ISOLATION AND CLONING OF HUMAN VEGF PROMOTER REGION IN PGL3 BASIC VECTOR

IMAN YAZE

A dissertation submitted in partial fulfillment of the requirements for the award of the degree of Master of Biotechnology

Faculty of Biosciences and Bioengineering

University Technology Malaysia

MAY 2011

This thesis is dedicated to my beloved mother and father,

Who have been my inspirations in whole my life.

ACKNOWLEDGEMENT

I would like to extend my sincere gratitude to many whom without them this research would not have been possible.

First of all, I would like to thank my thesis supervisor, Dr Razauden Zulkifli for his guidance throughout my research. His encouragement and advices gave me the inspiration to keep me on the accurate conducting during the work in the laboratory.

I also extend my appreciation to bon Fatima, the kindest staff, and other helpful staffs in my laboratory who deserve thanks for the assistance in supplying the relevant guideline.

I am also grateful to thanks Mrs salehinejad, my best lecturer in my degree. For sure without her useful advices, I would not have changed my major and my location to continue my education.

I express my gratitude to my friends who supported and encouraged me during my experimental works.

Last but definitely not least, I would like to give my special thanks to my lovely family. My parents' sincere support has allowed me to pursue my course aspirations without any hindrances, and I will be eternally grateful for that. Without their kindly support, I would not have been able to conduct my study abroad.

ABSTRACT

Worldwide mortality and morbidity from infectious diseases is being replaced by chronic diseases, such as cancer, obesity and type II diabetes, cardiovascular diseases, neurodegenerative diseases and aging which involve inflammation. Vascular endothelial growth factor (VEGF) as a potent pro-inflammatory cytokine is elevated in many human diseases, or animal models of human disease, which are mentioned above. Compounds derived from botanic sources, such as polyphenolic compounds, have shown anti-inflammatory activity. These natural compounds express anti-inflammatory activity by modulation of pro-inflammatory gene expression.

We hypothesized this effect may related to control regulation of VEGF gene promoter. In this study, 5` flanking region of the VEGF promoter were isolated using nested PCR to define the transcription factors binding sites. The human VEGF promoter region was cloned in DH5 alpha for future investigation.

ABSTRAK

Kadar kematian dan morbiditas di dunia disebabkan oleh penyakit sedang digantikan oleh penyakit kronik, seperti kanser, obesity dan diabetes jenis II, penyakit kardiovaskular, penyakit neurodegenerative dan penuaan yang melibatkan keradangan. Vascularfaktor pertumbuhan endotel (VEGF) sebagai sitokin pro-inflamasi diaplikasikan kepada berbagi jenis penyakit manusia, atau model binatang dari penyakit manusia, seperti yang disebut di atas. Sebatian yang berasal dari sumber botani, seperti sebatian polifenol,telah menunjukkan aktiviti anti-inflamasi. Sebatian semulajadi mengekspresikan aktiviti anti-inflamasi dengan modulasi ekspresi gen pro-inflamasi. Secara hipotesis kesan ini mungkin berkaitan dengan kawalan peraturan promoter gen VEGF. Dalam kajian ini, 5 ` flanking region dari promoter VEGF adalah dipencilkan menggunakan nested PCR untuk menentukan faktor-faktor transkripsi binding site. Wilayah VEGF promoter manusia pengklonan ke dalam alpha DH5 untuk penyelidikan pada masa depan.

.

TABLE OF CONTENTS

CHAPTER	TITLE	PAGE
	SUPERVISOR'S DECLARATION	**i**
	DECLARATION	**ii**
	DEDICATION	**iii**
	ACKNOWLEDGEMENTS	**iv**
	ABSTRACT	**v**
	ABSTRAK	**vi**
	TABLE OF CONTENTS	**vii**
	LIST OF TABLES	**xi**
	LIST OF FIGURES	**xii**
	LIST OF ABBREVIATIONS	**xiv**
1	**INTRODUCTION**	
	1.1 Introduction	1
	1.2 Problem statement	2

1.3 Objectives 4

1.4 Scope of study 4

1.5 Significance of study 4

2 LITERATURE REVIEW

2.1 Inflammation 5
2.2 Relationship between Polyphenols and Inflammations 11
2.3 VEGF 18
2.4 Transcription factors 26
2.5 PGL3-Basic Vector 29

3 MATERIALS AND METHODS

3.1 Isolation of genomic DNA from human blood 39

3.2 Agarose gel electrophoresis for I dentification of genomic DNA 41

3.3 Identification of DNA concentration and purity 42

3.4 Designing primers 43

3.5 PCR (polymerase chain reaction) 45
3.6 Agarose gel electrophoresis for identification of PCR products 46
3.7 Gel extraction and PCR product clean up using Qiagen Qiaquick Gel Extraction Kit 47

