Synopsis

Notch3 Receptor: Activation Mechanism & Association with Ovarian cancer

The Notch signaling influences a wide spectrum of cell fate decisions, both during development and in the adult organisms. This pleiotropic role of Notch signaling in humans is attributed partially to the presence of four Notch receptors and many canonical and non-canonical ligands (D'souza et al., 2008). Any alteration in the Notch receptors or ligands and the Notch signaling has been associated with several human diseases including cancer (Penton et al., 2012). This highlights the importance of understanding the molecular mechanism of Notch receptors activation in normal and pathophysiological conditions. In this thesis, different antibodies against Notch3 extracellular domain are used as a tool to understand the mechanism of Notch receptor activation. This work has provided insights into the activation mechanism of the Notch3 receptor by a non-canonical ligand Yb-1.Various conformational changes that Notch receptor undergoes on ligand binding for the transduction of signal have been investigated. Further, the antibodies were used to study the significance of Notch signaling in Ovarian cancer. This work highlights the role of Notch and FSH communication in ovarian cancer progression and proposes the combination of Notch and FSH receptor antagonists as potential therapeutics.

This thesis is divided into two parts: The Section I focuses on understanding the role of the Notch3 extracellular domain on receptor-ligand interactions and the conformational changes it undergoes for the transduction of signal to the intracellular domain using antibodies as a tool. In Section II, implications of these antibodies in therapeutic targeting of ovarian cancer have been investigated. Also, a crosstalk between FSH and Notch signaling in ovarian cancer perspective has been established.

Section I: Activation mechanism of Notch3 receptor

The Notch receptors have a large extracellular domain that comprises a number of EGF-like repeats (ELRs) and the Negatively Regulatory Region (NRR) which keeps the receptors in an auto-inhibited state (Gordon et al., 2007). Binding of ligands to different ELRs (Rebay et al., 1991b) bring about conformational changes in NRR (Jarriault et al., 1995) leading to series of proteolytic cleavages at sites S2 and S3 that ultimately result in the release of the Notch

intracellular domain and subsequent upregulation of the Notch target genes (Brou et al., 2000; Mumm et al., 2000). In the earlier studies from the laboratory, polyclonal and monoclonal antibodies against different domains of Notch1 were used to map the ligand-receptor interactions. Notch1 ELR 11-12 was demonstrated to be the primary ligand binding site and ELR 25-26 was found to be a cryptic ligand binding domain (Sharma et al., 2013). The same approach has been used in Notch3 receptor to map the binding sites for the canonical membrane bound ligand Jagged1 and a soluble non canonical ligand, Yb-1 that preferentially binds only to Notch3 (Rauen et al., 2009). Polyclonal antisera were raised against Notch3 ELR 1-12, ELR 17-27 and NRR, and were characterized for their specificity to recognize different Notch3 ELRs in various binding and signaling assays. Polyclonal a/s against Notch3 ELR 1-12 and ELR 17-27 inhibited Jagged1 binding to the full-length Notch3 receptor and consequent signaling. On the other hand, Yb-1 binding to the Notch3 receptor and subsequent signaling was unaffected by Notch3 ELR 1-12 a/s but was inhibited by ELR17-27a/s. These results suggested different binding sites for the canonical and non-canonical ligands on the Notch3 receptor. Further, taking advantage of the similarity between Notch1 and Notch3 ELRs, a polyclonal a/s against Notch1 ELRs 21-30 that was dissected into antibodies against different ELRs using affinity chromatography (Sharma et al., 2013), the probable binding site of Yb-1 was mapped to ELRs 17-24 of Notch3. So, this study suggests that the soluble ligand Yb-1 binds to ELRs in Notch3 that are distinctly different from the ELRs 7-10and 21-22 of Notch 3 (ELRs 11-12 and ELRs 25-26 of Notch1) that have been identified for Jagged1 or Delta4 binding (Lin et al., 2010). However, both immobilized Jagged1 and soluble Yb-1 stimulate Notch signaling through the NRR of Notch3 receptors as the polyclonal a/s against Notch3 NRR inhibited both Jagged1 and Yb-1 mediated Notch3 receptor activation.

The NRR is the key regulatory domain of Notch receptors as it keeps the receptors in an auto-inhibited conformation in the absence of the ligands. It has a compact conformation with three LNR domains (LNR-A, LNR-B, and LNR-C) wrapped around the two hetero-dimerisation domains, HD-N and HD-C (Xu et al., 2015). The HD-C domain harbors the S2 cleavage site that gets exposed on the binding of the ligands to different ELRs. To investigate the molecular details of NRR activation, Single chain Fragment variables (ScFvs) were generated against Notch3 NRR. All the ScFvs showed cross-reactivity to Notch1 NRR. Using bioinformatic tools, the putative epitopes recognized by different ScFvs were identified and confirmed by ELISA experiments. All ScFvs recognized distinctly different epitopes on

