Development of a High-throughput Screening Co-Culture Angiogenesis Assay System Using hTERT-immortalized Primary Cells

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Abstract

Angiogenesis is a multi-step physiological process that is involved in a large number of normal and disease state processes; *in vitro* angiogenesis models provide useful tools to study these processes, one of which is the analysis of tubule formation. Several papers report that tubules formed in co-culture assays that are composed of both endothelial and stroma-producing cells were significantly more heterogeneous and more closely resembled capillaries than mono cell culture models that utilized only endothelial cells to generate tubules in an extracellular matrix. Current co-culture models using primary cells have donor variability and inconsistent results due to lot-to-lot variation. In this study, we established an *in vitro* co-culture model system consisting of an assay-ready mixture of the aortic endothelial cell line TeloHAEC-GFP (GFP-tagged, human telomerase reverse transcriptase [hTERT]-immortalized human aortic endothelial cells) and an hTERT-immortalized, adipose-derived mesenchymal stem cell line (hTERT-MSCs) in a specially formulated medium containing VEGF supplement (Angio-***Ready***™ Angiogenesis Assay System). Both cell lines were immortalized by hTERT alone and have been well-characterized, showing that the cells retain the most important characteristic of their primary counterparts. The new co-culture system forms functional tubular structures in less than 7 days; additionally, the hTERT-MSC cells that surround the tubular structures have undergone transformation as evidenced by elevated positive αSMA staining (a marker of smooth muscle cells), indicating that the system has physiological relevance. Notably, our results showed that the co-culture system has minimal lot-to-lot variation as indicated by the treatment of three lots with the anti-cancer drug ramucirumab (Cyramza®), which targets the VEGF pathway. More importantly, the tubular formation efficiency is reduced or blocked by well-known anti-cancer drugs such as sunitinib (SUTENT®) and bevacizumab (Avastin®). We also tested four hypoxia inducible factor-1 (HIF-1) inhibitors identified in previous high-throughput screens and found that those compounds inhibited tubule formation in the Angio-***Ready***™ co-culture system. These results suggest that the co-culture system can mimic the hypoxic environment in solid tumors. Previously, the authors optimized the Angio-***Ready***™ system for 384-well performance, and here we report further optimization of the system into a 1,536-well, high-throughput format and a shortening of the assay time frame to 3 days. Using this format, we evaluated 2,816 drugs from The National Center for Advancing Translational Sciences (NCATS) Pharmaceutical Collection (NPC), and 35 potent inhibitors (IC_{50} ≤1 μM) were identified. Moreover, many known angiogenesis inhibitors were identified, such as topotecan, docetaxel, and bortezomib. Several potential novel angiogenesis inhibitors (*e.g.*, thimerosal and podofilox) were also identified from this study. Among the inhibitors, some compounds were proven to be involved in the hypoxia-inducible factor-1α (HIF-1α) and the nuclear factor-kappa B (NF-κB) pathways. These results demonstrate that the co-culture model described in this report provides a consistent and robust *in vitro* system for antiangiogenic drug screening.

Introduction

- Angiogenesis is a multi-step physiological process; it is also involved in a large number of disease states.
- *In vitro* angiogenesis models provide very useful tools to study angiogenesis; additionally, these model can be used in drug screening applications.
- Tubules formed in co-culture assays were significantly more heterogeneous and more closely resembled capillaries than tubules formed in Matrigel® matrix (Corning)[1].
- A few *in vitro* co-culture models have been developed using primary cells; however, donor variability, low cell quantity per lot, and the short lifespan of primary cells limit their usefulness and consistency.
- In this study, we established an *in vitro* co-culture model system using cell lines that were immortalized by hTERT alone.
- Systematic procedures have been employed to validate this co-culture model using VEGF pathway-related compounds.
- The assay has been optimized to a 384- or 1,536-well, high-throughput screening format and been used to identify 35 potent angiogenesis inhibitors out of a NIH drug library of 2,816 drugs.