3.8 Nested PCR (Nested Polymerase Chain Reaction) 48
3.9 Gel extraction and PCR product clean up 49
3.10 Isolation and purification of PGL3 Basic vector from *E.coli* DH5α 50
3.11 Restriction Enzymes and digestion condition 50
3.12 Ligation by the Promega T4 DNA ligase 51
3.12.1 Calf Intestinal Alkalin Phosphatase (CIAP) treatment 52
3.12.1.1 Composition of CIAP stor buffer 53
3.12.2 Phenol: chloroform extraction 53
3.12.3 Ethanol precipitation 53
3.13 Media 54
3.13.1 Luria Bertani (LB) media preparation 54
3.13.2 Preparation of ampicillin stock solution 55
3.14 Transformation of plasmid DNA into host cell 55
3.14.1 Preparation of competent cells 55
3.14.1.1 Transformation & Storage Solution (TSS) production 55
3.14.2 Transformation of VEGF promoter constructs into *E.coli* DH5α 56
3.15 Amplification of *E.coli* DH5α in broth and preparation of glycerol stocks 57
3.16 Glycerol stock 57
3.17 Purification of plasmid 57

4 RESULTS AN DISCUSSIONS

4.1 Isolation of genomic DNA from human blood 59

4.2 Polymerase chain reaction (PCR) 60
4.3 Nested Polymerase Chain Reaction (PCR) 61
4.4 DNA sequencing 64
4.4.1 Similarity search using Basic Local Alignment (BLAST) 66
4.5 Isolation and purification of PGL3 Basic Vector from *E.coli* DH5α 67
4.6 Digestion of the vector and insert 67
4.7 Ligation by the Promega T4 DNA ligase 71
4.7.1 CIAP treatment 71
4.7.2 Phenol/chloroform extraction 71
4.8 Transformation of VEGF promoter constructs into *E.coli* DH5alpha 73
4.9 Analysis of human Vascular Endothelial Growth Factor promoter region 76

5 CONCLUSION AND FUTURE WORKS

5.1 Conclusion 79
5.2 Future works 79

REFERNCES 80

Appendix (A-B-C-D-E) 96-100

LIST OF TABLES

TABLE NO.	TITLE	PAGE
2.1	Mediators involve in inflammation event	6
2.2	VEGF expression inducible factors	21
2.3	PGL3-Basic Vector Sequence Reference points	30
3.1	Primary materials of PCR and their functions	36
3.2	PCR cycle steps	37
3.3	Primer sequences for isolation of human VEGF promoter regions	44
3.4	Nested primer sequences for the introduction of restriction enzyme cutting sites.	44
3.5	Restriction enzyme condition and buffer system	51

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE
2.1	Rel/NF-kB signal transduction	8
2.2	Nuclear factor kB role in inflammation through oxidative stress and smoking	11
2.3	Basic structure and the system used for the carbon numbering of the flavonoid nucleus	12
2.4	Proposed model for the action mechanisms of oleic acid and minor compounds from olive oil	17
2.5	Schematic representation of interactions between VEGF family members and their receptors	25
2.6	Direct and indirect effects of VEGF on wound angiogenesis	24
2.7	Schematic model for Polyphenoles and flavonoids mediated modulation of cell signaling	28

2.8	PGL3 Vector multiple cloning regions	31
2.9	PGL3-Basic Vector (plasmid) circle map and its cutting sites for different digestion enzymes	32
3.1	Flow chart of experimental design	34
3.2	Cloning of PGL3-basic-Vegf into E.coli DH5α	35
3.3	Nested PCR Reaction Diagram	38
3.4	Gene Ruler Fermentase 1kb DNA Ladder	42
4.1	Gel electrophoresis of genomic DNA	60
4.2	Gel electrophoresis of PCR product	61
4.3	Gel electrophoresis of Nested PCR products	62
4.4	The sequence of human VEGF promoter	65
4.5	Transformed colonies containing PGL3 Basic Vector	67
4.6	Gel electrophoresis of digestion of insert	68
4.7	Gel electrophoresis of digestion of vector (PGL3 basic)	69
4.8	Gel electrophoresis of digested and treated vector	70
4.9	Transformed circular vector and digested vector (positive and negative controls)	73
4.10	Transformed colonies containing possible recombinant plasmid	74
4.11	Transcription factor binding sites	77-78

