THE POTENTIAL ANTI-TUMOR EXTRACT OF *OCIMUM BASILICUM* LAMIACEAE TOWARDS COLORECTAL CANCER VIA THE ANGIOGENESIS MECHANISTIC PATHWAY

IBRAHIM DEEB TAWFIQ AL DEEB

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by

IBRAHIM DEEB TAWFIQ AL DEEB

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This thesis is dedicated to

My beloved mother and father,

Memory of my mother in-law,

My beloved wife,

My children.

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بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيمِ

(وَفــَوْقَ كُلِّ ذِي عِلــْمٍ عَلِيــمٌ)

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Ang	Angiopoietin
CAM	Chorioallantoic membrane
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
CXCL12	Stromal cell derived factor 1
CXCL5	Epithelial-derived neutrophil-activating peptide
Dll4	Delta like ligand 4
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ENA-78	Epithelial-derived neutrophil-activating peptide
eNOS	Endothelial nitric oxide synthetase
ERK	Extracellular signal regulated kinase
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptors
GC-SF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRO α, β, γ	Chemokine (C-X-C motif) ligands 1, 2, 3
HIF-1	Hypoxia inducible factor
HSPGs	Heparin sulfate proteoglycans
I-309	Chemokine (C-C motif) ligand 1
IC50	Concentration that inhibits 50% of cell population growth
ICAM	Intracellular adhesion molecule

IFN-gamma	Interferon gamma
IGF-1	Insulin-like growth factor 1
IL-8	Interleukin 8
I-TAC	Chemokine (C-X-C motif) ligand 11
LD_{50}	Lethal dose for 50% of population
MAPK	Mitogen activated protein kinase
MCP-1, 3, 4	Chemokine (C-C motif) ligand 2, 7, 13
mCRC	Metastatic colorectal cancer
MIP-1α	Macrophage inflammatory protein-1
MMPs	Matrix metalloproteinases
mRCC	Metastatic renal cell carcinoma
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOTCH1	Neurogenic locus notch homolog protein 1
NSCLC	Non-small cell lung cancer
OS	Overall survival
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PE	Plate efficiency
PECAM	Platelet endothelial cell adhesion molecule 1
PFS	Progression free survival
PI3K	Phosphoinositide 3-kinase
PlGF	Placental growth factor
PTEN	Phosphatase and tensin homolog
RB	Retinoblastoma-associated proteins
RBCs	Red blood cells

ROS	Reactive oxygen species
SF	Surviving fraction
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinases
TKIs	Tyrosine kinase inhibitors
TKR	Tyrosine kinase receptors
TNF	Tumor necrosis factor
TSP-1	Thrombospondin-1
uPAR	Urokinase receptor
VCAM	Vascular cell adhesion molecule 1
VE-cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor
VEGFI	Vascular endothelial growth inhibitor
VEGFR-1	Vascular endothelial growth factor receptors 1
WHO	World Health Organization

LIST OF SYMBOLS

α	Alpha
β	Beta
δ	Delta
ε	Epsilon
κ	Kappa
π	Pi
℃	Degree Celsius
%	Percent

LIST OF UNITS

Å	Angstrom
cm	Centimeter
g	Gram
h	Hour
kg	Kilogram
L	Liter
m	Meter
M	Molar
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mm^3	Cubic millimeter
mM	Millimolar
mmol	Millimole
nm	Nanometer
rpm	Rounds per minute
U	Unit
µg	Microgram
µl	Microliter
µm	Micrometer
µmol	Micromole

LIST OF APPENDICES

Appendix A	Approval Letter from TAIF University/KSA Animal Ethics Committee
Appendix B	GC-MS Chemical Identification Report of OBL70 (10mg/ml)

KAJIAN ANTI-TUMOR BAGI *OCIMUM BASILICUM* LAMIACEAE TERHADAP KANSER KOLOREKTAL MELALUI LALUAN MEKANISME ANGIOGENESIS

ABSTRAK

Angiogenesis merupakan proses pertumbuhan salur darah baru yang memainkan peranan penting dalam kanser dan sangat dipengaruhi oleh keradangan yang mana kedua-duanya memberikan kesan terhadap kanser kolorektal. Bahan semulajadi yang mempunyai kesan anti-oksidan yang tinggi diketahui mempunyai aktiviti anti-keradangan dan anti-angiogenesis dan oleh hal yang demikian ianya adalah berguna dalam merawat kanser kolorektal. Dalam kajian ini, ektrak metanol bagi 19 spesis tumbuhan dari famili Lamiaceae yang dijumpai di Saudi Arabia telah disaring untuk kesan aktiviti anti-angiogenesis bagi memastikan potensi ektrak-ekstrak tersebut terhadap kanser kolorektal. Hanya enam jenis ekstrak didapati merencat angiogenesis sebanyak 50% atau lebih iaitu *Thymbra capitate* (Arial part) (97.93±2.63%), *Premna resinosa* (Stem) (85.03±16.84%), *Phlomis viscosa* Poir (Arial part) (81.47±22.95%), *Salvia samuelssonii* (Arial part) (55.69±12.72%), *Ocimum basilicum* (Leaves) (54.55 ± 4.27%) dan *Ocimum basilicum* (Arial part) (53.99 ± 8.75%). Walau bagaimanapun, ekstrak metanol bagi daun *Ocimum basilicum* (*OB*) menunjukkan kesan perencatan tertinggi terhadap proliferasi dan migrasi sel endotelial dikalangan ekstrak yang diuji. Daun (*OB*) diekstrak menggunakan 70% etanol (OBL70) pada 55 ^{0}C, menyebabkan peningkatan kesan anti-angiogenesis sebanyak 43% jika dibandingkan dengan ekstrak metanol. OBL70 dikaji secara *in vitro* terhadap proliferasi sel endotelial, kesan sitotoksik, migrasi sel, pencerobohan sel dan struktur

