CHAPTER 6

DISCUSSION

6.1 INTRODUCTION

Wnt proteins and the related pathways; have been studied in detail over past 3 decades and their role in development and patterning of embryo, proliferation (Nusse and Varmus 1992), differentiation of different cell types is well established (He et al 1998; Hsieh et al 1999; Finch et al 1997).

In 2001 a study by Taketo and colleagues, showed the involvement of 2 Wnt proteins; Wnt5a and Wnt10b, in growth of endothelial cells in yolk sac and placental angiogenesis (Ishikawa et al 2001). Since then there has been a steady progress in the understanding of Wnt in developing vasculature, different groups have shown the implication of Wnt in the development of the vasculature under different conditions, including embryonic angiogenesis (Uren et al 2000; Bafico et al 1999) and recently Wnt has been shown to be involved in development of vasculature in CNS (Daneman et al 2009).

Wnt inhibitor family; sFRP (secreted frizzled related protein) is instrumental in modulating angiogenesis. sFRP-1/FrzA has been shown to induce proangiogenic effect in vitro and in vivo (Pascale Dufourcq et al 2002). Other member of sFRP family; sFRP-2 has been shown to stimulate angiogenesis via calcineurin/NFAT pathway (Courtwright et al 2009).

In the present work we investigated the effect of sFRP4 on endothelial cell physiology and angiogenesis in vitro and in vivo.
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. There have been no published reports showing effect of sFRP4 on angiogenesis. In this work, for the first time we present the anti-angiogenic property of sFRP4, when our in vitro results show the inhibitory effect of sFRP4 on endothelial cells. Using different cell based models we investigated the effect of sFRP4 on endothelial cells. We used both primary endothelial cells and immortalized endothelial cell lines for the study.

The wound healing assay used to determine the effect of sFRP4 on migration of endothelial cells was performed with 3 different endothelial cells; PAEC, ECV-304 and Eahy926. All three cells showed a reduction in wound healing upon treatment with sFRP4; the decrease was dose dependent in PAEC (Figure 3.3). The possible explanation for better inhibitory response of sFRP4 treatment in primary cell line lies in the fact that, upon immortalization of endothelial cell the cellular expression levels of typical endothelial markers like; eNOS decreases (Chatterjee et al. 2003) which may in turn affect the physiological response of the cells to sFRP4 treatment. A dosage lower than 125pg/ml of sFRP4 failed to exhibit any pronounced effect on wound healing in endothelial cells, indicative that 125pg/ml was the lowest working dosage for in-vitro effect of sFRP4 (Figure 3.6). The effect of sFRP4 on endothelial monolayer physiology and integrity was also indicated by the increased leakyness of the endothelial monolayer under sFRP4 treatment (Figure 3.18).
We observed sFRP4 was able to exert inhibitory effects on wound healing for long period of time; upto 24hrs indicative that sFRP4 pronounced its effect on wound healing partly by inhibiting both proliferation and migration. This becomes more evident with the results of cell migration using the trans-well Boyden’s chamber which clearly shows that sFRP4 inhibits the chemitactistic migration of endothelialial cells. The drop in the migration index was significant in both ECV-304 and HUVEC cells under sFRP4 treatment (Figures 3.14 and 3.15).

The constant remodeling of the actin cytoskeleton into filopodia, lamellipodia, and stress fibers is essential for migration of endothelial cells. Filopodia are membrane projections that contain long parallel actin filaments arranged in tight bundles, Lamellipodia are cytoplasmic protrusions that form at the leading edge of spreading or migrating cells and Stress fibers are actin filaments of inverted polarity linked by $\alpha$-actin and myosin and distributed along contractile fibers. Filopodia act as sensors of motile stimuli. Classically, the formation of filopodia is regulated by activation of the small GTPase Cdc42 that associates with Wiskott–Aldrich syndrome proteins (WASPs) (Karni Schlessinger et al 2009). Formation of lamellipodia; the prima facie event in the initiation of cell migration, was inhibited 5 mins after treatment with sFRP4 (Figure 3.16). Extension of lamellipodia is universally coupled with local actin polymerisation (Myrto Raftopoulou and Alan Hall 2004). This local actin assembly in formation of lamellipodia and filopodia has been shown to impart effect on angiogenesis in vitro (Qian et al 2003). The effect of sFRP4 on reduction in number of lamellipodia was pronounced and evident even after 4hrs of treatment (Figure 3.17). These observations clearly indicate that sFRP4 implicates its inhibitory effects by inhibiting migration of endothelial cells at early stages, which is formation of lamellipodia.
The anti-angiogenic effect of sFRP4 was pronounced and evident in the ring stability experiments, where sFRP4 administration disrupted the integrity of 2D-ring structures within 10 mins (Figure 3.12). The matrigel tube assay, which is by far the most widely accepted in-vitro angiogenesis assay also validated the inhibitory effect of sFRP4 on endothelial cells, where the endothelial tube structures were disturbed at 30mins after sFRP4 administration. sFRP4 also inhibited and regressed the budding sprouts from the already formed endothelial tubes (Figure 3.13), these findings give clear evidences that sFRP4 inhibits endothelial function and angiogenesis in vitro.