LIST OF ABBREVATIONS

ALS	-	Amyloid lateral sclerosis
AMD	-	Age related macular degeneration
AP-1, 2	-	Activator protein1, 2
AP-4	-	Activating enhancer binding protein 4
BLAST	-	Basic Local Alignment Search Tools
bp	-	Base pair
BR-C	-	Broad-Complex
CDP CR	-	CCAAT Displacement Protein
CF1	-	Common factor 1
CIAP	-	Calf Intestinal Alkaline Phosphatase
COX-2	-	Cyclooxygenase
DNA	-	Deoxyribonucleic acid
dNTPs	-	DNA nucleotides
EB	-	Elution buffer
EC	-	Endothelial cell
E.coli	-	*Escherichia coli*
EDTA	-	Ethylene Diamine Tetra Acetic acid

EPC	-	Endothelial progenitor cell
eVOO	-	Extra virgin olive oil
GATA1, 2	-	Globin transcription factor 1, 2
GR	-	Glucocorticoid receptor
HDAC2	-	Histone deacetylase 2
H_2O_2	-	Hydrogen peroxide
HSF	-	Heat shock factor
Hb	-	Homebox
HNF-3b	-	Hepatocyte nuclear factor 3 beta
iNOS	-	Inducible nitric oxide synthase
IL	-	Interleukin
LB	-	Luria Bertani
Lyf-1	-	Lymphoid transcription factor 1
LIF	-	Leukemia inhibitory factor
LPS	-	Lipopolysaccharide
ml	-	Milliliter
µl	-	Microliter
MCP-1	-	Monocyte chemotactic protein-1
MgCl2	-	Magnesium chloride
MgSo4	-	Magnesium sulfate
MMPs	-	Matrix metalloproteinases

NFkB	-	Nuclear factor kappa-light-chain-enhancer of activated B cells
MyoD	-	Myogenic differentiation
MZF1	-	Myeloid zinc finger 1
NO	-	Nitric oxide
Nrp	-	Neuropilins
O^{2-}	-	Superoxide anion
OD	-	Optical dencity
PAI-1	-	Plaminogen activator inhibitor-1
Pbx1	-	Pre-B-Cell leukemia homeobox 1
PCR	-	Polymerase chain reaction
P: C: I	-	Phenol: chloroform: isoamyl
PGs	-	Prostaglandins
pmol	-	Picomolar
RTKs	-	Receptor tyrosine kinases
RA	-	Retinoic acid
RNA	-	Ribonucleic acid
RORalp	-	Related orphan receptora1
ROS	-	Reactive oxygen species
rpm	-	Rotation per minute
S1P	-	Sphingosine-1-phosphate

SDS	-	Sodium dodecyl sulfate
SGE	-	Grape skin polyphenol extract
SP1	-	Specificity Protein 1
SRY	-	Sex determining region Y
TAE	-	Tris-Acetate EDTA
TE	-	Tris- HCL-EDTA
TGF-α	-	Transforming growth factor α
TNF-α	-	Tumor necrosis factor α
Tris-HCL	-	Tris hydrochloric acid
TSS	-	Transformation & storage solution
uPA	-	Urokinase plasminogen activator
uPAR	-	Urokinase plasminogen activator receptor
UV	-	Ultraviolet
VEGF	-	Vascular endothelial growth factor
VEGFR	-	VEGF Receptor

CHAPTER 1

INTRODUCTION

1.1 Introduction

Inflammation is a biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. It is a protective effort by the organism in response to injurious stimuli as well as initiates the healing process for the tissue.

In the absence of inflammation, wounds and infections would never heal. However, chronic inflammation can also lead to a host of diseases, such as high fever, atherosclerosis and rheumatoid arthritis. One of the cells responsible in inflammation is endothelial cells. VEGF (Vascular endothelial growth factor) is an endothelial cell-specific mitogen that is structurally related to platelet-derived growth factor (Tischer *et al.*, 1991).

On the other hand Angiogenesis is the key process involved in normal development and wound repair and is motivated by different kinds of growth factors for instance vascular endothelial growth factor (VEGF) (Ushio-Fukai, 2006) which is crucial angiogenic growth factor. VEGF stimulates proliferation, migration, and tube formation; three different stages of Angiogenesis in endothelial cells (ECs) (Ushio-Fukai, 2006).

Analysis of VEGF transcripts in vascular smooth muscle cells by Polymerase Chain Reaction(PCR) and cDNA cloning revealed three different forms of the VEGF in human-coding region including 189, 165, and 121 amino acids in length (Tischer *et al*., 1991).

In brief VEGF main function is to produce new blood vessels throughout embryonic development. In adult human they are responsible in recovering vascular injuries, by creating new vessels to evade blocked vessels.

