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Magalam Raghavendra

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Magalam Raghavendra

Supervisor: Johanna Blomqvist, Swedish University of Agricultural Sciences, Department of Microbiology

Assistant Supervisors: Masood Kamali-Moghaddam, Uppsala University, Department of Immunology, Genetics and Pathology

Junhong Yan, Uppsala University, Department of Immunology, Genetics and Pathology

Examiner: Volkmar Passoth, Swedish University of Agricultural Sciences, Department of Microbiology

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Uppsala BioCenter
Department of Microbiology

ABSTRACT

Proximity ligation assay (PLA) is a novel affinity based method used to detect proteins, protein-protein interactions and post-translational modifications. The detection of target molecules is achieved with the help of oligonucleotide labeled antibodies. The oligonucleotides attached on antibodies can be joined by ligation when the conjugates are brought in proximity by binding on the same target molecule (Gullberg et al., 2003). The ligated products can be used as templates in qPCR for quantification. PLA is performed in two approaches, 1) solid surface; where a solid support is used to which a capture antibody is immobilized and target is incubated to capture antibody (Fredriksson et al., 2002). 2) The other approach is solution phase; where there is no solid support, hence no capture antibody is used nor any washing step, and target molecule is incubated directly with detection antibodies. In my project, I have utilized solution phase and solid phase PLA for detection of IGF-1 and detection of phosphorylation on platelet derived growth factor receptor (PDGFβr).

This thesis project aimed to develop an assay for IGF detection and post-translational modification detection in vitro using proximity ligation. My experiment could not establish PLA as a standard assay for detecting IGF-1 and phosphorylated-PDGFβr on solid surface and in solution phase. However, the recombinant PDGFr molecule was successfully detected with solid phase PLA. In this thesis, advantages of PLA and possible reasons for unsuccessful detection of phosphorylated site on PDGFβr are discussed.

TABLE OF CONTENTS

1. INTRODUCTION

The GENCODE (version 7) project published that there are only 20,687 protein- encoding transcripts in a human being (Harrow et al., 2012, Venter et al., 2001). Although there are only few protein coding transcripts in a human, yet it is very complex to understand the protein biological functions. Hence, the current focus of the research in the field of protein interactions, post translational modifications and alternative splice variants, is to achieve a novel protein detection method, as this would help the researchers to understand the complexity of proteins. The detection of proteins is more complicated than the detection of the genes as protein synthesis involves alternative splicing and post translational modifications, thus there is demand to achieve new methods to detect proteins and novel biomarkers to diagnose diseases. (Gullberg et al., 2004, Weibrecht et al., 2010).

Detection of proteins is an important strategy to reveal the complex mechanisms of the living organism; of course there are few methods which are already available in the market today that help in detecting the target proteins, but there is always a constant hunt in achieving a small limit of detection and also detecting multiple targets in same sample at very low concentration. Thus it is very crucial to invent a novel tool which detects the target proteins at low concentration with high sensitivity and with high affinity bonding between the detection probe and target protein (Fredriksson et al., 2002).

1.1 Protein detection methods

Mass spectrometry

Mass spectrometry (MS) is one of the methods used in studying proteins and has been very useful from decades in detecting biomarkers such as those of cancer (Diamandis, 2004, Zhang et al., 2004). Proteins can be ionized by two approaches, electron spray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI) (Domon and Aebersold, 2006). Firstly the intact proteins are ionized by one of the two methods mentioned above and then introduced to a mass analyzer. This approach is called “top bottom” strategy. The other approach is bottom up where the proteins are digested into small peptides by enzymes and introduced into mass spectrometry (MS) to characterize the protein in the sample (Domon and Aebersold, 2006).

MS basically measures the mass to charge ratio of gas phase ions. MS consists of an ion source which converts the analyte or sample into gas phase ions, the analyzer then separates the ions based on mass to charge ratio. The mass to charge ratio values of individual ions are recorded by a detector. In MS, the mass analyzer is the key for the complete technology and some of the examples of the analyzers are LTQ-orbitrap, time of flight (TOF) mass analyzer and Fourier transform ion cyclotron resonance (FTICR) mass analyzer (Han et al., 2008).

Mass spectrometry apparatus

The mass spectrometry process consists of four main stages; ionization, acceleration, deflection and detection. In the ionization step, the sample is bombarded by electrons coming from a high energy filament. During this step electrons from the sample are removed by electrons coming from heated filament. In the acceleration step ions are placed between a set of charged plates. These ions are repelled from one charged plate to another, finally there is slit in the plate to which ions are attracted at an accelerated rate. The acceleration of ions depends on charge on the plates. There is a highly powered magnet surrounding the apparatus. Deflection of sample ions is proportional to sample mass, lighter ions are deflected most. The ions which have correct charge reach to detector. In detection stage, ions are neutralized after hitting the detector and an amplifier would enhance the charge received into a detection signal, which ultimately would be converted to mass/charge ratio by computer software (Figure 1). http://chemicalinstrumentation.weebly.com/mass-spectrometry.html

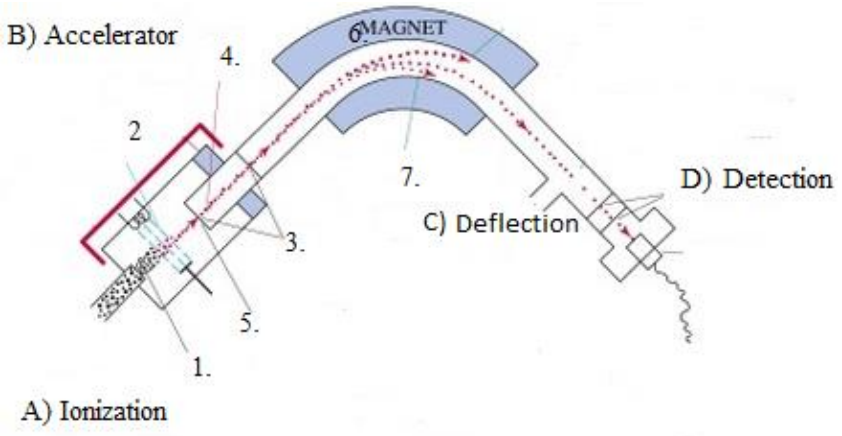

Figure 1. Mass spectrometry: A) ionization; 1) Sample, 2) Electron beam, 3) slits, 4) Ion beam.B) Accelerator; 5) Accelerator plate C) Deflection; 6) Magnet 7) Deflection of ions D) Detector. Modified from (http://chemicalinstrumentation.weebly.com/mass-spectrometry.html)

ELISA

Enzyme linked immuno-sorbent assay is a molecular tool which is extensively used in industries and laboratories as quality control and protein detection method (Green et al., 1997, Savige et al., 2003). ELISA was first developed by the research group of Peter Pearlman and Eva Engvall in Stockholm and by the research group of Anton Schuurs and Bauke Van Weemen in the Netherlands in 1960. The basic principle involves antigens from a sample that are coated to the surface of a well on a microtiter plate, then an antibody specific to the antigen (detection antibody) is added to surface of the well to bind to the antigen and this antibody is linked to an enzyme, and finally an enzyme substrate is added and upon when enzyme linked antibody is attached to desired antigen there will be a colour change due to the substrate (Hnasko et al., 2011). The sensitivity of this assay can be increased by adding a secondary antibody that is linked to an enzyme (Hnasko et al., 2011). This antibody detects the primary antibody which is bound to antigen. In this case between each step, it involves a washing step so that unbound antibody- antigen complexes are discarded and when finally enzyme substrate is added we can visibly see a colour change in the experimental wells, the amount of colour changes is directly proportional to the amount of antigen and antibody bound complex (Voller et al., 1978). This amount of colour change can be analyzed under spectrophotometer and give us actual amount of antigen present in the sample. (http://www.abnova.com/support/resources/ELISA.asp).