Examination of the effects of sFRP4 in an in vivo environment demonstrated that sFRP4 can significantly reduce the development of blood vessels in both the physiological setting (CAM assay) (Figure 4.1) and angiogenic implants (Figure 4.2) (cotton plug and matrigel implants). Furthermore, sFRP4 could halt the growth of the aggressive SKOV-3 ovarian tumor via its anti-angiogenic properties without affecting tumor cell viability (Figure 4.3), a finding similar to earlier studies (Fox and Dharmarajan 2006; Feng Han et al 2006).

SKOV-3 cells produce VEGF (Mesiano et al 1998) and can increase vessel permeability, thereby facilitating tumor growth by aiding the passage of macromolecules and growth factors directly into the tumor environment. Furthermore, VEGF promotes endothelial cell survival and angiogenesis (Naga et al 2007). The anti-angiogenic effect of sFPR4 in SKOV-3 ovarian tumor is supportive of the in-vivo findings where sFRP4 was seen to block wound healing and endothelial ring formation even under VEGF stimulation (Figures 5.8, 5.9 and 5.13). The VEGF induced effects in SKOV-3 ovarian tumors may act to counter sFRP4 inhibition of angiogenesis, but are insufficient to support further tumor growth. Thus sFRP4 treatment results in developmental stasis of the tumor. The in vivo tumor data
demonstrated no appreciable difference in numbers of apoptotic EC amongst all treatment groups (Figure 4.5). However, in previous research, TUNEL-positive EC were detected with very low frequency which did not correspond with the dramatic loss of capillarization present in the regressing corpus luteum (approximately 1 in 100 apoptotic cells) (Modlich et al 1996). The authors discovered this phenomenon was due to a combination of rounding and subsequent detachment of EC from the basement membrane together with contractive occlusion of blood vessels. Hence, it is not surprising that, due to the speed of the apoptotic process, EC can be rapidly dispersed into the blood stream in vivo and avoid being detected using apoptosis assays. This data also highlight the endothelial-specific action of sFRP4 observed as a significant reduction in the number of EC within the tumor mass (Figure 4.4). This finding is comparable to the anti-angiogenic properties demonstrated by Avastin in selectively reducing the number of EC (Kim et al 2008).

Reports by Goodwin et al. showed that endothelial cells migrating to scrape wound display more cytosolic and nuclear organization of β-catenin. Reports are indicative of important role of Wnt/β-catenin pathway in physiological and pathological angiogenesis (Goodwin et al 2006). Investigating the mechanism of action of anti-angiogenic effect of sFRP4, we activated the canonical Wnt/β-catenin pathway by inducing β-catenin recovery using 500μM LiCl (Figure 5.5) (Clement-Lacroix et al 2005). LiCl treatment did not recover back sFRP4 inhibition of wound healing or boyden chamber migration (Figures 5.1 to 5.4). Wnt-β-catenin pathway has been implicated in proliferation and differentiation of different cell types and also migration of cells, but the non- canonical Wnt pathway, c-JUN plays a major role in migration of different cell types, the results show the same trend. While LiCl treatment induced the recovery of β-catenin and activation of canonical Wnt signaling thereof, if was not able to recover the sFRP4
mediated inhibition, at the same time sFRP4 treatment reduced the nuclear localization of c-JUN (Figure 5.6). This propounds the idea that sFRP4 imparts its effects via more than one pathways.

ROS levels were significantly increased under external sFRP4 treatment and overexpression of CRD and sFRP4 plasmid in endothelial cells (Figures 5.21, 5.23 and 5.32). The antioxidant activity of the cells treated with sFRP4 was significantly reduced; exemplified by reduced Catalase and Thiol levels under sFRP4 treatment (Figures 5.24 and 5.25). Interestingly a potent superoxide quencher Superoxide dismutase (SOD) activity was significantly increased (Figure 5.22), this can be explained to be a feed back mechanism of the cell to increased superoxide species. Also the increased release of H$_2$O$_2$ from cells under sFRP4 treatment is better explained by the increased SOD activity, as SOD converts superoxide to hydrogen peroxides. Increased oxidative stress has been shown to relocalizes β-catenin to the nucleus where it promotes association of FOXO and β-catenin and competes with the binding of β-catenin to TCF. H$_2$O$_2$ promotes FOXO-mediated transcription at the expense of β-catenin/Tcf mediated transcription (Hoogeboom et al 2008; Almeida et al 2007). Nitric Oxide (NO) synthesized by endothelial NO synthase (eNOS) is essential for EC survival, migration and postnatal neovascularisation. The transcriptional repression of eNOS by FOXOs might also contribute to the anti-angiogenic effects of FOXOs on Endothelial Cell. In addition, changes in expression of several extracellular matrix proteins, such as collagen and MMPs, indicate that FOXOs might also be involved in regulating vessel remodeling (Potente et al 2005).