1.2 Problem statement

Excessive inflammation is considered critical factor in many human diseases, including cancer, obesity, type II diabetes, cardiovascular diseases, neurodegenerative diseases and aging.

VEGF has been shown to increase vascular leak of both proteins and particulates or permeability, which is necessary prerequisite for the induction of angiogenesis. It is proposed that VEGF levels increase before and/or during the angiogenic process. This changes in VEGF level is observed in many human diseases which are characterized by inflammation and vascular leak,

for instance cerebral ischemia, tumor ascites, trauma, early diabetic retinopathy, preeclampsia, ovarian hyper stimulation syndrome, and status epilepticus.

VEGF acts either directly or indirectly as a potent pro-inflammatory cytokine; and as it mentioned above that increases in vascular permeability and it is an index of inflammation precede VEGF-induced angiogenesis (Croll *et al*., 2004).

Some compounds extracted from botanic sources, such as phenolic compounds, have shown anti-inflammatory activity *in vitro* and *in vivo*. They express some anti-inflammatory activity such as cyclooxygenase, lipoxygenase, nitric oxide synthases and several cytokines, mainly by acting through nuclear factor-kappa B and mitogen-activated protein kinase signaling (Santangelo *et al*., 2007).

Polyphenols comprise one of the most numerous dispersed group of plant secondary metabolites, present in all plants that are commonly consumed in the Mediterranean diet containing fruits, vegetables, grains, legumes, tea, red wine and extra virgin olive oil (eVOO) (Santangelo *et al*., 2007). Although the function of VEGF is well studied the mechanism involve in its expression is vague. Hence the aim of this study is to isolate the promoter region that controls the expression of this important gene in order to investigate the factor that may influence its expression.

1.3 The specific objectives of this study are;

1) To characterize the regulatory regions of promoter by using bioinformatic tools
2) To isolate the promoter region of VEGF gene
3) To clone the promoter in PGL3 Basic vector
4) To transform VEGF promoter construct to DH5α competent cells

1.4 Scope of study

The scope of this study is to isolate and identify the human VEGF promoter which involves the genomic extraction from fresh blood and bioinformatic tools respectively. Designing suitable primer and cloned the VEGF promoter in PGL3 reporter vector.

1.5 Significance of study

Regarding that inflammation represents a major pathologic basis for the majority of human malignancies and some other kinds of disease such as type II diabetes, cardiovascular diseases and neurodegenerative diseases. Understanding of the mechanism involve is important in order to find a way to control this problem.

REFERENCES

Adamis, A. P., and Shima, D. T. (2005). The role of vascular endothelial growth factor in ocular health and disease. *Retina*. 25, 111 – 118.

Adcock, I. M., Tsaprouni, L., Bhavsar, P., Ito, K. (2006). Epigenetic regulation of airway inflammation. *Curr Opin Immunol*. 19, 694-700.

Argraves, K. M., and Argraves, W. S. (2007). HDL serves as a S1P signaling platform mediating a multitude of cardiovascular effects. *J. Lipid Res*. 48, 2325-2333

Ballermann, B. J. (2005). Glomerular endothelial cell differentiation. *Kidney Int*. 67, 1668– 1671.

Bertelli, A. A., Gozzini, A., Stradi, R., Stella, S., and Bertelli, A. (1998). Stability of resveratrol over time and in the various stages of grape transformation. *Drugs Exp Clin Res*. 24, 207–211.

Bertelli, A., Bertelli, A. A., Gozzini, A., and Giovannini, L. (1998). Plasma and tissue resveratrol concentrations and pharmacological activity. *Drugs Exp Clin Res*. 24, 133–138.

Brogan, I. J., Khan, N., Isaac, K., Hutchinson, J. A., Pravica, V., and Ian, V. (*1999*). Hutchinson. Novel Polymorphisms in the Promoter and 5 UTR Regions of the Human Vascular Endothelial Growth Factor Gene. *Human Immunology*. 60, 1245–1249.

Brogi, E., Wu, T., Namiki, A., Isner, J. M. (1994). Indirect angiogenic cytokines upregulate VEGF and bFGF gene expression in vascular smooth muscle cells, whereas hypoxia upregulates VEGF expression only. *Circulation* 90, 649–652.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., Nagy, A. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature.* 380, 435–439.

Chung, C. T., and Miller, R. H. (1993). Preparation and storage of competent *Escherichia coli* cells. *Methods in enzymology*. 218, 621-627.

Circulation Research.(2007). *American Heart Association, Inc*. 100, 607-21.

Coultas, L., Chawengsaksophak, K., Rossant, J. (2005). Endothelial cells and VEGF in vascular development. *Nature*. 438, 937–945.

