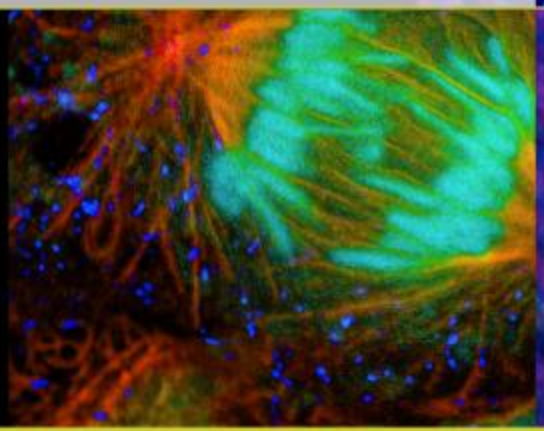
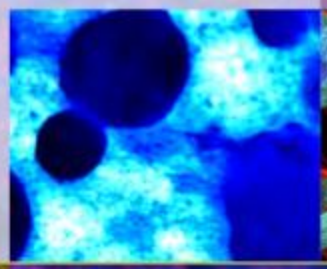
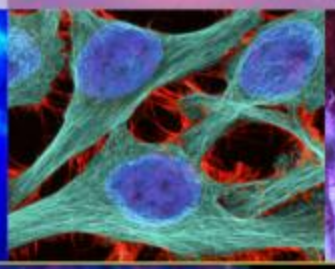
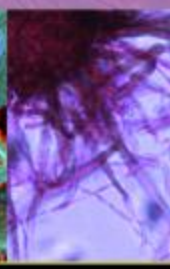
HTERT IMMORTALIZED CELL LINES – UNIQUE TOOLS FOR TISSUE-RELEVANT RESEARCH

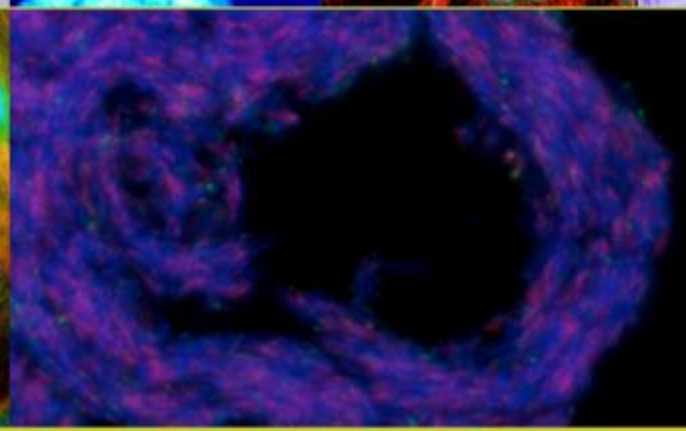
Chengkang Zhang, Ph.D.
Senior Scientist
ASCB Vendor Showcase
Dec. 15, 2013

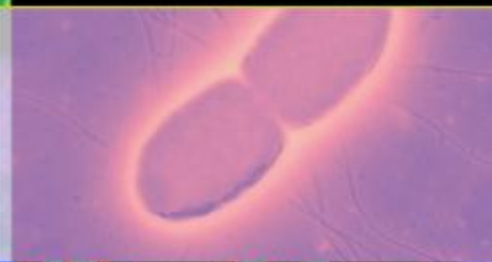


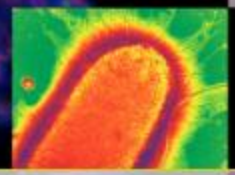












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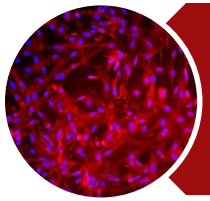
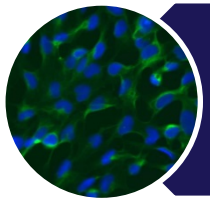
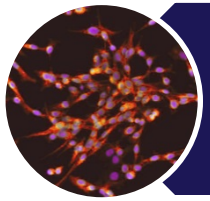
Outline



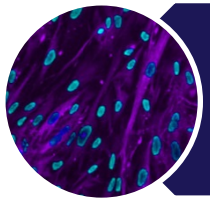
History of cell culture, telomerase, and cell immortalization



Create your own immortalized cell lines



Overview of hTERT immortalized cell lines from ATCC



Examples of hTERT immortalized cell lines

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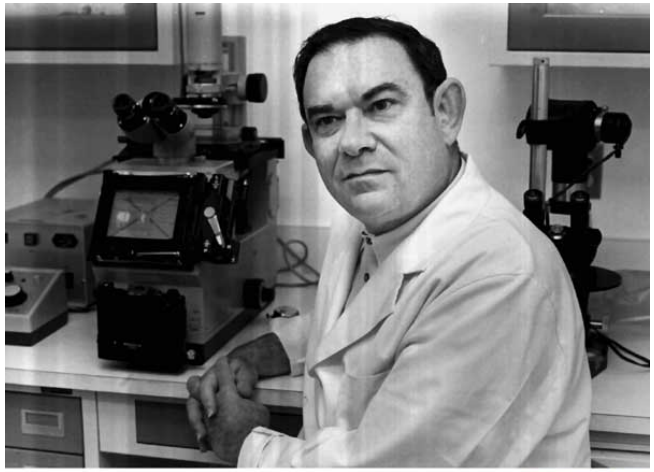
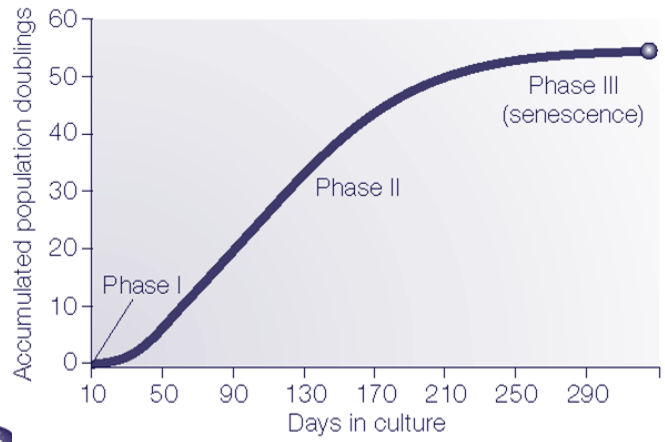
Inconvenience of primary cell culture

The Hayflick Limit



Nature Reviews | Molecular Cell Biology



Who: Leonard Hayflick
When: 1965
Methods: Normal diploid cells were serially passaged in culture until they stopped dividing
Institution: Wistar Institute
Where: Philadelphia, PA, U.S.A

Primary human cell strains each have a characteristic replicative lifespan or "doubling potential", and that this lifespan is an intrinsic characteristic that can differ between strains.

- **Phase I** is the primary culture.
- **Phase II** represents subcultivated cells during the period of exponential replication.
- **Phase III** represents the period when cell replication ceases but metabolism continues. Cells may remain in this state for one year before death occurs.

ATCC

Shay JW & Wright WE. *Nature Reviews Molecular Cell Biology* 1: 72-76, 2000

Telomere and telomerase: The history

1938/1941
The ends of chromosomes had to be capped by a special structure termed "telomere"
H. Muller/B. McClintock

1961/1965
Postulation of the "Hayflick limit"

1972
"End replication problem"
J. Watson

1978-1982
Sequence of *Tetrahymena* telomere TTGGGG
E. Blackburn, J Szostak

1985
Discovering of Telomerase.
C Greider, E. Blackburn

1989
Telomere hypothesis of cell senescence - yeast
J Szostak

1990
-human cells/cancer

1994
Development of the PCR-based"TRAP assay"

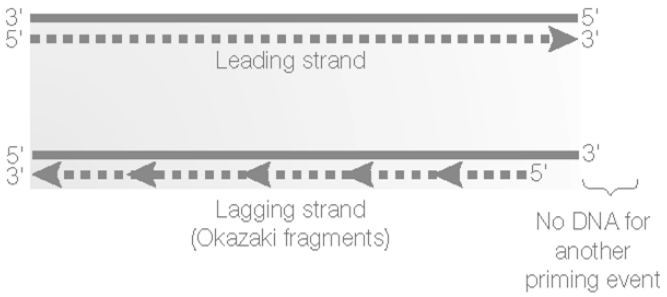
1995
Cloning of hTR

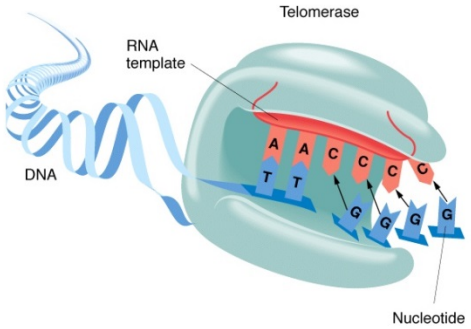
1995/1997
Characterization of TRF1/TRF2
Cloning of hTERT
Generation of the "telomerase knockout mouse«

1997

1998
Ectopic expression of telomerase in normal fibroblasts and epithelial cells bypasses the Hayflick's limit

1999/2000…
Telomerase/telomere dysfunctions and cancer





The Nobel Prize in Physiology or Medicine 2009

"for the discovery of how chromosomes are protected by telomeres and the enzyme telomerase"