NO is a potent redox species, which has been shown to react with superoxide to form highly reactive secondary redox species; peroxynitrite. On the flip side; NO is a potent pro-angiogenic stimulant and has been implicated in endothelial cell proliferation and vasomotor tone. Interestingly, sFRP4
induced NO production (Figure 5.14), as observed by Griess assay and fluorescent probe; DAF-FM. This contradiction can be attributed to the fact that Griess assay and DAF-FM both are not specific to NO, in turn they are indirect measurement of NO. While Griess assay measures the nitrite equivalent of NO levels, DAF-FM has been shown to react and get oxidized by peroxynitrites and superoxides as well. The increased fluorescence can be attributed to increased peroxynitrites. NO being a natural quencher of superoxide, is produced in abundance in endothelial cells. It becomes easy to assume that NO produced by endothelial cells reacts with increased superoxides to produce peroxynitrite, under sFRP4 treatment. It is plausible to think that the limitation of the assay system false detects peroxynitrites as increase in levels of NO.

Increased ROS levels, specifically superoxides are mostly attributed to the activity of NADPH oxidase, Xanthine oxidase and mitochondrial complex III. Under sFRP4 treatment NADPH oxidase plays the major part in generation of superoxide radicals as observed by experiments involving inhibitors for NADPH oxidase, Xanthine oxidase or mitochondrial complex III (Figures 5.28 to 5.30). Further, Superoxide dismutase is able to recover back sFRP4 inhibition and apoptosis in endothelial cells, showing yet again that sFRP4 impounds its effects on endothelial cells partly by inducing oxidative stress and activation of stress mediated cell death pathways.

Akt, serine threonine kinase is a known mediator for survival of different cell types including endothelial cells. Akt pathway blocks cell death and apoptosis (Franke et al 1997), provides survival signals under fluid shear stress (Dimmler 1998). Akt has been shown to impart protection against oxidative stress induced apoptosis and facilitate cell survival under oxidative stress conditions (Nair and Warren Olenow 2008). sFRP4 has been shown to abrogate PI3K-Akt pathway in epithelial cells (Lacher et al 2003). In
endothelial cells, sFRP4 administration abrogated Akt phosphorylation at 1hr after treatment (Figure 5.18), thus sFRP4 inhibits the Akt mediated survival pathways and potentiates oxidative stress mediated pathological condition in endothelial cells.

Results with different cell; HUVEC, bEnd3 and ECV-304 all showed that sFRP4 induces caspase-3 activation in endothelial cells. The activation of caspase-3 is central to both death receptor mediate apoptosis and also stress induced apoptosis. The activation of caspase-3 was seen to be in conjunction with decreased mitochondrial membrane depolarization (another marker for apoptosis). In immortalized cell lines; bEnd3 and ECV-304, did not show mitochondrial depolarization, but still these cells showed caspase-3 activation, further impounding the fact that sFRP4 mediates its effects on endothelial cells via different pathways. The study demonstrates sFRP4 facilitates selective apoptosis in endothelial cells in vitro (Figure 4.5), that is in part due to the activation of cellular reactive oxygen species which then facilitate nuclear entry of FOXO (which preferentially binds to the limited pool of nuclear β-catenin), thus driving the cell towards apoptosis and partly due to inactivation of Akt mediated survival pathways.

In summary, these studies demonstrate a previously unknown role of sFRP4 as an inhibitor of angiogenesis and illustrate sFRP4 inhibition of blood vessel formation both in vitro and in vivo. sFRP4 inhibits the migration and proliferation of endothelial cells, both crucial steps in angiogenesis. This unique anti-angiogenic effect of sFRP4 is not dependent on a single given pathway parse, but it’s a combined effect a complex signaling mechanism that includes:

a) Inhibition of Wnt/β-catenin pathway.

b) Inhibition of PCP signaling pathways.
c) Abrogation of migration by inhibition of Wnt-cJUN and Akt pathways.

d) Promotion of apoptosis by Akt inhibition, FOXO regulation and oxidative stress.

The finding has brought into light a novel candidate for regulation of angiogenesis. The data promotes sFRP4 as a potent angiogenesis inhibitor which has therapeutic potential for the control of angiogenesis-dependent pathologies like cancer and ENL.