.d. gas-permeable medical grade silicone tubing (Dow Corning Corp., Midland, Mich.) to create a closed perfusion circuit with an intervening reservoir of culture medium. The tubing between the reservoir and the input side of the chamber was passed through a 500-ml conical flask containing a mixture of 5% CO₂ in air to ensure correct gaseous exchange. Tumor cells (1×10^6) in CMEM (1.5 ml) were then injected into the outer chamber, and the entire apparatus was incubated at 37° in a walk-in hot room. For invasion assays, ¹²⁵I-labeled cells were injected into the outer chamber, and the number of cells penetrating the wall of the vessel was determined by measuring the radioactivity recovered in the inner perfusion circuit. For selection experiments, nonradiolabeled cells were introduced, and invasive cells harvested from the internal circuit were reinjected into chambers containing fresh vessels. The invasive cell populations recovered by serial passage through veins will be referred to by the prefix BV (blood vessel) followed by the number of times the cells have been passaged through veins.

Animals. Specific-pathogen-free C57BL/6 mice were obtained from the Animal Production Area, Frederick Cancer Research Center, and the West Seneca Laboratories, Roswell Park Memorial Institute.

Metastasis Formation. The ability of cells to form experimental metastases after i.v. injection was measured by injecting 5×10^4 to 5×10^5 viable cells as a single-cell suspension in 0.2 ml HBSS into the tail vein of unanesthetized adult C57BL/6 mice matched for age, weight, and sex. Mice were killed 18 or 21 days later, and the number of lung metastases was determined with a dissecting microscope as described previously (6, 9).

The following methods were used to assay the ability of cells to form spontaneous metastases: (a) C57BL/6 mice were given s.c. injections in the external ear of 5×10^4 cells in 0.1 ml HBSS, and the number of lung metastases was determined 4 weeks later; (b) C57BL/6 mice were given s.c. injections in the ear as above. The ear plus the growing tumor were then amputated 10 days later (7), and formation of regional lymph node and lung metastases was determined 4 to 6 weeks later; or (c) C57BL/6 mice were given i.m. injections of 2.5×10^4 viable cells in 0.5 ml HBSS in the footpad of the hind leg. In some animals, the "primary" tumor was amputated after 4 weeks, and metastasis formation was determined 4 weeks thereafter. In other animals, the "primary" tumor was not amputated, and metastasis formation was measured 6 weeks after initial tumor cell injection. In all of the protocols, mice were matched for age, weight, and sex.

RESULTS

In Vitro Selection of B16 Melanoma Cell Variants with Increased Ability to Invade CAM. The ability of B16-F1 melanoma cells to invade and penetrate CAM maintained *in vitro* and the invasive behavior of variants selected from B16-F1 cells by serial passage of cells that successfully penetrate the CAM are shown in Table 1. The results indicate that the proportion of cells in the parent cell population that penetrate through the CAM to reach the lower chamber (Fig. 2) is very low. Serial passage of the invasive cell fractions recovered from the lower chamber produced a significant increase in the proportion of cells penetrating the CAM. Maximum efficiency of invasion was achieved after 10 passages through the CAM (Table 1).

Confirmation that invasion in this system involves selection (enrichment) of subpopulations with increasing invasive properties was provided by the finding that the "nonpenetrating" cell fractions recovered from CAM's inoculated with early-passage populations contained significantly fewer invasive cells than did the starting inoculum (Table 1). With increasing CAM passage, however, the proportion of invasive cells recovered in the nonpenetrating fraction increased. After 10 passages, the efficiency of invasion by the reinoculated nonpenetrating fraction was similar to that of the original inoculum, suggesting that the cell population was now composed almost exclusively of invasive cells. Thus, in early passages, invasion was a nonrandom process which selects for invasive cell subpopulations. With subsequent passaging, the proportion of invasive cells in the population was enriched; by 10 passages, invasion had become a random process and the population consisted of cells with invasive properties.

The invasive behavior of variants at any given passage level is highly reproducible, and significant variation has not been detected using different CAM preparations. In addition, assays

Table 1

Invasiveness of B16-F1 mouse melanoma cells during serial passage in chick CAM in vitro

Alliquots of 1×10^6 viable B16-F1 melanoma cells in CMEM were added to the ectodermal surface of portions of CAM in invasion chambers. Cells penetrating the CAM were recovered from the bottom chamber of the invasion apparatus 7 days later and reinoculated onto fresh CAM preparations. This was repeated up to 14 times. At each passage level, [¹²⁵I]dUrd-labeled cells (penetration assay) and nonradiolabeled cells (serial passaging) derived from a common parent culture were used.

Passage no.	% of inoculated cells penetrating the CAM ^a	% of "nonpenetrating" cell fraction penetrating new CAM ^b
1	2.4 ± 0.6 ^c	Not detectable
2	4.1 ± 1.1	1.3 ± 0.4
4	11.6 ± 2.3	3.9 ± 0.8
6	17.4 ± 3.7	13.7 ± 2.1
8	23.7 ± 4.4	18.4 ± 2.7
10	31.6 ± 4.9	27.6 ± 4.3
14	30.6 ± 4.2	33.4 ± 4.6

^a Cell-associated [¹²⁵I]dUrd radioactivity recovered in the bottom chamber as a percentage of total radioactivity in the original cell inoculum. Inocula contained between 3.5 and 6×10^6 cpm/ 10^6 cells.

^b Nonradiolabeled cells (1×10^5) were added to CAM invasion chambers and incubated for 7 days at 37° after which "nonpenetrating" cells which failed to reach the bottom chamber were recovered from the culture medium in the upper chamber and from the CAM (see "Materials and Methods"). These were cultured *in vitro* to generate sufficient numbers of cells, labeled with [¹²⁵I]dUrd, and reinoculated onto fresh CAM to assay cell penetration as described in Footnote a.