Croll, S. D., Wei, T., Ransohoff, R. M., Kasselman, L. J., Yancopoulos, G. D., Cai, N., Kintner, J., Zhang, Q., Martin, F. J., Murphy, A. J., and Wiegand, S. J. (2004). VEGF-mediated inflammation precedes angiogenesis in adult brain. *Experimental Neurology*. 187, 388– 402.

Curin, Y., and Andriantsitohaina, R. (2005). Polyphenols as potential therapeutical agents against cardiovascular diseases. *Pharmacol*. 57, 97–107.

Donnelly, L.E., Newton, R., Kennedy, G.E., Fenwick, P.S., Leung, R.H., and Ito, K. (2004). Anti-inflammatory effects of resveratrol in lung epithelial cells: molecular mechanisms. *Am J Physiol Lung Cell Mol Physiol*. 287, 774–83.

Duvall, W. L. (2005) . Endothelial dysfunction and antioxidants. *Mt Sinai J Med*. 72, 71 –80.

Eminga, S. A., Brachvogelb, B., Odorisio, Teresa., Kocha, M., Stelzmann, J. (2007). Regulation of angiogenesis: Wound healing as a model.

Esser, S., Wolburg, K., Wolburg, H., Breier, G., Kurzchalia, T., and Risau, W. (1998). Vascular endothelial growth factor induces endothelial fenestrations in vitro. *J. Cell Biol*. 140, 947– 959.

Fang, J. G., Lu, M., Chen, Z. H., Zhu, H. H., Li, Y., Yang, L., Wu, L. M., and Liu, Z. L. (2002). Antioxidant effects of resveratrol and its analogues against the freeradical-induced peroxidation of linoleic acid in micelles. *Chemistry*. 8, 4191–4198.

Ferrara, N. (2004). Vascular endothelial growth factor: basic science and clinical progress. *Endocr. Rev*. 25, 581–611.

Ferrara, N. (2004). VEGF: basic science and clinical progress. *Endocrine Rev*. 25, 581–611.

Ferrara, N. (2002). Role of vascular endothelial growth factor in physiologic

and pathologic angiogenesis: therapeutic implications. *Semin. Oncol*. 29, 10–14.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K. J., Moore, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*. 380, 439–442.

Finnerty, H., Kelleher, K., Morris, G. E., Bean, K., Merberg, D. M., Kriz, R., Morris, J.C., Sookdeo, H., Turner, K. J., and Wood, C. R. (1993). Molecular cloning of murine FLT and FLT4. *Oncogene*. 8, 2293–2298.

Folkman, J. (2004). Endogenous angiogenesis inhibitors. *APMIS.* 112, 496–507.

Fong, G. H., Zhang, L., Bryce, D. M., and Peng, J. (1999) .Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. *Development*. 126, 3015– 3025.

Frankel, E., Waterhouse, A., and Kinsella, J. (1993). Inhibition of human LDL oxidation by resveratrol. *Lancet*. 341, 1103–1104.

Gilmore, T. D. (2006). The Rel/NF-kappaB Signal Transduction Pathway. *Boston University*.

Harborne, J.B. (1980). Plant phenolics. *Berlin: Springer-Verlag*. P, 329–95.

Hicklin, D. J., and Ellis, L. M. (2005). Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J. Clin. Oncol*. 23, 1011 – 1027.

Hynes, R. O. (2002). A reevaluation of integrins as regulators of angiogenesis. *Nat. Med*. 8, 918–921.

Iijima, K., Yoshizumi, M., Hashimoto, M., Akishita, M., Kozaki, K., Ako, J., Watanabe, T., Ohike, Y., Son, B., Yu, J., Nakahara, K., and Ouchi, Y. (2002). Red wine polyphenols inhibit vascular smooth muscle cell

migration through two distinct signaling pathways. *Circulation*. 105, 2404–2410.

Jakeman, L. B., Winer, J., Bennett, G. L., Altar, C. A., Ferrara, N. (1992). Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. *J. Clin. Invest*. 89, 244–253.

Jobin, C., Bradham, C.A., Russo, M.P., Juma, B., Narula, A.S., and Brenner, D.A. (1999). Curcumin blocks cytokine-mediated NF-kappa B activation and proinflammatory gene expression by inhibiting inhibitory factor I-kappaB kinase activity. *J Immunol*. 163, 3474–83.

Josko, J., and Mazurek, M. (2004). Transcription factors having impact on vascular endothelial growth factor (VEGF) gene expression in angiogenesis. *Med Sci Monit*. 10, 89-98.

