Letter to Editor

Secreted Frizzle-Related Protein (sFRP4) can abrogate pregnancy – a new dimension in its biological role

Jasna J Mohan*[1], Rajesh Kumar Jha[1], Pradeep Kumar G[1], Arun M Dharmarajan[2] and Malini Laloraya[1]

[1] Utero-Embryo Repromics Laboratory, Molecular Endocrinology & Reproduction Division, Rajiv Gandhi Centre for Biotechnology, Thycaud PO, Poojappura, Thiruvananthapuram - 695 014, Kerala, India.
[2]School of Anatomy & Human Biology, University of Western Australia,35 Stirling Highway, Crawley,WA 6009.

Summary

Successful implantation is dependent on precisely orchestrated and reciprocal signaling between the implanting blastocyst and the receptive uterus. A key signaling mechanism that is operative during implantation is the Wnt/ Beta - catenin signaling pathway. Secreted frizzled-related proteins (sFRPs) are reported to be antagonist to these pathways and are group of secreted glycoproteins, structurally similar to Wnt receptors [frizzled (FZD) proteins] but lack the transmembrane domains. SFRPs inhibit Wnt action through competitive binding to the ligand-binding domain of the frizzled receptor complex. *In silico* analysis using PSORTII has revealed mouse sFRP4 to be predominantly mitochondrial (43.5%) and nuclear (34.8%) and not extracellular like human sFRP4 (44.4 %). Our western blotting and immunohistochemical studies unraveled the sub-cellular localization of the sFRP4 molecule and its significant presence in the nucleus and the mitochondrial fraction during the peri-implantation stage. The nuclear presence of sFRP4 during pregnancy adds new dimension to its potential modes of action and biological function. Studies are underway to explore the structure and function of sFRP4 using molecular modeling.

Key words: Implantation, sFRPs, Wnt signaling

Introduction

During pregnancy the uterine stromal compartment undergoes dramatic remodeling, particularly at the time of implantation. Proper utero-embryo communication at implantation is transduced by various uterine and embryonic factors. It is an intriguing observation that embryo implantation and tumor metastasis share striking similarities in biological processes involving cell adhesion by trophinin, tastin, and bystin, immune escape, angiogenesis and invasion and includes, destruction of host tissue, erosion of blood vessels and tumor metastasis-related gene expression. Unlike the normal placenta, tumors ignore tissue boundaries and spread aggressively and in a destructive manner. Thus identification of implantation-specific proteins and the elucidation of novel regulatory pathways that ensure maintenance of the boundary between controlled cell growth during embryogenesis and its dysregulation during carcinogenesis are of prime importance.

Importantly, Wnt/catenin signaling is common to both cancer metastasis and embryo implantation. Wnt5a expression and PKC activation are directly correlated to increasing tumor grade in humans (Weeraratna et al., 2002) and Wnts secreted by the embryo participate in the molecular cross talk of implantation (Mohamed et al., 2004; Paria et al., 2002).

Inhibition of the Wnt pathway provides an inherent mechanism to control the excessive signaling leading to unfavorable consequences. Secreted frizzle-related proteins (sFRP) are well known antagonists to the Wnt signaling pathway and can either bind to ligand binding domain of frizzle receptor to compete with Wnt ligand or directly bind to Wnt thereby blocking its interaction with the receptor. sFRP4 is identified as a prognostic marker in localized prostate cancers (Horvath et al., 2004). Feng et al. (2006) had shown that sFRP4 and beta-catenin are up-regulated in colorectal tumors. Increased levels of sFRP4 were also reported in the nonproliferative placental basal

Correspondence to be addressed to: Dr. Malini Laloraya Email: laloraya@gmail.com; laloraya@rgcb.res.in

zone but not in the rapidly growing labyrinth zone of the placenta (Hewitt et al., 2006). Cellular localization of the putative secretory protein, sFRP4, and its expression profile during window of implantation is analyzed in this study.

Methodology

SDS-PAGE, Western blotting and immunoreaction

Mouse uteri of different stages of pregnancy were excised, cleared of blood and adhering fat and homogenized in appropriate extraction buffer using a polytron homogenizer. Differential centrifugation was used to isolate the different subcellular fractions. The proteins separated were then electroblotted onto PVDF membrane (0.2m, BioRad, USA). The membranes were then probed with sFRP4 primary antibody and goat anti-rabbit HRP secondary antibody. The immunopositive bands were detected using diaminobenzidine (DAB, USA) as the color-developing substrate in the presence of 0.04% $NiCl_2$ and 30% H_2O_2 in PBST. The images were captured adopting BioRad (USA) FluorS Multi-Imager.

Localization of sFRP4 on uterine sections

Uterine sections (6 mm thick) of different stages of pregnancy were deparaffinized, rehydrated and rinsed in PBS. Slides were autoclaved in sodium citrate buffer (pH 6) for 10 min, placed in a blocking solution and then incubated in primary antibody. Immunostaining was performed using antirabbit IgG conjugated with FITC and were couterstained with propidium iodide to stain the nuclei. The sections were imaged under Leica TCS SP2 Confocal Laser Scanning Microscope.