Results

Angio-*Ready*™ Kit enables a "thaw, seed, and go" process

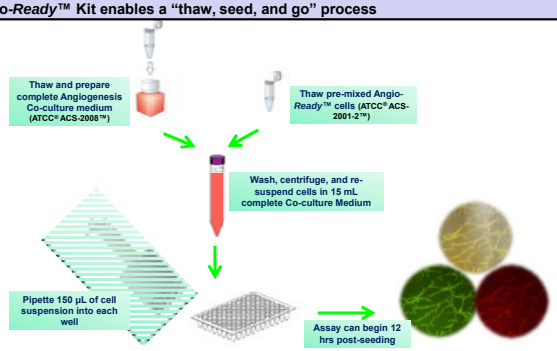

Figure 1. Angio-*Ready*™(ATCC® ACS-2001-2™) Assay overview: "thaw, seed, and assay".

TeloHAEC-GFP and hTERT-MSC co-culture model is similar to *in vivo* physiology

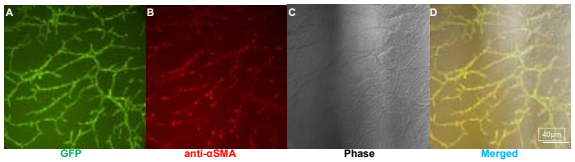

Figure 2. Establishment of TeloHAEC-GFP and hTERT-MSC co-culture angiogenesis. TeloHAEC-GFPs co-cultured with hTERT-MSCs for 7 days in the optimized angiogenesis medium displayed a long branching organization (A) and exhibited immuno-reactivity to an αSMA antibody (B), which co-localized with the TeloHAEC-GFPs (D). Phase contrast microscopy indicated the 3-dimensional structure of the tubes (C).

The Angio-*Ready*™ kit is a robust system for compound screening and is very consistent between multiple different kit lots

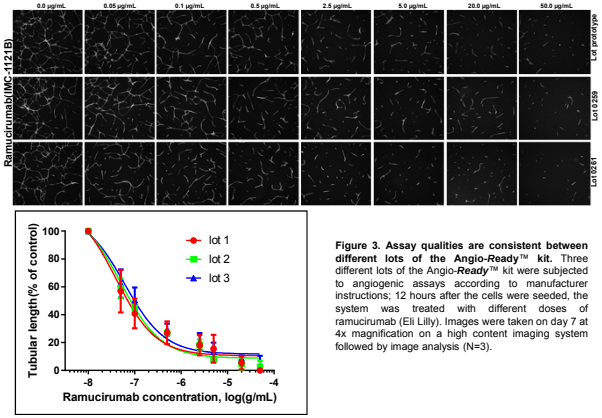

Figure 3. Assay qualities are consistent between different lots of the Angio-*Ready*™ kit. Three different lots of the Angio-***Ready***™ kit were subjected to angiogenic assays according to manufacturer instructions; 12 hours after the cells were seeded, the system was treated with different doses of ramucirumab (Eli Lilly). Images were taken on day 7 at 4x magnification on a high content imaging system followed by image analysis (N=3).

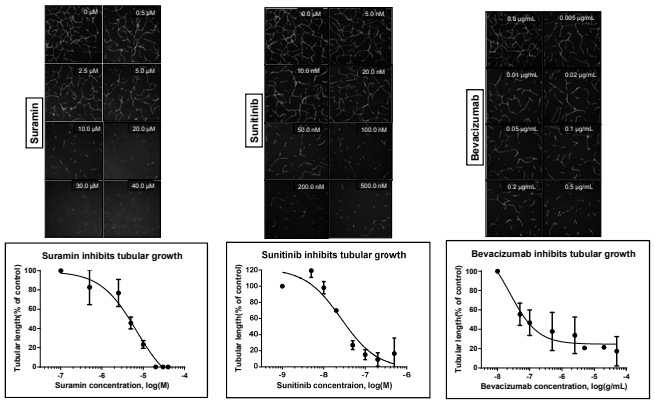

Figure 4. The Angio-*Ready*™ assay system is sensitive to well-known VEGF inhibitors, including two clinically approved prescription cancer drugs. The Angio-***Ready***™ assay was conducted according to manufacturer instructions; 12 hours after cells were seeded, different doses of compounds such as suramin, sunitinib (Pfizer), and bevacizumab (Genentech) were added. Images were taken on day 7 at 4x magnification (N=3).