Photo: Gerbil, Licensed by Attribution Share Alike 3.0

Elizabeth H. Blackburn


Photo: Gerbil, Licensed by Attribution Share Alike 3.0

Carol W. Greider


Photo © Harvard Medical School

Jack W. Szostak

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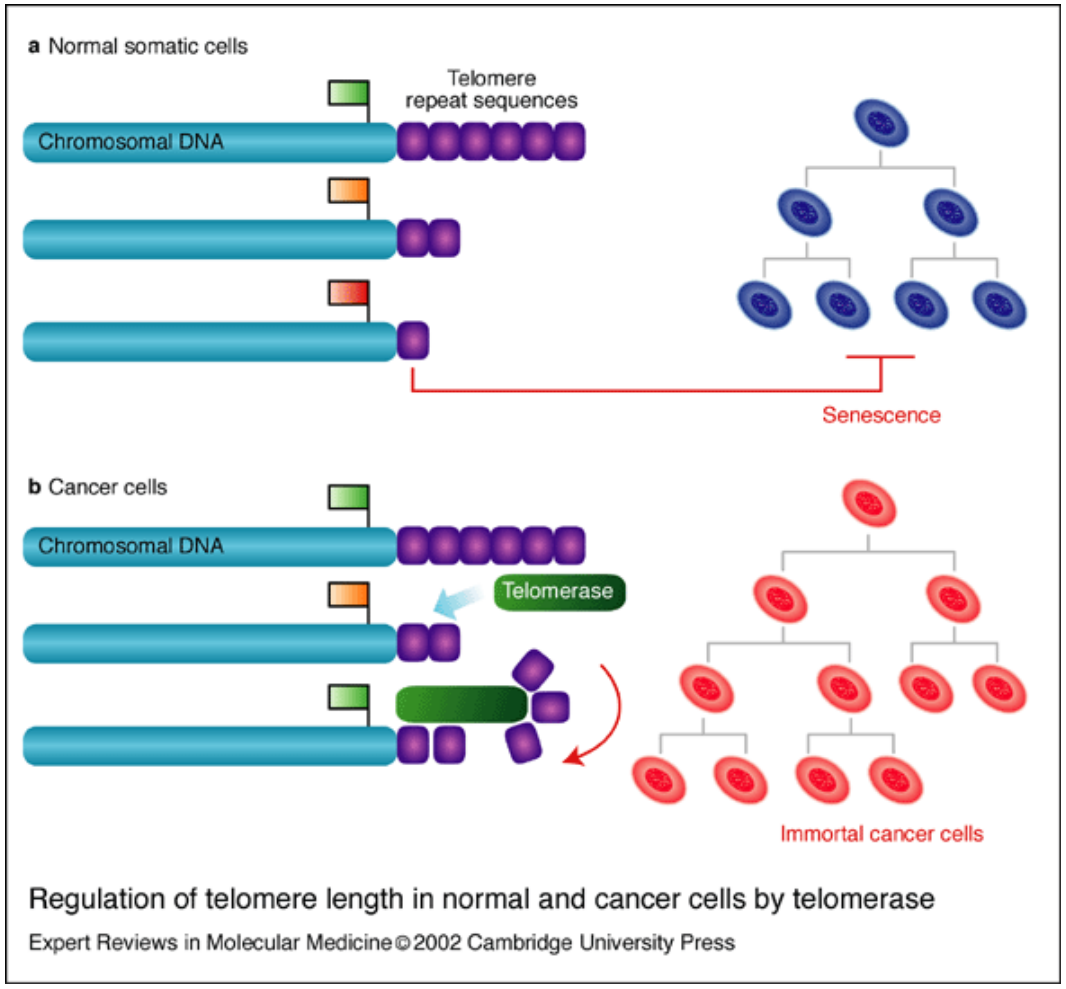
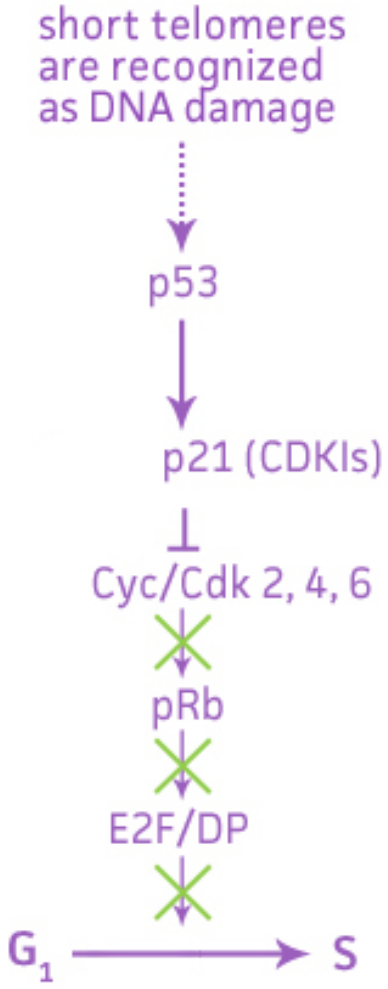
Shaw JW, Wright WE. *Nature Reviews Molecular Cell Biology* 1: 72-76, 2001.

Bypass replicative senescence by telomerase



Regulation of telomere length in normal and cancer cells by telomerase

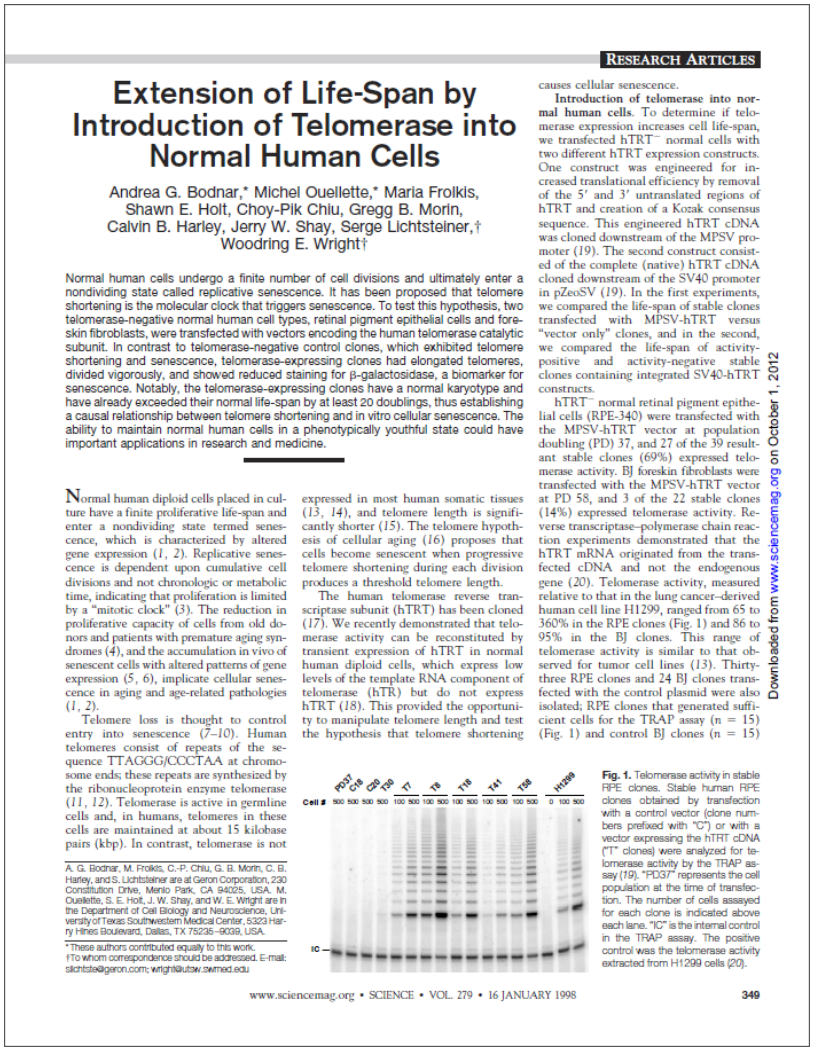
Expert Reviews in Molecular Medicine ©2002 Cambridge University Press



Keith WN, *et al*. *Expert Reviews in Molecular Medicine*, April 22, 2002.

http://www.senescence.info/telomeres_telomerase.html

Immortalization of normal human cells by hTERT



RESEARCH ARTICLES

Extension of Life-Span by Introduction of Telomerase into Normal Human Cells

Andrea G. Bodnar,* Michel Ouellette,* Maria Frolkis, Shawn E. Holt, Choy-Pik Chiu, Gregg B. Morin, Calvin B. Harley, Jerry W. Shay, Serge Lichtsteiner,† Woodring E. Wright†

Normal human cells undergo a finite number of cell divisions and ultimately enter a nondividing state called replicative senescence. It has been proposed that telomere shortening is the molecular clock that triggers senescence. To test this hypothesis, two telomerase-negative normal human cell types, retinal pigment epithelial cells and foreskin fibroblasts, were transfected with vectors encoding the human telomerase catalytic subunit. In contrast to telomerase-negative control clones, which exhibited telomere shortening and senescence, telomerase-expressing clones had elongated telomeres, divided vigorously, and showed reduced staining for β-galactosidase, a biomarker for senescence. Notably, the telomerase-expressing clones have a normal karyotype and have already exceeded their normal life-span by at least 20 doublings, thus establishing a causal relationship between telomere shortening and in vitro cellular senescence. The ability to maintain normal human cells in a phenotypically youthful state could have important applications in research and medicine.

Normal human diploid cells placed in culture have a finite proliferative life-span and enter a nondividing state termed senescence, which is characterized by altered gene expression (*1*, *2*). Replicative senescence is dependent upon cumulative cell divisions and not chronologic or metabolic time, indicating that proliferation is limited by a "mitotic clock" (*3*). The reduction in proliferative capacity of cells from old donors and patients with premature aging syndromes (*4*), and the accumulation in vivo of senescent cells with altered patterns of gene expression (*5*, *6*), implicate cellular senescence in aging and age-related pathologies (*1*, *2*).

Telomere loss is thought to control entry into senescence (*7*–*10*). Human telomeres consist of repeats of the sequence TTAGGG/CCCTAA at chromosome ends; these repeats are synthesized by the ribonucleoprotein enzyme telomerase (*11*, *12*). Telomerase is active in germline cells and, in humans, telomeres in these cells are maintained at about 15 kilobase pairs (kbp). In contrast, telomerase is not expressed in most human somatic tissues (*13*, *14*), and telomere length is significantly shorter (*15*). The telomere hypothesis of cellular aging (*16*) proposes that cells become senescent when progressive telomere shortening during each division produces a threshold telomere length.

The human telomerase reverse transcriptase subunit (hTRT) has been cloned (*17*). We recently demonstrated that telomerase activity can be reconstituted by transient expression of hTRT in normal human diploid cells, which express low levels of the template RNA component of telomerase (hTR) but do not express hTRT (*18*). This provided the opportunity to manipulate telomere length and test the hypothesis that telomere shortening causes cellular senescence.