There are different types of ELISA; 1) Direct Elisa, 2) Indirect Elisa, 3) Sandwich Elisa, 4) Competitive Elisa.

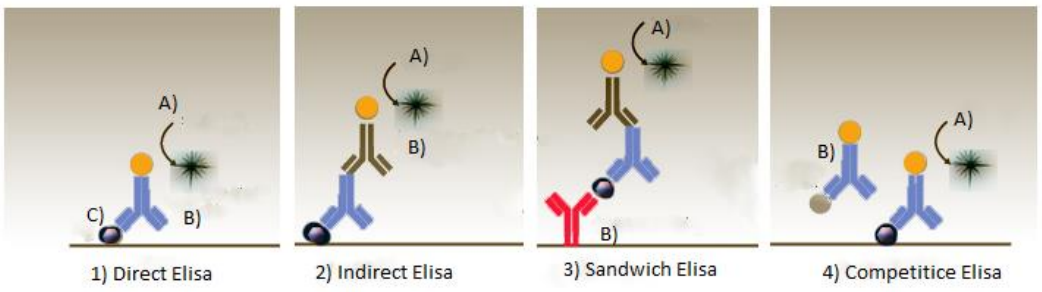

Figure 2. Elisa:
1) direct Elisa: A) Substrate, B) Primary antibody conjugate, c) Antigen.
2) Indirect Elisa: A) substrate, B) secondary antibody conjugate.
3) Sandwich Elisa: A) Substrate, B) Capture antibody.
4) Competitive Elisa: A) Inhibitor antigen, B) Substrate.

1) Direct Elisa; antigen is recognized by a fluorescent tagged detection antibody, 2) indirect ELISA; antigen is recognized by a detection antibody and this antibody is further detected by fluorescent tagged antibody,

3) sandwich ELISA: the capture antibody detects the antigen and this antigen is further detected by detection antibody and finally this antibody is detected by a fluorescent tagged antibody and 4) target sample along with purified antigen competes to bind to secondary antibody immobilized on solid surface, then later primary antibody binds to this complex. (modified from **http://www.abnova.com/support/resources/ELISA.asp)**

1.2 Proximity Ligation Assay

The mystery of cellular function is yet to be unraveled to gain insights about proteins, post translational modifications. Although existing methods give us better understanding of proteins, researchers are constantly exploring new ways to implement the best method to detect protein and post translational modifications. In recent decades there have been major technological advancements such as making monoclonal antibodies, mass spectrometry, green fluorescent protein fusions, which enabled researchers to achieve insights into proteomics.

Proximity ligation assay (PLA) is a method which uses monoclonal antibody and polyclonal antibodies technology to detect proteins. PLA also uses the same principle as ELISA, where antibodies are used as detector probes to detect target protein. But PLA stands apart from ELISA and other affinity based targeting methods with its dual recognition and high specificity (Gullberg et al., 2003). Proximity ligation assay also circumvents the cross-reactivity errors and non-specific adsorption. In this assay the antibodies are linked with an oligonucleotide using biotin-streptavidin chemistry. To achieve this interaction, the oligonucleotide is covalently linked to streptavidin through maleimide conjugation chemistry. Then this modified streptavidin oligonucleotide is linked to a biotinylated antibody. This antibody attached with oligonucleotide is directed towards the target in the sample (Söderberg et al., 2008, Gullberg et al., 2004). The main advantage of using this modified antibody is that streptavidin and biotin have high affinity to each other and that they are stable even at 95˚C (Green, 1990). When two or more detection probes bind to the same target molecule, the oligonucleotides present on these probes get ligated and form a single stranded DNA sequence when they are in close proximity. The ligated products are then amplified in qPCR. This nucleotide sequence will serve as template for primers and produce a single stranded circular nucleotide sequence and for every successful template formation a signal is generated and this signal is recorded by PCR machine software.

Proximity ligation assay can be performed in two different approaches, solid phase PLA and solution phase PLA. In solid phase the main feature is that a primary antibody is

immobilized on solid surface, preferably magnetic beads (figure 3). Further when the immobilized primary antibody is incubated with the sample the antibody binds to the protein molecule. Finally the secondary antibodies detect the target molecule and generate a novel DNA strand which helps in providing detection signals in qPCR. In solution phase PLA primary and secondary antibodies are directly added to protein analyte solution without any immobilization of primary antibody on solid support (figure 3).

Solid phase PLA method gives a broader dynamic range and low limit of detection (LOD) when compared to solution phase PLA or any other protein detection methods (Fredriksson et al., 2007). The another main advantage of this method is, during washing steps the unbound probes and non-specific molecules or any contaminants can be eliminated therefore reducing background noise. The solution phase is a very direct approach and less protein analyte solution is required and also this avoids any washing steps further reducing the sample consumption. Both approaches have proven to be successful in detecting multiple targets, while solid phase PLA provides low cross reactivity and detecting sample at very low concentration, solution phase PLA reduces sample amount and can detect multiple targets with single batch of polyclonal antibodies (Lundberg et al., 2011)

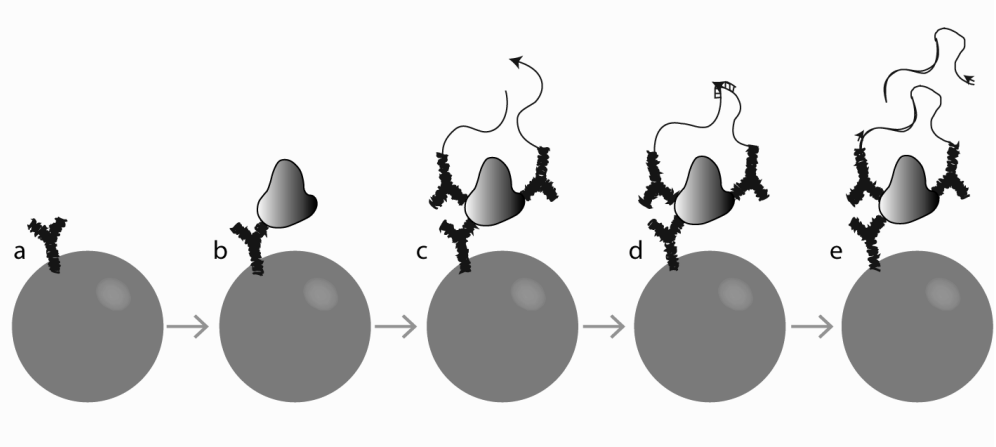

Figure 3. Solid phase PLA. a) The capture antibody is immobilized on the solid surface, b) target antigen is captured by capture antibody, c) detection probes detect the target antigen, d) when detection probes are in close proximity the oligonucleotides present on the detection probes get hybridized and form a template sequence and e) primers amplify the template sequence and generate detection. Modified from (Darmanis et al., 2010).