tiub kapilari menggunakan MTT, pembentukan koloni, esei pencalaran, esei pencerobohan sel dan esei pembentukan tiub. Nilai IC_{50} selepas 96 jam rawatan adalah 79.78± 2.35 µg/ml. OBL70 pada 100 µg/ml mampu merencat pembentukan koloni, migrasi, pencerobohan sel dan pembentukan tiub dengan peratusan seperti berikut, 71.6±4.1, 59.1±1.17, 31.51± 2.37 dan 100±0.0 % dibandingkan dengan kumpulan kawalan dan secara berpandukan dos. Kesan anti-tumor OBL70 bagi kultur *in vitro* bersama melalui model kultur 2D spheroid *in vitro* bersama menunjukkan bahawa perencatan pertumbuhan spheroid pada kepekatan 200 µg/ml adalah 40.15 ± 9.99 % dibandingkan dengan kumpulan tanpa rawatan selepas 13 hari rawatan. Pengenalpastian kimia GC-MS telah mengenalpasti 77 sebatian. Daripada jumlah ini, tiga sebatian dengan peratusan tertinggi, dinamakan (+)-Epi-bicyclosesquiphellandrene (7.83%), 2-Methoxy-4-vinylphenol (3.54 %) dan Eugenol (3.3 %). Mekanisme molekul bagi OBL70 terhadap sel endotelial menunjukkan perencatan yang signifikan dengan perlepasan IL-8, Leptin dan VEGFR-2 sebanyak 82, 65 dan 55% dibandingkan dengan kumpulan tanpa rawatan menggunakan kit susunan angiogenesis antibodi manusia. Akhir sekali, (+)-Epi-bicyclosesquiphellandrene menunjukkan afiniti pengikatan terhadap semua protein target dengan kesan yang lebih baik melalui kajian ‘docking’. Secara kesimpulannya, OBL70 bagi ekstrak *Ocimum basilicum* menunjukkan kesan anti-tumor yang signifikan terhadap kanser kolorektal dan aktivitinya di buktikan melalui perencatan sel endothelial, faktor angiogenesis; IL-8, Leptin dan VEGFR-2 yang diramalkan melalui sebatian utamanya. Ini menyarankan potensi OBL70 dalam aplikasi klinikal bagi penyakit kanser kolon.

THE POTENTIAL ANTI-TUMOR EXTRACT OF *OCIMUM BASILICUM* LAMIACEAE TOWARDS COLORECTAL CANCER VIA THE ANGIOGENESIS MECHANISTIC PATHWAY

ABSTRACT

Angiogenesis is a process of new blood vessel development which can be triggered by inflammation. Both processes play crucial role in colorectal cancer. Natural products that have high level of antioxidants are known to have anti-inflammatory and anti-angiogenic activity and hence could be useful to treat colon cancer. In this work present here, methanolic extracts of 19 plant species of lamiaceae family found in Saudi Arabia were screened for their anti-angiogenic activity to ascertain their potency towards colon cancer. Only six extracts inhibited neovascularization formation by 50% or more, namely *Thymbra capitate* (Arial part) (97.93±2.63%), *Premna resinosa* (Stem) (85.03±16.84%), *Phlomis viscosa* Poir (Arial part) (81.47±22.95%), *Salvia samuelssonii* (Arial part) (55.69±12.72%), *Ocimum basilicum* (Leaves) (54.55 ± 4.27%) and *Ocimum basilicum* (Arial part) (53.99 ± 8.75%). However, methanolic extract of *Ocimum basilicum* (*OB*) leaves shown the highest inhibition towards endothelial cells proliferation and migration among other extracts. (*OB*) leaves were extracted with 70% ethanol (OBL70) at 55 ^{0}C, causing a 43% increase in the anti-angiogenic properties compared to methanolic extract. OBL70 was studied *in vitro* against endothelial cells proliferation, cytotoxicity, migration, invasion and capillary-like structures using MTT, colony formation, scratch, invasion and tube formation assays, respectively. Its IC50 value after 96 h treatment was 79.78± 2.35 µg/ml. OBL70 at 100 µg/ml was able to inhibit colony

formation, migration, invasion and tube formation by 71.6±4.1, 59.1±1.17, 31.51± 2.37 and 100±0.0 % in respect to untreated groups, in a dose-dependent manner, respectively. The anti-tumor properties of OBL70 against *in vitro* co-cultured 2D spheroids model revealed that 200 µg/ml concentration could inhibit the spheroids growth by 40.15 ± 9.99 % compared to untreated group after 13 days treatment. The GC-MS chemical identification revealed the presence of 77 compounds. Of these, three compounds with highest abundance, namely (+)-Epi-bicyclosesquiphellandrene (7.83%), 2-Methoxy-4-vinylphenol (3.54 %) and Eugenol (3.3 %). The molecular mechanism of OBL70 against endothelial cells revealed significant inhibition for the release of IL-8, Leptin and VEGFR-2 by 82, 65 and 55% compared to untreated group. (+)-Epi-bicyclosesquiphellandrene also showed the highest binding affinities against all targeted proteins with more potent inhibitory effect in molecular docking studies. In conclusion, OBL70 of *Ocimum basilicum* extract shows potential anti-tumor properties against colorectal cancer via the inhibition of endothelial cells angiogenic factors; IL-8, Leptin and VEGFR-2 by its major compounds (+)-Epi-bicyclosesquiphellandrene (7.83%), 2-Methoxy-4-vinylphenol (3.54 %) and Eugenol (3.3 %) . This highlights its potential application in clinical setting for treating colon cancer.

CHAPTER 1

INTRODUCTION

1.1 Cancer

1.1.1 Overview of Cancer

Cancer is a metabolic syndrome characterized by uncontrolled proliferation of normal cells that precipitate genetic instabilities and alterations that accumulate within cells and tissues leading to transformation of normal cells into malignant cells (He, Gu, Lim, Yuan, & Mo, 2016).

Douglas Hanahan & Weinberg (2017) reported that most and maybe all types of human cancers are sharing six essential alterations in cell physiology that collectively lead to malignant growth, these are; self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of programmed cell death (apoptosis), limitless replication potential, sustained angiogenesis and tissue invasion and metastasis.

1.1.2 Cancer Epidemiology

Cancer is considered a leading cause of death in both developing and developed countries. The number of deaths is expected to increase worldwide as the population age and number is increasing, especially in less developed countries that reflect about 82% of the world's population (Torre et al., 2015). Besides the growth in population, there is an increase of some lifestyle behaviors that are known to increase cancer risk, such as smoking, unhealthy food and less physical activities that play a role in increasing cancer risk in these less developed countries.

Despite the huge inquiries and rapid development in dealing with cancer; it is still a worldwide killer.

According to the World Health Organization (WHO), cancer is expected to hit the highest death-causing disease and the single most important fence against increasing life expectancy in every country of the world in the 21st century (W. H. Organization). It accounts for about 23% of total deaths in USA and is considered the second cause of death after heart diseases (Jemal et al., 2011). It is estimated that almost 18.1 million new cancer cases were diagnosed and 9.6 million deaths were occurred in 2018 worldwide (Bray et al., 2018). Among these, almost one-half new cases and over one-half of the deaths are happened in Asia.

1.1.3 Causes and Risks of Cancer

Cancer occurs because of internal factors (inherited mutations, hormones and immune conditions) and environmental/acquired factors or lifestyle factors (smoking, food, exposure to radiations and infectious organisms (Anand et al., 2008).

Although all cancer cases are due to multiple mutations or genetic defects, only about 5-10% of all cancer cases are linked directly to these inherited gene defects (Hahn & Weinberg, 2002; Loeb & Loeb, 2000). The rest of cancer cases are happened due to mutations that are linked to interactions with environmental factors (Mucci, Wedren, Tamimi, Trichopoulos, & Adami, 2001).

