In Vitro Models for Studying Angiogenesis

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Overview

- Angiogenesis
- Important considerations for studying angiogenesis *in vitro*
- BD *in vitro* angiogenesis models
  - EC tube formation
  - EC migration
  - EC invasion
Angiogenesis

- **Angiogenesis** – formation of new blood vessels (sprouting, branching etc.) from existing vessels (e.g. wound healing, fertility etc.)

- **Vasculogenesis** – formation of new blood vessels from angioblasts or progenitor stem cells (i.e. *de novo* vessel synthesis)
Diseases Associated With Angiogenesis

Excessive
- Cancer
- Rheumatoid Arthritis
- Blindness (Diabetic Retinopathy)
- Aids Complications
- Psoriasis

Insufficient
- Stroke
- Heart Disease
- Infertility
- Scleroderma
- Ulcers
Tumor Angiogenesis

- Localized solid tumor
  - Necessary for tumor growth and metastasis
  - Inhibition of angiogenesis represents a critical point of intervention for cancer treatment
  - Circumvents problems of cellular toxicity and tolerance development seen with chemotherapy

- Tumor that can grow and spread
Angiogenesis Research

• **Basic research to elucidate molecular mechanisms of angiogenesis:**
  - Identify and characterize regulatory pathways that mediate various steps of angiogenesis such as endothelial cell migration, invasion, and tubulogenesis

• **To develop treatments for cancer and other diseases associated with angiogenesis**
  - Identification of compounds that inhibit or stimulate key steps in the angiogenesis process
Progression of Angiogenesis

1. Pro-angiogenic factors released from hypoxic cells
   - VEGF
   - bFGF

2. Factors Bind to EC receptors

3. MMps activated and released
   - Basement membrane degradation

4. Proliferation
5. Invasion
6. Adhesion
7. Migration

8. Tube formation
9. Highly permeable

10. Pericyte recruitment
11. Vessel stabilization
**Table 2** Anti-angiogenic drugs approved for clinical use and phase of clinical trials for other indications