^c Mean ± S.E. derived from measurements on 8 replicate chambers at each passage.

Invasion of Different Tissues by Variants Selected in CAM, Bladder, and Vein. Selection of cells for invasiveness in one tissue may not necessarily enable them to invade other tissues. This was demonstrated by showing that CAM-passaged invasive variants did not differ from unselected parent F1 cells in their ability to invade bladder or vein (Table 4). In contrast, bladder- and vein-passaged variants were highly invasive in the CAM, urinary bladder, and vein preparations (Table 4).

Formation of Spontaneous and Experimental Metastases by Invasive Variants. Assay of the ability of CAM-passaged invasive cell variants to form experimental metastases after i.v. injection or spontaneous metastases after s.c. injection into the external ear revealed that their metastatic activity did not differ significantly from parental B16-F1 cells (Table 5). In contrast, cells from invasive variants selected by passaging in bladder organ culture (BP series) or perfused vein (BV series) produced significantly more pulmonary metastases than did parent B16-F1 cells following both i.v. (experimental metastasis) and s.c. (spontaneous metastasis) injection (Table 5). Indeed, the metastatic activity of these invasive variants is comparable to that of the highly metastatic B16-F10 line which was selected from the B16-F1 line for its ability to produce lung metastases following i.v. injection (5).

Table 3

Invasiveness of B16-F1 mouse melanoma cells during serial passage in segments of dog femoral vein maintained in a perfusion apparatus in vitro

Segments of dog femoral vein in a perfusion culture apparatus were injected with 1×10^6 B16-F1 cells and maintained for 2 weeks at 37°. Cells that penetrated the blood vessel wall were recovered from the inner perfusion circuit (see "Materials and Methods") and reinjected onto fresh vein preparations. This process was repeated up to 10 times. Replicate populations of [¹²⁵I]dUrd-labeled cells (penetration assay) and nonlabeled cells (serial passaging) from the same parent culture were used at each passage.

Passage no.	% of inoculated cells penetrating vessel wall ^a	% of "nonpenetrating" cell fraction penetrating new vessel ^b
1	1.6 ± 0.4 ^c	0
2	19.3 ± 4.2	11.3 ± 2.9
3	32.4 ± 4.9	20.7 ± 3.6
4	36.9 ± 5.6	30.7 ± 4.8
6	35.8 ± 3.7	31.3 ± 4.2
8	31.6 ± 4.4	32.7 ± 5.0

^a Cell-associated [¹²⁵I]dUrd radioactivity recovered in the inner perfusion circuit as a percentage of the total radioactivity in the original cell inoculum.

^b Cells were injected into the outer chamber as in Footnote a, and the chamber was incubated for 4 days at 37°. Cells in the outer chamber which failed to attach to the vessel were aspirated, cultivated *in vitro* for a short period to provide sufficient cells, labeled with [¹²⁵I]dUrd, and reinjected onto fresh vessels, and their penetration was assayed as in Footnote b.

^c Mean ± S.E. derived from measurements on 2 chambers at each passage.

Table 4

Invasiveness of B16 melanoma cell variants in different tissues in vitro

Cell ^a	% of cell penetration ^b		
	CAM	Bladder	Vein
Parent B16-F1	2.3 ± 0.5 ^c	1.3 ± 0.4	1.6 ± 0.4
B16-F1-CP10	29.8 ± 4.3	1.9 ± 0.6	2.3 ± 0.5
B16-F1-BP8	38.2 ± 5.1	30.7 ± 4.3	24.7 ± 3.9
B16-F1-CP10, BP4	30.9 ± 4.0	22.6 ± 4.6	14.3 ± 3.1
B16-F1-BV6	47.2 ± 6.8	26.5 ± 4.1	33.2 ± 4.5

^a The suffix designations for B16-F1 cells are: CP10, 10 passages in CAM; BP8, 8 passages in bladder organ cultures; CP10, BP4, 10 passages in CAM followed by 4 passages in bladder organ cultures; BV6, 6 passages through vein preparations.

^b Preparations of the indicated cell types labeled with [¹²⁵I]dUrd were assayed for their ability to penetrate CAM, mouse bladder organ cultures, and perfused dog femoral vein as described in Tables 1, 2, and 3, respectively.

^c Mean ± S.E. derived from measurements on 4 replicate assay preparations for CAM and bladder and 2 for vein.

The BL6 variant selected from B16-F10 cells by passaging in intact bladder is also more efficient than the parental B16-F10 cells in producing spontaneous metastases. However, unlike the BP variants passaged in bladder organ culture, the BL6 variant is less efficient than the parent B16-F10 line in producing experimental metastases after i.v. injection (Table 6).

DISCUSSION

A variety of *in vitro* systems have been used to study invasion of normal tissues by malignant tumor cells. Attempts to study invasion by allowing normal and tumor cell populations to interact in monolayer culture (1-3, 13, 15) are probably oversimplified since tumor cells are not required to invade an organized tissue matrix. Invasion of tumor cells into aggregates of normal cells (10, 16) suffers from similar disadvantages. In addition, doubts must be expressed about the functional significance of tumor cell infiltration into aggregates in view of

Table 5
Metastasis formation by cell variants selected from B16-F1 melanoma cells

Cell ^a	Metastasis formation	
	Spontaneous ^b	Median no. of pulmonary colonies ^c
B16-F1	2/20 (10) ^d	9 (2-27) ^e
B16-F10	12/20 (60)	111 (23-196)
B16-F1-CP10	2/19 (11)	7 (0-31)
B16-F1-BP8	14/20 (70)	92 (18-210)
B16-F1-CP10, BP4	10/19 (53)	47 (12-129)
B16-F1-BV4	13/18 (72)	104 (31-224)
B16-F1-BV8	17/20 (85)	123 (27-185)

^a The suffix designations for B16-F1 cells are as in Table 4, Footnote a.

