Deciphering the Interplay Between Notch and Growth Factor Signaling in the Complex Regulation of Vascular Morphogenesis

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Abstract

Angiogenesis is a multi-step, complex process regulated by growth factors, enzymes, and extracellular matrix molecules. In vivo, the angiogenic process involves multiple cell types acting in concert to cause endothelial cell proliferation, migration, differentiation, and, ultimately, micro-vascular arrays. Angiogenesis inhibitors are demonstrably effective in both preclinical models and clinical use, but their value can be transitory due to evasive and intrinsic resistance. The discovery of new classes of anti-angiogenic drugs has proven difficult, as very few in vitro systems adequately model the entire process. Here, we investigated the relationship between two major regulatory pathways, Notch and VEGF, using a kinetic co-culture model of angiogenesis with a compact fluorescent imaging instrument. Treatment with the Notch ligand, DLL4, specifically inhibited VEGF-mediated branching. This was reversed by γ-secretase inhibitors, indicating the presence of endogenous regulatory mechanisms restricting VEGF-mediated angiogenesis. Additionally, the contribution of endogenous VEGF on tube formation could be inhibited using pharmacological agents, which exhibited both time- and concentration-dependent effects. Interestingly, in a scratch wound model of cell migration, both DLL4 and VEGF altered the rate of endothelial cell migration. Ongoing studies are designed to develop in vitro models of resistance to anti-angiogenic drugs by using combinations of pro-angiogenic factors that more closely mimic the condition found in vivo.

GFP-AngioKit Co-culture Model

- GFP-AngioKit allows for:
  1) Continuous monitoring of tube formation via GFP fluorescence.
  2) Quantitation of drug effects while experiment is ongoing.
- Co-culture of HUVECs and Normal Human Dermal Fibroblasts.
- Lentiviral-GFP vector homogeneously labels HUVECs
  1) Viral infected cells grow similar to mock infected.
  2) Form tubes in Matrigel similar to mock infected.
  3) Form tubes in co-culture similar to mock infected.
- Compatible with IncuCyte™ acquisition and tube analysis processing using AngioKit Analysis Module.

Incucyte Technology

- Fits in an incubator; HD Phase optics
- LED light source; 50,000 hrs; relatively little heat
- Fluorescent optics optimized for measuring GFP in media
- Automated data acquisition and analysis
- Export images and movies

Automated Tube Analysis

Fig. 1. A, Representative images of HUVECs infected with lentiviral-GFP at the indicated MOI and grown for 48h at 37°C to allow for GFP expression. B-D, Co-culture of NHDFs and GFP-infected HUVECs in a 24-well format and grown for 1, 7, or 11 days, respectively. E, GFP-AngioKit components as offered by Essen Instruments, Inc.

DLL4 Enhances HUVEC Migration

Fig. 2. A-D, IncuCyte-FLR angiogenesis images at days 1, 5, 9, and 14 of the assay, respectively. Scale bar: 300μm E-H, Computational analysis of tube formation using the AngioKit Analysis Module. I-J, Graphical analysis of tube length using media optimized for high (I) or low (J) endothelial tube formation.

EGF & bFGF Potentiate Angiogenesis

Fig. 3. A-C, Treatment with three growth factors (VEGF, FGF, and EGF) potentiate tube formation in a concentration-dependent manner. Representative images indicate growth factor potentiation (right) over control (left). Scale bar is 400μm. D-F, Anti-VEGF treatment inhibits growth factor potentiation, as indicated in representative images (below each graph). Anti-VEGF treatment in the presence of respective growth factor is shown on the right of each image set.

DLL4 Inhibits VEGF-mediated Branching

Fig. 4. A, DLL4 inhibits branching of tubules in the presence of 4ng/mL, but not 16 ng/mL, VEGF. B, The DLL4 effect on tube length occurs later, and to a lesser extent, than its effect on branching. C-D, Treatment with the γ-secretase inhibitors (GSI), DAPT or L-685,458, reverse the DLL4 inhibition on tube branching, with very little effect on overall tube length, as shown in F and G, respectively. H-K, Histogram analysis of branch point formation at day 6 (H and J) and day 11 (I and K) of DAPT and L-685,458 treated cocultures, respectively. Analysis at day 6 reveals that DLL4, but neither GSI, is affecting branch point formation. However, at day 11, the GSIs reverse the DLL4 effect in a concentration dependent manner.

Summary and Conclusions

Summary:
- IncuCyte™ FLR quantitatively assessed the time- and concentration-dependence of pharmacological agents in GFP co-culture and cell migration assays.
- bFGF and EGF potentiated VEGF-stimulated tube formation.
- Neutralizing VEGF antibodies inhibited bFGF and EGF effects.
- DLL4 selectively inhibited branching of tubules and the effect was reversed with γ-secretase inhibitors.
- VEGF, EGF, and DLL4 enhanced the rate of VEGF-mediated wound closure in a concentration-dependent manner.

Conclusions:
- The GFP co-culture angiogenesis assay provides the means of quantitating the temporal effects of pharmacological agents on microvascular tubule formation to gain an in-depth understanding of their mechanism of action.
- Downstream assays such as cell migration can assist in decoding the full spectrum of the biological activity of these agents.