Influence of Environment on HDF Cell Survival and Function *In Vitro*

April 9, 2008

Objectives of Study

- Determine best conditions for establishing & maintaining HDF (Human Dermal Fibroblast) cells *in vitro* by observing environment's effects on cell survival & function
  - Assess attachment of HDF cells based on surface type
    - Quantitative Cell Attachment Assay
  - Assess sensitivity of HDF cells to toxic substance
    - Live/Dead Fluorescence Assay
  - Assess effect of serum on HDF cell division
    - Anti-PCNA Staining Assay, Cell Proliferation Assay

Materials and Methods

- Quantitative Cell Attachment Assay
  - Seed HDF cells in DMEM 10% FBS onto untreated & TC-treated polystyrene plates at equal concentration
  - At 0.5, 1.25, 2.5 and 4 hrs, use light microscope to count # of attached cells in gridded 0.01cm^2 representative area
- Live/Dead Fluorescence Assay
  - Incubate HDF cells (50-60% confluent) for 30 min. in…
    - A: PBS only, B: Ethanol only, or C: PBS & 2 drops Ethanol (n=2)
    - All contain Live/Dead Stain (2μM Calcein AM in 4μM EthD-1/PBS)
  - Observe cell morphology with light microscope and cytoplasm/nucleus color with fluorescent microscope
    - Green: Live cell cytoplasm; Red: Dead cell nucleus

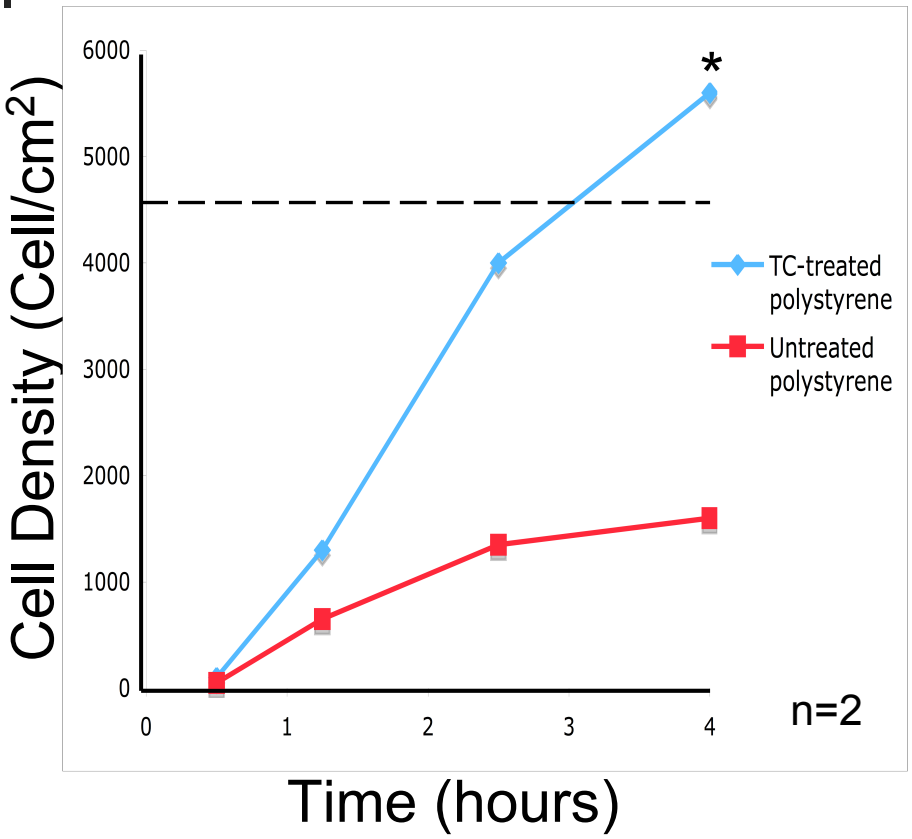
Materials and Methods

- Anti-PCNA Staining Assay
  - Seed HDF cells in DMEM 1%, 5% or 10% FBS
  - At 48 hrs, fix cells in formalin & react with H_2O_2
  - Test cells (incubated in DMEM 1%, 5% or 10% FBS, n=1)
    - Add 1° Ab (Anti-PCNA Mouse IgG), 2° Ab (Anti-Mouse IgG-HRP), & AEC chromogenic substrate solution
  - Control cells (incubated in DMEM 10% FBS, n=3)
    - A: 1° Ab only; B: 2° Ab only; C: no Ab
  - Stain all cells with hematoxylin
  - Observe color of cell nucleus with light microscope and note % of cells with red nuclei
    - Red nuclei: elevated PCNA (in S phase), Blue: non-dividing