^b Number of animals with lung metastases and total number of animals given injections. C57BL/6 mice were given injections of 5×10^4 viable cells in the external ear (s.c.), the injected ear was amputated 10 days later, and metastasis formation was assayed 4 weeks after amputation.

^c C57BL/6 mice given i. v. injections of 5×10^4 cells, and metastasis formation was assayed 18 days later. Results derived from measurements on 10 animals/group.

^d Numbers in parentheses, percentage.

^e Numbers in parentheses, range.

Table 6

Metastasis formation by an invasive cell variant (BL6) derived from B16-F10 melanoma cells

Route of injection	Cell	No. of mice bearing lung tumor nodules	
		Experiment 1	Experiment 2
s.c. ^a (external ear)	B16-F10	3/9 (33) ^b	1/12 (17) ^b
	B16-BL6	6/8 (75) ^b	7/10 (70) ^b
i.m. (footpad)	B16-F10	2/7 (29) ^{b,c}	1/9 (22) ^{b,d}
	B16-BL6	7/9 (78) ^{b,c}	4/5 (80) ^{b,d}
i.v.	B16-F10	441 (170-500) ^{e,f}	231 (66-481) ^{e,g}
	B16-BL6	148 (43-274) ^{e,f}	83 (16-258) ^{e,g}

^a Mice were given injections of 5×10^4 viable cells in the external ear s.c. and autopsied 4 weeks after injection.

^b Numbers in parentheses, percentage.

^c Mice given injections of 2.5×10^4 viable cells in the footpad; primary lesion amputated after 4 weeks and mice killed 4 weeks later.

^d Autopsied 6 weeks after injection without amputation.

^e Number of lung nodules formed 3 weeks after i.v. injection of 5×10^3 cells median. Numbers in parentheses, range.

^f Experiment 1, 8 animals/group.

^g Experiment 2, 15 animals/group.

or i.m., where invasiveness is a prerequisite for metastasis, it is able to exploit its advantage over the F10 cells and metastasize more effectively.

The importance of the selection procedure in determining the invasive phenotype is also illustrated by the present results in the properties of CAM-passaged cells. These variants do not differ from parental B16-F1 cells in their ability to invade other tissue (i.e., bladder or vein). In addition, invasive variants selected in the CAM do not differ from the B16-F1 parent cells in their metastatic behavior *in vivo*. Comparison of CAM-selected variants, in which invasiveness and metastatic ability are functionally dissociated, with variant sublines selected in bladder and vein in which these traits are coupled could provide insight into the contribution of specific cellular alterations to these particular behavior properties.

As emphasized elsewhere (8, 27), the search for properties uniform to all invasive cells may well be unproductive. We consider, however, that the likelihood of identifying properties which are expressed with a relatively high degree of consistency in invasive tumor cells will be substantially increased by studying variant cell populations selected specifically for this phenotype.

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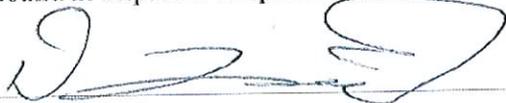
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Subculturing:	Protocol: <ol style="list-style-type: none"> 1. Remove and discard culture medium. 2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin - 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. 3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. 4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting. 5. Add appropriate aliquots of the cell suspension to new culture vessels. An inoculum of 5×10^3 to 7×10^3 viable cells/sq. cm. is recommended. 6. Incubate cultures at 37°C.
	Interval: Subculture when cells reach a concentration of 4×10^4 cells/sq. cm. Subcultivation Ratio: A subcultivation of 1:6 to 1:8 is recommended Medium Renewal: Two to three times weekly Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO Storage temperature: liquid nitrogen vapor phase
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L6 cells fuse in culture to form multinucleated myotubes and striated fibers. The extent of cell fusion declines with passage and the cells should be frozen at low passage and periodically recloned with selection for fusion competent cells.

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Tumorigenic:	No			
Age:	newborn			
Comments:	The L10BIOBR-GFP cell line was derived as a negative control for CRL-2771. The immortalized murine melanocyte cell line L10BIOBR was transduced with pD1VA-GFP and subjected to puromycin selection. Together, L10BIOBR-GFP (CRL-2770) and L10BIOBR-MAPKK (ATCC © CRL-2771) are valuable cell models for oncogenic transformation and signal transduction studies for melanoma [PubMed: 12514183]. Please note , although the L10BIOBR-GFP cell line harbors the <i>gfp</i> gene, as verified by PCR analysis, the cell line does not express sufficient GFP protein for detection of GFP fluorescence by flow cytometry or fluorescence microscopy.			
Propagation:	ATCC complete growth medium: Ham's F10 medium supplemented with 50 ng/ml TPA (Sigma Catalogue No. P-8139) and 7% horse serum Atmosphere: air, 95%; carbon dioxide (CO ₂), 5% Temperature: 37.0°C			

