CHAPTER 1

INTRODUCTION

1.1 GENERAL

Angiogenesis, is the term used to describe the growth of new capillary blood vessels in the body. Angio means, “of blood or lymph vessel” and genesis means, “beginning or origin”. Briefly, it’s a physiological process involving the growth of new blood vessels from pre-existing vessels. It is a normal process in growth and development, as well as in wound healing. During the development of embryo, blood islands and vessels are the structures that develop first. The flow of nutrients and oxygen depends on the intercalating network of capillaries and vessels that fenestrate all through the body. However, this is also a fundamental step in the transition of tumors from a dormant state to a malignant state, as the tumors warrants robust vasculature for growth. With involvement in progression of tumors and metastasis, angiogenesis has emerged as a great prospective for research.

The revolutionary works from Judah Folkman in the field of tumor angiogenesis have brought to light a new field of research and a new regime of cancer treatment known as Anti-angiogenesis therapy. He first introduced the concept of tumor angiogenesis in his published works in 1971 (Folkman 1971). The enormous volumes of works in the field have showed that, pathological angiogenesis is implicated not only in the development of tumors but in many other non-neoplastic diseases (Folkman 2007). For the last 3 decades there has been extensive work in the area of therapeutic use of anti-
angiogenic drugs for the treatment of angiogenesis related diseases. In 2004, FDA approved anti-angiogenesis as a therapy for treatment of tumors. Avastin, a VEGF blocker was the first marketed anti-angiogenic drug. Presently there are only handful inhibitors of angiogenesis known and great body of research is going on in this field. Anti-angiogenesis therapy is gaining much importance in recent times because of the ease of administration as the drug does not have to cross any barrier. Moreover physiological angiogenesis in adults is observed only during wound healing and reproductive ovarian cycle, thus anti-angiogenic therapy promises to have less pronounced side effects.

The development of blood vessels is a complex process which involves different cell types including endothelial cells, pericytes and smooth muscle cells. The process involves action of various endothelial specific growth factors such as VEGF (Connolly 1991), PDGF, FGF, TGF-β, Bradykinin, Angiopoietin-1, GCSF and HGF. Broadly three different mechanisms have been attributed to new blood vessel formation:

**Vasculogenesis,** a term used for *de novo* blood-vessel formation as seen in case of embryonic development and vascularisation.

**Intussusception,** a term for new blood vessel formation by splitting off existing ones, as seen in most cases in adults. Blood vessels are formed in adults from pre-existing ones.

**Sprouting angiogenesis,** is a type of vasculogenesis that occurs in adults (female menstrual cycle) where endothelial cell in response to stimulus get detached from pre-existing blood vessels and proliferate in the surrounding matrix forming solid sprouts attaching neighboring blood vessels.
Of late there has been a growing body of research going on in the field of angiogenesis and many new secretory factors are being discovered to have role in the angiogenesis process. Secreted signaling molecules like members of FGF, TGF-β, BMP, Hedgehog and Wnt have emerged as major players in these processes (Figure 1.1).

**Figure 1.1 Different types of Angiogenesis**

From the mother vessel the new vessels can be formed either by splitting the mother vessel into new vessels: Intussusception or by sprouting of a new blood vessel from the lumen of parent vessel: sprouting angiogenesis.

1.2 WNT

Wnts are a family of highly conserved secreted cysteine-rich glycoproteins, which act as signaling molecules. With other secreted factors like FGF, TGF-β and Hedgehog proteins, Wnts are involved in regulating diverse signaling pathways; from tumourigenesis, to early mesodermal patterning of the embryo, proliferation and differentiation of different cell
types (Nusse and Varmus 1992; He et al 1998; Hsieh et al 1999; Finch et al 1997) including endothelial cells (Masckauchan 2006; Goodwin 2007). Wnt play a crucial role in the development of the vasculature under different conditions, including embryonic angiogenesis (Uren et al 2000; Bafico et al 1999) and recently Wnt have been shown to be involved in development of vasculature in CNS (Daneman et al 2009).

The first Wnt gene was isolated from mouse genome as a relatively obscure proto-oncogene in 1982 (Nusse and Varmus 1982). A total of 19 different Wnt proteins have been identified in humans till date. Wnt proteins released from signaling cells bind to Frizzled (Frz) / Low Density Lipoprotein (LDL) Related Receptor Protein (LRP) complex on the cell surface. Further down stream to the receptor, Wnt signals are transduced via several intracellular proteins and different pathways (Logan and Nusse 2004). There are at least 3 different arms of Wnt signaling pathways:

1) Canonical, or Wnt-β-catenin pathway, which targets a key cellular regulatory molecule β-catenin,

2) The Wnt-Calcium-mediated pathway which mobilizes intracellular calcium to activate calcium/calmodulin-dependant protein kinase II and protein kinase C,

3) The Wnt-planar cell polarity pathway which signals through Rho-associated kinase and c-Jun-N-terminal kinase (Turashivili et al 2006) (Figure 1.2).
Figure 1.2 Wnt pathways (a) Canonical Wnt pathway involving β-catenin, (b) Non-canonical Wnt pathway involving either Rho kinase, JNK pathway or Ca^{2+} and Calmodulin dependent kinase