<table>
<thead>
<tr>
<th>Drug (Trade name; company)</th>
<th>Approved*</th>
<th>Phase I</th>
<th>Phase II</th>
<th>Phase III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bevacizumab (Avastin; Genentech)</td>
<td>Multiple myeloma (2003)</td>
<td>NSCLC, multiple myeloma, NHL</td>
<td>Multiple myeloma, NHL, NSCLC, lymphoma, melanoma, Waldenstrom's macroglobulinemia, prostate, head and neck, breast, liver, non-Hodgkin's, gastric, pancreatic, colorectal, ovarian, mesothelioma cancer, and others</td>
<td>Lymphoma, myelodysplasia, multiple myeloma, NHL, solid tumours, head and neck, colorectal, ovarian, prostate cancer and others</td>
</tr>
<tr>
<td>Thalidomide (Celgene Corporation)</td>
<td>Multiple myeloma (2006)</td>
<td>Soft tissue sarcoma, multiple myeloma, ALS, melanoma, neuroendocrine tumours, head and neck, glioma, glioblastoma, pancreatic neuroendocrine tumours, NSCLC, RHL, pemetrexed solid tumours, myelofibrosis, myelodysplastic syndrome, AML, CML, SCLC, Hodgkin's disease, melanoma, breast, lung, colorectal, kidney, mesothelioma, gastrointestinal, thyroid, uterine, ovarian cancer, and others</td>
<td>Solid tumours, glioma</td>
<td></td>
</tr>
<tr>
<td>Erlotinib (Tarceva; Genentech; OSI Pharmaceuticals; Roche)</td>
<td>Lung cancer (2004)</td>
<td>NSCLC, colorectal, pancreatic, ovary, head and neck, oral cancer</td>
<td>NSCLC, mesothelioma, glioblastoma, glioma, gall bladder, GST/billirubin tumours, bladder cancer, prevention of malignant peripheral nerve sheath tumours, colorectal, pancreatic, breast, renal cell, prostate, ovarian, head and neck, gastric, oesophago, liver cancer, and others</td>
<td>NSCLC, glioblastoma, solid tumours, colorectal, pancreatic, head and neck cancer</td>
</tr>
<tr>
<td>Bevacizumab (Avastin; OSI Pharmaceuticals)</td>
<td>Age-related macular degeneration (2004)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eribulin (Halaven; Eisai)</td>
<td>Lung cancer (2010)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sunitinib (Sutent; Pfizer)</td>
<td>Kidney cancer (2005)</td>
<td>Kidney, melanoma, hepatocellular cancer</td>
<td>Melanoma, glioblastoma, GST, SCLC, thyroid, neuroendocrine, colorectal, soft tissue sarcoma, NSCLC, CML, multiple myeloma, cholangiocarcinoma, NHL, kidney, colorectal, prostate, ovarian, peritoneal, pancreatic, breast, gastric, head and neck, stomach, gall bladder, bladder cancer, and others</td>
<td>Solid tumours, melanoma, glioblastoma, BHL, glioma, multiple myeloma, Kaposi's sarcoma, ALL, CML, MDS</td>
</tr>
<tr>
<td>Lenalidomide (Revlimid; Celgene Corporation)</td>
<td>Myelodysplastic syndrome (2005)</td>
<td>Multiple myeloma, myelodysplastic syndrome</td>
<td>NSCLC, RHL, multiple myeloma, CML, myelofibrosis, myelodysplastic syndrome, glioblastoma, ovarian neoplasms, AML, mantle-cell lymphoma, Waldenstrom's macroglobulinemia, monocytes, peritoneal, thyroid, primary pancreatic cancer</td>
<td>Multiple myeloma, prostate cancer, melanoma, myelodysplastic syndrome, solid tumours, postradiation oncotherapy, CNS tumours</td>
</tr>
<tr>
<td>Sorafenib (Nexavar; Pfizer)</td>
<td>GST, Kidney cancer (2006)</td>
<td>Renal cell cancer, GIST</td>
<td>Melanoma, VHL, solid tumour, NSCLC, CML, hepatocellular, colorectal, prostate, breast, renal cell, gastric, neuroendocrine cancer, and others</td>
<td>Melanoma, solid tumours, colorectal, breast cancer</td>
</tr>
</tbody>
</table>

*Year of first approval by the US Food and Drug Administration. (c) 2008 American Society Clinical Oncology. All rights reserved. To obtain permission to reproduce or redistribute this content, please contact Permissions. () Annals of Oncology. All rights reserved. To access the full-text article, please visit The Lancet. () 2008 Nature Publishing Group. All rights reserved. To access the full-text article, please visit Nature Reviews Drug Discovery. Folkman, J. Nature Reviews Drug Discovery 6:273-286 (2007).
Clinical Relevance of Endothelial Cell Based *in vitro* Angiogenesis Assays