Karin, M., and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol*. 18, 621-63.

Karin, M., Yamamoto, Y., and Wang, Q.M. (2004). The IKK NF-kappa B system: a treasure trove for drug development. *Nat Rev Drug Discov*. 3, 17-26.

Kim, I., Ryan, A. M., Rohan, R., Amano, S., Agular, S., Miller, J. W., and Adamis, A. P. (1999). Constitutive expression of VEGF, VEGFR-1, and VEGFR-2 in normal eyes. *Invest Ophthalmol. Visual Sci*. 40, 2115–2121.

Kris-Etherton, P., Eckel, R. H., Howard, B.V., St Jeor, S., and Bazzarre, T. L. (2001). Lyon Diet Heart Study: Benefits of a Mediterranean-Style. *Ciculation.*103, 1823–1825.

Larsen, G.L., Henson, P.M. (1983). Mediators of inflammation: *Annu Rev Immunol*. 1, 335-59.

Lashkari, K., Hirose, T., Yazdany, J., McMeel, J. W., Kazlauskas, A., and Rahimi, N. (2000). Vascular endothelial growth factor and hepatocyte growth factor levels are differentially elevated in patients with advanced retinopathy of prematurity. *Am. J. Pathol*. 156, 1337–1344.

Lederberg, J. (1994). The transformation of Genetics by DNA: An Anniversary Celebration of AVERY, MACLEOD and MCCARTY in Anecdotal, Historical and Critical Commentaries on Genetics. *Genetics*. 136, 423-426.

Leiro, J., Arranz, J.A., Fraiz, N., Sanmartin, M.L., Quezada, E., and Orallo, F. (2005). Effect of cis-resveratrol on genes involved in nuclear factor kappa B signaling. *Int Immunopharmacol*. 5, 393–406.

Li, J., Hampton, T., Morgan, J. P., Simons, M. (1997). Stretch-induced VEGF expression in the heart. *J. Clin. Invest*. 100, 18–24.

Loureiro, R. M., D'Amore, P. A. (2005). Transcriptional regulation of vascular endothelial growth factor in cancer. *Cytokine Growth Factor Rev*. 16, 77–89.

Maharaj, A. S. R., and D'Amore, P. A. (2007). Roles for VEGF in the adult. *Microvascular Research*. 74, 100–113.

Maharaj, A. S., Saint Geniez, M., Maldonado, A. E., D'Amore, P. A. (2006). Vascular endothelial growth factor localization in the adult. *Am. J. Pathol*. 168, 639–648.

Manna, S.K., Mukhopadhyay, A., and Aggarwal, B.B. (2000). Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappaB, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. *J Immunol*. 164, 6509–19.

Marsilio, V., Campestre, C., and Lanza, B. (2001). Phenolic compounds change during California-style ripe olive processing. *Food Chem*. 74, 55–60.

Martinez-Domingues, E., de la Puerta, R., and Ruiz-Gutierrez, V. (2001). Protective effects upon experimental inflammation models of a polyphenolsupplemented virgin olive oil diet. *Inflamm Res*. 50, 102–106.

Miller, N. J., and Rice-Evans, C. A. (1995). Antioxidant activity of resveratrol in red wine.*Clin Chem*. 41, 1789–1794.

Minchenko, A., Salceda, S., Bauer, T., and Caro, J. (1994). Hypoxia regulatory elements of the human vascular endothelial growth factor gene. *Cell Mol Biol Res.* 40, 35.

Miquerol, L., Langile, B.L., Nagy, A., (2000). Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. *Development*. 127, 3941–3946.

Mueller, M. M. (2006). Inflammation in epithelial skin tumours: Old stories and new ideas. *Im Neuenheimer Feld. Heidelberg, Germany*. 280, 735-7447.

Nam, N.H. (2006). Naturally occurring NF-kappaB inhibitors. *Mini Rev Med Chem*. 6, 945-51.

Nanji, A.A., Jokelainen, K., Tipoe, G.L., Rahemtulla, A., Thomas, P., and Dannenberg, A.J. (2003). Curcumin prevents alcoholinduced liver disease in rats by inhibiting the expression of NF-kappaB dependent genes. *Am J Physiol Gastrointest Liver Physiol*. 284, 321–7.

Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J.* 13, 9–22.

Ng, Y. S., Rohan, R., Sunday, M. E., Demello, D. E., and D'Amore, P .A. (2001). Differential expression of VEGF isoforms in mouse during development and in the adult. Dev. *Dyn*. 220, 112–121.

Olsson, A. K., Dimberg, A., Kreuger, J., and Claesson-Welsh, L. (2006). VEGF receptor. Signalling – in control of vascular function. *Nat Rev Mol Cell*. 7, 359–71.