Subculturing:	<p>Protocol:</p> <ol style="list-style-type: none"> 1. Remove and discard culture medium. 2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin - 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. 3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. 4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting. 5. Add appropriate aliquots of the cell suspension to new culture vessels. An inoculum of 5×10^3 to 7×10^3 viable cells/sq. cm. is recommended. 6. Incubate cultures at 37°C. <p>Interval: Subculture when cells reach a concentration of 2×10^4 cells/sq. cm. Subcultivation Ratio: A subcultivation ratio of 1:3 to 1:4 is recommended Medium Renewal: Two to three times weekly Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO Storage temperature: liquid nitrogen vapor phase</p>
Preservation:	
Doubling Time:	29 hours
Related Products:	<p>recommended serum: ATCC 30-2040 derived from same cell line: ATCC CRL-2771 Cell culture tested DMSO: ATCC 4-X Erythrosin B vital stain solution: ATCC 30-2404 Trypan Blue vital stain solution: ATCC 30-2402</p>
References:	<p>89472: Govindarajan B, et al. Malignant transformation of melanocytes to melanoma by constitutive activation of mitogen-activated protein kinase (MAPKK) signaling. J. Biol. Chem. 278: 9790-9795, 2003. PubMed: 12514183</p>

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Product Description

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Cell Biology

ATCC® Number: CRL-1772™ [Order this Item](#)

Price: \$256.00

Designations: C2C12

Related Links ▶

[Biosafety Level:](#) 1

[NCBI Entrez Search](#)

Shipped: frozen

[Cell Micrograph](#)

Medium & Serum: [See Propagation](#)

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Growth Properties: adherent

[Frequently Asked Questions](#)

Organism: *Mus musculus* (mouse)

[Material Transfer Agreement](#)

Morphology: myoblast

[Technical Support](#)

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Source:



Strain: C3H

Tissue: muscle

Cell Type: myoblast;

Permits/Forms:

In addition to the [MTA](#) mentioned above, other [ATCC and/or regulatory permits](#) may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please [click here](#) for information regarding the specific requirements for shipment to your location.

Applications:

transfection host ([Nucleofection technology from Lonza Roche FuGENE® Transfection Reagents](#))

Comments:

This is a subclone (produced by H. Blau, et al) of the mouse myoblast cell line established by D. Yaffe and O. Saxel. [\[22903\]](#)

The C2C12 cell line differentiates rapidly, forming contractile myotubes and producing characteristic muscle proteins. [\[22953\]](#)

Treatment with bone morphogenic protein 2 (BMP-2) cause a shift in the differentiation pathway from myoblastic to osteoblastic. [\[23427\]](#)

Tested and found negative for ectromelia virus (mousepox).

Propagation:

ATCC complete growth medium: The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.

Temperature: 37.0°C

Subculturing:	<p>Protocol: IMPORTANT - DO NOT ALLOW CULTURES TO BECOME CONFLUENT. Cultures must not be allowed to become confluent as this will deplete the myoblastic population in the culture. Myotube formation is enhanced when the medium is supplemented with 10% horse serum instead of fetal bovine serum.</p> <ol style="list-style-type: none"> 1. Remove and discard culture medium. 2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum which contains trypsin inhibitor. 3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. 4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting. 5. Add appropriate aliquots of the cell suspension to new culture vessels. Inoculate at a cell concentration between 1.5×10^5 and 1.0×10^6 viable cells/75 cm². 6. Incubate cultures at 37°C.
Preservation:	<p>Medium Renewal: Every two to three days Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO</p>
Related Products:	<p>Storage temperature: liquid nitrogen vapor phase Recommended medium (without the additional supplements or serum described under ATCC Medium): ATCC 30-2002 recommended serum: ATCC 30-2020</p>
References:	<p>22903: Yaffe D, Saxel O. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. <i>Nature</i> 270: 725-727, 1977. PubMed: 563524 22953: Blau HM, et al. Plasticity of the differentiated state. <i>Science</i> 230: 758-766, 1985. PubMed: 2414846 23427: Katagiri T, et al. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage [published erratum appears in <i>J Cell Biol</i> 1995 Feb;128(4):following 713]. <i>J. Cell Biol.</i> 127: 1755-1766, 1994. PubMed: 7798324 28236: Chow YH, et al. Improvement of hepatitis B virus DNA vaccines by plasmids coexpressing hepatitis B surface antigen and interleukin-2. <i>J. Virol.</i> 71: 169-178, 1997. PubMed: 8985336 32828: Kessler PD, et al. Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. <i>Proc. Natl. Acad. Sci. USA</i> 93: 14082-14087, 1996. PubMed: 8943064 33069: Hsu DK, et al. Identification of a murine TEF-1-related gene expressed after mitogenic stimulation of quiescent fibroblasts and during myogenic differentiation. <i>J. Biol. Chem.</i> 271: 13786-13795, 1996. PubMed: 8662936</p>

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~~Steva Getelli~~
Ruchi Bhalla
Jared Churko
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Isabelle Planie
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- * 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.

Classification: 2

Date of last Biohazardous Agents Registry Form: Mar 27, 2009

Signature of Permit Holder: 

BioSafety Officer(s): Stanley April 21/09

Chair, Biohazards Subcommittee: 

Modification Form for Permit BIO-UWO-0017

Permit Holder: Dale Laird

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. *
Approved Microorganisms	E coli DH5 alpha, JM109	
Approved Cells	Human (primary), skin biopsies, Rodent (primary), transgenic alpha mutant mouse, Human (established), HELA, 293T, 293 Hek, Tumour cell lines, Rodent (established), Keratinocytes, NRK BiCR, M1RK, N2A,	B16 - F0 B16 - F10
Approved Use of Human Source Material	Human blood (whole), Human tissues (unpreserved), Human tissues (preserved), ODDD Patients and relatives	
Approved GMO	JM109, T-EASY, PcDNA3, pEGFP, retrovirus AP-2, SV 40 Large T antigen, HEK, E1A oncogenes, HeLa	PGFP - U-RS VECTOR
Approved use of Animals	mice	
Approved Toxin(s)		

New cells to be used for connexin and pannexin studies. New vectors to be used to silence Panx1. Additional Biohazards are level 1.