<table>
<thead>
<tr>
<th>Drug</th>
<th>Endothelia-cell target</th>
<th>Clinical trials</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiostatin</td>
<td>Binds to ATP synthase, angiomotin and annexin II on endothelial cells to inhibit endothelial-cell proliferation and migration</td>
<td>Phase I</td>
<td>69,156,157</td>
</tr>
<tr>
<td>Bevacizumab (Avastin)</td>
<td>Recombinant humanized monoclonal antibody against vascular endothelial growth factor (VEGF)</td>
<td>Phase II and III</td>
<td>158</td>
</tr>
<tr>
<td>Arresten</td>
<td>Believed to bind integrin-α,β3, to inhibit endothelial-cell proliferation, migration, tube formation and neovascularization</td>
<td>No</td>
<td>159</td>
</tr>
<tr>
<td>Canstatin</td>
<td>Believed to bind integrin-α,β3, to inhibit endothelial-cell proliferation, migration and tube formation</td>
<td>Should start this year</td>
<td>160</td>
</tr>
<tr>
<td>Combretastatin</td>
<td>Microtubules: induces reorganization of the actin cytoskeleton and early membrane blebbing in human endothelial cells</td>
<td>Completed Phase I</td>
<td>161</td>
</tr>
<tr>
<td>Endostatin</td>
<td>Believed to target integrin-α,β3, to inhibit endothelial-cell proliferation and migration, and induce apoptosis of proliferating endothelial cells (R. Kalluri, personal communication); endostatin does not affect wound healing</td>
<td>Phase I and II</td>
<td>57,72</td>
</tr>
<tr>
<td>NM-3</td>
<td>An isocoumarin small-molecule inhibitor of VEGF. It was shown to selectively inhibit endothelial-cell proliferation, sprouting and tube formation <em>in vitro</em></td>
<td>Phase I</td>
<td>162</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>Blocks endothelial-cell migration and neovascularization in the cornea, but might not be specific for endothelial cells</td>
<td>No</td>
<td>9</td>
</tr>
<tr>
<td>Turnstatin</td>
<td>Binds to integrin α,β3, on endothelial cells; inhibits endothelial-cell proliferation and neovascularization</td>
<td>No</td>
<td>163,164</td>
</tr>
<tr>
<td>2-methoxyestradiol</td>
<td>Inhibits microtubule function in proliferating endothelial cells, resulting in endothelial-cell apoptosis</td>
<td>Phase I and II</td>
<td>97</td>
</tr>
<tr>
<td>Vitaxin</td>
<td>A humanized monoclonal antibody against integrin α,β3</td>
<td>Phase I and II</td>
<td>165</td>
</tr>
</tbody>
</table>
Important Considerations for Developing Angiogenesis Studies

• Incorporate appropriate extracellular matrix (ECM) protein(s) to facilitate cell functionality and assay outcome
• Choose appropriate endothelial cell source
• Choice of angiogenesis assay
• Establish acceptable dynamic range to measure stimulation and/or inhibition of angiogenesis
Extracellular Matrix

ECM provides a physiological substrate that supports key cellular functions

- Structural organization of cells and tissue
- Cell attachment, survival, and proliferation
- Induction and maintenance of cell differentiation
- Can influence signal transduction and regulation of gene expression

Examples: gelatin, fibronectin, vitronectin, laminin, collagen, BD Matrigel™ matrix
• HUVEC—most commonly studied human EC type in angiogenesis
• Source, isolation procedure, and initial culturing conditions can influence response to pro-angiogenic factors (e.g. VEGF, bFGF)
Sources of Endothelial Cells

- **Large vessel**
  - aortic (e.g., HAEC)
  - umbilical vein (e.g., HUVEC)
  - pulmonary artery
- **Microvascular (e.g., HMVEC)**
  - brain
  - lung
  - dermis (e.g., HDMEC)
  - myocardium
Human Umbilical Vein Endothelial Cells

- Most commonly used human EC type for studies of angiogenesis
- Source, isolation procedure, and initial culturing conditions can influence response to pro-angiogenic factors (e.g. VEGF, bFGF)
- BD™ Human Umbilical Vein Endothelial Cells (HUVEC-2) (cat. no. 354151)
  - Pre-qualified for responsiveness to VEGF in endothelial cell migration assay
  - Tested for presence of von Willebrand factor (vWF), CD31, uptake of Dil-Ac-LDL, and absence of alpha actin
BD BioCoat™ Angiogenesis Systems

BD HUVEC-2 cells
• Pre-qualified for VEGF responsiveness and for use with endothelial cell migration assay

Endothelial Cell Tube Formation
• Composed of a 96-well black/clear plate coated with BD Matrigel matrix (non-insert system)

Endothelial Cell Migration
• 24- or 96-Multiwell BD FluoroBlok™ insert (3 µm pore size)
• Coated with human fibronectin

Endothelial Cell Invasion
• 24-Multiwell BD FluoroBlok insert (3 µm pore size)
• Coated with BD Matrigel matrix

