Reporter Cell Lines Derived From Immortalized Dermal Microvascular Endothelial Cells As Improved Cell Models For Vascular Biology Research

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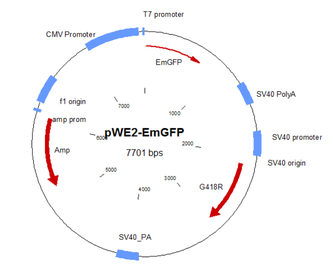
Introduction

Replicative senescence and donor variability among different isolations of primary endothelial cells can restrict the potential usefulness of this cell model in the study of vascular biology. Immortalized cell lines can provide useful alternatives for primary cells particularly when applications call for consistency of cell characteristics over time. The utility of these immortalized cell lines can be further enhanced through specific genetic modifications, such as the introduction of a reporter gene. These improved cell lines are especially useful for screening and other high throughput applications where repeatability is key. We have generated two reporter cell lines based on immortalized dermal microvascular endothelial cells for use as models for vascular biology study.

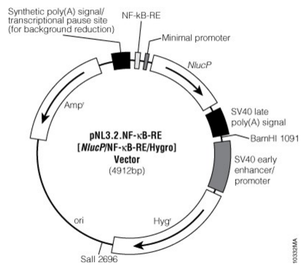
The Telomerase-Immortalized Microvascular Endothelial GFP-expressing reporter cell line TIME-GFP, ATCC® CRL-4045™ was generated using TIME, ATCC® CRL-4025™ as the parental line, which was established by hTERT-immortalization of neonatal foreskin microvascular endothelial cells. These cells exhibit a normal diploid karyotype, extended lifespan in culture and endothelial characteristics that make them an ideal model to create reporter cell lines for investigating many aspects of endothelial cell biology. The clonal reporter cell line (TIME-GFP, ATCC® CRL-4045™) stably expresses green fluorescence protein (GFP) and retains angiogenic potential for at least 12 passages. The GFP-expressing cells migrate and coalesce into networks of vessel-like structure within 8 hours after being plated onto Cell Basement Membrane Gel (ATCC® ACS-3035™). The stable expression of GFP in these cells enables detection and analysis of the fragile endothelial structures to occur without post-assay fixation and/or staining.

We have also developed a NanoLuc® luciferase reporter cell line (NFKB-TIME, ATCC® CRL-4049™) in which the expression of NanoLuc® luciferase is regulated by multiple copies of the NFκB response element. When the cells are exposed to inflammatory cytokine such as TNFα, activation of the NFκB signaling pathway results in increased NanoLuc® luciferase activity, which correlates to the canonical activation of intercellular adhesion molecule 1 (ICAM-1) assays. The high sensitivity, excellent signal/background ratio and simple single-addition assay makes this reporter cell line an ideal replacement for the cumbersome and highly variable ICAM-1 activation assays. The TIME cell line and derivative reporter cell lines described in this report provide valuable

I. Cell Line Development

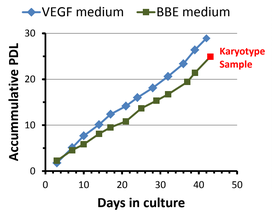


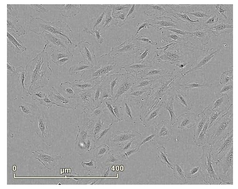
TIME-GFP (ATCC® CRL-4045™), a clonal cell line with stable expression of the Vivid Colors™ EmGFP fluorescence protein is established after G418 selection of TIME (ATCC® CRL-4025™) cells that have integrated the expression vector pWE2-EmGFP, in which the EmGFP expression is driven by a CMV promoter.

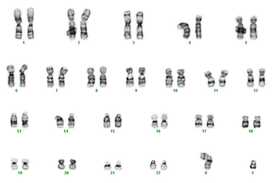


NFKB-TIME (ATCC® CRL-4049™) cell line is established after hygromycin selection of TIME (ATCC® CRL-4025™) cells that have integrated the NanoLuc® vector pNL3.2.NF-κB-RE[NlucP/NF-κB-RE/Hygro] that contains five copies of an NF-κB response element (NF-κB-RE) that drives transcription of a destabilized form of NanoLuc® luciferase.