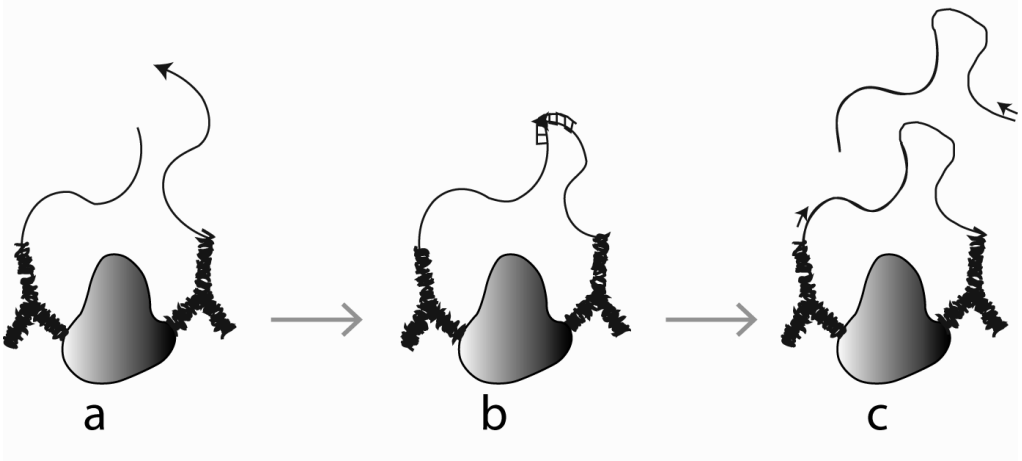

Figure 4. Solution phase PLA: solution phase PLA does not require a micro-particle, thus no capture antibody. A) Target antigen is recognized by the detection probes, B) As detection probes are in close proximity the oligonucleotides present on the detection probes are hybridized with the help of ligase and C) the primers amplify the oligonucleotide sequence and amplify thus generating detection signal. Modified from Darmanis et al., 2010.

1.3 Proximity Probes

Proximity probes are the most important part of proximity ligation assay. There are different types of probes.

Aptamers: Initially when PLA was designed, aptamers with 3' and 5' DNA extension were the first generation of proximity probes (Frederickson et al., 2002).. These aptamers are single stranded RNA or DNA oligonucleotides which are selected *invitro* and these aptamers are highly specific and shown to detect complex proteins in pico-molar to nano-molar concentrations (Bock et al., 1992), and are specific to the target single epitope site and are used to detect high complex and pure proteins (Fredriksson et al., 2002), although these have high specificity, it is not recommended to use in sandwich. since there are only very few suitable aptamers available, the most commonly used probes are mono clonal antibodies, polyclonal antibodies or immuno- globulins, which is combination of heavy chain and light chain (fb or fv) and these prove to be better option.

Antibody probes

In PLA most commonly used probes are monoclonal and polyclonal antibodies and these antibodies are attached with oligonucleotides. These probes are made by two different ways 1) covalent conjugate antibodies and 2) non-covalent conjugate antibodies (Lundblad, 2005).

Covalently Conjugated Antibodies

The preparation of covalent conjugation of antibody with oligonucleotide is achieved with the help maleimide conjugate chemistry. In maleimide conjugation chemistry the primary amines on antibody are activated by a cross linker called SMCC (succinimidyl-4{n-maleimidomethyl}cyclohexane-1-carboxylate). In conjugation, an antibody is treated with a small molecule like SMCC (succinimidyl-4{n-maleimiedomethyl}cyclohexane-1-carboxylate), it exposes an activte ester to form amide bonds with amine groups on lysine chains and N terminus (Hermanson, 2008), (Kalia and Raines, 2010), This activated antibody is then attached to a thiol reduced oligonucleotide. The conjugation between oligonucleotide with antibody is confirmed with the help of SDS-page. Finally using HPLC (high performance liquid chromatography) the conjugate antibody can be purified.

Non-covalent conjugation antibody

In case of non-covalent conjugate antibody, the streptavidin modified oligonucleotide is attached to a biotinylated antibody. The Streptavidin molecule is attached to the oligonucleotide with the help of maleimide conjugate chemistry. These streptavidin modified oligonucleotides can be purchased from several companies, and these oligonucleotides can be directly attached to the biotinylated antibody without any purification steps. This streptavidin and biotin conjugate chemistry is used because of their high binding affinity to each other and they can with stand high pH and high temperature.

During biotynylation of antibody the primary amines present on the antibody react with the biotin reagent. This biotin reagent consists of three parts, a biotin molecule, NHS sulfo group and a spacer arm. An amine bond is formed between biotin molecule and primary amine on antibody while NHS-sulfo group leaves and this enhances the solubility of the antibody in water. In this non-covalent conjugation reaction between biotin and antibody the spacer arm is very important as it enhances the binding between the biotin molecule on antibody and streptavidin molecule on oligonucleotide, and longer spacer arm also reduces the steric hindrance.

Advantages and disadvantages of PLA probes

While conducting experiments, the suitable probe is taken into consideration and pros and cons are weighed among each type of probes. The biotinylated probes are better suitable for conducting direct experiment as they require no purification step. Unlike

biotinylated probes, conjugated probes require excessive purification steps before conducting PLA experiment. Conjugated probes give better results in experiments where the target molecule is very small when compared to the biotinylated probes, because the biotin and streptavidin molecules add size to probes and hence may cause difficulties in detecting small molecules. Moreover, the biotin probes may cause hindrance and give high signals if the strep and biotin bond breaks. However, biotin probes are also very efficient in giving results because of the washing steps which will remove the unbound probes to target and because of biotin and streptavdin bonding. The washing step will not affect the already bound molecules and also these probes are very stable in solutions and also at high temperatures and high pH. But conjugated probes are very useful in multiplex PLA experiments, (when detecting different protein in parallel) as we can design different nucleotide sequences and make probes more efficient and reduce cross reactions (Fredriksson et al., 2007).

1.4 IGF-1

IGF-1, insulin-like growth factor is also known as **somatomedin c.** This is produced in liver and plays vital part in apoptosis, growth development or cellular differentiation. Three main somatomedins have been characterized: somatomedin C (IGF-1), Somatomedin A (IGF-2) and somatomedin B (Rosenfeld, 2003). IGF-1 is studied in this thesis as this protein is involved in cancer related diseases (Baserga et al., 2003), and muscular diseases (Musaro and Rosenthal, 2002). As the PLA has shown to detect molecules at very low concentrations (Blokzijl et al.,) it could be a very important assay if a standard assay is established to detect the IGF-1 molecule at low concentrations in order to diagnose early IGF-1 related diseases.

IGF-1 is a cytokine that is activated when attached to its receptors, IGF-1R, whose effects are altered by its binding proteins, and there are six different insulin like growth factor binding proteins (IGFBP) (Forbes et al., 2012).

Insulin like growth factor-1 is a single chain 70 amino acid based protein consisting of a hydrophobic core having 3 helices and 3 disulphide bonds which help in structure conformation. (Figure 5) (Vajdos et al., 2001). IGF-1 has a total molecular mass of only 7.6 kD. IGF-1 also shows significant sequence identity to insulin (Rinderknecht et al., 1978). Although IGF-1 has homology to insulin structure, it still has very specific interaction with

its receptor and insulin does not show any interaction with IGF-1 receptor (Zhu et al,, 1997).