Shipped on dry ice, store at -20ºC
Do not store in frost free or -70ºC freezer
BD Fluorescent Dyes

BD™ Fluorescent Dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>Application</th>
<th>Abs/Em (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD™ Calcein AM</td>
<td>pre- or post-labeling</td>
<td>494/517</td>
</tr>
<tr>
<td>BD™ DiIC₃(3)</td>
<td>pre-labeling</td>
<td>549/565</td>
</tr>
</tbody>
</table>

Quality Control
BD Calcein AM Fluorescent Dye
- Purity >95%
- Extinction Coefficient >6,000 M⁻¹cm⁻¹

BD DiIC₃(3) Fluorescent Dye
- Purity >95%
- Extinction Coefficient >150,000 M⁻¹cm⁻¹
(Extinction Coefficients are compound in methanol)

Storage and Stability
Product is shipped on dry ice. Upon receipt, store immediately at -20°C. Stable for at least three months from date of shipment.

Ordering Information

<table>
<thead>
<tr>
<th>Cat. No.</th>
<th>Description</th>
<th>Qty.</th>
</tr>
</thead>
<tbody>
<tr>
<td>354216</td>
<td>BD™ Calcein AM Fluorescent Dye</td>
<td>10 x 50 µg</td>
</tr>
<tr>
<td>354217</td>
<td>BD™ Calcein AM AM Fluoroscent Dye</td>
<td>1 mg</td>
</tr>
<tr>
<td>354218</td>
<td>BD™ DiIC₃(3) Fluorescent Dye</td>
<td>100 mg</td>
</tr>
</tbody>
</table>

NG-108 neuroblastoma cells stained with BD™ DiIC₃(3) Fluorescent Dye.
Angiogenesis Tube Formation Assay
Angiogenesis Tube Formation Assay
Simplified Steps in Angiogenesis Process

1. EC lysis of basement membrane and extracellular matrix
2. EC invasion/migration
3. EC proliferation
4. Capillary tube formation and differentiation

Angiogenic Stimulators: VEGF, FGF

BD
BD BioCoat Angiogenesis System: Endothelial Cell Tube Formation

BD Falcon™ 96-well black/clear microplate

BD Matrigel matrix coating
- Optimized manufacturing process generates flat surface; elimination of meniscus
- Screened for ability to promote tube formation

Compatible with automated image acquisition and data processing

Reproducible
- Consistent endothelial cell tube formation: well-to-well, lot-to-lot, assay-to-assay
- Z' value = 0.6, suitable for compound screening

Mat Cover and Lid
- Ensures product stability

Shipped on dry ice, store at -20°C
Do not store in frost free or -70°C freezer
Day 1

Thaw, 6-24 hours

Gel, 30 min.

Frozen 96-well tube formation microplate

Trypsinize and resuspend cells in assay medium

Day 2

37°C/5% CO₂ incubation

Fluorescent dye labeling

BD Matrigel Matrix

Endothelial cells

Pro-angiogenic factors

Automated Image Acquisition & Data Processing

BD Pathway™ 855 Bioimager
Endothelial Cell Tube Formation

Collagen I
2D

BD Matrigel Matrix
Bright Field

BD Matrigel Matrix
Fluorescence

Human Microvascular Endothelial Cells
Image Analysis on BD Pathway Bioimager

BD™ Tube Formation Module
BD™ Image Data Explorer (Software)
Endothelial Cell Tubulogenesis: High Resolution Detection Using Confocal Collapsed Stack Imaging

BD HUVEC-2 cells, stained with calcein AM, 4X confocal images on BD Pathway Bioimager
Angiogenesis Segmentation

- Confocal improves images resulting in better tubule detection
- Fewer “broken” tubules
- More accurate count of tube length and number (arrow)
Angiogenesis—Dose Response to Suramin