1.3 sFRP: SECRETED FRIZZLED RELATED PROTEIN

sFRP: secreted frizzled related proteins are a family of 5 glycoproteins that share homology with the extracellular domain of the membrane-bound frizzled receptors (White et al 2006). sFRP was first discovered as a gene family encoding secreted proteins that contain homology with cysteine-rich ligand binding domain found in frizzled family of transmembrane receptors (Rattner et al 1997). The sFRPs are approximately 30kDa in size and each contains a putative signal sequence, a frizzled like cysteine rich domain and a conserved hydrophilic carboxy terminal domain. They lack the domain encoding the putative seven transmembrane segments (Hoang et al 1998).
sFRP4 is a member of secreted frizzled related protein family of Wnt- inhibitors that bind directly to Wnt and antagonize both canonical and non-canonical Wnt pathway (Kawano and Kypta 2003). sFRP4 has been shown to induce apoptosis independent of β-catenin (Biao He et al 2005). Downregulation of sFRP4 has been implicated in various types of malignancies including prostate and cervix cancer, the anti-proliferative and pro-apoptotic role of sFPR4 is well documented (White et al 2009; Constantinou et al 2008; Drake et al 2003; Hsieh et al 2003). Surprisingly enough in pathologies like mesothelioma (Fox and Dharmarajan 2006) and colorectal carcinoma (Feng Han et al 2006) sFRP4 has not been shown to induce apoptosis in tumor cells. Other member of the sFRP family; sFRP1 has been shown to control vascular cell proliferation in-vitro and in-vivo (Ezan et al 2004). Recent report by Goodwin et al. has shown that sFRP4 and sFRP2 are not expressed in most of the endothelial cells (Goodwin et al 2006).

1.4 ENDOTHELIAL CELLS AND WNT

Endothelial cells lining the inner lumen of blood vessels were initially considered as a sheet of cells often referred to as “sheet of nucleated cellophane” (Florey Lord 1966; Kaiser and Sparks 1987). For long there were several unsuccessful attempts to culture endothelial cells, the first attempt dates back to 1922 (Lewis 1922). The fact that endothelial cells in culture readily transform into fibroblasts was first reported by Toro (1937). The study by Folkman in 1974 (Gimbrone et al 1974) had a catalytic effect, with many research groups started working in the field of endothelial cell biology. Over last 35 years, endothelial cell culture has set new paradigm in angiogenesis studies. Endothelial cell culture has provided us with the ease of studying blood vessel formation on the bench top. Study of angiogenesis in-vitro
involves studying the various properties of endothelial cells and identifying the physiological behavior patterns of these cells that modulate blood vessel formation. Recently Wang et al (2007) have shown that canonical Wnt pathway is involved in endothelial cell commitment from embryonic stem cells (Wang et al 2007). Also different reports have shown the role of Wnt in the development of vasculature and blood vessels during development (Nestor et al 2005), central nervous system (Daneman et al 2009) and in angiogenesis (Dufourcq et al 2002; Goodwin et al 2006).

1.5 WNTS IN ANGIOGENESIS

Proliferation and migration of endothelial cells are the benchmark events in development of blood vessels. The Wnt pathway has been implicated in proliferation, cell survival, differentiation and migration of various cell types including endothelial cells; it’s believed to exert its effect by modulating both the cellular and transcriptional events (Moon et al 2004, Reya and Clevers 2005). Genetic studies of embryonic mutations evidence the role of Wnt signaling in vascular development (Monkley et al 1996). A growing body of evidence shows that Wnt plays an important role in the development of vasculature under different conditions that include embryonic angiogenesis as well (Nestor 2005). There are reports that document the role of Wnt signaling and requirement of the canonical Wnt pathway in endothelial cell commitment from embryonic stem cells (Wang et al 2007).

Furthermore, co-expression of Wnt proteins and Wnt pathway inhibitors by endothelial cells is implicated in the regulation of angiogenesis (Dufourcq et al 2002; Goodwin et al 2006). Exact mechanism by which Wnt affects angiogenesis is not well understood till date. There are reports indicating pro-angiogenic role of FrzA; a Wnt inhibitor (Dufourcq et al 2002). Interestingly a recent publication by Goodwin et al (2006) showed that endothelial cells display endogenous activation of canonical Wnt pathway.
along with expression of Wnt inhibitors like; sFRP1 and DKK1. It has been suggested that the co-expression of Wnt proteins and Wnt pathway inhibitors by endothelial cells carefully regulate the process of angiogenesis (Goodwin et al 2006).

Till date there is no published data to demonstrate the involvement of sFRP4 in angiogenesis. The present study investigates the role of sFRP4 on the endothelial cell physiology using in vitro models and effect on physiological and tumor angiogenesis using in ovo and in vivo models. This study also dissects the mechanism of action of sFRP4 and the effects of sFRP4 on various angiogenic pathways.

In summary the study addresses following questions:

1) Identification of sFRP4 mediated effects on endothelial cell physiology.

2) Evaluation and reconfirmation of the effect of sFRP4 on tumor angiogenesis, in ovo neovascularisation and in vivo angiogenesis.

3) Mechanistic insight of sFRP4 mediated inhibition of angiogenesis.