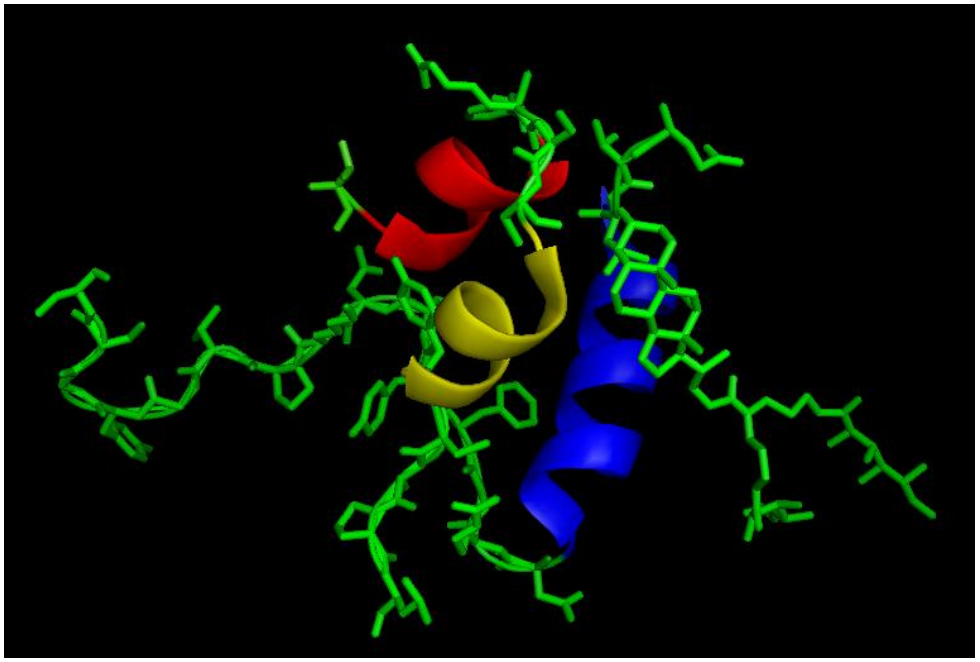

Figure 5. IGF-1 is a 70 amino acid protein which has a hydrophobic core having 3 helices, 3 disulhide bonds which stabilize structural conformation. The C-terminus is largely responsible for IGF-1R specificity and this region is not homologous to insulin.
Modified from (Vajdosetal.,2001) and (http://www.rcsb.org/pdb/explore/explore.do?structureId=1IMX)

1.5 PDGF

Platelet derived growth factor is a protein which regulates cell growth and division of cells, helping in formation of new blood vessels. In responses to injury like wound repair and in tissues that maintain themselves by continual cell turnover such as body cavities and in the hematopoietic system, PDGF helps in cell proliferation and in cell development (Andrae et al., 2008). The basis of cell proliferation whether it is normal or abnormal proliferation, lies within a group of hormones or growth factors (Ross et al., 1974).

The PDGF is a potent mitogen for mesenchymal cells which consists of PDGF-1 and PDGF-11 polypeptide chains and these are linked by disulphide bonds and they have a molecular weight of about 31-33 kDa. (Andrae et al., 2008, Sariban and Kufe, 1988). There are three members of the PDGF family – PDGFAA, PDGFAB and PDGFBB (Hannink and Donoghue, 1989). PDGFs are expressed in the heart, the brain, skeletal muscle and placenta. They are required for the recruitment of pericytes and vascular smooth muscle cells and contribute to neural regeneration. PDGFs modulate intracellular signalling through the MAPK, PI3-K and PKC-gamma pathways via PDGF receptor (Heldin et al., 1992).

PDGF and interaction with PDGF isoforms

The three different PDGF isoforms PDGFAA, PDGFAB, PDGFBB interact with two different PDGF receptors, alpha (α) and beta (β) (Claesson-Welsh, 1996). The alpha, (α) type receptor binds to PDGF-AA, PDGF-BB and PDGF-AB isoforms, whereas beta (β) type binds to PDGF-BB and PDGF-AB. In PDGF there are eight cysteine residues which are conserved and among these, six form intra chain disulphide bonds, resulting in a knot like structure and rest of the two form intrachain bonds with other subunits. (Heldin et al.,1998, Jaumann et al., 1991). There are three different loops present in the knot like protein structure which is stabilized by β-sheets pointing in different directions, and these just not only stabilize but also have an active role in receptor binding (Heldin et al., 1998, Andersson et al., 1995). Although there is abundant knowledge about PDGF, but unfortunately there is little advancement in detecting the protein or phosphorylated PDGF. Hence in this experiment, it is emphasized on detecting the phosphorylated PDGFβ-r on solid phase PLA to achieve a leap in molecular techniques.

Receptor

The bivalency of PDGF facilitates dimerization (Heldin et al., 1998) and the PDGF receptors are activated due to the dimerization which triggers kinase activation and maximal kinase activity is achieved as a result of tyrosine phosphorylation on PDGF receptor. Different types of PDGF receptors of homo or heterodimers are formed depending on the PDGF isoform (Ostman and Heldin, 2007). Actual sites for phosphorylation on α-receptor are not known, but the PDGF-β receptor is a tyrosine kinase protein which becomes dimerized and auto-phosphorylated by binding of an isoform like PDGF-BB which leads to signaling pathways (Oefner et al., 1992, Jarvius et al., 2007).

1.6 qPCR

The PLA method detects proteins with the help of antibodies similar to ELISA or any other assay which uses immuno binders, but unlike other assays here the detection probes are modified into single stranded DNA template upon detecting the target protein, which can be later amplified with the help POLYMERASE CHAIN REACTION (PCR). In qPCR the single stranded DNA template is multiplied for every cycle just like normal PCR. In qPCR the amplified molecules are measured through two strategies one is through fluorescent dyes such as sybr green and other is through TAQ-MAN probes. In this method TAQMAN probe

is utilised, which uses a fluorophore and a quencher, and when the primer is extended and forms a new strand, the fluorophore is released from the proximity of quencher molecule which emits fluorescence which is measured by a detector. In qPCR, X-axis is the number of amplification cycles and Y axis is the fluorescence of each cycle. As the curve of the amplification plot reaches exponential phase a threshold for fluorescence is set in background. Threshold cycle (Ct value) is the number of cycles at the intersection between amplification curve and a threshold line of fluorescence. The Ct value is inversely proportional to the initial concentration of the target which means the Ct value is higher when the initial template concentration is low. In PLA the concentration of the unknown target is measured from lowest to the highest, which is in amplification plot the higher concentration of target in sample the lower the Ct. In PLA the unknown target sample is serially diluted with target compatible matrix and a standard curve is drawn with Ct value of each sample against the concentration each sample. Along with standard curve a LOD (limit of detection) is measured which is the lowest amount of concentration of target detected with little amount of error.

2. AIM

Aim of the project was to develop the already existing technology, Proximity Ligation Assay (PLA) and achieve the detection of biomarker IGF-1 and phosphorylated PDGF receptor molecule in solid phase PLA and solution phase PLA using biotinylated proximity probes and conjugate probes.

3. MATERIALS AND METHODS

3.1 Buffers, proteins and oligonucleotides

1x PBS buffer had a pH 7.4, 137 mM NaCl, 2.7 mM KCL, 10 mM sodium phosphate dibasic, 2mM potassium phosphate monobasic. PLA buffer consisted 1 mM D-biotin, 0.1% purified BSA, 0.5% tween-20, 10 mM goat IgG (Invitrogen), 0.1 μg/μl salmon sperm DNA (Invitrogen), 5mM ETDA in 1x PBS. All the salts were ordered from Sigma Aldrich.

Washing buffer, PBS-Tween consists of 0.05% tween-20 in 1xPBS and the storage buffer were 1x PBS in 0.1% BSA.

Antibodies*:

Human PDGF R beta affinity purified antibody, goat IgG

Phospho-PDGF R beta (Y751) affinity purified Ab, Rabbit IgG

Human IGF-1 Mono-clonal Ab (Clone 33255), Mouse IgG

Human/ Mouse IGF-1 polyclonal antibody

Biotin anti Human IGF-1 recombinant

*all antibodies were purchased from R&D systems.