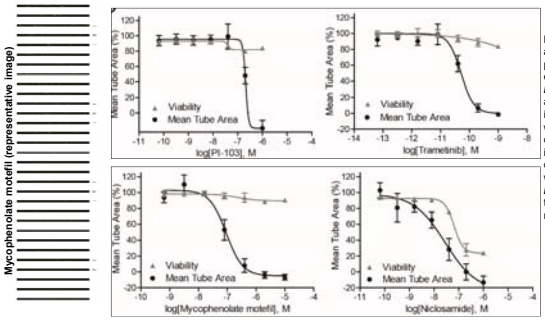

Figure 5. The Angio-*Ready*™ assay system displays a potent response to anti-HIF-1 compounds. The Angio-***Ready***™ assay was conducted according to the manufacturer's instructions; 12 hours after cells were seeded, 4 anti-HIF-1 compounds that had been identified through other high content screening methods were tested using the Angio-***Ready***™ system. Images were taken on day 3 at 4x magnification (N=3).

The actual use of the Angio-*Ready*™ system for screening an NIH drug library of 2,816 drugs

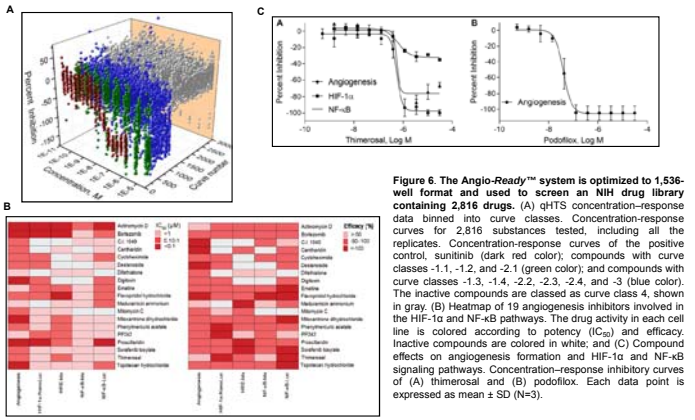

Figure 6. The Angio-*Ready*™ system is optimized to 1,536-well format and used to screen an NIH drug library containing 2,816 drugs. (A) qHTS concentration–response data binned into curve classes. Concentration-response curves for 2,816 substances tested, including all the replicates. Concentration-response curves of the positive control, sunitinib (dark red color); compounds with curve classes -1.1, -1.2, and -2.1 (green color); and compounds with curve classes -1.3, -1.4, -2.2, -2.3, -2.4, and -3 (blue color). The inactive compounds are classed as curve class 4, shown in gray. (B) Heatmap of 19 angiogenesis inhibitors involved in the HIF-1α and NF-κB pathways. The drug activity in each cell line is colored according to potency (IC_{50}) and efficacy. Inactive compounds are colored in white; and (C) Compound effects on angiogenesis formation and HIF-1α and NF-κB signaling pathways. Concentration–response inhibitory curves of (A) thimerosal and (B) podofilox. Each data point is expressed as mean ± SD (N=3).

Summary

- Measurable, fine, tubular structures form in a minimum of 3 days in the TeloHAEC-GFP/hTERT-MSC co-culture system; the MSCs that surrounded the tubular structures in an *in vivo*-like manner expressed a marker of smooth muscle cells.
- Tubular formation efficiency is increased by VEGF stimulation and decreased by the VEGF inhibitors suramin, sunitinib, and bevacizumabin in a dose-dependent manner.
- No lot-to-lot variation is seen between 3 different lots of the Angio-***Ready***™ system.
- Test compounds identified through external high-content screening methods as being antiangiogenic blocked tubule formation in the Angio-***Ready***™ assay.
- This co-culture model provides a consistent and robust *in vitro* system for studying vascular biology and tissue engineering, as well as for high-throughput compound screening.

References

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