* 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.

Classification: 2

Date of last Biohazardous Agents Registry Form: Mar 27, 2009

Signature of Permit Holder: 

BioSafety Officer(s): Alanney Sept 21/09

Chair, Biohazards Subcommittee: Steve Kelder

HuSH shRNA Plasmid, pGFP-V-RS

pGFP-V-RS shRNA Cloning Plasmid
Catalog # TR30007

Product Description:

- Plasmid vector for cloning shRNA expression cassettes
- Designed for long term gene silencing studies
- Kanamycin (25ug/ml) and Puromycin resistance markers for easy selection of transformed or transfected cells
- U6 polymerase III promoter for shRNA expression
- MMLV LTR sequences for packaging into retroviral particles
- EcoRI and HindIII sites convenient for shuttling existing HuSH cassettes

Content: Each vial contains 5 ug of dried and purified plasmid DNA.

Storage and Stability: The plasmid is stable for at least 1 yr at -20°C from the date of shipment.

Guarantee: This product is guaranteed for the correct sequences and listed functions.

Related Products: Specific HuSH constructs are available at OriGene covering the full human, mouse and rat genomes.

Quality Control Assays

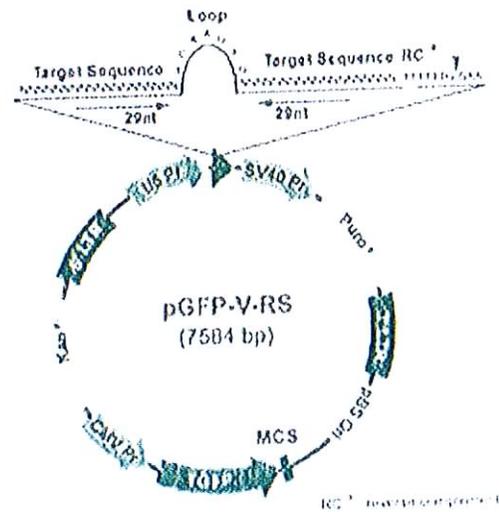
DNA Quantitation: The concentration of the purified plasmid was determined at OD_{260} by a UV spectrometer.

DNA Sequence Analysis: The final purified plasmid was sequenced to confirm its identity.

Functional Analysis:

1. Cloning: the pGFP-V-RS plasmid was digested with BamHI and HindIII and the digested fragment isolated. Multiple shRNA expression cassettes were cloned into this plasmid.
2. Inhibition of target gene: shRNA constructs cloned into pGFP-V-RS were verified for inhibition of target genes.
3. Stable cell lines: pGFP-V-RS was verified to generate stable cell lines using direct transfection.

Figure 1: Map of shRNA Cloning Vector pGFP-V-RS



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Re: Biohazard Modification Form: Laird lab

Subject: Re: Biohazard Modification Form: Laird lab
From: Dale Laird <Dale.Laird@schulich.uwo.ca>
Date: Mon, 21 Sep 2009 08:24:54 -0400
To: Jennifer Stanley <jstanle2@uwo.ca>

Hi Jennifer, These vectors are for transfections only. We do not anticipate making viral particles with them. Dale

Dale W. Laird, Ph.D.
Professor
Canada Research Chair in Gap Junctions and Disease
Department of Anatomy and Cell Biology
University of Western Ontario
Dental Science Building, Rm 00077
London, Ontario, Canada, N6A-5C1
Tel: (519) 661-2111 x86827
Fax: (519) 850-2562
Dale.Laird@schulich.uwo.ca
www.uwo.ca/anatomy/laird/index.htm

>>> Jennifer Stanley <jstanle2@uwo.ca> 18/09/2009 5:19 pm >>>
Hi Dr. Laird:

Thank you for your recent Biohazard Modification Form submission.
Please clarify, as soon as possible, whether or not the retrovirus particles are being produced.

Thanks,
Jennifer



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Cell Biology

ATCC® Number:	CRL-6475™	<input type="button" value="Order this Item"/>	Price:	\$264.00
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Shipped:	frozen		Cell Micrograph	
Medium & Serum:	See Propagation		Make a Deposit	
Growth Properties:	adherent		Frequently Asked Questions	
Organism:	<i>Mus musculus</i> (mouse)		Material Transfer Agreement	
Morphology:	melanocyte		Technical Support	
Source:	 Organ: skin Strain: C57BL/6J Disease: melanoma		Related Cell Culture Products	
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.			
Applications:	transfection host (technology from amaxa)			
Propagation:	ATCC complete growth medium: The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. Temperature: 37.0°C Atmosphere: air, 95%; carbon dioxide (CO2), 5%			
Subculturing:				

Protocol:

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:10 is recommended

Medium Renewal: Every 2 to 3 days

Preservation: Freeze medium: culture medium, 95%; DMSO, 5%

Storage temperature: liquid nitrogen vapor phase

Related Products: Recommended medium (without the additional supplements or serum described under ATCC Medium): ATCC [30-2002](#)
recommended serum: ATCC [30-2020](#)

References: 22151: Fidler IJ. Biological behavior of malignant melanoma cells correlated to their survival in vivo. *Cancer Res.* 35: 218-224, 1975. PubMed: [1109790](#)
22191: Fidler IJ, et al. Immunological properties of mouse macrophages activated with mediators from rat lymphocytes stimulated with concanavalin A. *Cancer Res.* 36: 3608-3615, 1976. PubMed: [953987](#)
22192: Fidler IJ, Bucana C. Mechanism of tumor cell resistance to lysis by syngeneic lymphocytes. *Cancer Res.* 37: 3945-3956, 1977. PubMed: [908034](#)
22243: Fidler IJ, Kripke ML. Metastasis results from preexisting variant cells within a malignant tumor. *Science* 197: 893-895, 1977. PubMed: [887927](#)
23224: Biles EB, Kornfeld S. Isolation and metastatic properties of detachment variants of B16 melanoma cells. *J. Natl. Cancer Inst.* 60: 1217-1222, 1978. PubMed: [418183](#)
23362: . . . *Nat. New Biol.* 242: 148-149, 1973.