Oligonucleotides

Taq-man probe 5'-TET TGTACGTGAGTGGGCATGTAGCAAGAGG-3'-TAMRA

((TET, tetra-chlorfluorescein; TAMRA, carboxytetramethylrhodamine)

SLC 1 Streptavidin-5'-CGCATCGCCCTTGGACTACGACTGACGAACCGCTTTGC CTGACTGATCGCTAAATCGTG-3'

SLC2 5'-GTGTCTAAAGTCCGTTACCTTGATTCCCCTAACCCTCTTGAAA AATTCGGCATCGGTGA-3'-Streptavidin

Bio FWD 5'-CATCGCCCTTGGACTACGA-3'

Bio REV 5'-GGGAATCAAGGTAACGGACTTTAG-3'

Bio-Splint 5'-TACTTAGACACGACACGATTTAGTTT-3'

3.2 Antibody Immobilization

In solid phase method, dyna magnetic streptavidin beads were used as solid surface on which the antibody was immobilized as capture molecule. To immobilize the antibodies on beads, 100 µl (10 mg/ml) of dynabeads was taken and storage solution from the beads was removed with the help of a magnet and a pipette and then the beads were washed twice with 500 µl of washing buffer. 200 µl of already prepared 50 mM antibody was added to the magnetic beads and then incubated for one hour, after one hour of incubation the antibody would be attached to the solid surface. The remaining liquid was removed

from the beads with the help of magnetic stand and a pipette. Now finally the beads were washed again with washing buffer and PBS was added as storage liquid into beads and then the solution was stored at 4 ºc.

3.3 Preparation of proximity probes

Commercially bought biotinylated antibody was added in two tubes and then incubated for 45 minutes with SLC1 and SLC2 oligonucleotides separately with the final concentration of 50nM and incubated them. During the incubation the streptavidin oligonucleotides and biotin antibody are attached. This biotin antibody and oligonucleotide complex was added with PBS/BSA separately so that these antibodies do not cross react with each other, and finally these probes were diluted to either 250 pM or 500 pM with the PLA buffer for solid phase PLA and 60 pM for solution phase PLA.

3.4 Solid Phase

Serial dilutions were prepared, preferably six serial dilutions from highest to lowest from 1 nM to 0.01 pM and a negative control without target protein sample was made. The immobilized antibodies on beads were diluted 5 times with PLA buffer. On a 96 well PCR plate, 45 µl of recombinant target protein was added to 5 µl of beads except for negative control, where only PLA buffer was added instead of protein and then the reaction mixture was incubated at room temperature on a rotator for 90 minutes. After the incubation process was finished the reaction wells were washed. The PCR plate was placed on a magnetic stand and washed twice with PBStween solution to remove unbound molecules. Then 50 µl of 500 pM concentrated probe (detection antibodies) was added to each well including to the negative control and the PCR plate was incubated at room temperature for 90 minutes. Then the washing step was carried again to remove unbound probe molecules.

Finally 50 µl of PCR mix was added in to each well and placed in a qPCR thermo cycler. The PCR mix consisted of 5 µl of 10x PCR buffer, 2.5 µl $MgCl_2$ 50mM, 1.1 µl Taqman-probe 10 µM, 0.5µl Biofwd 10 µM, 0.5 µl Biorev10 µM, 0.5 µl Bio splint 10 µM, 0.04 ATP 10 mM, 0.4 µl DNTP's 25 mM with dUTP, 0.3 µl Taq-pol 5 units/µl, 0.02µl T_4 DNA Ligase 30 U/µl, 0.10 µl UNG 1units/µl and water was added to make the mixture to 50 µl for each reaction. The qPCR was programmed to following conditions: 95 ºC for 2 min, followed by 40 cycles of 15sec 95 ºC and 1 min 60 ºC.

3.5 Solution Phase

Unlike solid phase in solution phase only 60 pM conc. probes were used. Here a dilution series was prepared from 1 nM to 0.01 pM, and a negative control. In this method the antibodies were not immobilized on solid surface hence there was no capture antibody like in solid phase, therefore this method was not as accurate as solid phase. In this method the antigen or target sample was incubated directly with 60 pM probe at 1:1 ratio for 90 minutes.

After incubation, 2 µl of antigen + probe sample was added to 48 µl of PCR mix (similar to solid phase) in a new well and then the PCR plate was placed in a qPCR thermo cycler. The qPCR was programmed to following conditions: 95 ºC for 2 min, followed by 40 cycles of 15sec 95 ºC and 1 min 60 ºC.

3.6 Data Analysis

The qPCR results were analysed by Mx-pro software and the achieved results were transferred to an excel sheet to plot a graph. In the graph the Ct values were determined for each well and these values were taken down and a mean and standard error were calculated and with these numbers a graph was drawn with x-axis as concentration values from lowest to highest and y-axis ct values. R^2 was calculated from the slope of the graph curve and a limit of detection (LOD). Limit of detection, defined as the concentration at rolling circle amplification (RCA) counts 2×SD over background was also calculated. Dynamic range is the total log difference between highest sample concentrations to the negative control.

4. RESULTS

4.1 Detection of IGF-1 on solid phase PLA

The main objective of this experiment was to detect IGF-1 protein in animal tissue sample and human cerebral spinal fluid (CSF) in solid phase PLA and solution phase. Before trying to detect IGF-1 in a patient CSF sample, the PLA experiment was performed to detect recombinant IGF-1in buffer with the help of biotin antibodies. In this solid phase assay the antibody against IGF-1 was immobilized on magnetic beads and detection antibodies were incubated with capture antibody and target molecule complex. The Ct values were obtained using qPCR for 1 nM to 0.001 pM serial dilutions of recombinant IGF1 sample and positive control vascular endothelial growth factor recombinant protein (VEGF). VEGF

was used as a positive control in order to validate the results as detection of VEGF using this solid phase assay was already reported (Schallmeiner et al., 2007).

A graph was plotted using Ct values versus sample dilution concentrations (figure 6). The IGF-1 protein was not detected in low concentrations (0.001 pM to 1 pM), also the difference in the Ct value between the 0.001pM and 1nM was not significant enough to conclude the detection experiment as successful. In contrast, the positive control, which was conducted under same the conditions as IGF-1 got good signals and showed difference of eleven Ct values. This experiment has proved me that the detection of IGF-1 in patient CSF sample could not be possible, because the antibodies could not detect the recombinant IGF-1 protein. Limit of detection (LOD) for IGF-1 (with r^2=.812) was ~10 pM and with only 5 ct values difference in dynamic range. LOD for VEGF (r^2=0.929) was less than 1pM and respectively and with dynamic range of ~ 9 Ct difference.

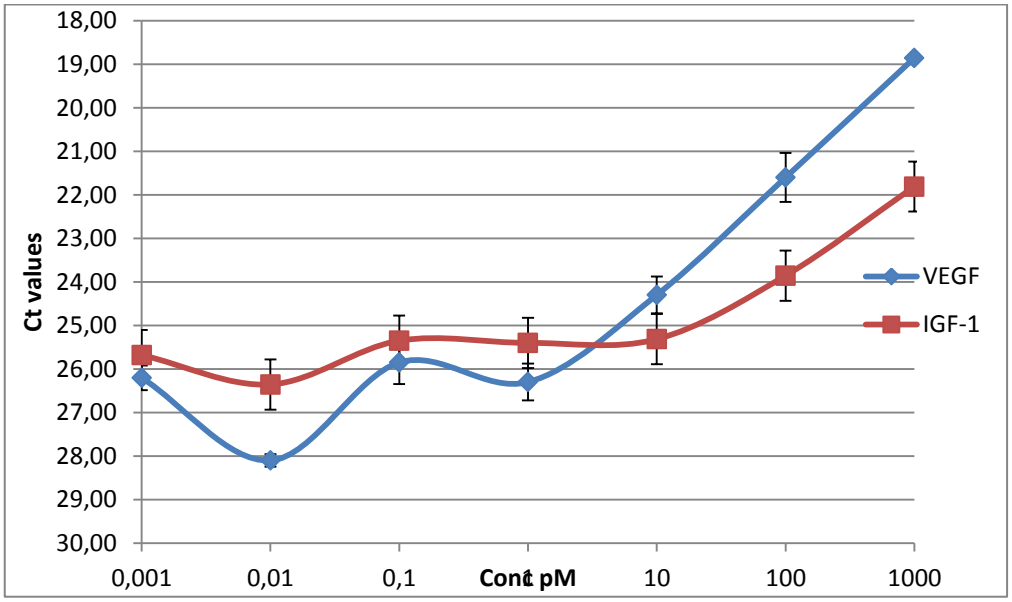

Figure 6. Detection of IGF-1 biomarker using PLA on solid phase in PLA buffer using biotinylated IgG antibodies. VEGF served as positive control. The X- axis shows the concentration of the IGF-1 dilution series from 1nM to 0.001pM. At 0.001pM there is no antigen in it, but only detection probes. On Y-axis, the average of Ct values of the PCR results are plotted, and along the slope there are error bars which give the standard deviation from same sample replicate measurements of several independent dilutions.