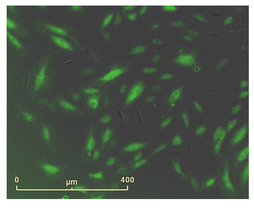
II. Characterization of TIME-GFP

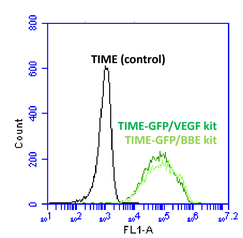


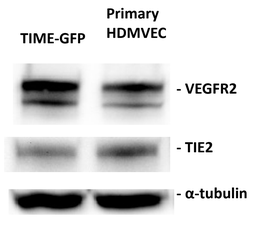




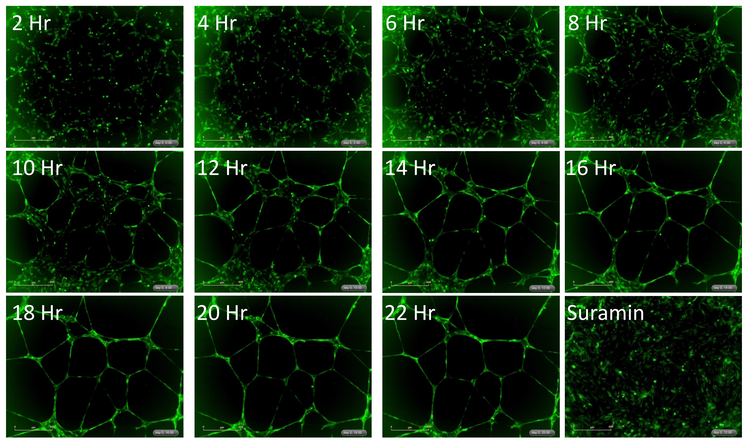
TIME-GFP (ATCC® CRL-4045™) cells exhibit typical "cobblestone" endothelial morphology, and maintain consistent growth rate in either Vascular Cell Basal Medium (ATCC® PCS-100-030™) supplemented with Microvascular Endothelial Cell Growth Kit-BBE (ATCC® PCS-110-040™) or Microvascular Endothelial Cell Growth Kit-VEGF (ATCC® PCS-110-041™). The TIME-GFP cells retain normal 46 chromosomes after extended culture in both culture media.







TIME-GFP (ATCC® CRL-4045™) cells stably express the Vivid Colors™ EmGFP fluorescence protein as examined by fluorescence microscopy (overlaid with phase contrast images) as well as flow cytometry. The TIME-GFP cells express essential cell-surface receptors, VEGFR2 and TIE2, which play important roles in endothelial cell growth and angiogenesis at comparable levels to primary human dermal microvascular endothelial cells (HDMVEC). The TIME-GFP cells also express endothelial cell markers such as CD31/PECAM-1 and VE-cadherin, and are capable of uptaking low-density lipoprotein (LDL) from the culture medium.

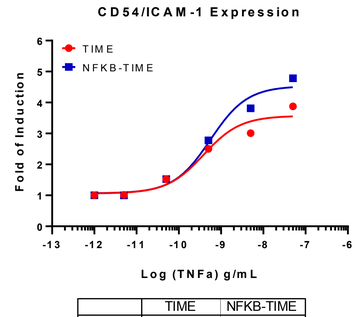


TIME-GFP cells migrate and coalesce into networks of vessel-like structure within 8 hours after being plated onto basement membrane gel. The stable expression of GFP in these cells enables detection and analysis of the fragile endothelial structures to occur without post-assay fixation and/or staining. The tubulogenesis of TIME-GFP cells is completely blocked by the addition of 30nM Suramin in the culture medium.