Mutations in proto oncogenes resulting in their dominant gain of function (*MYC, RAF, Bcl-2* and *RAS*), or tumor suppressor genes leading to their loss of function (*p53, NF1, NF2* and *RB*), or DNA repair genes (*p21, p22, p27, p51, p53* and tool box for DNA) and genes involved in cell growth metabolism (Krishnamurthi, 2007).

Turning from a normal cell to a cancer cell is a multistep process. This means that genetic mutation will happen step by step leading eventually to the transformation of normal human cell to highly malignant cell (Hanahan & Weinberg, 2000). For human population, four to seven rate limiting events are required for this to occur (Balmain, Barrett, Moses, & Renan, 1993). Each event will add a growth advantage for the next event leading to the conversion of normal cell to cancer cell (Foulds, 1954; Nowell, 1976).

1.1.4 Classification of Cancer

Cancers are classified on the basis of tissue of origin into six types; carcinomas for cancers that derived from epithelial cells; sarcomas for those arising from connective tissue; lymphoma and leukemia for those resulting from blood-forming cells; germ cell-tumors for those derived of pluripotent cells (mostly testicles and ovaries); blastomas for cancers derived from precursor or embryonic cells and melanomas for cancers that resulting from cells that produce skin pigment (Yarbro, Wujcik, & Gobel, 2010).

Usually, cancer is named as the organ or tissue of origin followed by its classification. For example, a cancer in the gland which arising from epithelial cells in the colon is called colorectal adenocarcinoma.

1.1.5 Genetics and Molecular Regulators of Cancer

The most important genetic mutations that drive cancer cells is that leading to withstand chronic proliferation. Cancer cells can increase this function through several alternative ways; they can produce their own growth factors, or enhancing stromal cells to release growth factors, or by elevating the level of receptors expressed at the surface of cancer cells or converting these receptors to a more sensitive even with

limited quantities of the growth factor, or by structural conformation of the receptor to produce ligand-independent firing receptor. Another growth factor independent way is through the activation of downstream signal transducers, for example Ras signal transducer activation, B-Raf that activates mitogen activated protein kinase (MAPK) pathway, and Akt/PKB signal transducer that activates phosphoinositide 3-kinase (PI3K) (B. Jiang & Liu, 2009; Yuan & Cantley, 2008).

From another side, the gain of proliferation ability could result from defects in negative feedback mechanisms. One example is loss-of-function mutation in Phosphatase and tensin homolog (PTEN), which counteract PI3K signaling, leading to tumorigenesis in a verity of cancer models (B. Jiang & Liu, 2009; Yuan & Cantley, 2008).

In the other hand, cancer must escape from growth suppressor regulators which depends on the action of tumor suppressor genes. The two prototypical tumor suppressors encode the RB (retinoblastoma-associated) and TP53 proteins; which play the central role of cell choice whether to proliferate or to die through apoptotic program (Burkhart & Sage, 2008; Deshpande, Sicinski, & Hinds, 2005).

Moreover, cancer must evade 'contact inhibition' mechanism, which is regulated by tumor suppressor gene *NF2*. It is clear now that mutations of *NF2* or its product merlin, would prevent cell-to-cell attachment between cell-surface adhesion molecules (e.g., E-cadherin) and tyrosine kinases receptors (e.g., EGFR). Missing this link will promote cell proliferation without a stop (Curto, Cole, Lallemand, Liu, & McClatchey, 2007; Okada, Lopez-Lago, & Giancotti, 2005). Another example is mutations of tumor suppressor gene *LKB1* or its product LKB1 protein, which saves

the integrity of epithelium and allow contact inhibition mechanism even in the upregulation of other oncogenes (Partanen, Nieminen, & Klefstrom, 2009).

The following table summarizes the most important regulators involved in carcinogenesis:

Table 1.1: Examples on Mutations Affect Oncogenes and Tumor Suppressor Genes or Their Proteins That Regulate Carcinogenesis.

Oncogene/ Oncoprotein	**Function**	**Example**	**Reference**
Ras	Cell proliferation	Adenocarcinoma of pancreas and colon	(Marshall, 1995)
Myc	Cell proliferation	Breast cancer	(Bouchard, Staller, & Eilers, 1998)
Bcl-2	Inhibitors of apoptosis	Leukemia	(Mah et al., 1993)
PI3K/Akt	Proliferation, metabolism, survival, and angiogenesis	Ovarian cancer	(Meng, Xia, Fang, Rojanasakul, & Jiang, 2006)
N-cadherin	Metastasis	Metastatic stage	(Suyama, Shapiro, Guttman, & Hazan, 2002)
Telomerase	immortalization	Most cancers	(Condon et al., 2002)
IL-1α	Cell proliferation, angiogenesis, inflammation, metastasis	Cancer-related inflammation	(BenEzra, Hemo, & Maftzir, 1990)
B-catenin	Cell proliferation	Colorectal cancer	(Polakis, Hart, & Rubinfeld, 1999)
BCR-ABL1	Fusion protein with always on tyrosine kinase activity	Chronic myeloid leukemia	(Ernst, La Rosée, Müller, & Hochhaus, 2011)
Erb-B1/ErbB-2	Tyrosine kinase activity receptors	Breast cancer	(Carlomagno et al., 1996)

Table 1-1. Continued

Tumor Suppressor Gene or Its Protein	**Function**	**Example**	**Reference**
PTEN phosphatase	Counteract cell proliferation	Prostate cancer	(J. Li et al., 1997)
Rb	Activate cell senescence or apoptosis	Bone and breast cancer	(Sharma et al., 2010)
TP53/p53	Activate cell senescence or apoptosis	Most cancers	(Fromentel & Soussi, 1992)
NF2 or Merlin protein	Control contact inhibition	Most cancers	(Morrison et al., 2001)
LKB1 or LKB1 protein	Conserve epithelium integrity, control contact inhibition	Cervical, breast and skin cancer	(Katajisto et al., 2007)
TGF-β	Anti-proliferative effect	Prostate cancer	(Assinder, Dong, Kovacevic, & Richardson, 2009)
Bax and Bak	Trigger apoptosis	Ovarian cancer	(Scorrano et al., 2003)
BECN1/ Beclin-1	Trigger apoptosis and autophagy	Ovarian cancer	(Ju, Zhao, Ye, Yang, & Zhang, 2016)
E-cadherin	Maintain contact inhibition, prevent metastasis	Breast cancer	(Sundfeldt et al., 1997)
APC	Control β-catenin accumulation and interact with E-cadherin	Colorectal cancer	(Fodde, 2002)

1.1.6 Colorectal Cancer

For both sexes, colorectal cancer (CRC) is the second and third cancer for mortality and incidence, respectively. It is one of the ten top cancers worldwide and it represents about 6.1 % of all newly diagnosed cancer cases (Bray et al., 2018).