Calcein staining, 4X confocal
Different Analysis Parameters
Similar Results

IC$_{50}$ = 2.93E-05

IC$_{50}$ = 2.94E-05

IC$_{50}$ = 2.97E-05
Effect of Cell Number
Endothelial Cell Tube Formation

**HMEC-1 Cell Number Titration**

**HUVEC Cell Number Titration**
Tube Formation Using Various Human Endothelial Cells

<table>
<thead>
<tr>
<th></th>
<th>Total tube length (pixel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVEC</td>
<td>12000 ± 200</td>
</tr>
<tr>
<td>HMVEC</td>
<td>12000 ± 200</td>
</tr>
<tr>
<td>HMEC-1</td>
<td>10000 ± 200</td>
</tr>
</tbody>
</table>

CV

- HUVEC: 7.6%
- HMVEC: 5.1%
- HMEC-1: 5.4%
HMVEC are seeded onto BD Matrigel matrix-coated 96-well plates on three different days and incubated at 37°C for 24 hours. Images are obtained using a 4X objective. Tube area is calculated for each plate (n=90).
Stimulation and Inhibition of Tube Formation

Table 2 Effects of resistin on tube-like structure formation and on VEGF secretion in the culture medium by HUVEC.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Resistin 50 ng/ml</th>
<th>Resistin 100 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube-like structures</td>
<td>68 ± 14</td>
<td>78 ± 6*</td>
<td>90 ± 12*</td>
</tr>
<tr>
<td>Total length of tubules</td>
<td>2.8 ± 8</td>
<td>4.0 ± 10*</td>
<td>4.3 ± 8*</td>
</tr>
<tr>
<td>VEGF secretion</td>
<td>411 ± 73</td>
<td>721 ± 16*</td>
<td>702 ± 20*</td>
</tr>
</tbody>
</table>

Results are means ± SE, n=5 separate experiments for the number and total length (in mm) of the tubules and n=4 separate experiments for VEGF secretion (expressed as pg VEGF/5 × 10⁶ cells). *P<0.01 compared with control.

Endothelial Cell Tube Formation

- **Assay setup** to data within 24 hours
- **Validated Protocols**
- **High-throughput**: 96-well format
- **Automated image acquisition and data processing**
  - Labeling with fluorescent dye
  - Morphometric analysis
  - Can use fluorescent microscope
- **Reproducible**
  - Consistent endothelial cell tube formation:
    - Well-to-well, lot-to-lot, assay-to-assay
  - $Z'$ value = 0.6, good for screening
BD BioCoat Angiogenesis Systems

BD HUVEC-2 cells
• Pre-qualified for VEGF responsiveness and for use with endothelial cell migration assay

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• Composed of a 96-well black/clear plate coated with BD Matrigel matrix (non-insert system)

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• Coated with human fibronectin

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• Coated with BD Matrigel matrix

Shipped on dry ice, store at -20°C
Do not store in frost free or -70°C freezer
BD Falcon FluoroBlok 24- and 96-Multiwell Insert Systems

- Unique fluorescence-blocking PET membrane
- Available in 1.0, 3.0, and 8.0 µm pore sizes
- Ease-of-use
- Real-time kinetic analyses
- Automation compatible
What is BD FluoroBlok?

The dyed membrane physically and visually separates cells and/or molecules on top of membrane from those below the membrane.

Blocks light @ 490-700 nm
1.0, 3.0, and 8.0 μm pore diameter
Individual, 24-Multiwell, and 96-Multiwell Formats

Cross section of an insert system – not to scale
Analysis of Cell Migration and Invasion Using Fluorescence Blocking PET Membrane Cell Culture Inserts

- Attractant
- Excitation (485 nm)
- Emission (530 nm)
- Fibronectin (migration)
- or
- BD Matrigel™ Matrix (invasion)
- BD FluoroBlok™ PET Membrane (3 μm pores)
Detection Instrument

- A fluorescent plate reader with bottom-reading capability, and an inverted fluorescent microscope for confirmation and troubleshooting
- A fluorescence imager
- Set Up Guidelines and Dimensional Templates for Fluorescence Plate Readers Used With BD Falcon HTS FluoroBlok Insert Systems and BD BioCoat Multiwell Insert Cell-Based Assays, *Technical Bulletin # 436*
Cell Labeling Dyes