Introduction of telomerase into normal human cells. To determine if telomerase expression increases cell life-span, we transfected hTRT⁻ normal cells with two different hTRT expression constructs. One construct was engineered for increased translational efficiency by removal of the 5′ and 3′ untranslated regions of hTRT and creation of a Kozak consensus sequence. This engineered hTRT cDNA was cloned downstream of the MPSV promoter (*19*). The second construct consisted of the complete (native) hTRT cDNA cloned downstream of the SV40 promoter in pZeoSV (*19*). In the first experiments, we compared the life-span of stable clones transfected with MPSV-hTRT versus "vector only" clones, and in the second, we compared the life-span of activity-positive and activity-negative stable clones containing integrated SV40-hTRT constructs.

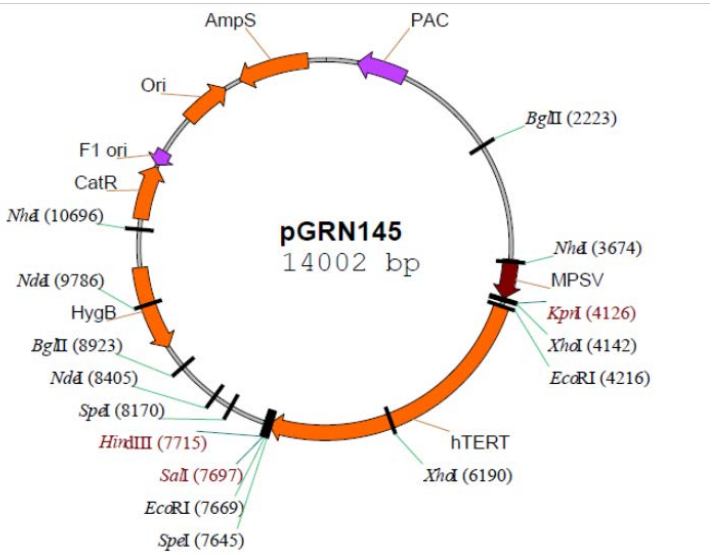
hTRT⁻ normal retinal pigment epithelial cells (RPE-340) were transfected with the MPSV-hTRT vector at population doubling (PD) 37, and 27 of the 39 resultant stable clones (69%) expressed telomerase activity. BJ foreskin fibroblasts were transfected with the MPSV-hTRT vector at PD 58, and 3 of the 22 stable clones (14%) expressed telomerase activity. Reverse transcriptase–polymerase chain reaction experiments demonstrated that the hTRT mRNA originated from the transfected cDNA and not the endogenous gene (*20*). Telomerase activity, measured relative to that in the lung cancer–derived human cell line H1299, ranged from 65 to 360% in the RPE clones (Fig. 1) and 86 to 95% in the BJ clones. This range of telomerase activity is similar to that observed for tumor cell lines (*13*). Thirty-three RPE clones and 24 BJ clones transfected with the control plasmid were also isolated; RPE clones that generated sufficient cells for the TRAP assay (n = 15) (Fig. 1) and control BJ clones (n = 15)

Fig. 1. Telomerase activity in stable RPE clones. Stable human RPE clones obtained by transfection with a control vector (clone numbers prefixed with "C") or with a vector expressing the hTRT cDNA ("T" clones) were analyzed for telomerase activity by the TRAP assay (*19*). "PD37" represents the cell population at the time of transfection. The number of cells assayed for each clone is indicated above each lane. "IC" is the internal control in the TRAP assay. The positive control was the telomerase activity extracted from H1299 cells (*20*).

A. G. Bodnar, M. Frolkis, C.-P. Chiu, G. B. Morin, C. B. Harley, and S. Lichtsteiner are at Geron Corporation, 230 Constitution Drive, Menlo Park, CA 94025, USA. M. Ouellette, S. E. Holt, J. W. Shay, and W. E. Wright are in the Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235–9039, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: slichtste@geron.com; wright@utsw.swmed.edu

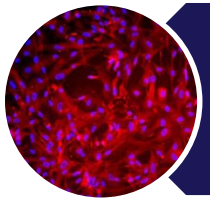
RPE1	Retinal Pigment Epithelial Cell	CRL-4000™	hTERT-RPE1
BJ	Foreskin Fibroblast	CRL-4001™	BJ-5ta



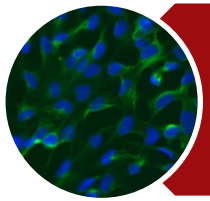
pGRN145, plasmid in *E. coli*
ATCC® MBA-141

Bodnar AG, *et al*. *Science* 279: 349-352, 1998.

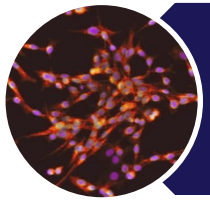
Outline



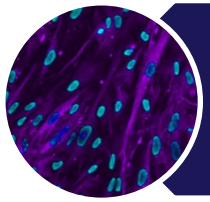
History of cell culture, telomerase, and cell immortalization



Create your own immortalized cell lines



Overview of hTERT immortalized cell lines from ATCC



Examples of hTERT immortalized cell lines

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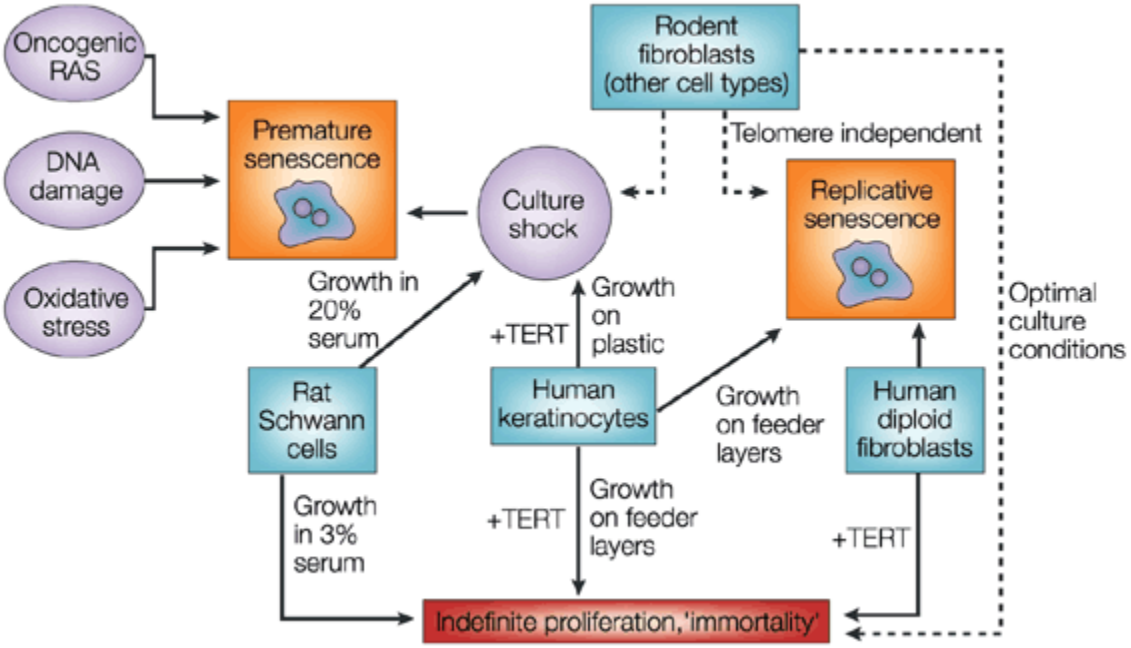
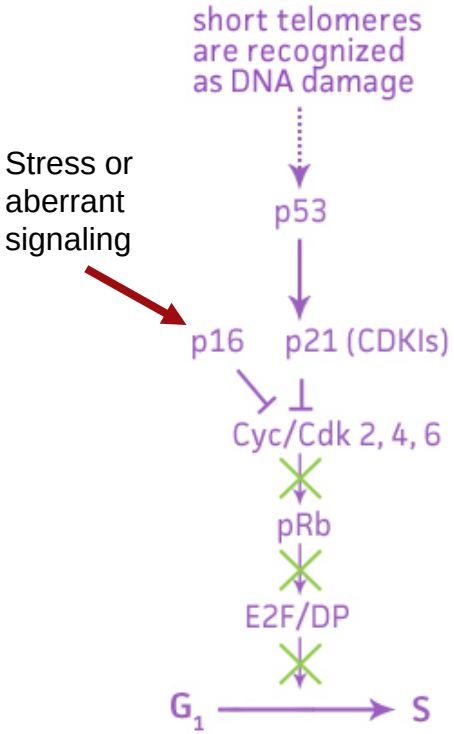
Roads to cell immortalization



Mathon NF, Lloyd AC. *Nature Reviews Cancer* 1: 203-213, 2001.



http://www.senescence.info/telomeres_telomerase.html

↗ Telomerase
- hTERT
- HPV-16 E6
- Myc T58A

↘ p53/p21
- SV40T
- HPV-16 E6

↘ p16/pRB
- HPV-16 E7
- CDK4
- Bmi-1

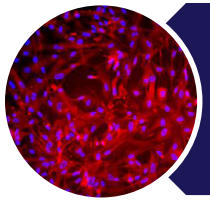
Other Methods
- Feeder culture (3T3)
- Rho-associated kinase inhibitor (Y-27632)
- Physiological Oxygen (2-5%)

ATCC

Tools for cell immortalization

Plasmids and Reagents	ATCC® No.
hTERT	MBA-141™
SV40-Baylor	VRMC-3™
HPV-16 E6/E7	CRL-2203™, 45113D™
CDK4	MGC-19704™, MGC-4678™, MGC-3719™
Bmi-1	81582D™, MGC-12685™
3T3 Feeder Cells	CCL-92™, 48-X™
ROCK Inhibitor Y-27632	ACS-3030™

Outline



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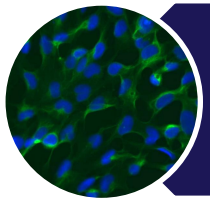

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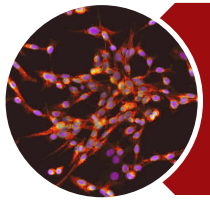

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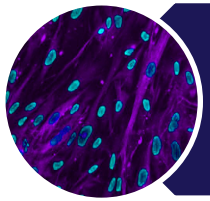

Examples of hTERT immortalized cell lines

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hTERT immortalized cell lines from ATCC