Materials and Methods

- Cell Proliferation Assay
  - Seed HDF cells in DMEM 1% FBS at 7,000 cell/mL
  - At 4 hrs…
    - Determine # of attached cells via trypsinization and Coulter Counter cell counting (n=6)
    - Change media of cells to DMEM 1%, 5% or 10% FBS
  - At 48, 120 & 168 hrs, repeat determination of # of attached cells with trypsinization and Coulter Counter cell counting (n=3 for each DMEM 1%, 5% & 10% FBS at each time point)

HDF Cell Attachment is Affected by Surface Type



* significant difference between untreated and TC-treated cell density at 4 hours ($p<0.05$, Student's T-test)
- - - Denotes theoretical max cell density

- TC-treated surface enhances HDF cell attachment
  - ↑ attachment rate
  - ↑ cell density
  - ↑ cell extension with pseudopodia
- 4 hr incubation: long enough to ensure attachment
  - Density > Theoretical (error in choosing representative area)

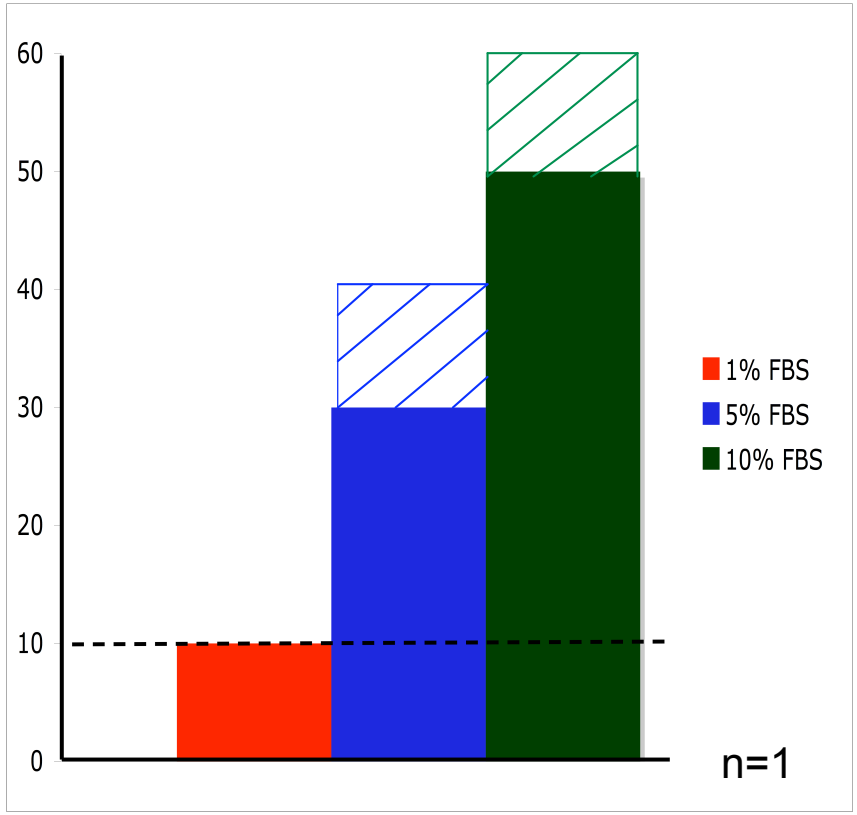
HDF Cells are Sensitive to Toxic Substance

Condition	Color*	Cell Viability	Cell Morphology
PBS	Green	Live	extended, distinct membrane, spindle
Ethanol	Red	Dead	flat, fuzzy membrane, rounded spindle
PBS + Ethanol	Green & Red	Live & Dead	clusters of above two morphologies

*indicates color viewed with fluorescent microscope

- Nontoxic PBS maintains healthy culture of HDF cells in short-term
- HDF cells are sensitive to toxicity of ethanol
  - Evidence of cell death
    - Morphology (flatter with less distinct membranes)
    - Red Staining (can only enter membranes of dead cells)
- PBS + Ethanol condition suggests ethanol kills cells with which it comes into direct contact