- Any fluorescent dye derived from the fluorescein, rhodamine, and cyanine families can be used with this system
  - emission wavelength must be between 490-700 nm

- Ultraviolet-inducible dyes tend to be incompatible with the BD FluoroBlok Insert since they tend to emit light in the blue range

- For more information on spectra and alternative fluorophore choices, consult the BD FluoroBlok Insert Cross Reference Chart: Technical Bulletin #451

Endpoint or Real-Time Kinetic Assay

**POST-LABELING**

**PRE-LABELING**

For this, cell may be pre-labeled or intrinsically express a fluorescent protein.

Cell migration

Calcein AM
BD HUVEC-2 Cells Exhibit Concentration-Dependent Migration Towards VEGF

VEGF-induced cell migration assessed using the **BD BioCoat angiogenesis system: endothelial cell migration** (fibronectin-coated BD FluoroBlok membrane, 96-Multiwell format).
- Human fibronectin-coated BD FluoroBlok 24- and 96-Multiwell 3 μm pore insert systems; non-occluded pores
- Optimum endothelial cell migration
Migration Activity Using Different EC Types

HAEC Cells

<table>
<thead>
<tr>
<th>VEGF (ng/ml)</th>
<th>Fold increase over control (mean ± se)</th>
<th>8-10 fold stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>1.000</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>3.125</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>6.250</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>12.500</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>25.000</td>
<td>8.0</td>
<td></td>
</tr>
</tbody>
</table>

HUVEC-2 Cells

<table>
<thead>
<tr>
<th>VEGF (ng/ml)</th>
<th>Fold increase over control (mean ± se)</th>
<th>2-3 fold stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>1.000</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>3.100</td>
<td>2.0</td>
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</tr>
<tr>
<td>6.200</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>12.500</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>25.000</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>
This assay is well suited for medium throughput drug screening.
BD BioCoat Endothelial Cell Invasion System

- Cross section of one of 24 inserts of a Multiwell Insert plate

BD FluoroBlok membrane, 3 µm pores, 24-Multiwell insert plates
Human Primary Vascular Endothelial Cell Invasion through BD Matrigel Matrix

Control

Chemoattractant
Effect of MMP inhibitor 1'10' Phenanthroline on HMVEC Invasion

- BD Matrigel matrix-coated BD FluoroBlok 24-Multiwell 3 μm pore size insert system optimized for endothelial cell invasion
Quantitative analysis of HUVEC (A) migration or (B) invasion toward hMSC-conditioned media. Data presented are the mean of four inserts SD., *p .05 compared with hMSCs; #, *p .05 compared with hMSC SPH1. Abbreviations: DMEM, Dulbecco. Potpova, I.A., et al., *Stem Cells* **25**:1761-1768 (2007).
Time Course of MCP-1 Induced Chemotaxis in THP-1 and Primary Monocytes

- Pre-labeled cells in the inserts were incubated with 25 nM MCP-1 in the bottom chamber
- Bottom fluorescence was measured at varying time points
- Data on the graph are means ±S.D. from a typical experiment (n=4 wells)
- BD Biosciences Technical Bulletin #457
Criteria for a Robust Migration/Invasion Assay on the BD FluoroBlok Insert System

• Definition:
  – Positive control: Cells with chemoattractant
  – Negative control: Cells without chemoattractant
  – Background fluorescence:
    • Pre-labeled cells: zero time point
    • Post-labeled cells: Unlabeled cell

• Assay criteria:
  – % CV
  – Dynamic range (signal-to-noise ratio)
  – \[ Z' = 1 - \frac{3\text{SD of POS} + 3\text{SD of NEG}}{\text{Mean of POS} - \text{Mean of NEG}} \]
    • > = 0.5 is preferred
  – *Zhang, etc. 1999
Angiogenesis


Questions?

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