Tissue	Cell Type	ATCC® No	Designations	Comments
Breast	Mammary Epithelial	CRL-4010™	hTERT-HME1	Normal adult
Bone	Bone Cartilage Fibroblast	CRL-2846™, CRL-2847™	CHON-001, CHON-002	Normal fetal
Esophagus	Barrett's Esophageal Epithelial	CRL-4027™, CRL-4028™, CRL-4029™, CRL-4030™	CP-A, CP-B, CP-C, CP-D	Pre-malignant sample
Eye	Retinal Pigment Epithelial	CRL-4000™	hTERT-RPE1	Normal
Kidney	Angiomyolipoma	CRL-4004™	UMB1949	Angiomyolipoma
		CRL-4008™	SV7tert PDGF tumor-1	Autocrine transformation and epigenetic changes
	Proximal Tubule Epithelial	CRL-4031™	RPTEC/TERT1	Normal adult
Lung	Bronchial Epithelial	CRL-4011™	NuLi-1	Normal adult
		CRL-4013™, CRL-4015™, CRL-4016™, CRL-4017™	CuFi-1, CuFi-4, CuFi-5, CuFi-6	Cystic Fibrosis
		CRL-4051™	HBEC3-KT **(coming soon)**	Normal adult
	Small Airway Epithelial	CRL-4050™	HSAEC1-KT **(coming soon)**	Normal adult
Pancreas	Pancreatic Duct	CRL-4023™	hTERT-HPNE	Normal adult
		CRL-4036™, CRL-4037™, CRL-4038™, CRL-4039™	hTERT-HPNE E6/E7, E6/E7/st, E6/E7/K-RasG12D, E6/E7/K-RasG12D/st	Stepwise oncogenic transformation
Skin	Foreskin Fibroblast	CRL-4001™	BJ-5ta	Normal neonatal
	Keratinocyte	CRL-4048™	Ker-CT **(just released)**	Normal neonatal
	Dermal Fibroblast	CRL-4005™	TelCOFS02MA **(just released)**	COFS
Uterus	Endometrium Stromal	CRL-4003™	T HESCs	Normal adult
Vascular	Microvascular Endothelial	CRL-4025™	TIME	Normal neonatal
	Microvascular Endothelial	CRL-4045™	TIME-GFP **(just released)**	Stable GFP expression
	Microvascular Endothelial	CRL-4049™	NFKB-TIME **(coming soon)**	NanoLuc reporter line
	Aortic Endothelial	CRL-4052™	TeloHAEC **(coming soon)**	Normal adult

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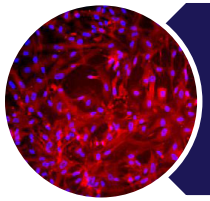
Outline



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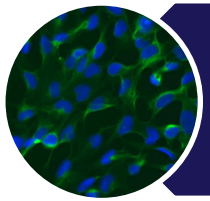

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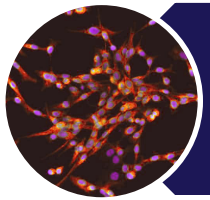

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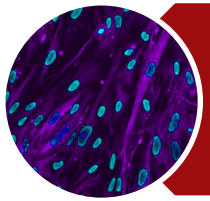

Examples of hTERT immortalized cell lines

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RPTEC/TERT1 (CRL-4031™) – A new cell model

Limitations of existing *in vitro* renal cell cultures

Primary Cells

Obtaining primary cultures from the kidney is hampered by the fact that there are 15-20 cell types that comprise the kidney cortex and the nephron. Homogeneous cultures retaining physiological functions are hard to obtain.

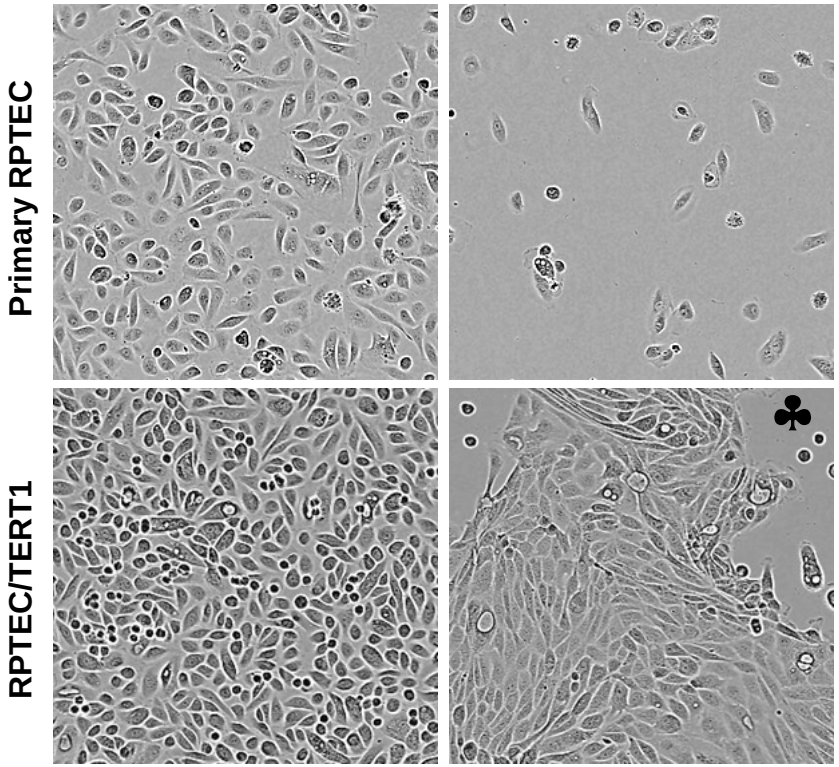
Immortal renal epithelial cell lines

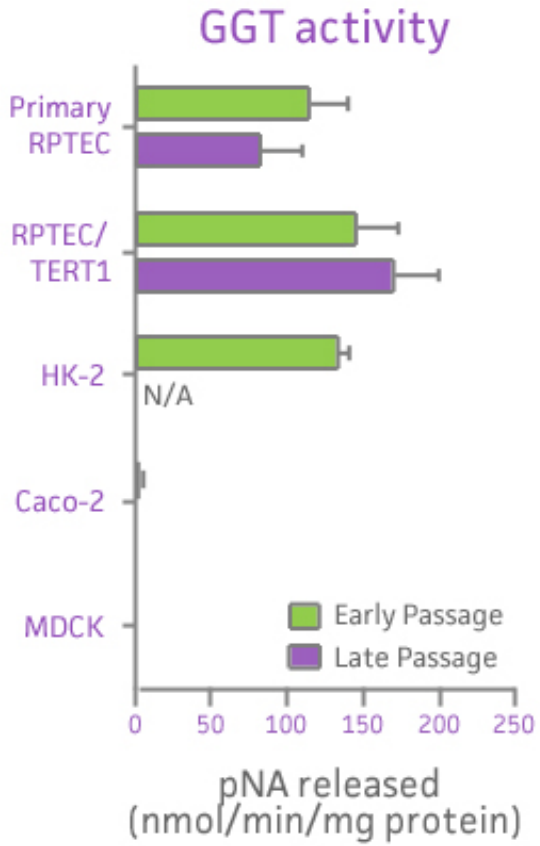
Cell Line	Derived from	Nephron Segment of origin
LLC-PK1	Yorkshire Pig	Proximal nephron
OK	North American Opossum	Proximal nephron
JTC-12	Monkey	Proximal nephron
MDCK	Dog	Collecting duct
A6	Xenopus laevis	Distal tubule
HK-2	Human	HPV16-transformed, Proximal/Distal?
Caki-1	Human	Kidney carcinoma
HEK293/OATs	Human	OATs over-expressing lines

None of the continuous renal epithelial cell lines fully express all the needed differentiated functions known from the ancestor cells *in vivo*

ATCC®

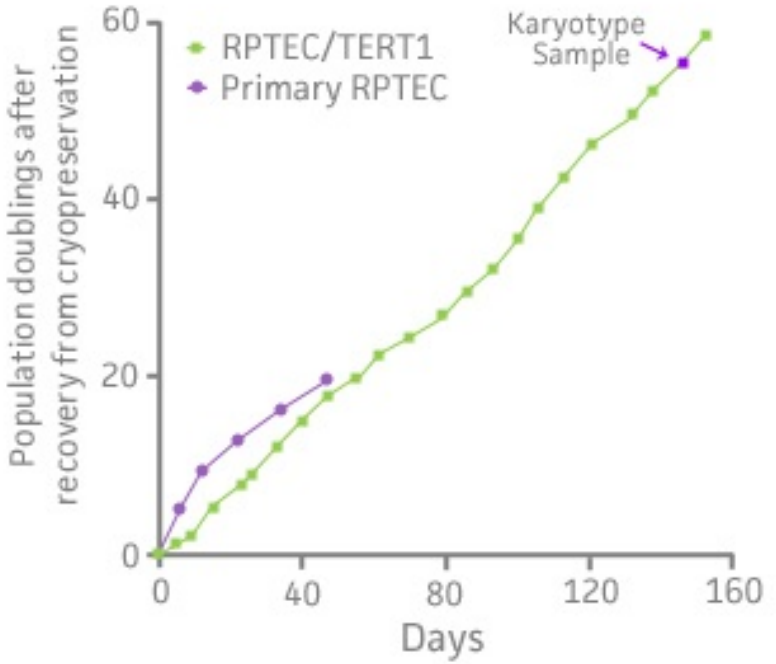
RPTEC/TERT1- Characteristic morphology and biochemical markers

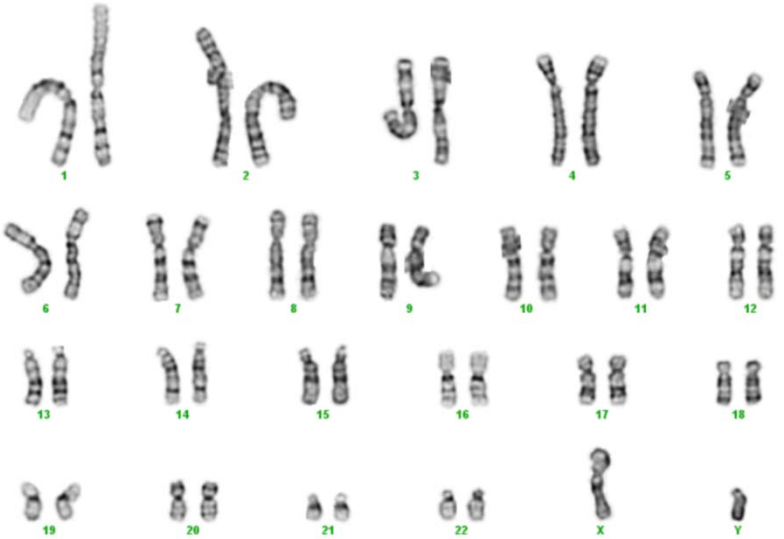




The RPTEC/TERT1 cells exhibit characteristic epithelial morphology only in the serum-free medium (♣) and express γ-Glutamyl Transferase (GGT), a marker protein located in the brush border of the renal proximal tubule epithelia.