4.2 Detection of IGF-1 in solution phase

The main difference between solution phase and solid phase is that the detection of molecules is direct in solution phase and does not require any additional washing step and can be done very easily. In solution phase the target molecule is directly incubated with the conjugate probes. In this experiment, IGF-1 molecule was incubated with 60 pM conc. detection probes. Later, the samples along with probes were amplified in

qPCR. The results obtained with the solution phase shown in figure 7 indicated that the solution phase proved to be less effective than solid phase experiment. Figure 7 shows that all IGF-1 serial dilutions had the same Ct values indicating that detection of recombinant IGF-1 was unsuccessful. In contrast, the positive control (VEGF) (r^2=0.8847) gave good signals achieving low limit of detection of 0.01 pM and a dynamic range of ~9 ct values difference.

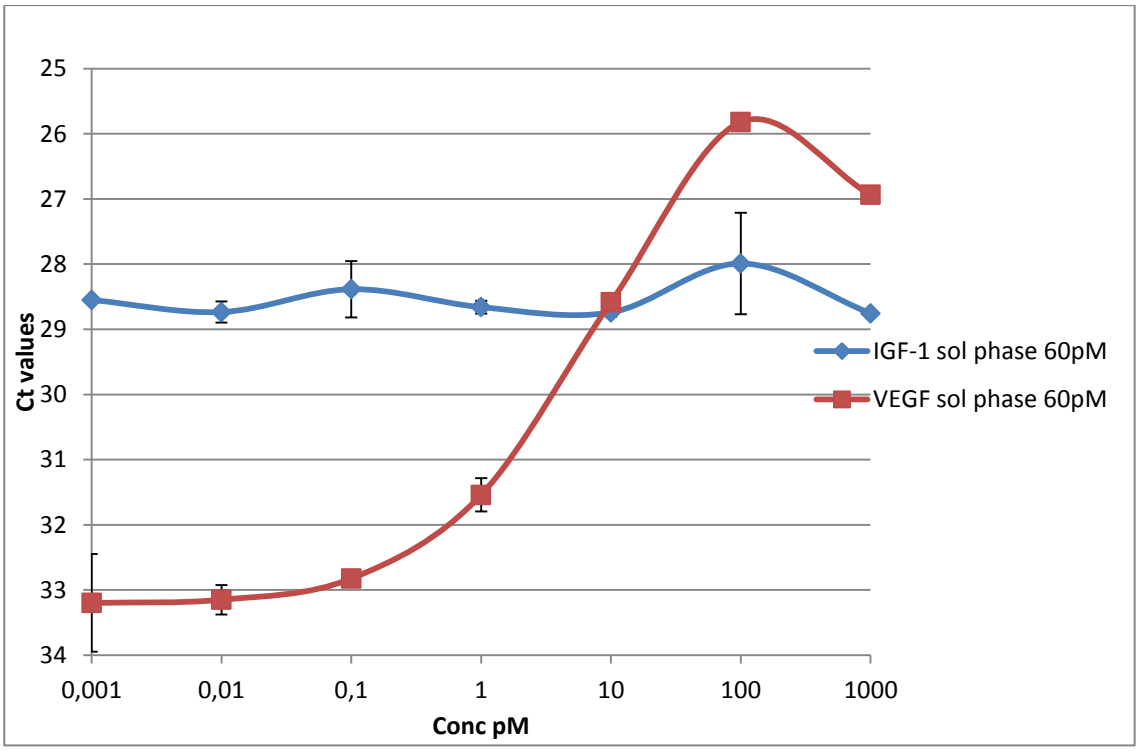

Figure 7. Detection of IGF-1 biomarker using PLA on solution phase in PLA buffer with smcc conjugated IgG antibodies. VEGF served as positive control. The X- axis shows the concentration of the IGF-1 dilution series from 1 nM to 0.001 pM. At 0.001pM there was no antigen present, but only detection probes. On Y-axis, the average of Ct values of the PCR results are plotted, and along the slope there are error bars which give the standard deviation from same sample replicate measurements of several independent dilutions.

4.3 Detection of phosphorylated PDGFr in cell lysates on solid phase

The cell lysate sample containing activated PDGFβr was incubated with detection antibody that was immobilized on solid particles (dyna magnetic beads). Proximity probes or detection probes to detect the PDGFr were attached with oligonucleotides using maleimide conjugation chemistry. Two different batches of phosphor detecting antibodies were made, each having different oligonucleotides attached to them. One of these two phospho-site detecting antibodies served as ARM1 and other antibody served as ARM2. ARM 2 phospho detection antibody which detects the phosphorylated tyrosine 750 site on PDGFβr was used (Figure 8). This antibody serves as secondary detection antibody which detects phosphorylated site and the other polyclonal antibody as primary antibody which

detects PDGFr protein. Similarly ARM 1 of phospho detection antibody was used to detect the phosphorylated site on PDGFβr and anti PDGFr antibody served as ARM2 (Figure 9).

In this solid phase experiment to detect phosphorylated PDGFr, cultured cells were treated with PDGF-BB cytokine to phosphorylate PDGFr.

Recombinant PDGFr was used as a positive control and lysis buffer was used as negative control and stimulated (+) cell lysate was the target. Figure 8 and 9 show that there was a linear increase in detection values of recombinant PDGFr and there was a 12 Ct value difference (dynamic range) from 0.001 pM to 1nM with LOD of (r^2 =0.966) at 0.01pM

However, for stimulated (+) cells, the Ct values obtained for all dilutions did not differ from each other. Similarly, it was also observed in non-stimulated cells that the Ct values were in same range from 0.0001 pM to 1 nM. Both the experiments have resulted in unsuccessful detection of phosphorylated PDGFβr.