Notch NRR. Many ScFvs inhibited the basal and ligand-stimulated activation of Notch receptors. Four ScFvs - 22, 42, 52 and 72, based on their effects on Notch3 signaling and their distinct epitopes on NRR were selected for investigating the conformational changes in the NRR on activation by soluble ligand Yb-1. ScFv22 recognized the LNR-C which did not undergo any change in the presence of Yb-1. Binding of ScFv42, which, was mapped to HD-N domain of Notch3 NRR, was decreased in the presence of the ligand suggesting major alterations in the HD-N domain during receptor activation. The HD-C domain of the NRR was identified as the binding site for the ScFv72. Binding of this ScFv72 to Notch3 increased significantly in presence of Yb-1 suggesting ligand induced conformational change in HD-C with the epitope being more exposed upon activation. Finally, binding of ScFv52, whose predicted epitope appeared to be different from the actual ScFv52 binding in ELISA, did not alter in presence of Yb-1. Interestingly, the receptors with the Gain-of-Function mutations in the NRR exhibited the ScFv binding patterns similar to those of the ligand-activated NRR. Overall, these results provided the novel insights into the mechanism of conversion of NRR domain from the protease resistant to sensitive state on stimulation by the ligands.

The link between Notch signaling and many cancers is well established and the Notch inhibitory ScFvs characterized in this study are the potential therapeutic tools for treatment of many cancers. The link between ovarian cancer and Notch signaling and the potential of usage of the ScFvs as therapeutic antibodies is explored in the next section.

Section II: Association of Notch signaling with Ovarian cancer

A growing body of literature confirms an increased Notch signaling in ovarian cancer (Groeneweg et al., 2014). The role of Notch signaling in maintenance of the ovarian cancer stem cell population (Park et al., 2010a), angiogenesis (Lu et al., 2007) and epithelial to mesenchymal transition (Gupta et al., 2013) has also been reported. Additionally, higher exposure of the ovary to gonadotropins during menopause, ovulation, or infertility therapy has also been implicated as a possible risk factor for ovarian cancer development (Choi et al., 2007a).With the progression of ovarian cancer, there is an excess accumulation of ascites in the peritoneal cavity which acts as a reservoir of soluble factors and cellular components that provide tumor-promoting microenvironment to the ovarian cells (Ahmed and Stenvers, 2013b). Differing reports indicate the presence of gonadotropins in ascites of ovarian cancer patients (Chen et al., 2009). However, the exact role of gonadotropins and the molecular mechanism involved in ovarian cancer progression has not been fully characterized. Sporadic

reports propose that effect of FSH on ovarian cancer development might be mediated via Notch signaling (Park et al., 2010b). Therefore, the link between Notch signaling and FSH in ovarian cancer cells was investigated. The ovarian cancer cell lines, Ovcar-3, Skov-3, and Ovcar-4 exhibited functional FSH and Notch receptors. FSH increased Notch signaling is the dose dependent manner in the ovarian cancer cell lines. There was also an increase in Notch1 and Notch3 receptors, Notch ligands Delta1 and Delta4 both at the transcript and protein levels in the ovarian cancer cells treated with FSH. Interestingly, Delta4 is also reported to be present on the stromal cells of ovarian cancer (Kuhnert et al., 2015).When Ovcar-3 cells were incubated with Delta 4 along with FSH, there was an additive increase in Notch signaling. This increase could be inhibited by an antiserum (RF5 a/s) against FSH receptors. The Notch NRR specific ScFv42, characterized as an antagonistic antibody in the Section I, inhibited the FSH induced Notch signaling activity in Ovcar-3 cells.

FSH and exogenous Notch ligand Delta4 individually and in combination up-regulated proliferation of the ovarian cancer cell lines. The NRR specific ScFvs along with the FSH receptor antibodies inhibited this proliferation. Thus, the ovarian tumor cell proliferation could be regulated by the Notch ligands present in the stromal cells and FSH. To identify the potential source of FSH that may be driving cancer progression, FSH levels in ascites were estimated and it was observed that the levels of the hormone in the ascitic fluids of ovarian cancer patients were significantly higher compared to those from the non-ovarian cancer patients. The origin of this FSH appears to be the cellular aggregates called spheroids obtained from the ascitic fluids of cancer patients that expressed FSHβ mRNA. Further, when the ovarian cancer cells (Ovcar-3 and Skov-3) were cultured under non-attachment conditions that facilitated spheroid formation, there was an expression of FSHβ mRNA together with the secretion of the hormone into the medium. In contrast, there was no expression of FSH when the cells were cultured in the attached conditions.

The size of the spheroids found in the ovarian cancer patients' ascites has been positively correlated with the chemo-resistance and these spheroids have been considered as a source of relapse (Gong et al., 2015). The Notch NRR ScFv and FSHR antibodies in combination significantly inhibited the spheroid formation and also disaggregated the already formed spheroids. It is rather intriguing how FSH expression is turned on in the spheroids. However, the present study demonstrates that FSH is one of the driving factors in ovarian cancer and its effects are mediated via Notch signaling. The study also demonstrates that antibodies against

Notch and FSHR in combination are the potential immunotherapeutics for ovarian cancer. Thus, this study demonstrates that antibodies against Notch provide an insight into its activation while serving as potential immunotherapeutic tools for ovarian cancer treatment.