RPTEC/TERT1 – Extended lifespan and stable karyotype

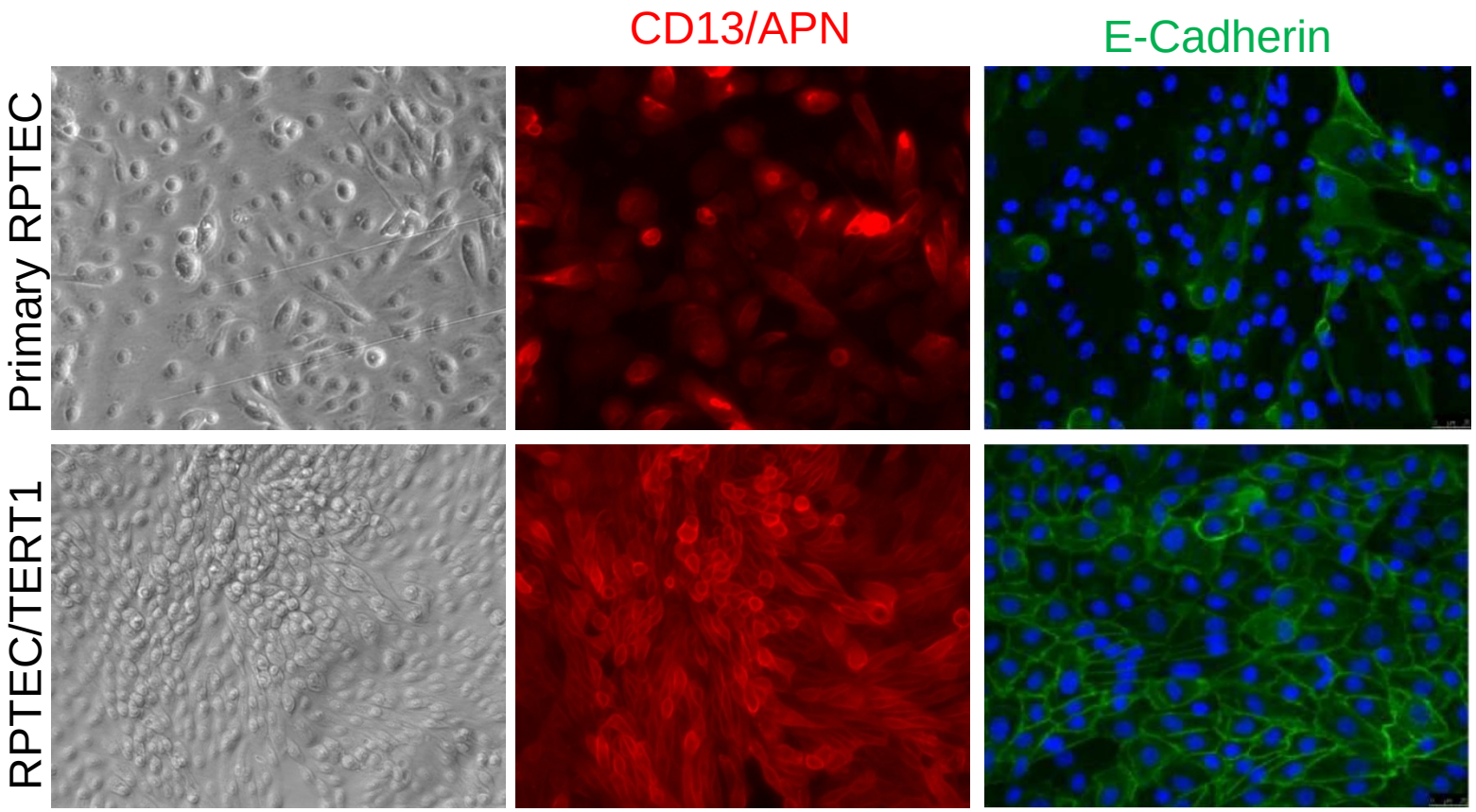




RPTEC/TERT1 at P21
After recovery from cryopreservation

The RPTEC/TERT1 cells propagate well and retain a normal male karyotype after extended culture in serum-free medium.

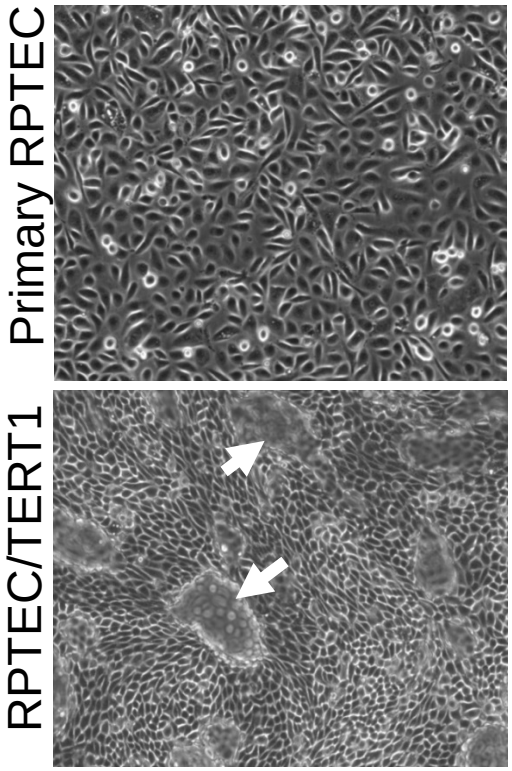
RPTEC/TERT1 – Homogenous population

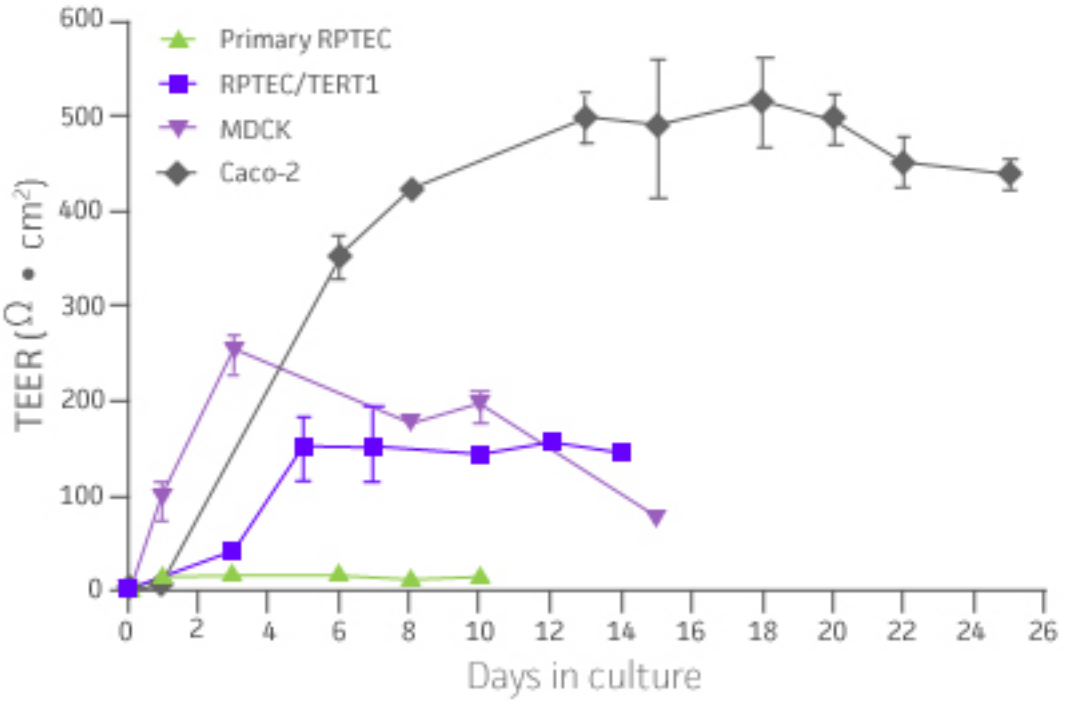


The RPTEC/TERT1 cells show uniform expression of E-cadherin and CD13(Aminopeptidase N), while primary RPTEC cells expression of these markers are highly variable.