Results and Discussion

In-silico analysis

Subcellular distribution of human and mouse sFRP4 was predicted using PSORT II. Mouse sFRP4 showed a 43.5%: mitochondrial, 34.8%: nuclear, 8.7%: extracellular, cell membrane, 8.7%: cytoskeletal and 4.3%: cytoplasmic distribution. Thus the PSORT II predictor for mouse sFRP4 contradicts its role as a known secretary protein. The prediction showed it to be 43.5%: mitochondrial, 34.8%: nuclear and just 8.7%: extracellular, cell membrane. Human sFRP4 subcellular localization distribution was predicted to be 44.4%: extracellular, cell membrane, 11.1%: mitochondrial, 22.2%: nuclear, 11.1%: cytoskeletal, 11.1%: cytoplasmic using PSORT II. To deduce the domains present in the molecule SMART analysis was used and mouse sFRP4 were predicted to contain a CRD and a netrin domain (Figure 1).

Spatiotemporal distribution of sFRP4 in the uterus :

Western blot analyses were used to evaluate the expression of sFRP4 in the three subcellular fractions studied – cytosolic, nuclear and mitochondrial**.** Different constitutively expressed proteins were used as markers for normalizing the protein concentration for Western analyses. The sFRP4 antibody detected two immunopositive bands (a 40kDa and 70kDa) in all the three fractions. In the cytosolic fraction 40kDa and 70kDa protein expressions were visible at the pre-implantation stage (Day 4, 10am), with a decrease in their expression in the succeeding stages (data not presented). A significant increase in their expression was evident at the post-implantation stage (Day 5, 10am). In the nuclear and mitochondrial fractions appreciable amounts were expressed during the late peri (Day5, 5am) and post-implantation stages (Day 5, 10am) (Figure 2).

Immunofluorescence results revealed an obvious nuclear presence of sFRP4 at late peri-implantation stage (Day 5, 5am) thereby corroborating our Western data. Figure 3 shows yellow fluorescence in Day 5, 5 am indicating a complete overlay of the green (sFRP4-FITC) fluorescence with the red fluorescence (Propidium iodide - Nuclear stain). This is in sharp contrast to distinct green (sFRP4-FITC) fluorescence due to no nuclear sFRP4 and red fluorescence (Propidium iodide -Nuclear stain) at Day 4, 4 pm.

Our wet-lab experiments comprising of western blot analyses and immunocytochemistry confirmed nuclear mouse sFRP4 predictions (PSORTII analysis) and showed its nuclear presence in the peri-implantation uteri (Day 5, 5am). This study adds a new dimension to the biological role of sFRP4 and provides evidence for the presence of mouse sFRP4 in the nucleus, cytoplasm and mitochondrion. The presence of sFRP4 in the nucleus increases potential biological activities for this protein and we are currently exploring the implication of its nuclear localization.