Osawa, Y., Uchinami, H., Bielawski, J., Schwabe, R. F., Hannun, Y. A., and Brenner, D. A. (2005). Roles for C16-ceramide and sphingosine 1-

phosphate in regulating hepatocyte apoptosis in response to tumor necrosis factor-alpha. *J. Biol. Chem*. 280, 27879–27887.

Pace-Asciak, C. R., Rounova, O., Hahn, S. E., Diamandis, E.P., and Goldberg, D. M. (1996). Wines and grape juices as modulators of platelet aggregation in healthy human subjects. *Clin Chim Acta*. 246, 163–182.

Pajusola, K., Aprelikova, O., Korhonen, J., Kaipainen, A., Pertovaara, L., Alitalo, R., and Alitalo, K. (1992). FLT4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines. *Cancer Res*. 52, 5738– 5743.

Pendurthi, U. R., Williams, J. T., and Rao, L. V. (1999). Resveratrol, a polyphenolic compound found in wine, inhibits tissue factor expression in vascular cells: a possible mechanism for the cardiovascular benefits associated with moderate consumption of wine. *Arterioscler Thromb Vasc Biol*. 19, 419–426.

Perona, J. S., Cabello-Moruno, R., Ruiz-Gutierrez, V. (2006). The role of virgin olive oil components in the modulation of endothelial function. *4 Nutrition and Lipid Metabolism Group*. *Nutritional Biochemistry*. 17, 429–445.

Pertovaara, L., Kaipainen, A., Mustonen, T., Orpana, A., Ferrara, N., Saksela, O., Alitalo, K. (1994). Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. *J. Biol. Chem*. 269, 6271–6274.

Peters, K. G., Vries, C. D., and Williams, L.T. (1993).Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. *Proc. Natl. Acad. Sci. U S A*. 90, 8915–8919.

Petroni, A., Blasevich, M., Salami, M., Papini, N., Montedoro, G. F., and Galli, C. (1995).

Inhibition of platelet aggregation and eicosanoid production by phenolic components of olive oil. *Thromb Res*. 78, 151–160.

Petrova, T. V., Makinen, T., and Alitalo, K. (1999) Signaling via Vascular Endothelial Growth Factor Receptors. 253, 117–130.

Prats, A. C., and Prats, H. (2002). Translational control of gene expression: role of IRESs and consequences for cell transformation and angiogenesis. *Prog. Nucleic Acid Res. Mol. Biol.* 72, 367–413.

Primrose S. B., and Twyman R. M. (2007). Principles of Gene Manipulation and Genomics, 7th edn. *Blackwell publishing, Victoria, Australia.*

Rahman, I., Biswas, S. K., and Kirkham, P. A. (2006). Regulation of inflammation and redox signaling by dietary polyphenols. *biochemical pharmacology*. 72, 1439–1452.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature*. 386, 671-674.

Roccaro, A. M., Russo, F., Cirulli, T., Di Pietro, G., Vacca, A., Dammacco, F. (2005). Antiangiogenesis for rheumatoid arthritis. *Drug Targets Inflamm. Allergy*. 4, 27– 30.

Robinson, C. J., and Stringer, S. E. (2001). The splice variants of vascular endothelial growth factor (VEGF) and their receptors. *J. Cell Sci*. 114, 853–865.

Rothwarf, D. M., Karin, M. (1999). The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus. *Sci. STKE*.

Ruhrberg, C., Gerhardt, H., Golding, M., Watson, R., Ioannidou, S., Fujisawa, H., Betsholtz, C., Shima, D. T. (2002). Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. *Genes Dev*. 16, 2684–2698.

Santangelo, C., Varì, R., Scazzocchio, B., Benedetto, R. D., Filesi, C., and Masella, R. (2007). Polyphenols, intracellular signalling and inflammation. 4, 394-405.

Sawano, A., Iwai, S., Sakurai, Y., Ito, M., Shitara, K., Nakahata, T., and Shibuya, M. (2001). Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. *Blood*. 97, 785– 791.

Seko, Y., Fujikura, H., Pang, J., Tokoro, T., Shimokawa, H. (1999). Inductionvascular endothelial growth factor after application of mechanical stressretinal pigment epithelium of the rat in vitro. *Invest. Ophthalmol. Visual Sci*. 40 , 3287–3291.

Selleck, S. B. (2006). Signaling from across the way: transactivation of VEGF receptors by HSPGs. *Mol. Cell*. 22, 431–442.