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Cell Biology

ATCC® Number:	CRL-6322™	Order this Item	Price:	\$318.00
Designations:	816-F0		Related Links ▶	
<u>Biosafety Level:</u>	1		NCBI Entrez Search	
Shipped:	frozen		Make a Deposit	
Medium & Serum:	See Propagation		Frequently Asked Questions	
Growth Properties:	adherent		Material Transfer Agreement	
Organism:	<i>Mus musculus</i> (mouse)		Technical Support	
Morphology:	Spindle shaped		Related Cell Culture Products	
Source:	Organ: skin Strain: C57Bl/6J Disease: melanoma			
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.			
Applications:	transfection host (Roche FuGENE® Transfection Reagents technology from amaxa)			
Tumorigenic:	Yes			
Propagation:	ATCC complete growth medium: The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. Atmosphere: air, 95%; carbon dioxide (CO ₂), 5% Temperature: 37.0°C			
Subculturing:				

Protocol:

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:4 to 1:10 is recommended

Medium Renewal: Every 2 to 3 days

Preservation:

Freeze medium: Complete growth medium; 95% DMSO, 5%

Storage temperature: liquid nitrogen vapor phase

Related Products:

Recommended medium (without the additional supplements or serum described under ATCC Medium): [ATCC 30-2002](#)

recommended serum: [ATCC 30-2020](#)

References:

22151: Fidler IJ. Biological behavior of malignant melanoma cells correlated to their survival in vivo. *Cancer Res.* 35: 218-224, 1975.

PubMed: [1109290](#)

22191: Fidler IJ, et al. Tumoricidal properties of mouse macrophages activated with mediators from rat lymphocytes stimulated with concanavalin A. *Cancer Res.* 36: 3608-3615, 1976. PubMed: [953987](#)

22192: Fidler IJ, Bucana C. Mechanism of tumor cell resistance to lysis by syngeneic lymphocytes. *Cancer Res.* 37: 3945-3956, 1977. PubMed: [908034](#)

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22424: Fidler IJ. Immune stimulation-inhibition of experimental cancer metastasis. *Cancer Res.* 34: 491-498, 1974. PubMed: [4812256](#)

23224: Briles EB, Kornfeld S. Isolation and metastatic properties of detachment variants of B16 melanoma cells. *J. Natl. Cancer Inst.* 60: 1217-1222, 1978. PubMed: [418183](#)

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 Approved Biohazards Subcommittee: November 21, 2008
 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 Health Canada (HC) 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, Health Canada (HC) 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. If there are changes to the information on this form (excluding grant title and funding agencies), modifications must be submitted to Occupational Health and Safety. See website: www.uwo.ca/humanresources/biosafety/

PRINCIPAL INVESTIGATOR DALE W. LAIRD
 SIGNATURE [Signature]
 DEPARTMENT ANATOMY & CELL BIOLOGY
 ADDRESS DSB 00077
 PHONE NUMBER x 86827
 EMAIL DALE.LAIRD@SCHULICH.UWO.CA

Location of experimental work to be carried out: Building(s) DSB Room(s) 00076 / 00070

*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 Occupational Health and Safety (See Section 12.0, Approvals). For research being done at Lawson Health Research Institute, London Regional Cancer Program, Child and Parent Research Institute, or Robarts Research Institute, a University Biosafety Committee member can also sign as the Safety Officer for the Institution.

FUNDING AGENCY/AGENCIES: CIHR
 GRANT TITLE(S): CX43 MUTATIONS LINKED TO HUMAN DISEASE

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:

<u>DR. QING (CINDY) SHAO</u>	<u>JARED CHURKO</u>
<u>DR. STEPHANIE LANGLOIS</u>	<u>RUCHI BHALLA</u>
<u>DR. SILVIA PANUBIA</u>	<u>STAVIA CELETTI</u>
<u>DR. ISABELLE PLANT</u>	<u>MATHERINE TOYH</u>
<u>DR. GREGORY GOING</u>	<u>JENNIFER SIU</u>
	<u>JAMIE SIMEK</u>

1.0 Microorganisms

Laird, Dale W.