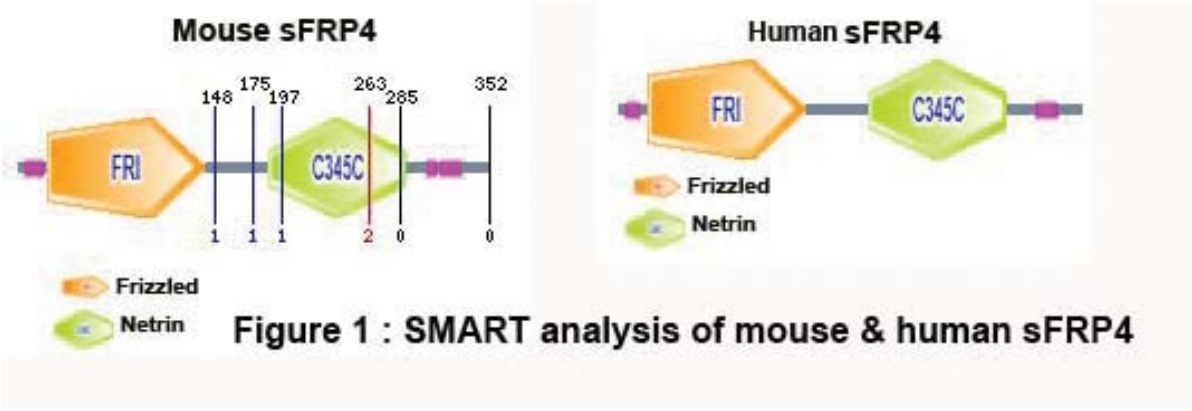

Figure 1 : SMART analysis of mouse & human sFRP4

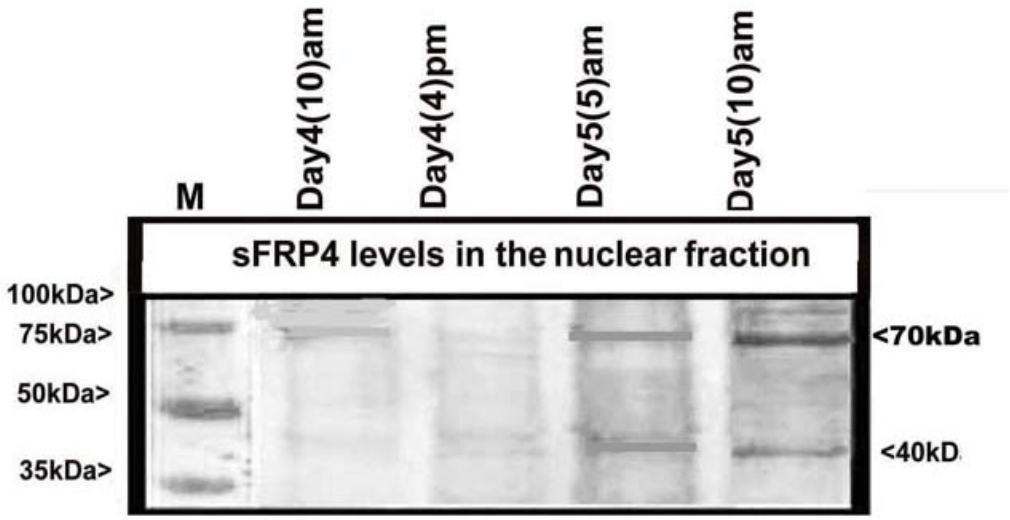

Figure 2 : Western blot of nuclear sFRP4 expression

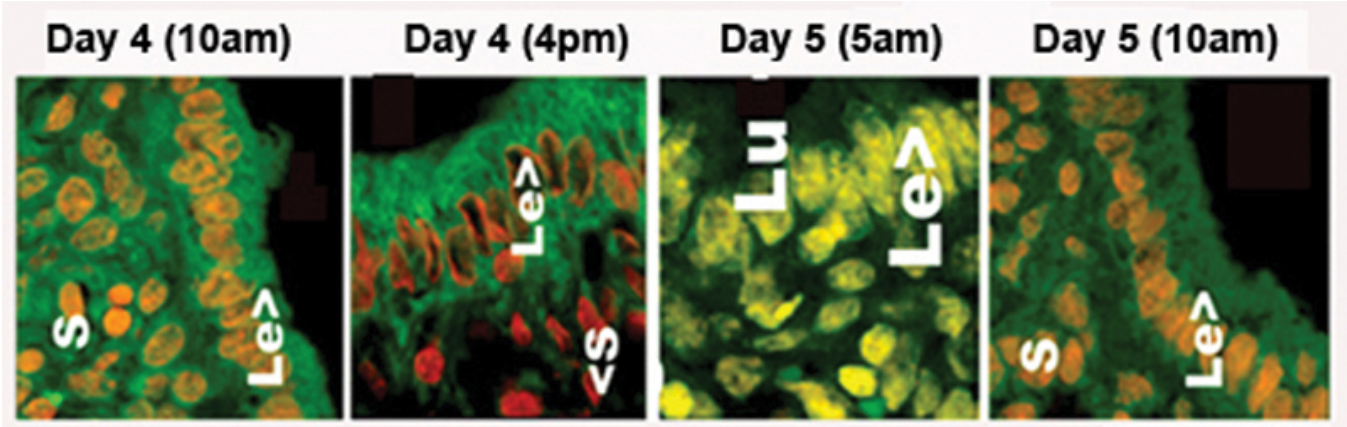

Figure 3: sFRP4 localization in uterus during 'implantation window'

References

1 Feng HQ, Zhao W, Bentel J, Shearwood AM, Zeps N, Joseph D, Iacopetta B, Dharmarajan A (2006) Expression of sFRP-4 and beta-catenin in human colorectal carcinoma. *Cancer Lett* **231**: 129-137.

2 Hewitt DP, Mark PJ, Dharmarajan AM, Waddell BJ (2006) Placental expression of secreted frizzled related protein-4 in the rat and the impact of glucocorticoid-induced fetal and placental growth restriction. *Biol Reprod* **75:** 75-81.

3 Horvath LG, Henshall SM, Kench JG, Saunders DN, Lee CS, Golovsky D, Brenner PC, O'Neill GF, Kooner R, Stricker PD, Grygiel JJ, Sutherland RL (2004) Membranous expression of secreted frizzled-related protein 4 predicts for good prognosis in localized prostate cancer and inhibits PC3 cellular proliferation in vitro. *Clin Cancer Res* **10**: 615-625.

4 Mohamed OA, Dufort D, Clarke HJ (2004) Expression and estradiol regulation of Wnt genes in the mouse blastocyst identify a candidate pathway for embryo-maternal signaling at implantation. Biol Reprod **71**: 417-424.

5 Paria BC, Reese J, Das SK, Dey SK (2002) Deciphering the cross-talk of implantation: advances and challenges. Science **296**: 2185-2188.

6 Weeraratna AT, Jiang Y, Hostetter G, Rosenblatt K, Duray P, Bittner M, Trent JM (2002) Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. *Cancer Cell* **1**: 279-288.