Globally, total number of deaths in both sexes reached 880, 792 in 2018, of which 52.4% in Asia which represents the highest followed by Europe with almost half number of deaths (27.5%). Interestingly, number of deaths were increased ascendingly according to the human development index, which may reflect the influence of lifestyle behavior. Moreover, high and upper middle-income levels represent the highest percentage of deaths by colorectal cancer. According to the latest WHO data published in 2018 Colon-Rectum Cancers Deaths reached 1.89% or 1.28% in Malaysia or Saudi Arabia of total deaths, respectively.

In Malaysia, reports indicate that CRC incidence is still less than other eastern Asian countries such as, Japan, South Korea, and Singapore. However, the incidence and mortality rates are increasing, while decreasing or does not change in other eastern Asian countries. Moreover, most cases in Malaysia are diagnosed in the late stages (Rashid, Aziz, Ahmad, Shah, & Sagap, 2009).

An important factor which is strongly linked to the high incidence rates is the limited health resources and infrastructures which will affect early screening and diagnosed cancer among females and males, respectively (Ferlay et al., 2015). Although, the higher incidence rates are occurred in western developed countries, there is a remarkable increase in its incidence in several areas which was formerly considered at low risk, such as eastern Asia countries (Allemani et al., 2015). This deterioration may be due to the changes in lifestyle habits, increase smoking tobacco and drinking alcohol, and fast food consumption (Sung et al., 2015).

1.2 Angiogenesis

1.2.1 Definition of Angiogenesis

Angiogenesis is the formation of new blood vessel from pre-existing vessel during physiological tissue homeostasis or tumorigenesis. It is a complex process including extracellular rebuilding, endothelial cell (EC) migration, proliferation, capillary differentiation and vascular anastomosis (Blood & Zetter, 1990). Tumor angiogenesis will provide essential oxygenation and nutrients to growing tumor, besides enabling transfer of tumor cells to other organs to establish secondary tumors (Carmeliet & Jain, 2000; Ferrara, 2010).

1.2.2 Angiogenesis in Physiological and Pathological Aspects

During embryogenesis, the formation of new blood vessels requires both, the proliferation of new endothelial cells and their arrangement as tubes (vasculogenesis), and the sprouting of new vessels from pre-existing blood vessel (angiogenesis). After morphogenesis, the normal blood vessels become highly quiescent. In the adult stage, and during physiological processes such as wound healing or female reproductive cycle, the angiogenesis is turned on again, but only transiently. The switch off is regulated by endogenous angiogenesis inhibitors, such as thrombospondin-1 (TSP-1), angiostatin and endostatin. Reports revealed that these inhibitors serve as regulators under physiological circumstances for transitory angiogenesis during wound healing and tissue remodeling, or as intrinsic barriers to resist angiogenesis induced by tumors (Seppinen et al., 2008).

In contrast, during tumor progression, angiogenesis is almost remained activated, causing continuous sprouting of new blood vessels to help supporting tumor growth (Douglas Hanahan & Folkman, 1996).

1.2.3 Cancer Angiogenesis

1.2.3(a) Types of Angiogenesis

Tumors have set of strategies to enhance their blood supply, including sprouting angiogenesis, vessel co-option, intussusception of existing vessel, and mobilization of bone marrow-derived endothelial progenitor cells into growing vessels (Adams & Alitalo, 2007; Rafii, Lyden, Benezra, Hattori, & Heissig, 2002).

Vessel co-option usually refers to the tumor, particularly in the case of metastases, where tumor cells grow in proximity to the pre-existing vessel and getting nutrients from it (Holash et al., 1999).

Vessel intussusception is a process where vessels split longitudinally. It is a fast process and increases vascular density without need of endothelial cells proliferation.

Sprouting angiogenesis is a slow, invasive process capable of formation of new blood vessel from pre-existing vessel and reach avascular areas. This process initially requires releasing proteases to degrade extracellular matrix (ECM) proteins, where endothelial cells can migrate, proliferate and join pre-existing vessel tell the formation of a lumen and recruit pericytes that stabilize the new formed vessels (Adams & Alitalo, 2007). It is triggered by the lack of oxygen in the adjacent growing tissues (hypoxia), which in turn will release pro-angiogenic factors such as vascular endothelial growth factor (VEGF) into the environment. Established blood vessels will respond to these growth factors by generating new blood vessels which are directed to the pro-angiogenic factors gradient. In more details, within the sprouting blood vessels, tip-cells will respond to the VEGF by forming protruding and actin-rich filaments. Tip-cells don’t proliferate, they just migrate toward the source of VEGF thus giving

the direction for new forming blood vessels. While, stalk-cells will proliferate behind the leading tip-cells allowing the extension of sprouting blood vessels. Then, rearrangement of stalk-cells cytoskeleton will give the tube shape of blood vessels in response, in part, to hydrodynamic forces (Charpentier & Conlon, 2014; Gebala, Collins, Geudens, Phng, & Gerhardt, 2016; Ribatti & Crivellato, 2012) (Figure 1.1).

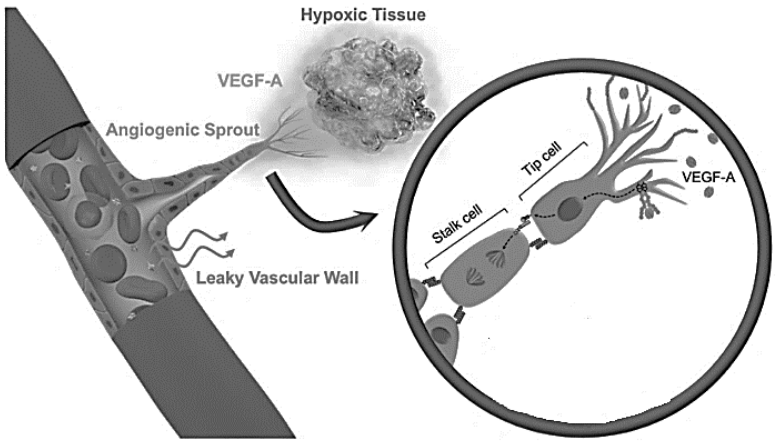

Figure 1.1. Sprouting Angiogenesis. As tumor grows and more oxygen is needed, a hypoxic situation will be created inside the tumor cells, triggering release of pro-angiogenic factors such as VEGF. Resulting in migration of tip-cells toward VEGF gradient which lead stalk-cells behind. The stalk-cells will proliferate to generate the extension of sprouting angiogenesis as well as forming vessel lumen upon cytoskeletal rearrangement (Pauty et al., 2018, P.2).