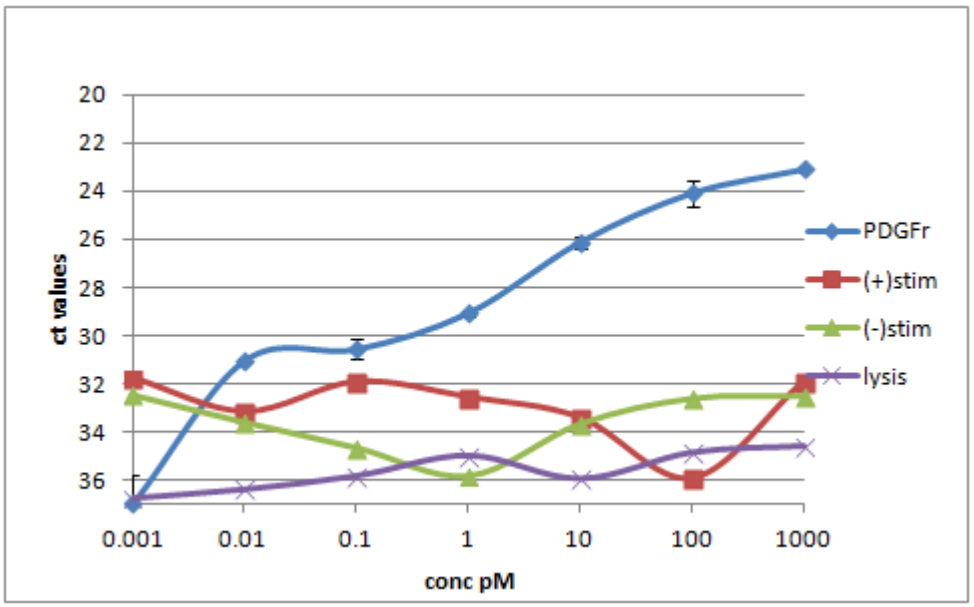

Figure 8. Detection of phosphor-PDGFr using PLA on solid phase in PLA buffer with smcc conjugated IgG antibodies. Recombinant-PDGFβr served as positive control. The X- axis shows the concentration of the sample dilution series from 1 nM to 0.001 pM. At 0.001 pM there was no antigen in it, but only detection probes. On Y-axis, averages of Ct values of the PCR results are plotted, and along the slope there are error bars which give the standard deviation from the same sample replicate measurements of several independent dilutions.

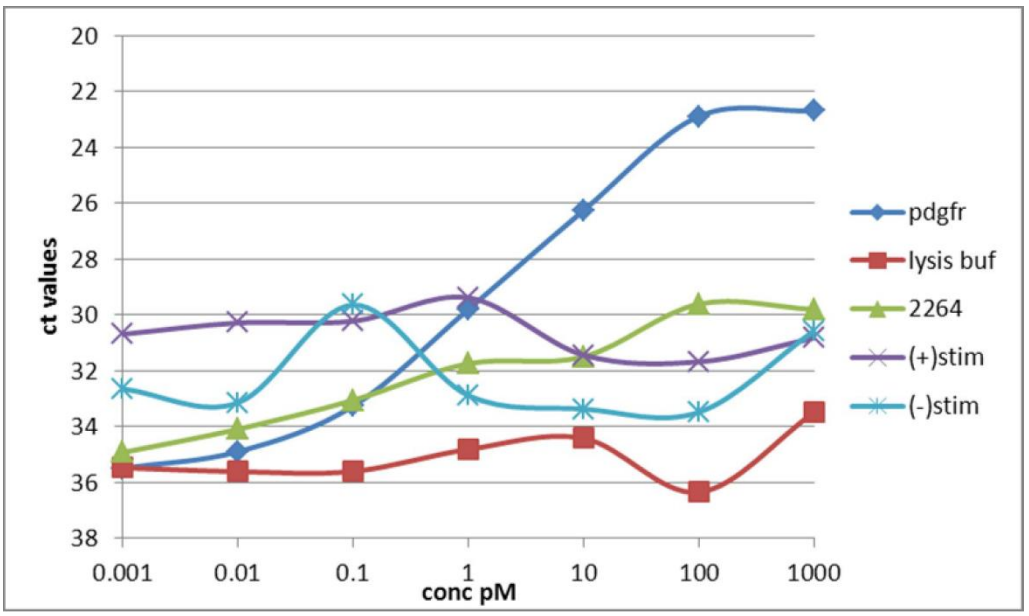

Figure 9. Detection of phospho-PDGFr using PLA on solution phase in PLA buffer with smcc conjugated IgG antibodies. Recombinant-PDGFβr served as positive control. The X- axis shows the concentration of the sample dilution series from 1nM to 0.001pM. At 0.001pM there was no antigen in it, but only detection probes. On Y-axis, averages of Ct values of the PCR results are plotted, and along the plot there are error bars which give the standard deviation from same sample replicate measurements of several independent dilutions.

4.4 Comparison of detection signals of phosphorylated PDGFβr in diluted samples

To study the phosphorylation of PDGFβr, the tyrosine 751-Phophosphrylated site detection antibody was purchased from R&D systems. This antibody was made into two different batches attached with different oligonucleotides. They are named as ARM1 (Y751 13042) and ARM2 (Y751 130582). For a successful detection of PDGFβr protein, I have used a Human PDGF R beta Affinity Purified Polyclonal Ab, Goat IgG as a primary antibody to detect the PDGFr protein, followed by Phospho-PDGF R beta (Y751) Affinity Purified Polyclonal Ab, Rabbit IgG as a secondary antibody to detect the tyrosine phosphorylated site on the PDGFβr protein. In experiments mentioned above (Figure 8,9) it was observed that the antibodies were unsuccessful in detecting the phosphorylated PDGFβr site, hence in this experiment I wanted to investigate whether the Phospho-PDGF R beta (Y751) ARM1 and ARM2 antibodies were contaminated or there was any cross reaction activity between the antibodies.

In an ideal PLA experiment, Ct value for a sample without target (protein) is usually high and Ct value for a sample with target protein is low. In other words, the higher the sample concentration, the lower the Ct value. However, in this experiment the lysis

buffer Ct value was unusual. At 100x dilution lysis buffer Ct value was lower than 10x dilution (Figures 10, 11). As 100x was more diluted than 10x, the Ct value of higher diluted sample should be more than the lesser diluted sample.

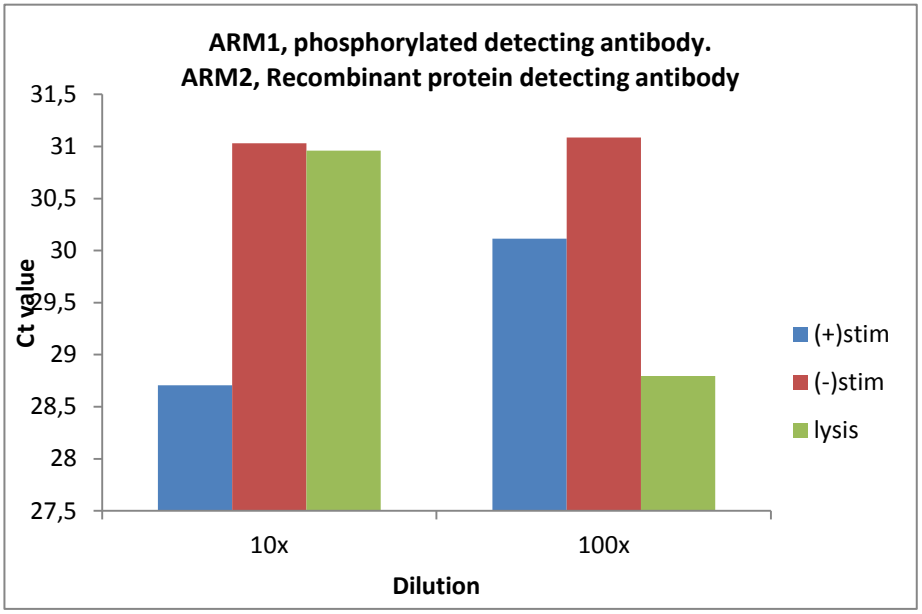

Figure 10. Detection of phosphor-PDGFr using solid phase in PLA buffer with smcc conjugated anti-PDGFβr IgG antibodies from R&D systems control. The X- axis is the dilution in times of the sample. On Y-axis, the average of Ct values of the PCR results are plotted. (+) represents; cell lysate sample with phosphorylated PDGFβr, (-) represents non phosphorylated PDGFβr cell lysate. Lysis is the lysis buffer.