Serum affects tendency of HDF cells to enter S phase

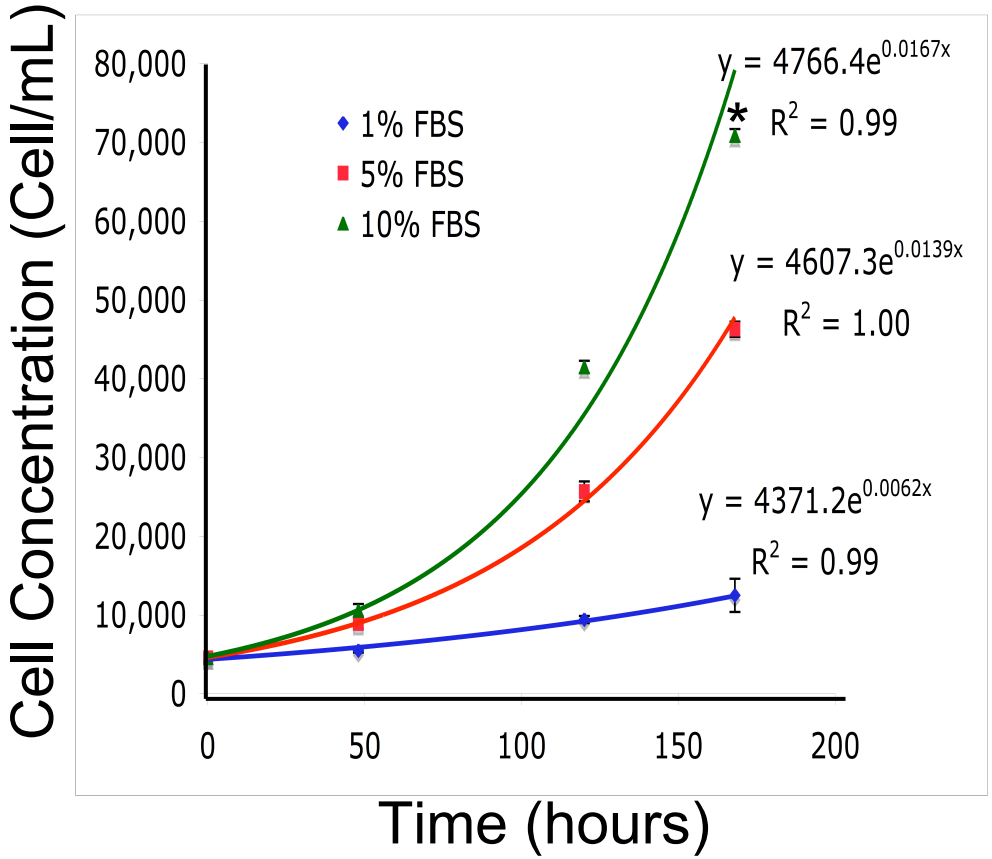


Note: Shaded region represents estimated range of % cells with red nucleus

- - - - - - Denotes % cells in controls with red nuclei

- As ↑ level of serum, ↑ # of cells w/ red nuclei (elevated PCNA)
  - ↑ # cells in S phase
- All controls contained approx. 10% cells w/ red nuclei
  - Error, H_2O_2 might have been too weak
  - Not enough to invalidate observed trend

Serum affects HDF cell proliferation rate



- HDF cells exhibited exponential growth
  - As ↑ serum level, ↓ cell doubling time
- Elevated serum increased rate of cell proliferation

*Significant differences in cell concentration between 1%, 5% and 10% serum at 168h and in cell concentration in 10% serum between 4, 48, 120 and 168 hrs ($p<0.05$) (Student's t-test)

Serum enhances cell growth & division

- Anti-PCNA Staining Assay & Cell Proliferation Assay both suggest that elevated serum promotes cell division
  - Anti-PCNA Staining Assay measures cell # in S phase, committed to undergo cell division
  - Cell Proliferation Assay measures change in cell concentration over time, indicating rate of cell proliferation & doubling time
- Both assays give quantitative measurements to indicate cell division; however, Cell Proliferation Assay uses Coulter Counter, while Anti-PCNA Staining Assay relies on manual counting through microscope
- Anti-PCNA Staining Assay provides ratio of dividing cells/non-dividing cells at single point in time, while Cell Proliferation Assay provides rate of cell division over time

Conclusions

- Identified conditions to promote HDF cell survival & function *in vitro*
  - HDF cells attach better to TC-treated than untreated surface
    - Need charged, wettable TC-treated surface to promote initial attachment of HDF cells *in vitro*
  - HDF cells are sensitive to toxic substance, ethanol
    - Need healthy, nontoxic environment to promote viability
  - Serum enhances cell growth and division
    - Need serum (which contains growth factors) to promote cell proliferation *in vitro*

Implications

- Knowledge gained from study will help to create optimal environment to support establishment and maintenance of healthy HDF cells *in vitro*
- A proper *in vitro* model that exhibits maximal cell attachment, viability and proliferation will improve conditions for *in vitro* experimentation to create a successful model of *in vivo* cell function