1.2.3(b) Regulation of Angiogenesis via VEGF/VEGFR Pathway

The process of angiogenesis is driven by pro-angiogenic factors such as VEGF, basic fibroblast growth factor (bFGF), and interleukin-8 (IL-8), which are released by tumor and tumor stroma cells. The balance between endogenous pro-angiogenic and anti-angiogenic factors will control the EC growth and angiogenesis (Ferrara, 2010).

VEGF has the major key for inducing angiogenesis and endothelial mitogen activity. VEGF was found upregulated in several types of human tumors, besides

responsibility for poor prognosis. The VEGF family consist of VEGF A-E and Placental growth factor (PIGF) 1 and 2 (Dvorak, Brown, Detmar, & Dvorak, 1995).

VEGF A is the most active pro-angiogenic factor. It can bind to both important tyrosine kinase receptors (TKR); vascular endothelial growth factor receptors (VEGFR-1) and (VEGFR-2). However, signaling via VEGFR-2 is more potent than via VEGFR-1 regarding inducing endothelial cells (Gille et al., 2001). Upregulation of VEGF can be induced by hypoxia inducible factor (HIF-1α), epidermal growth factor (EGF) and platelet derived growth factor (PDGF) (Petit et al., 1997; Reinmuth et al., 2001).

Both VEGFR-1 and -2 are type III TKRs (Shibuya & Claesson-Welsh, 2006). Dimerization of VEGF and VEGFR-2 and subsequent phosphorylation of several tyrosine kinases will initiate downstream pathways causing EC proliferation, survival, migration and capillary tube formation (Holmqvist et al., 2004; Olsson, Dimberg, Kreuger, & Claesson-Welsh, 2006).

Inhibition to the binding of VEGF and VEGFR-2 will prevent phosphorylation of other tyrosine kinases leading to decrease the level of the extracellular signal regulated kinase (ERK), p-Akt, focal adhesion kinase (FAK) and MAPK which are all mediators of endothelial survival and proliferation as well as for cancer cells (Rathinavelu, Alhazzani, Dhandayuthapani, & Kanagasabai, 2017).

1.2.3(c) Other Angiogenesis Regulatory Pathways

1.2.3(c)(i) Alternative Pro-Angiogenic Factors

1- PDGF:

This growth factor and its receptor (PDGFR) are involved in blood vessel maturation and recruitment of pericytes (Lindahl, Johansson, Levéen, & Betsholtz,

1997). PDGF is expressed by endothelial cells and act in paracrine matter, to activate PDGFR with tyrosine kinase activity. PDGFR has two forms PDGFR-α and -β, which are expressed mainly by pericytes and smooth muscle cells in order to develop the vessel (Andrae, Gallini, & Betsholtz, 2008). Poor prognosis of ovarian cancer was found to be associated with overexpression of PDGFR, which likely indicates the role of PDGF pathway in human cancers (Dabrow, Francesco, McBrearty, & Caradonna, 1998).

2- FGF:

The FGF ligands are among the earliest discovered potent angiogenic factors which have a role in stimulating EC proliferation, migration and differentiation (Abraham et al., 1986). FGF has high affinity toward heparin sulfate proteoglycans (HSPGs), which are co-receptors bind both FGF and one of the four fibroblast growth factor receptors (FGFR) (Korc & Friesel, 2009). FGFRs have tyrosine kinase activity and expressed in most cell types including endothelial cells leading to potent angiogenic activity (Lindner, Majack, & Reidy, 1990). Overexpression of either FGF ligands (specially FGF-2) or FGFRs has been documented in different types of human cancers and was linked with poor outcomes and prognosis of tumors, such as non-small cell lung cancer (NSCLC) and bladder carcinoma (Gazzaniga et al., 1999).

3- EGF:

Binding of EGF to epidermal growth factor receptor (EGFRs), which have tyrosine kinase activity (except HER3), was found to enhance metastasis, angiogenesis, cell proliferation, migration, adhesion and differentiation (Yarden & Sliwkowski, 2001). Because the activation of EGFR pathway will upregulate the release of other pro-angiogenic factors, such as VEGF, it is considered as indirect

regulator of angiogenesis rather than direct regulator, emphasizing that the role of EGF/EGFR pathway is less important to angiogenesis than other regulatory pathways, such as VEGF and PDGF systems (De Luca et al., 2008).

4- Transforming growth factor-β (TGF-β):

TGF-β and their correspondence receptors are expressed by most cell types, and involved in the processes of angiogenesis, cell differentiation, wound healing as well as growth inhibition properties (Blobe, Schiemann, & Lodish, 2000).

TGF-β is found to have both pro- and anti-angiogenic properties, depending on its levels. Low levels of TGF-β are involved in angiogenesis and production of protease, while the high levels inhibit endothelial cells growth (Carmeliet, 2003). Tumor cells were found to resist its inhibitory properties and will exploit the ability of TGF-β to regulate angiogenesis and cell invasion behavior (Bernabeu, Lopez-Novoa, & Quintanilla, 2009). Overexpression of TGF-β1 was reported in gastric, colon, breast, hepatocellular, lung and pancreatic cancer and was linked to angiogenesis, metastasis, poor prognosis and progression (Bierie & Moses, 2006).

TGF-β receptors has serine/threonine kinase domains (except TFG-β3), which upon activation will stimulate downstream pathways, specifically SMADs pathway. other activated downstream pathways are MAPK and PI3K (Bierie & Moses, 2006a; Blobe et al., 2000).

5- Angiopoietins and their receptor Tie (Ang/Tie):

The ligand Ang and Tie receptors which have tyrosine kinase activity, are of know rule in vasculature development and angiogenesis (Augustin, Koh, Thurston, & Alitalo, 2009). The Ang family (Ang-1, -2,-3, and -4) bind to Tie-2 receptor. Ang-1

has anti-tumorgenic effects as its binding to Tie-2 will stimulate vascular maturation and normalization (Winkler et al., 2004). However, some studies indicated that it stimulates tumor growth (Augustin et al., 2009).

In the other hand, Ang-2 has different effects which depend on the environmental context. It was reported that in the presence of VEGF, Ang-2 will promote angiogenesis, while in the absence of VEGF, Ang-2 will cause vessel regression (Holash et al., 1999).

Attempts to target Tie pathway in angiogenesis by neutralizing Ang-2 have led to tumor growth inhibition and suppress EC proliferation in preclinical studies, indicating the possibility of targeting Ang/Tie for anti-angiogenesis effects (Oliner et al., 2004).

The following table summarizes the most important angiogenic inducers or inhibitors with their suggested mechanism of action (Table 1.2).

Table 1.2: Angiogenic Regulators. Normal Angiogenic Process Is Highly Regulated Through Balance Between Angiogenic Inducers and Inhibitors. Any Dysregulation of This Balance Toward Angiogenic Inducers Will Lead to Uncontrolled Angiogenesis Such as Tumor Angiogenesis.