Schuh, A. C., Faloon, P., Hu, Q. L., Bhimani, M., and Choi, K. (1999). In vitro hematopoietic and endothelial potential of flk-1 embryonic stem cells and embryos. *Proc. Natl. Acad. Sci. U S A*. 96, 2159– 2164.

Shibuya, M., Yamaguchi, S., Yamane, A., Ikeda T., Tojo, A., Matsushime, H., and Sato, M. (1990). Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. *Oncogene*. 5, 519– 524.

Siemann, E., and Creasy, L. (1992). Concentration of the phytoalexin resveratrol in wine. *Am J Enol Vitic*. 43, 49–52.

Soares, R., and Azevedo, I. (2006). Apigenin: is it a pro-or anti-inflammatory agent? *Am. J. Pathol*. 168, 1762.

Soker, S., Takashima, S., Miao, H. Q., Neufeld, G., and Klagsbrun, M. (1998). Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell.* **92,** 735–745.

Surh,Y. J., Chun, K. S., Cha, H. H., Han, S. S., Keum,Y. S., Park, K. K., and Lee, S. S. (2001). Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. *Mutat. Res*. 480–481, 243–268.

Terman, B. I., Carrion, M. E., Kovacs, E., Rasmussen, B. A., Eddy, R. L., and Shows, T. B. (1991). Identification of a new endothelial cell growth factor receptor tyrosine kinase. *Oncogene.* 6, 1677–1683.

Terman, B. I., Dougher-Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, D. C., Gospodarowicz, D., and Bohlen, P. (1992). Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem. Biophys. Res. Commun*. 187, 1579–1586.

Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J.C., and Abraham, J.A. (1993). The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem.*

Tischer., Mitchel, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J.C., and Abrahamll, J.A. (1991). The Human Gene for Vascular Endothelial Growth Factor ,*Multiple Protein Forms Are Encoded Through Alternative Exon Splicing*. 25, 11947-11954.

Trela, B.C., and Waterhouse, A.L. (1996). Resveratrol: isomeric molar absorptivities and stability. *J Agric Food Chem*. 44, 1253–7.

Triggle, C. R., Hollenberg, M., Anderson, T. J., Ding, H., Jiang, Y., and Ceroni, L. (2003). The endothelium in health and disease : a target for therapeutic intervention. *J Smooth Muscle Res*. 39, 249– 67.

Ushio-Fukai, M. (2006). Redox signaling in angiogenesis: role of NADPH oxidase. *Cardiovasc. Res*. 71, 226–235.

Veikkola, T., and AlitaloU, K. (1999). VEGFs, receptors and angiogenesis.*CANCER BIOLOGY*. 9, 211-220.

Visioli, F., Bellomo, G., and Galli, C. (1998). Free radical-scavenging properties of olive oil polyphenols. *Biochem Biophys Res Commun*. 247, 60–64.

Visioli, F., Borsani, L., and Galli, C. (2000). Diet and prevention of coronary heart disease: the potential role of phytochemicals. *Cardiovasc Res*. 47, 419–425.

Voetsch, B., Jin, R. C., and Loscalzo, J. (2004). Nitric oxide insufficiency and atherothrombosis. *Histochem Cell Biol*. 122, 353–67.

Vries, C. D., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N., and Williams, L. T. (1992). The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science*. 255, 989– 991.

Wallerath, T., Deckert, G., Ternes, T., Anderson, H., Li, H., Witte, K., Forstermann, U. (2002). Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. *Circulation*. 106, 1652–1658.

Webster, C. M., Kellyc, S., Koikea, M. A., Chock, V. Y., Giffard, R. G., Midori, A. (2009). Inflammation and NFκB activation is decreased by

hypothermia following global cerebral. *ischemia: Yenari Neurobiology of Disease*. 33, 301–312.

Xia, P., Gamble, J. R., Rye, K. A., Wang, L., Hii, C. S., Cockerill, P., Khew-Goodall, Y., Bert, A. G., Barter, P. J., and Vadas, M. A. (1998). Tumor necrosis factor-alpha induces adhesion molecule expression through the sphingosine kinase pathway. *Proc. Natl. Acad. Sci. U. S. A*. 95, 14196–14201.

Yancopoulos, G. D., Klagsbrun, M., and Folkman, J. (1998). Vasculogenesis, angiogenesis, and growth factors: Ephrins enter the fray at the border. *Cell*. 93, 661–664.

Yasuhara, T., Shingo, T., and Date, I. (2004). The potential role of vascular endothelial growth factor in the central nervous system. *Rev Neurosci*. 15, 293– 307.

WEBSITES: http://www.promega.com/tbs/tm033/tm033.html

http://www.cbrc.jp/research/db/TFSEARCH.html