ATCC

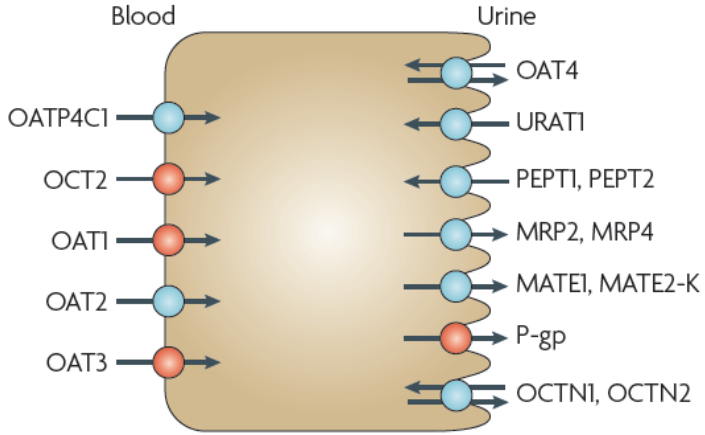
RPTEC/TERT1 – Intact epithelial barrier



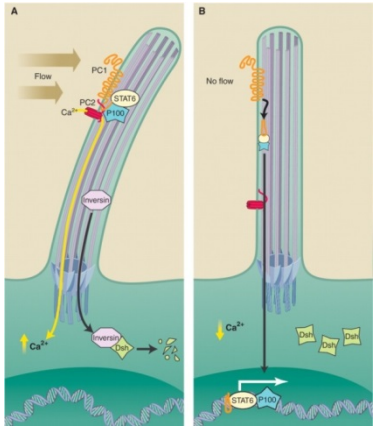


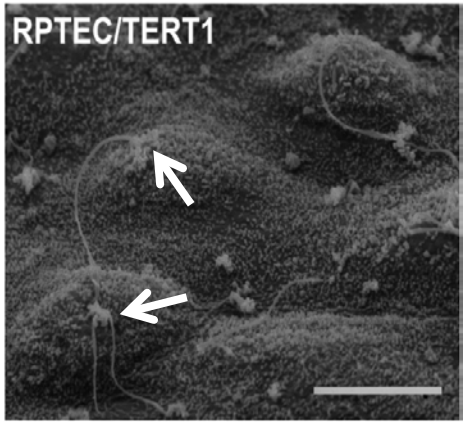
Dome-like structures (arrows) form as water and solutes are transported across the cell layer and become trapped, the development of these structures is a good indicator of epithelial formation. Similarly, the formation of an intact epithelium can be demonstrated by stabilized Trans-Epithelial Electrical Resistance (TEER). RPTEC/TERT1 cells exhibit both dome-like structures and stabilized TEER, while the primary RPTEC cells do not possess either feature of intact epithelial formation.

RPTEC/TERT1 – Other interesting features

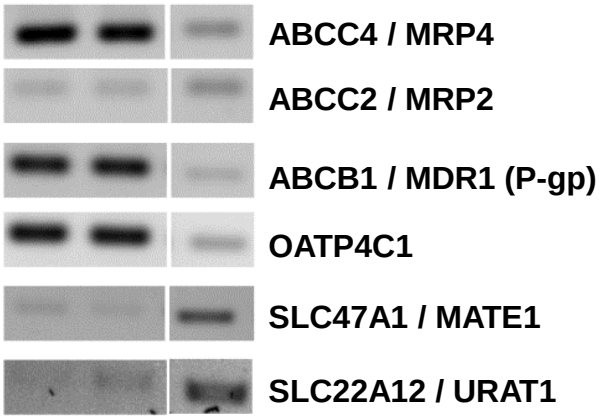


Giacomini KM, *et al. Nature Reviews Drug Discovery* 9: 215-236, 2010.

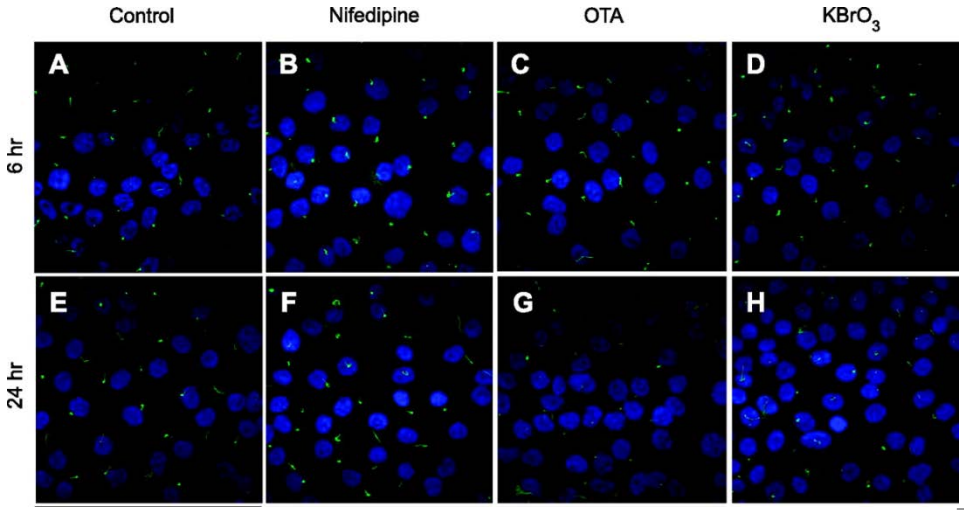




Wieser M, Stadler G, Jennings P, *et al*. *Am J Physiol Renal Physiol* 295(5): F1365-75, 2008.



T. RPTEC/TERT1 (CRL-4031)
P. Parental primary RPTEC
K. Kidney tissue



Radford, *et al. AJP - Renal Physiol* 302(8): F905-F916, 2012.

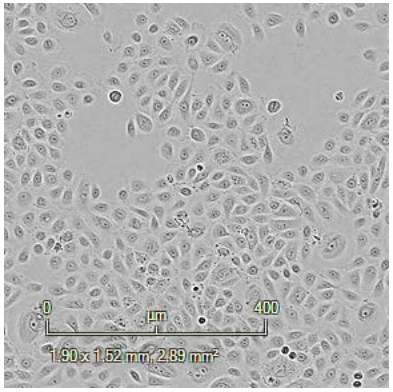
Ker-CT (CRL-4048™) – Immortalized keratinocyte

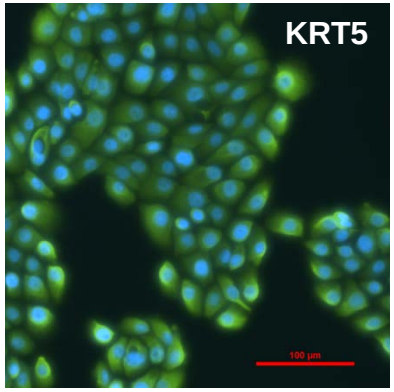
Retain intact differentiation capability

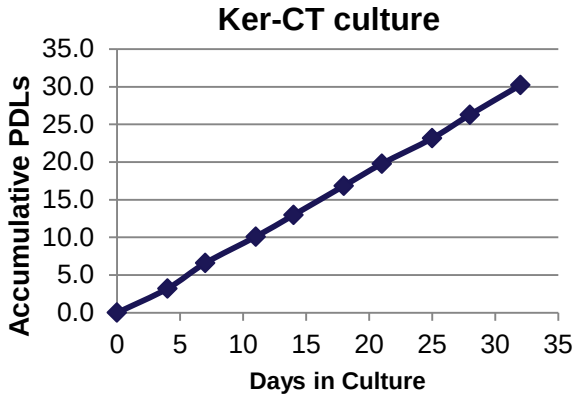
- Ker-CT cell line was immortalized by human telomerase and CDK4 from neonatal foreskin keratinocyte culture (Deposited by Dr. Shay, UTSW)

Ramirez R, et al. *Oncogene* 22(3): 433-44, 2003.

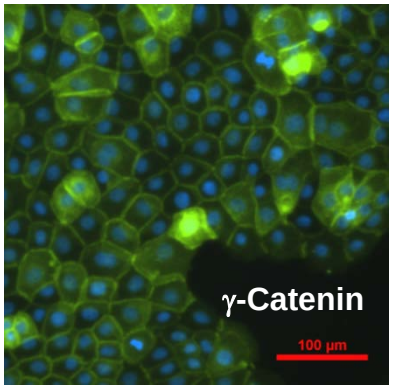
Proliferating

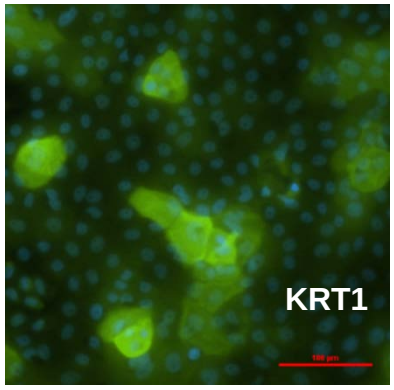


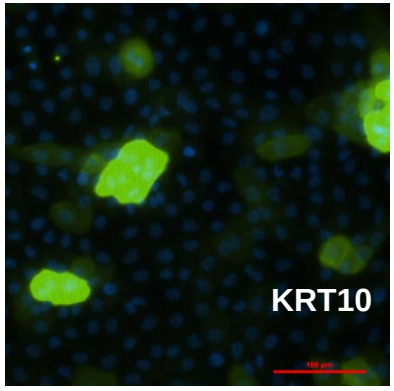


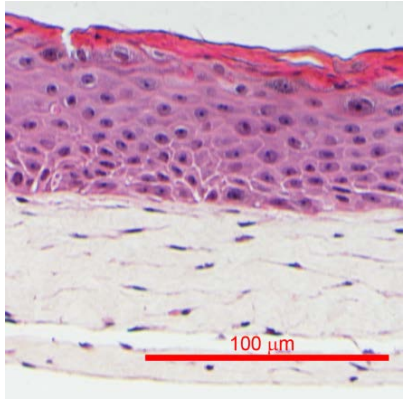


Differentiation









BJ + Ker-CT

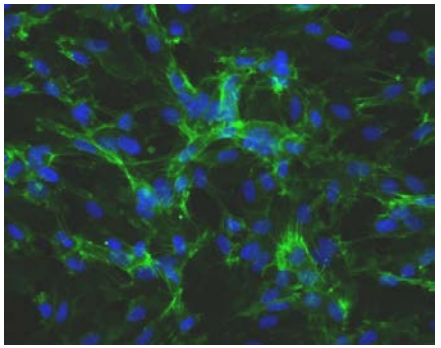
2D differentiation

3D differentiation

TIME (CRL-4025™) – Good endothelial cell model

TERT Immortalized Microvascular Endothelial cells

- Immortalized from neonatal foreskin microvascular endothelial cells
- Proliferation to at least 200 population doublings
- Normal diploid karyotype
- Normal endothelial cell phenotype/function
  - Surface marker (PECAM-1/CD31, VEGFR2, Tie-2)
  - Ac-LDL uptake
  - Tubule formation on basement membrane gel
  - Support infection by endotheliotropic Kaposi's sarcoma-associated herpes virus (KSHV/HHV-8)
  - Anoikis (apoptosis upon detachment from ECM)

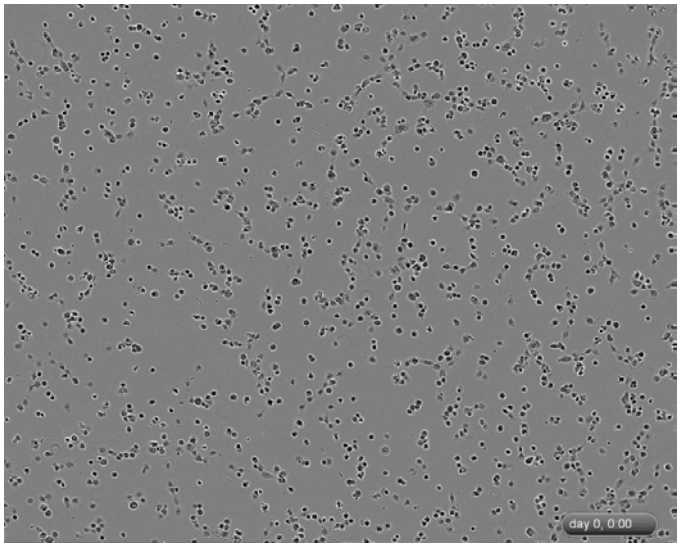


ATCC®

Venetsanakos E., et al. *Exp Cell Research*, 273:21-33, 2002.