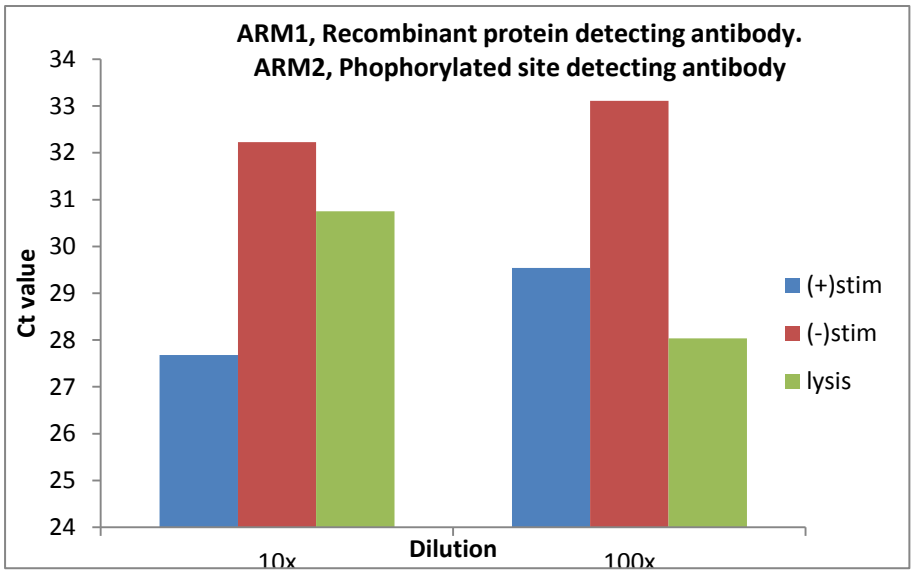

Figure 11. Detection of phosphor-PDGFr using solid phase in PLA buffer with smcc conjugated anti-PDGFβr IgG antibodies from R&D systems control. The X- axis shows the dilution in times of the sample. On Y-axis, the average of Ct values of the PCR results are plotted. (+) represents; cell lysate sample with phosphorylated PDGFβr, (-) represents non phosphorylated PDGFβr cell lysate. Lysis is the lysis buffer.

5. DISCUSSION

Proximity ligation assay (PLA) is an evolving method to study the interactions between biomolecules at protein level. Understanding the post transcriptional modifications of proteins would enable us to understand human diseases (Wilkins et al., 1999, Mann et al., 2003). The goal of proteomics is to identify individual proteins at low concentrations in a tissue or in fluids, which requires high accuracy and sensitivity to investigate biomarkers. There are already existing protein detection methods such as microarray, chip, or mass spectrometry, by which many biomarkers have been identified, but PLA allowed looking into the relation of proteins to human diseases.

In this thesis, I have employed solid phase and solution phase approaches to study IGF1 and phosphorylated PDGFβR, to establish as a biomarker using PLA for cancer related diseases and to understand cell-cell signaling respectively.

Simon Fredriksson et, al have reported plasma biomarkers in pancreatic cancer and ovarian cancer, a study which included IGF-2 (Fredriksson et al., 2007). IGF-1 structurally homologous to IGF-2, is an anabolic hormone produced in liver and taking part in protein catabolism and glucose utilization. But recently it was also reported as a possible biomarker for cancer (Borofsky et al., 2002, Rosenfeld, 2003). Recently, IGF-1 has been reported as a biomarker for cystic fibrosis (CF), (Gifford et al., 2013) with ELISA. However, the ELISA used in this study could only detect the target protein at more than 1ng/ml which is higher limit of detection than PLA. But the PLA could detect the protein at less than 1pM concentration and also it uses very low amount of detecting antibodies, therefore reducing the cross reaction between antibodies. Thus it would be a remarkable assay to study the target protein and its association to cancer or any other disease. PLA not only detects low concentrated protein, but also other proteins in same sample at same time called as multiplexing, thus enabling researchers to study other cytokines related to target protein (Chang et al., 2009, Tavoosidana et al., 2011).

In my experiments, I have tried to detect recombinant IGF-1 protein and later to establish an assay to detect IGF-1 in serum samples. In Figure 6, it was observed that, PLA could not detect the target protein (IGF-1), but it was successful in detecting recombinant VEGF, in the positive control. But still at higher sample concentration it was observed that anti-IGF-1 antibodies were able to detect IGF-1. The possible explanation for not detecting protein may be due to the fact that the IGF-1 molecular weight is 7.6 kD, which would make it difficult for detecting antibodies to detect the appropriate epitope site of the target

protein molecule, as the detecting antibodies have molecular weights of 150 kD excluding the molecular mass of streptavidin and biotin. To overcome this problem I have employed solution phase PLA to reduce the amount of capture antibodies, so that this technique could expose several epitopes for detection probes to recognize the IGF-1. But this effort did not give positive results (Figure 7), where positive control (VEGF) was detected but IGF-1 protein data showed only detection antibodies signals.

Detection of phospho-PDGFβr

Another crucial effort made with PLA was to establish a standard assay which could detect the specific phophorylation site on a receptor, so that it would enable researchers to study the molecular mechanism, course of events that would take place in a cell after a successful activation of specific phosphorylated site on a receptor.

In my experiments, I have used solid phase PLA to detect the PDGFβ receptor, which is found to be essential for cell growth and chemotaxis, (Heldin, 1992). PDGFβr detection with PLA *in situ* with dual recognition was already reported (Jarvius et al., 2007). According to their study a primary antibody detects PDGFβr site and a secondary antibody detects the phosphorylated site. In my thesis I have employed the same technique to detect the phophorylated PDGFβr, but on solid surface in HEK293 cell lysate samples.

In this thesis, I have tried to detect the PDGFβr with dual recognition, where one primary antibody detects the PDGFβr and the other primary antibody detects the phosphorylation site on PDGFβr. Before conducting the experiment to detect the receptor, the HEK293 cells were pre-treated with PDGF-BB isoform to activate the PDGFβr and to auto phosphorylate at tyrosine 750. The experimental results showed that the detection of phosphorylated PDGFβr was unsuccessful (Figure 9). This could be due to the differences in levels of ligand-induced receptor phophorylation (Jarvius et al., 2007). Another possible explanation for unsuccessful detection could be due to cell lysis buffer. In cell lysis buffer to inhibit the phosphatase enzyme action, phosphatase inhibitor was added. The unsuccessful detection of the phosphorylated receptor may be because of inadequate amount of phophatase inhibitor in lysis buffer.

Another reason for unsuccessful detection of PDGFβr may because of anti-phospho PDGFβr antibodies. In my experiment I have used polyclonal antibodies for detecting positive control (recombinant PDGF receptor) and the same antibody along with anti-phospho PDGFβr

antibody was also used to detect phosphorylated PDGFr. The antibody which was used in detection of recombinant PDGFr was effective in recognizing recombinant-PDGFr (figure 9). But the same antibody along with anti-phophorylated PDGFβr antibody was proved to be unsuccessful in detecting phosphor-PDGFβr, suggesting that Phospho PDGFβr antibody could be the reason for unsuccessful detection.

In order to assess the possible reason for antibodies not detecting receptor, I have compared (+) ARM1 and (-) ARM2 antibodies of anti- phospho-PDGFβr and tried to find which ARM was better in detecting phospho-PDGFβr. However, both ARM's have showed linear Ct values for whereas lysis buffer showed high Ct values with high background (figure 10, 11), suggesting again lysis buffer could be one of possible reasons for showing high background. However, it is still unclear why the detection of phosphorylated receptor on solid phase PLA in cell lysate samples was unsuccessful, even though PLA showed remarkable ability to recognize multiple phosphorylated receptors *in situ* (Chen et al., 2013, Jarvius et al., 2007).

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