Angiogenic inducers	**mechanisms**	**reference**	**Angiogenic inhibitors**	**mechanisms**	**reference**
VEGFA, B, C, D And receptors VEGFR2 and neuropilin-1	Increase endothelial cells permeability, proliferation, and survival	(Rathinavelu et al., 2017)	TSP-1, -2	Inhibits endothelial cells migration, proliferation, adhesion and survival	(Seppinen et al., 2008)
FGF (1-2)	Induce endothelial cells, smooth muscle cells and fibroblasts proliferation and differentiation	(De Luca et al., 2008)	Angiostatin	Inhibits endothelial cells migration, proliferation, adhesion and survival	(Seppinen et al., 2008)
PDGF And PDGFR	Recruit pericytes and smooth muscle cells	(Andrae et al., 2008)	endostatin	Inhibits endothelial cells migration, proliferation, adhesion and survival	(Seppinen et al., 2008)
Ang	Regulates blood vessels	(Augustin et al., 2009)	IL-4, -12, -18	Downregulates bFGF,	(Doyle et al., 2014)
TGF-α, TGF-α receptor	Enhance ECM production	(Bernabeu et al., 2009)	Tumstatin and canstatin (fragments of collagen IV)	Inhibits endothelial cells migration and proliferation and inhibits PI3K, mTOR and FAK pathways	(Eikesdal et al., 2008; Kamphaus et al., 2000)
integrins	Facilitate binding to matrix macromolecules and proteinases	(Mitra & Schlaepfer, 2006)	Arrestin	Inhibit cell cycle and induce apoptosis	(V Rosca et al., 2011)

Table 1-2. Continued

Angiogenic inducers	**mechanisms**	**reference**	**Angiogenic inhibitors**	**mechanisms**	**reference**
VE-cadherins	Enhance endothelial cells junctions	(Gavard & Gutkind, 2006)	Vascular endothelial growth inhibitor (VEGI)	Inhibits endothelial cells proliferation and migration	(Hou, Medynski, Wu, Lin, & Li, 2005)
Matrix metalloproteinases (MMPs)	Degrades blood vessels wall and ECM	(Adams & Alitalo, 2007)	Soluble VEGFR	Trap receptor for VEGF and PIGF	(Lähteenvuo et al., 2009)
Endothelial nitric oxide synthetase (eNOS) and cyclooxygenase-2 (COX-2)	Generate NO inside cells and increase expression of VEGF	(Kevil et al., 2004)	Interferons -α, -β, -γ	Inhibits endothelial cells migration	(Jablonska, Leschner, Westphal, Lienenklaus, & Weiss, 2010)
Cytokines, chemokines (IL-1, IL-8)	Induce chemotaxis in endothelial cells	(Brocker, Thompson, Matsumoto, Nebert, & Vasiliou, 2010)	Ang-2	Antagonist Ang-1	(Hata et al., 2002)

1.2.3(c)(ii) Hypoxic Pathway

Oxygen level is considered a switch key that regulates synthesis and secretion of growth factors as well as inflammatory mediators within the tissue. Hypoxia that happened in tumors will stimulate the expression of several growth factors, among them VEGF through the transcription factor HIF-1α (Terzuoli et al., 2010). In oxygen presence, HIF is degraded and inactive, while the presence of hypoxic conditions HIF will be activated and lead to transcription of target genes. There are other factors that activate and increase expression of HIF other than hypoxia. This includes several growth factors and cytokines such as tumor necrosis factor (TNF-α), IL-1β, EGF, and insulin-like growth factor-1 (IGF-1) (Fukuda et al., 2002; Hellwig-Bürgel, Rutkowski, Metzen, Fandrey, & Jelkmann, 1999; B.-H. Jiang et al., 2001; Stiehl, Jelkmann,

Wenger, & Hellwig-Bürgel, 2002). Moreover, oncogenes can trigger HIF expression and activity. For example, mutant *ras* will enhance angiogenesis due to upregulation of VEGF expression through activated HIF (J Rak et al., 1995). Other examples such as, *V-Src* and *HER2* as well as activated PI3K and MAPK signaling pathways shown increased activity or expression of HIF (Blancher, Moore, Robertson, & Harris, 2001; B.-H. Jiang, Agani, Passaniti, & Semenza, 1997; Laughner, Taghavi, Chiles, Mahon, & Semenza, 2001).

1.2.3(c)(iii) Inflammatory Pathway

Angiogenesis and inflammation are two linked processes that support tumor growth and progression (Albini, DeCensi, Cavalli, & Costa, 2016; Albini, Tosetti, Li, Noonan, & Li, 2012).

It is reported that chronic inflammatory process will initiate tumor development as well as pathological angiogenesis (Carmeliet & Jain, 2011). This link is correlated to the complex interaction between immune, tumor and endothelial cells.

Briefly, when cells are damaged due to any reason such as cell stress or infection, these cells will express on their cell membranes endogenous molecules known as (DAMPs), which in turn will sensed mainly by leukocytes (Nguyen, Pang, & Masters, 2017). This interaction will enhance the inflammatory process through the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway to release pro-inflammatory cytokines such as VEGF, IL-1α, IL-1β and TNFα along with chemokines IL-8, macrophage inflammatory protein-1 (MIP-1α), RANTES, COX-2 and prostaglandins (Afonina, Zhong, Karin, & Beyaert, 2017).

The target cells for the previous cytokines and chemokines are mainly endothelial cells which when activated will lead to vascular hyperpermeability and

vasodilation, in addition to further expression of adhesion molecules such as E-selectin, P-selectin, intracellular adhesion molecule (ICAM)-1 and -2 and vascular cell adhesion molecule (VCAM)-1 which in turn will alter cell-cell junctions, exacerbates the inflammatory response and promote angiogenesis.

Finally, this dysregulated inflammatory process will turn on cell survival signaling pathways in tumor cells leading to release panel of angiogenic factors including FGF-2, CXCL8, WNT7b, ANGPT-2, IL-1β, IL-6, IFN-α, CXCL9/10 and MMP2/9 (De Palma, Biziato, & Petrova, 2017).

Therefore, targeting inflammatory mediators and linked angiogenesis molecules could improve treatment of tumors (Douglas Hanahan & Weinberg, 2011).

1.2.3(c)(iv) Stress Oxidation Pathway

It is known that reactive oxygen species (ROS) act as inducer for angiogenesis through upregulation of VEGF, VEGFR-2, Ang-1, and Tie-2 expressions (Colavitti et al., 2002). ROS which originate from mitochondria, control the innate immunity response through two major pathways, including either direct activation of inflammatory mediators or upregulation redox sensitive transcription factor such as NF-κB (Ferrero-Miliani, Nielsen, Andersen, & Girardin, 2007). The later will activate expression of genes encoding inflammatory cytokines, adhesion molecules, enzymes and angiogenic factors (Karin, 2006). Thus, targeting NF-κB will block both macrophages and endothelial recruitment and downregulates expression of ICAM, thus inhibiting angiogenesis indirectly (Hagemann et al., 2008).