CHIR Operating Grant

Cx43 mutations linked to human disease

Intercellular gap junction channels allowing the direct passage of small molecules and secondary messengers between most contacting cells are built from a library of 21 connexin (Cx) family members. At present, 8 distinct genetic diseases ranging from sensorineural deafness to developmental disorders have been linked to germ line mutations in the genes encoding connexins. To date, 42 mutations in the *GJA1* gene encoding the gap junction protein, Cx43 have been linked to the human developmental disorder known as **odontodigital dysplasia (ODDD)**. This primarily autosomal dominant disease is typically characterized by syndactyly, camptodactyly, craniofacial abnormalities, enamel loss, incontinence and ophthalmic defects. Given that Cx43 is the predominant connexin expressed in over 35 distinct cell types, it is remarkable that patients have moderate to severe defects in some organs while other organs appear to remain free of developmental abnormalities and disease. Although many mutants are distributed on the cell surface, not unlike wild-type Cx43, all mutants tested to date exhibit complete or substantial loss-of-function and dominant-negative effects on co-expressed wild-type Cx43 with respect to gap junctional intercellular communication (GJIC). Mechanistically, at least some of these mutants appear to act as dominant-negatives by direct co-oligomerization with wild-type Cx43, although their effects *in vitro* and *in vivo* on other co-expressed connexins remain unknown. **Thus, we hypothesize that different ODDD-linked Cx43 mutants exhibit distinct cellular phenotypes and effects on co-expressed connexins manifesting in a loss of GJIC and perturbed cell differentiation, ultimately resulting in variable disease load.**

Aim 1: Examine the functional status, fate, dynamics and inter-connexin interactions of dominant and recessive ODDD-linked Cx43 mutants in reference cells and in cells obtained from ODDD patients. Dominant and recessive Cx43 mutants will be characterized with respect to (1) their ability to exert dominant and transdominant effects on GJIC, and (2) hemichannel function. These parameters will be assessed in defined reference cell models and primary cell cultures from ODDD-linked mutant mice that co-express Cx43 or other connexins typically co-expressed with Cx43 *in vivo*. FRET, co-immunoprecipitation and pulse-chase studies will be used to determine if the mutants establish direct interactions with co-expressed connexins and regulate their functional half-life. Finally, the expression, localization, phosphorylation status, and turnover of Cx43 will be examined in fibroblasts and/or tissue biopsies obtained from a cohort of patients harbouring Cx43 gene mutations.

*Aim 2: Characterize transgenic mouse models of ODDD that harbour missense or truncation mutations in distinct regions of the *Gja1* gene, and analyze the consequences of the mutants on cell differentiation.* Interestingly, current mouse models harbouring different ODDD-linked mutants exhibit both similar and distinct phenotypes that echo the diversity of symptoms observed in ODDD patients. Thus, we will first finish the generation and characterization of a "knock-in" gene-targeted mouse in which the endogenous Cx43 coding sequence is replaced either by the human wild-type Cx43 coding sequence or the Cx43^{G26D} mutant coding sequence. Together with the Cx43^{G60S}, Cx43^{H36I}, Cx43^{G138R} and littermate control mice, we will compare the phenotypes of these 4 mutant mouse models of human ODDD by performing *in situ* analysis using combinations of microCT and microMRI analysis, in addition to immunohistochemistry for connexins and tissue specific differentiation markers. Cell differentiation and function will be further assessed in primary cell cultures and/or explants from mineralized tissue (osteoblasts and ameloblasts), where abnormalities are consistently prevalent in patients; smooth muscle cells and myofibroblasts from the bladder, as approximately 30-50% of the patient cohort report urinary problems; and the mammary gland (myoepithelial and luminal cells), where sub-clinical disease may be present. Lastly, BrdU incorporation and Ki67 immunolabeling as well as TUNEL and caspase 3 staining will be used to assess the effect of Cx43 mutants on cell proliferation and death, respectively.

These studies will combine reference cell models expressing ODDD-mutants, mouse models of ODDD and direct ODDD patient data to establish genotype/phenotype relationships and the role of Cx43 in cell differentiation and function.

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	Health Canada or CFIA Containment Level
DHS E. coli	<input type="radio"/> Yes <input checked="" type="radio"/> No	<input type="radio"/> Yes <input checked="" type="radio"/> No	<input type="radio"/> Yes <input checked="" type="radio"/> No	500 mL	INUITROGEN	<input checked="" type="radio"/> 1 <input type="radio"/> 2 <input type="radio"/> 3
JM109	<input type="radio"/> Yes <input checked="" type="radio"/> No	<input type="radio"/> Yes <input checked="" type="radio"/> No	<input type="radio"/> Yes <input checked="" type="radio"/> No	500 mL	PROMEGA	<input checked="" type="radio"/> 1 <input type="radio"/> 2 <input type="radio"/> 3
	<input type="radio"/> Yes <input type="radio"/> No	<input type="radio"/> Yes <input type="radio"/> No	<input type="radio"/> Yes <input type="radio"/> No			<input type="radio"/> 1 <input type="radio"/> 2 <input type="radio"/> 3
	<input type="radio"/> Yes <input type="radio"/> No	<input type="radio"/> Yes <input type="radio"/> No	<input type="radio"/> Yes <input type="radio"/> No			<input type="radio"/> 1 <input type="radio"/> 2 <input type="radio"/> 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	<input checked="" type="radio"/> Yes <input type="radio"/> No	SKIN BIOPSIES	Not applicable
Rodent	<input checked="" type="radio"/> Yes <input type="radio"/> No	TRANSGENIC & MUTANT MICE	2006-191
Non-human primate	<input type="radio"/> Yes <input checked="" type="radio"/> No		
Other (specify):	<input type="radio"/> Yes <input checked="" type="radio"/> No		

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	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No	HELA, 293T, 293 HEK TUMOR CELL LINES	ATCC
Rodent	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No	KERATINO CYTES, NRK DICK. MURKIN, WRA	CLONTECH, ATCC VINCIA, HASCALL
Non-human primate	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No		
Other (specify)	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No	MDCK	ATCC

*Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org) THESE ARE ALL STANDARD WELL USED CELL LINES