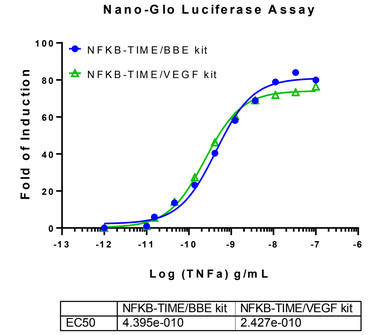
III. Characterization of NFKB-TIME

Similar to TIME-GFP (ATCC® CRL-4045™), the NFKB-TIME (ATCC® CRL-4049™) cells also retain essential microvascular endothelial cell function over extended culture period, including:

- Consistent growth rate in Vascular Cell Basal Medium (ATCC® PCS-100-030™) supplemented with Microvascular Endothelial Cell Growth Kit-BBE (ATCC® PCS-110-040™) or Microvascular Endothelial Cell Growth Kit-VEGF (ATCC® PCS-110-041™).
- Stable normal karyotype (46,XY) over >25 population doublings.
- Express endothelial cell markers such as CD31/PECAM-1 and are capable of uptaking low-density lipoprotein (LDL) from the culture medium.
- Able to migrate and coalesce into networks of vessel-like structure after being plated onto Cell Basement Membrane Gel (ATCC® ACS-3035™).

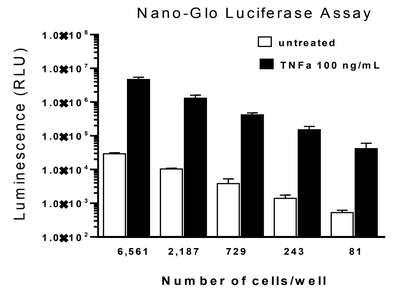


	TIME	NFKB-TIME
EC50	3.868e-010	5.590e-010



	NFKB-TIME/BBE kit	NFKB-TIME/VEGF kit
EC50	4.395e-010	2.427e-010

The expressions of CD54/ICAM-1 molecule on the surface of both TIME (ATCC® CRL-4025™) and NFKB-TIME (ATCC® CRL-4049™) cells significantly increase upon treatment of inflammatory cytokine such as TNFα. The NFKB-TIME cells express NanoLuc® luciferase regulated by multiple copies of the NFκB response element. When the cells are exposed to TNFα, activation of the NFκB signaling pathway results in increased NanoLuc® luciferase activity. The half maximal effective concentrations (Ec50) of TNFα measured by traditional CD54/ICAM-1 activation assay and Nano-Glo® luciferase assay are very similar in NFKB-TIME cells, yet the reporter cell line exhibits a much higher fold of induction for the activation of the NFκB signaling pathway.



Cells/well	Luminescence (RLU)		FOI
	Untreated	100 ng/mL TNFα	
6,561	29,228 ± 2,153	4,664,244 ± 786,091	160
2,187	10,347 ± 578	1,297,479 ± 301,887	125
729	3,812 ± 1,482	418,410 ± 57,006	110
243	1,394 ± 349	150,531 ± 38,821	108
81	528 ± 89	41,651 ± 18451	79

The use of NanoLuc® luciferase reporter greatly enhances assay sensitivity in the NFKB-TIME cells (ATCC® CRL-4049™). Variable number of NFKB-TIME cells were seeded into 96-well microplate and incubated for 24 hours in culture medium. The cells were then exposed to 100 ng/mL TNFα for 3 hours to activate the NFκB signaling pathway. Comparable fold of induction (FOI) of luminescence was observed within a wide range of initial cell seeding densities. Less than 100 cells/well produced significant activation of the reporter gene expression.

Summary

Human telomerase (hTERT) immortalized cell lines retain most differentiated cell functions and offer extended proliferative capacity *in vitro*. These cell lines can provide useful alternatives for primary cells particularly when consistency of cell characteristics is key. The utility of the hTERT-immortalized cell lines can be further enhanced through specific genetic modifications, such as the introduction of a reporter gene. The TIME cell line and derivative reporter cell lines (TIME-GFP and NFKB-TIME) described in this report provide valuable model systems for vascular biology, drug screening and tissue engineering.