Methods Relating to Tissue Culture of G1E Cells

G1E cells are a GATA^{-} (null) cell line derived from targeted disruption of GATA-1 in embryonic stem cells. These cells propagate in culture with a doubling time of about twelve hours. They resemble proerythroblasts and do not differentiate. They are dependent on Kit and Erythropoietin, but not IL-3. In our studies of erythroid differentiation, we have employed a G1E subclone designated G1E-ER4 which stably expresses a fusion product combining GATA-1 with the estradiol receptor ligand binding domain. Addition of either estradiol or taxoxifen (4-OHT) to the medium results in the functional activation of GATA-1. This triggers erythroid differentiation which becomes morphologically apparent at about 12 hours. By twenty-four hours, hemoglobin can be detected by benzidine staining. Microarray analysis indicates that transcriptional changes take place within the first hour of GATA-1 activation, however. By 30 hours, G1E cells reach a stage developmentally equivalent to an orthrochromatic erythroblast. They continue to hemoglobinize up to 72 hours, but then undergo apoptosis. They do not extrude their nuclei. Cells remain in log phase when maintained between 1 x 10^5 and 1 x 10^6 cells/mL; effectively this means passaging daily with splits between 1:4 and 1:10.

G1E Medium

Component	Store At	Volume
Iscove's MDM	4C	500 mL
FCS (ES-grade)	-20C	75 mL
Penicillin/Streptomycin stock	-20C	10 mL
Monothioglycerol (MTG)	4C	6.2 uL
Kit-ligand conditioned medium	-20C	3 mL
Erythropoetin 10,000 U/mL	-20C	100 uL

Puromycin is maintained as 1000X stock at -20C and can be added directly to culture flasks to maintain selection pressure on GIE-ER4. When a new batch is thawed, it is routine passed for one or two generations in media containing puromycin to select against revertants lacking GATA-1-ER.

Freezing cells

<u>2x Freezing Medium</u>

Component	Store At	Volume
FCS	-20C	4 mL
DMSO	Room Temp, Dark	1 mL

- Mix well, keep cold.
- Good for a few days at 4C.
- Make 2X medium and keep on ice. Fill freezing container with isopropanol and prechill to 4C.
- Count cells; should be in log phase 0.5 - 0.8 x 10^6/mL
- Calculate volume necessary for n number of 1 mL vials with 5-10 x 10^6 per vial.
- Centrifuge in 50 mL tubes for 5 minutes at 1200 rpm.
- Aspirate supernatant and resuspend n vials x 0.5 mL GIE medium (e.g., 5 vials = 2.5 mL)
- Add equal volume of cold 2X Freezing Medium. Mix gently, aliquot 1 mL per vial.
- Quickly place vials into freezing container. Put freezing container in -80C freezer overnight. The next day, transfer to liquid nitrogen.

Cell Staining

Benzidine Staining (to assess hemoglobin content)

Dissolve 60 mg of o-diansidine (Sigma D-9143) in 29.7 mL H2O and 0.5 mL glacial acetic acid. Note o-diansidine is a toxic carcinogen; wear gloves and work in fume hood. To dissolve, heat gently and stir for 30-60 minutes. Centrifuge to eliminate undissolved particulate matter. Store supernatant at 4C in light-proof container. Reagent is good for several months, but should be discarded when it turns brown.

Prior to staining, prepare fresh benzidine reagent: one part hydrogen peroxide to 10 parts benzidine reagent.

<u>Quantitation of cells in suspension</u>

- Add one part of above preparation to 10 parts of the media containing cells; pH change turns media yellow-green.
- After 1-2 minutes, observe with phase contrast microscope with filter removed, or on hemocytometer, a wet-prep slide or in the flask itself. Positive cells are red; negative are clear. Induced GIE and MEL are positive controls. About 0.05-0.1% of uninduced MEL cells are positive. After 10 minutes, large air bubbles make observation difficult.

<u>To create slides</u>

- Dilute one part of above preparation into 10 parts media containing cells. Ideally, 2-5 x 10^5/mL in 50 to 100 uL. Do not exceed 150 uL.
- Allow cells to stain 1-2 minutes. Load onto cytospin chamber and spin 4 minutes at 400 rpm.
- Air-dry slides. Counterstain in May-Grunwald for 5 seconds. Dip twice, quickly, in distilled water.
- Positive cells will be black, negative will be a light purple.

- Note: May-Grunwald contains methanol and will fix the cells. MG stain is reusable.

May-Grunwald/Giemsa Stain (for morphology)

- Prepare fresh Giemsa stain daily by 1:20 dilution from stock.
- Cytospin about $5x10^5$/mL in 100 uL.
- Air dry
- Stain in MG for 2 minutes
- Stain in Giemsa for 10 minutes
- Dip twice in water, allow slides to dry
- Add coverslips.

Protocols
G1E Microarray Home

Please direct all comments to: *John Welch, M.D., Ph.D.*
Last modification: August 16, 2003