2.4 For above named cell types(s) indicate HC 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)	HC or CFIA Containment Level (Select one)
Human Blood (whole) or other Body Fluid	ODDD PATIENTS AND RELATIVES	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No		<input type="checkbox"/> 1 <input checked="" type="checkbox"/> 2 <input type="checkbox"/> 3
Human Blood (fraction) or other Body Fluid		<input type="checkbox"/> Yes <input type="checkbox"/> No		<input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 3
Human Organs or Tissues (unpreserved)	ODDD PATIENTS AND RELATIVES	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No		<input type="checkbox"/> 1 <input checked="" type="checkbox"/> 2 <input type="checkbox"/> 3
Human Organs or Tissues (preserved)	ODDD PATIENTS AND RELATIVES	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No		<input checked="" type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> 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
SM109	T-CASY P _{CMV} DNAT (4)(-) P _{EGFP}	PRONEGA INVITROGEN CLONTECH	CONNEXIN GENES PANNEXIN GENES	CELL LINES EXPRESSING PLASMIDS TO BE GROW SLOWER AND FORM CELL-CELL OR CELL CHANNELS.

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

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

Virus Used for Transduction *	Vector(s) *	Source of Vector	Gene Transfected	Describe the change that results
RETROVIRUS	AP-2	DR. J. GARIBAYO MAGILL UNIVERSITY	CONNEXINS PANNEKINS	CELLS COMMUNICATE BETTER

* Please attach a Material Safety Data Sheet or equivalent.

SEE BIO-040-007 AS INFORMATION
IT'S CURRENTLY ON RECORD WITH
SAFETY OFFICE

4.4 Will genetic sequences from the following be involved?

- HIV YES, please specify _____ NO
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens YES, specify _____ NO
- SV 40 Large T antigen YES T.H.E. cells NO NO NO
- E1A oncogene YES HeLa cells NO NO
- Known oncogenes YES, please specify _____ NO
- Other human or animal pathogen and/or their toxins YES, please specify _____ NO

4.5 Will virus be replication defective? YES NO

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

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

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials be conducted using the viral vector in 4.0? 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? YES 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? YES, number _____ NO PENDING

6.0 Animal Experiments

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

6.2 Name of animal species to be used Mice

6.3 AUS protocol # 2006-101

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

10.0 Plants Requiring CFIA Permits

10.1 Do you use plants that require a permit from the CFIA? 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? Grow and maintain a crop "One-time" use

10.6 Do you do any modifications to the plant? YES 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? YES 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 _____
If no, please proceed to Section 10.0 NO

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

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

11.4 Has the import permit been sent to OHS? YES, please provide permit # _____ 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 _____

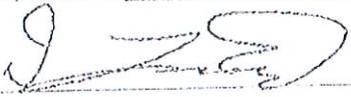
13.0 Containment Levels

14.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. 01 02 03

13.2 Has the facility been certified by OHS for this level of containment?
 YES, permit # if on-campus BIO-UWO-0017
 NO
 NOT REQUIRED

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:  Date: March 4, 09

15.0 Approvals

UWO Biohazard Subcommittee: SIGNATURE: 
Date: 31 March 2009

Safety Officer for Institution where experiments will take place: SIGNATURE: 
Date: March 27, 09

Safety Officer for University of Western Ontario (if different from above): SIGNATURE: _____
Date: _____

Approval Number: BIO-UWO-0017 Expiry Date (3 years from Approval): March 26, 2012

Special Conditions of Approval:

----- Original Message -----

Subject: questions

Date: Tue, 03 May 2005 09:17:07 -0400

From: Dale Laird <Dale.Laird@fmd.uwo.ca>

To: jstanle2@uwo.ca

CC: Cindy Shao <Cindy.Shao@fmd.uwo.ca>

Dear Jennifer, I apologize for the delay in responding to your questions and comments on my last Biohazardous Agents Registry Form. Your email was sent to dwlaird@uwo.ca instead of dale.laird@fmd.uwo.ca and got held back for some reason. I found 650 emails on my UWO account of which many were not forwarded to my FMD account (as they should be). Anyway here is the additional information you requested.

1. Section 4.3 - please note that HEK 293 contains E1A oncogene
Response: Thank you this is noted.

2. Section 4.5

Response: -The AP-2 retroviral vector is the main one we have been using for several years. The documentation, vector source and description was placed on file with the Safety office several years ago. See manuscript reference Galipeau et al., 1999, Cancer Research 59; 2384-2394. The 293GPG packaging cells which produce replication-defective virus are described in this same paper. The 293GPG packaging cells were originally described in Ory et al., Proc Natl Acad Sci U S A. 1996 Oct 15;93(21):11400-6. Both the AP-2 vector and packaging cells are from Dr. Jacques Galipeau in Montreal. The MTA for these cells and vectors were done in collaboration with Dr. Chris Maus several years ago.

-More recently we have obtained the pHL1.1-QCXLH retroviral vector (from GenScript) for shRNA studies. See (Barton and Medzhitov, 2002, PNAS 99; 14943-14945). The HEK293 derived packaging cells produce replication-incompetent viral particles. (See AmphoPack293 from BD Biosciences).

3: Section 1.2 - confirm E. coli DH5alpha,
Response: -Yes this is the E.coli we use,

4: Section 6.0 - do you have an animal protocol?

Response: -The animal studies being performed in this study are in conjunction with the Co-Principle Applicants, Drs. Kidder and Dr. Bernier. Their animal protocols have been modified to include this new grant. I do not have a separate animal protocol.

5: Section 8.3 - description of lindane use:

Response: -Lindane is dissolved in DMSO at 50um and used as a gap junction channel inhibitor at a final concentration of 50nM.

I trust this answers all your questions. Again sorry for the delay.

Dale Laird

Dale W. Laird, Ph.D.

Professor

Canada Research Chair in Gap Junctions and Disease