1.2.3(c)(v) Downstream Signaling Pathway

VEGFR-2 is the major TKR which mediates angiogenesis activity of VEGF via several signaling pathways including PLCγ-PKC-Raf kinase-MEK-MAPK and PI3K-Akt-mTOR pathways and activation of the Src tyrosine kinases, which in turn regulate EC migration, invasion, cell adhesion and survival (Mitra & Schlaepfer, 2006; Qi & Claesson-Welsh, 2001).

The dysregulation in the activity of VEGFR2 or other TKRs can occur through different mechanisms, which includes; amplification or overexpression of TKRs; gain of function mutations that eventually lead to continuous active kinase activity; genomic transfusions that produce proteins with active kinase activity; and continuous stimulation of TKRs by high levels of pro-angiogenic factors (Madhusudan & Ganesan, 2004).

This dysregulation will activate downstream signaling pathways, which in turn will determine the final biological outcomes of the receptor activation.

1.2.3(c)(vi) Cell Surface-Associated Proteins Pathway

Several studies revealed that EC adhesion molecules are involved in angiogenesis (Kwon et al., 1998). Reports shown that diseases such as atherosclerosis and diabetic retinopathy exposed an increased adhesion molecule expression with neovascularization (Carmeliet & Jain, 2000). Moreover, soluble forms of E-selectin, VCAM-1 and ICAM-1 have been all induced angiogenesis through unknown pathway (Gho, Kleinman, & Sosne, 1999).

Going deeply, during the sprouting of a growing vessel, Stromal cell derived factor-1 (CXCL12)and CCL2 play an important role to guide endothelial cells, their invasion, mobilization, migration, extravasation, directional migration, homing and cell survival (Izhak et al., 2012; Kioi et al., 2010; Müller et al., 2001).

In addition, the transcription level of cell associated surface protein such as platelet endothelial cell adhesion molecule-1 (PECAM-1) is an important driver for cell migration (Woodfin, Voisin, & Nourshargh, 2007).

In the other hand, targeting the interaction between CXCL12 with its receptor PECAM-1 considered a potential factor to prevent tumor angiogenesis (Janssens, Struyf, & Proost, 2017). Additionally, targeting TNF-α will prevent adhesion of monocytes to the endothelial cells monolayer leading to inhibition of inflammatory angiogenesis (Baci et al., 2018).

In the other hand, it is well known that activation of VEGFR-2 will stimulate vascular endothelial (VE)-cadherin pathway resulting in strong cell-cell junction and opens of adherens junctions leading to induction of endothelial permeability (Gavard & Gutkind, 2006).

The next figure shows different interactions between endothelial, tumor or stromal cells (figure 1.2):

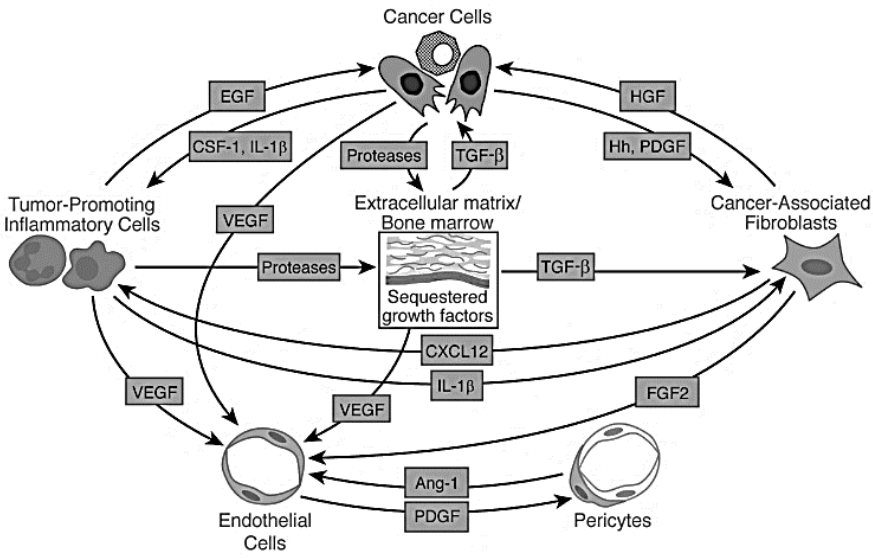

Figure 1.2. Tumor cells are not the only factor that regulates endothelial cells. Other cells that participate in angiogenesis regulation; such as cancer-associated fibroblasts which release FGF2; pericytes which release Ang-1 and help in blood vessels maturation; extra growth factors released from ECM degradation due to proteases activity; and other inflammatory cells which release VEGF and proteases during their activity (Douglas Hanahan & Weinberg, 2017, P.666).

1.3 Role of Endothelial Cells and Tumor Vasculature Environment in Angiogenesis

Tumor vessels are not like normal vessels. They are leaky, snaky, deficient in pericyte coverage, and abnormal arrangement of arterioles, capillaries and venules (Baluk, Hashizume, & McDonald, 2005). Moreover, lack of lymphatic vessels besides poor perfusion tend to increase interstitial pressure of tumor which will affect delivery of chemotherapies (Duda, Jain, & Willett, 2007). Those factors will create hypoxic and acidic tumor environment which will affect physiology, morphology and gene expression of tumor endothelial cells (Adams & Alitalo, 2007; Helmlinger, Yuan, Dellian, & Jain, 1997).

endothelial cells that cover lumen of blood vessel have an important role in angiogenesis, where they change their mechanics and physiological components

during vascular sprouting (Ausprunk & Folkman, 1977), stalk cell proliferation (Keegan et al., 1982), tip cells migration (Zetter, 1980), tube formation (J Folkman & Haudenschild, 1980), and vascular stabilization (Baluk, Morikawa, Haskell, Mancuso, & McDonald, 2003). The mechanics and physiological components process includes cytoskeletal rearrangement (J. Cao et al., 2017), focal adhesion formation (Abedi & Zachary, 1997) and contractile force (J. Hu et al., 2016).

In the other hand, it was found that activation of VEGFR-2 receptor via VEGF determines the tip/stalk-cell destiny through the Delta like ligand 4 (Dll4)/neurogenic locus notch homolog protein 1 (NOTCH1) (Hellström et al., 2007; Simons, Gordon, & Claesson-Welsh, 2016). So, when VEGF binds to VEGFR-2 will activate intracellular kinase activity of the receptor leading to cascades that results in choosing the cell as tip-cell which in turn will express Dll4 ligand on its surface. Consequently, Dll4 will bind to its receptor NOTCH1 on the surface of adjacent EC leading to cascades that will decrease the later sensitivity to VEGF and allow just its proliferation making it a stalk-cell (Blanco & Gerhardt, 2013; Simons et al., 2016).