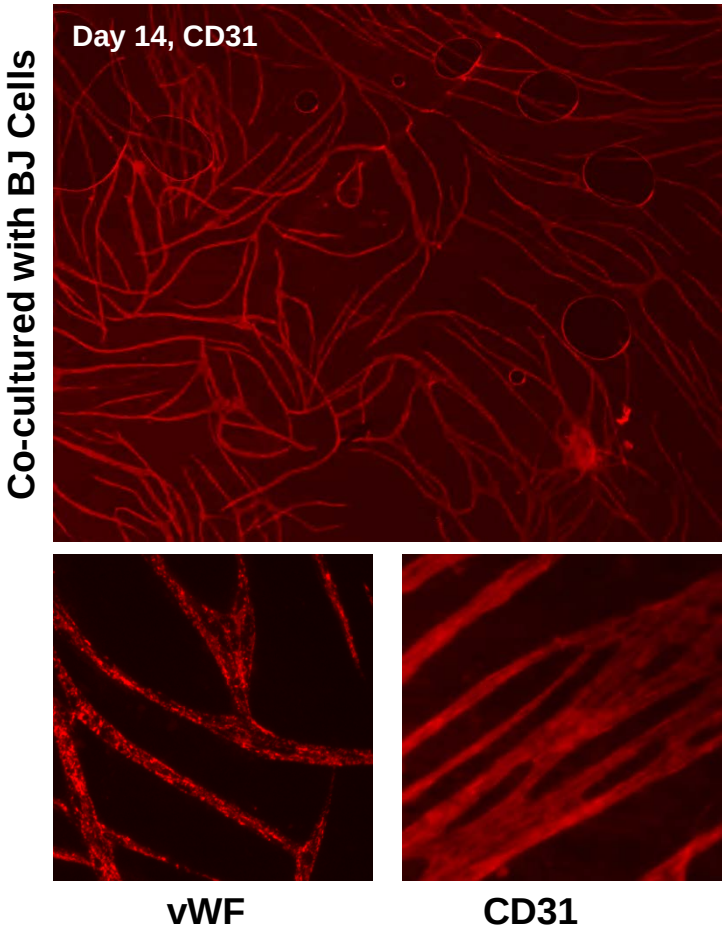
TIME – Capable of forming vascular structure

Tubule formation on Cell Basement Membrane Gel



TIME	CRL-4025™
Media	PCS-100-030™
	PCS-110-040™ (BBE Kit)
	PCS-110-041™ (VEGF Kit)
Basement Membrane Gel	ACS-3035™

Tubule formation on co-culture



Genetic engineered cell lines derived from TIME

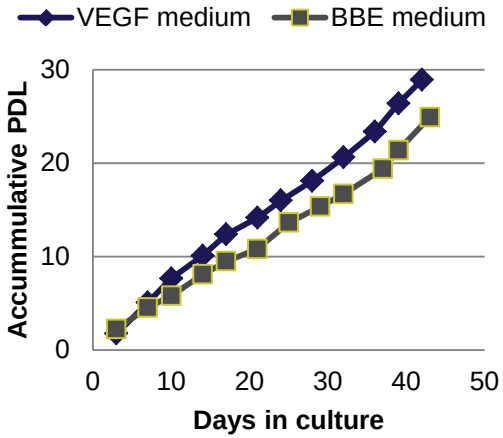
TIME-GFP (ATCC® CRL-4045™)

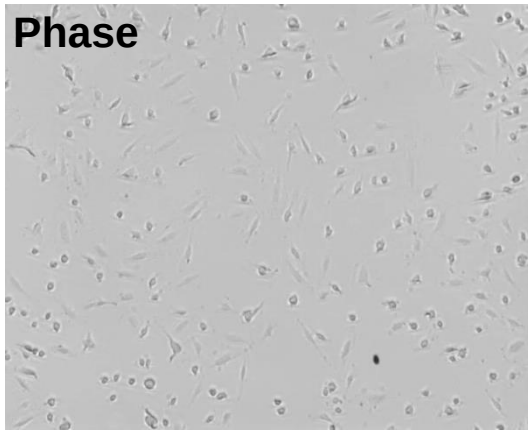
- Derived by transfecting TIME (ATCC® CRL-4025™) cells with linearized pWE2-EmGFP plasmid
- Clonal cell line selected based its stably expression of GFP driven by CMV promoter
- Diploid cell line of male origin with a chromosome number of 46
- Positive for endothelial cell markers as the parental TIME cell line (CD31, AcLDL uptake, VEGFR-2, Tie-2)
- Tested for at least 15 population doublings after recovery from cryopreservation
- Tubule formation on Cell Basement Membrane

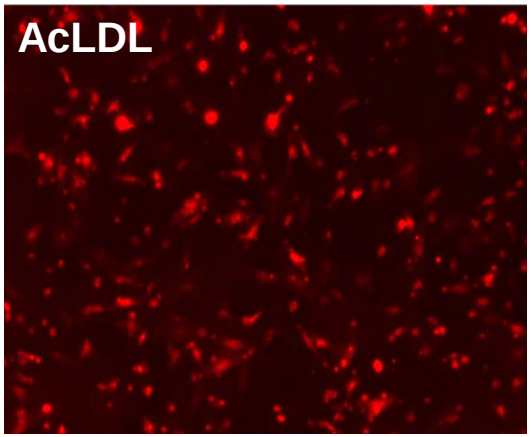
Poster# B1332, Monday, Dec 16th, 1:30-3:00 PM