1.4 Cancer Treatment

There are several methods for cancer treatment such as surgery, radiotherapy, chemotherapy, hormonal therapy, photodynamic therapy and stem cell transformation or special combinations (Patra, Mukherjee, & Kotcherlakota, 2014).

These treatments have side effects including limited bioavailability, toxicity, non-specificity, fast clearance or special precautions with metastasis. Choosing the treatment method depends on the type of cancer, stage and location (Mukherjee & Patra, 2016).

1.4.1 Surgery

Non-hematological cancers are theoretically can be cured if removed entirely. However, this is not always possible especially if cancer has metastasized or moved to another organ. Another obstacle is that removing all cancer cells is considered impossible.

Examples of surgeries are mastectomy for breast cancer, prostatectomy for prostate cancer and lung cancer surgery for NSCLC. The goal of surgery is to remove only tumor cells or the whole organ.

Surgery is also important for detecting the stage of cancer and whether it metastasized to reginal lymph nodes. Surgery can be performed before or after chemotherapy but the advantages for this protocol is still needs investigation.

1.4.2 Radiotherapy

Radiotherapy is the use of ionizing radiation to kill cancer cells. It can be administered externally or internally. It works by damaging the genetic material of cells to render their growth. However, the killing will affect both cancer and normal cells. Usually, normal cells can regrow again if radiotherapy is given in fractions.

Radiotherapy can be used to treat almost all solid malignancies as well as hematological cancers such as leukemia and lymphoma. The dose of radiation depends on the sensitivity of cancer to radiation and whether there are tissues or organs nearby the cancer cells. The radiation is used is high-energy rays, such as x-ray.

1.4.3 Chemotherapy

This category involves agents that have cytotoxic effect against cancer cells with limited or low activity against normal cells. They show a promising effect as monotherapy or in combination with other treatment options. Several groups belong

to chemotherapies such as topoisomerase inhibitors (e.g. irinotecan and doxorubicin), alkylating agents (e.g. cisplatin and cyclophosphamide) and microtubule-disturbing agents (e.g. vinblastine and paclitaxel) (Caruso et al., 2000; Weaver, 2014).

Although the previous agents show a high efficacy against several cancer types, but they have many limitations including toxicity against rapidly growing normal cells, high cost price, developing resistance and many other side effects (Aung, Qu, Kortschak, & Adelson, 2017).

1.4.4 Hormonal Therapy

This type of treatment is useful for cancers that use hormone to grow in order to slow or stop its growth, such as breast and prostate cancers.

1.4.5 Anti-Angiogenic Therapy

The concept of anti-angiogenic therapy appeared from the important observation of Judah Folkman and colleagues, where the tumor induces formation of new blood vessels from pre-existing vessels and that this process is important for the growth of tumor beyond 2-3 mm in size. After this, it was proposed that inhibition of angiogenesis could suppress tumor growth in humans (Judah Folkman, 1971). At the same time, studies revealed that VEGF (specially VEGFA) is the key regulator of angiogenesis, expressed in most solid tumors and its inhibition can lead to the tumor growth suppression in animal models (Carmeliet & Jain, 2011; de Oliveira, Hamm, & Mazzone, 2011; Ellis & Hicklin, 2008). Based on these observations, many therapeutics have been studied extensively to evaluate their anti-angiogenic effect through interfering with the VEGF/VEGFR-2 signaling pathway (Carmeliet, De Smet, Loges, & Mazzone, 2009; Kerbel, 2000, 2008; Olsson et al., 2006) (Figure 1.3).

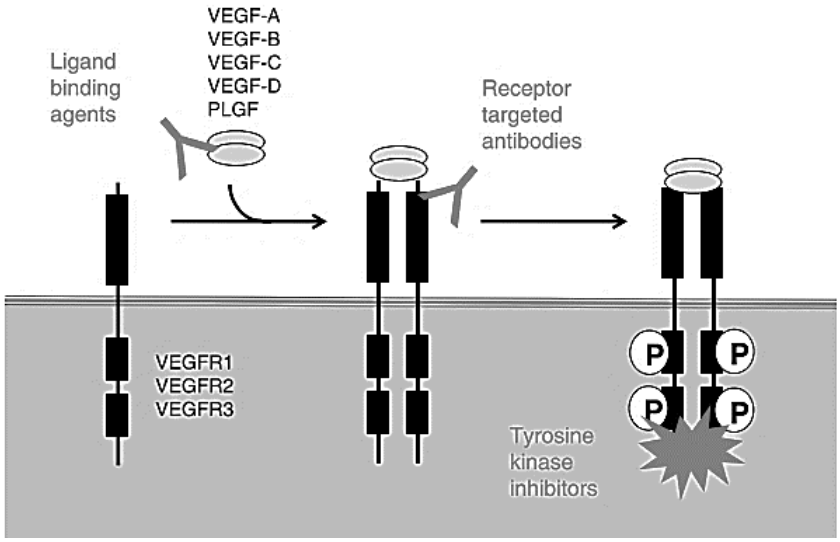

Figure 1.3. Simplified diagram shows the mechanism of action for agents that interfere with VEGF/VEGFR-2 signaling pathway. a) Ligand binding agents; which bind to VEGF members and prevent their binding to VEGFR-2 receptor (e.g. bevacizumab which blocks binding of VEGFA and aflibercept which blocks binding of VEGFA, VEGF-B and PlGF). b) Receptor targeted antibodies; are antibodies that block signaling of VEGFR-2 (e.g. ramucirumab which blocks VEGFR-2). c) Tyrosine kinase inhibitors; which blocks tyrosine kinase activity of VEGFR-1, VEGFR-2 and VEGFR-3 besides other receptors with tyrosine kinase activity such as PDGFR, c-Kit, EGFR and FGFR. In addition, this class inhibits downstream signaling of growth and survival cell kinases such as Raf, MEK and ERK signaling pathways (e.g. sorafenib, sunitinib, pazopanib) (Naveen S. Vasudev & Reynolds, 2014, P.2).

Bevacizumab, the first FDA approved anti-angiogenic therapy as first- and second-line treatment for mCRC in combination with 5-flurouracil based chemotherapy. It is a humanized anti-VEGF antibody. Its effect is mainly due to vascular normalization rather than angiogenesis blocking activity (Jain, 2005). It is well tolerated therapy with some manageable adverse effects including, hypertension, proteinuria, nose bleeding, upper respiratory infections, gastrointestinal symptoms and headache. In general, all adverse effects has been related to the blocking ability of normal activity of VEGF in the vessels (van der Zee et al., 1997). Bevacizumab has at least three suggested mechanisms of action. The first is an anti-angiogenic mechanism by blocking angiogenesis which supported by *in vitro* xenograft models. The second is based on preventing circulating endothelial and endothelial progenitor cells to