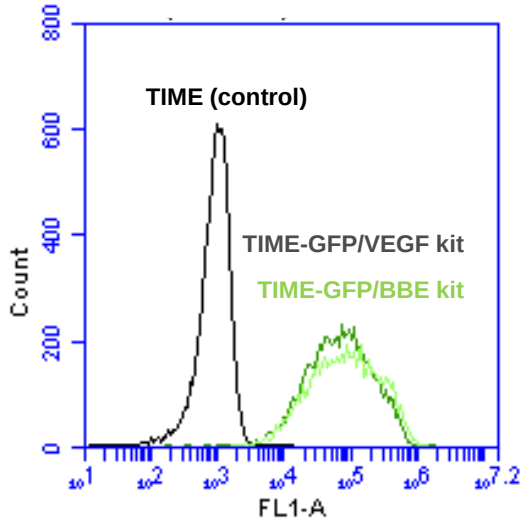
ATCC

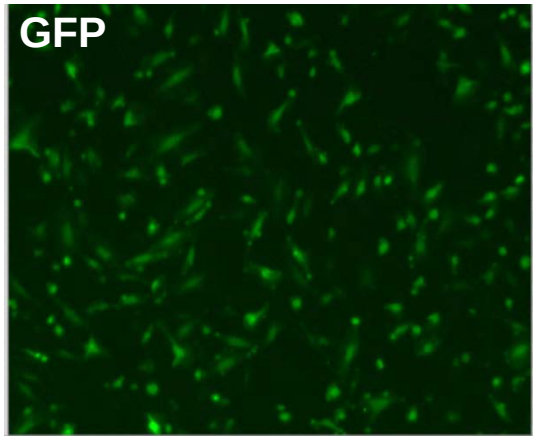
Characteristics of TIME-GFP cell line

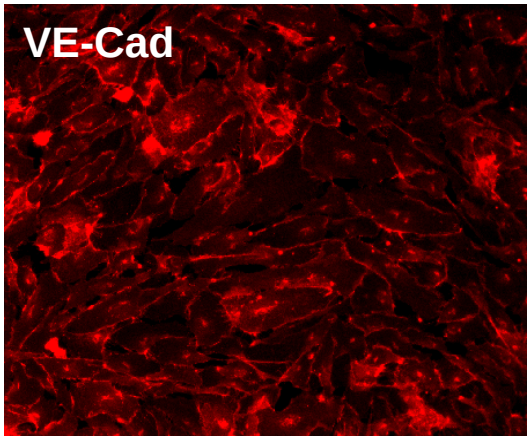




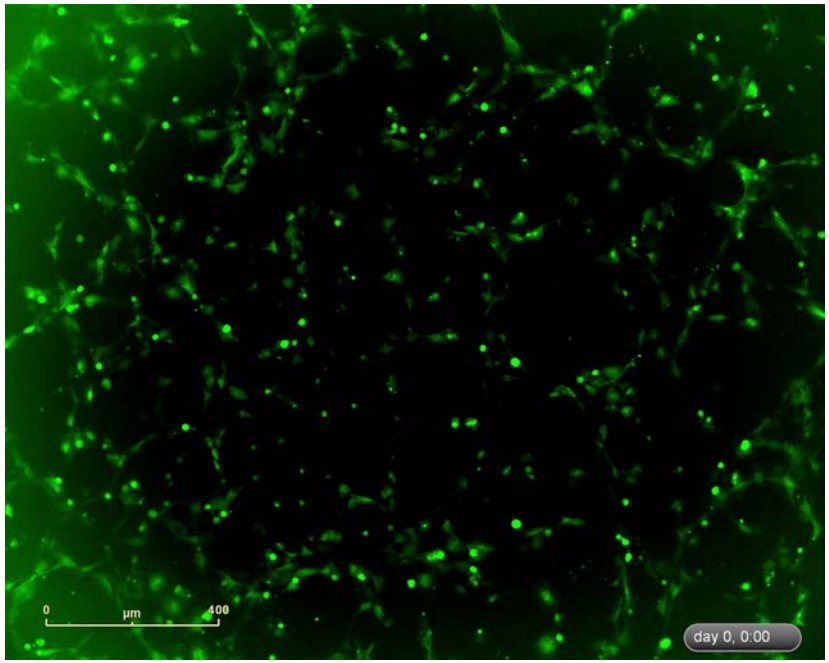




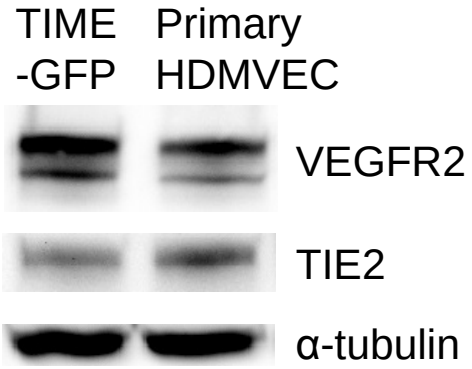


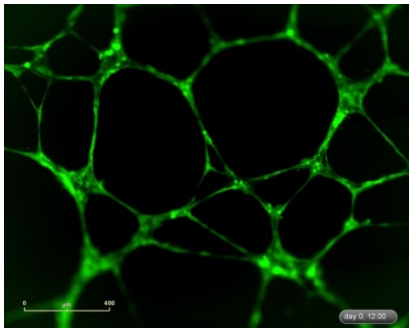


GFP expression facilitates real-time analysis

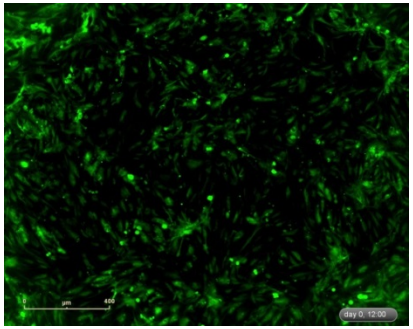


The GFP-expressing cells migrate and coalesce into networks of vessel-like structure within 10 hours after being plated onto Cell Basement Membrane Gel (ATCC® ACS-3035™). The stable expression of GFP in these cells enables detection and analysis of the fragile endothelial structures to occur without post-assay fixation and/or staining.





Vehicle

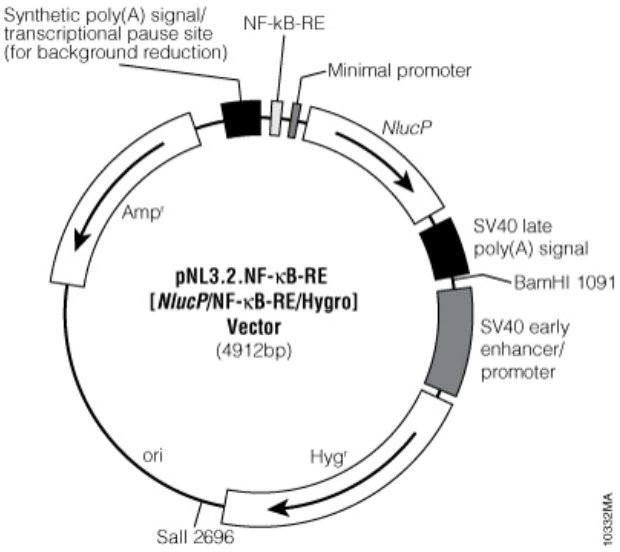


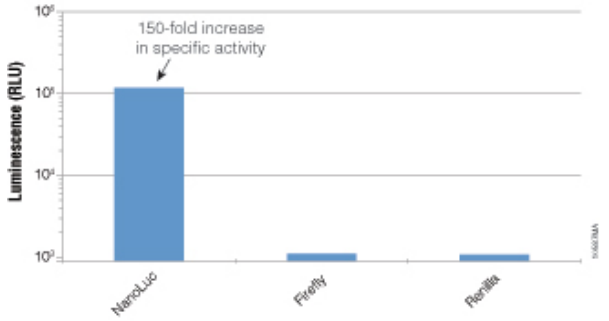
30nM Suramin

Genetic engineered cell lines derived from TIME

NFKB-TIME (ATCC® CRL-4049™)

- Derived by transfecting TIME (ATCC® CRL-4025™) cells with linearized pNL3.2-Nluc/NF-kB-RE/Hygro plasmid
- Clonal cell line selected based its high response to TNFα
- Normal diploid karyotype
- Positive for parental cell characteristics
  - TIME cell markers (CD31, AcLDL uptake)
  - Endothelial cell functions
- Tested for at least 15 population doublings after recovery from cryopreservation

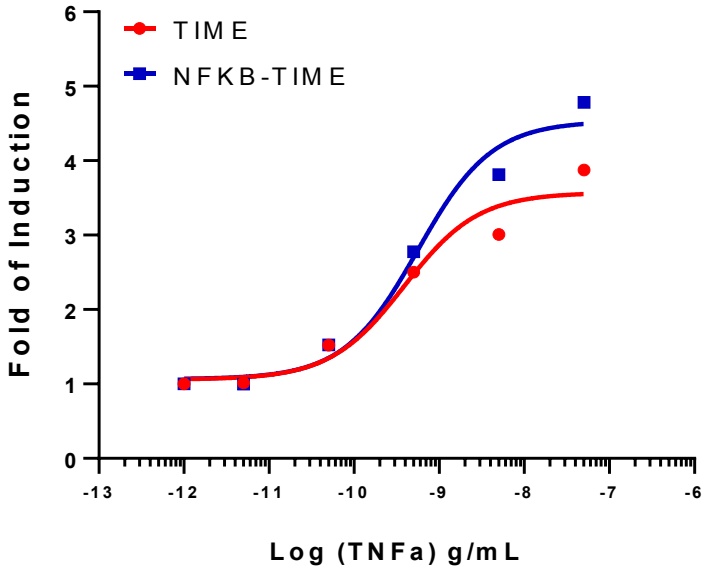




https://www.promega.com/products/pm/nanoluc/

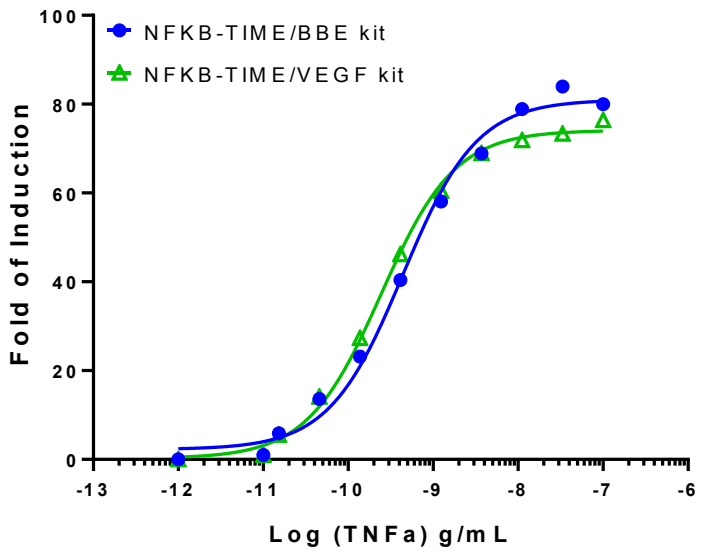
NFKB-TIME (CRL-4049™) reporter cell line

CD54/ICAM-1 Expression



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

Nano-Glo Luciferase Assay

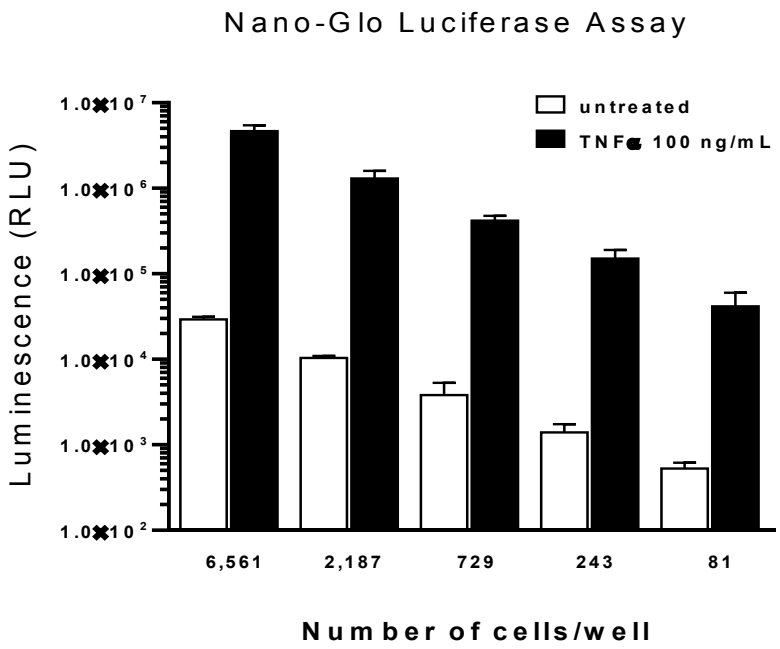


	NFKB-TIME/BBE kit	NFKB-TIME/VEGF kit
EC50	4.395e-010	2.427e-010

NFκB-TIME (ATCC® CRL-4049™) expresses NanoLuc® luciferase 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. 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 CD54/ICAM-1 activation assays.

ATCC®

Use of NanoLuc® increases assay sensitivity



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

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

hTERT immortalized cells provide unique tools

	Primary cells	hTERT immortalized	Oncogene, viral immortalized	Cancer cell lines
Mimic *in vivo* Tissue Phenotype	++++	+++	++	+
Genotypic Stability	Diploid	Diploid / Near diploid	Near diploid / Aneuploid	Aneuploid
Proliferative Capacity	+	+++	+++	+++
Supply	+	+++	+++	+++
Inter-Experimental Reproducibility	Low	Good	Good	Good
Cost	High	Medium	Low	Low
Ease of Use	+	++	++	+++

Pros and cons of different cell models for tissue-relevant functional studies

hTERT immortalized cells combine the *in vitro* nature of primary cells and the ability to be cultured continuously, avoiding the limitations of both types while still reaping their benefits.

ATCC