Investigating the role of FOXA1 protein in cancer

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ABSTRACT

The aim of this research project is the study of the FOXA1 protein, which has been related with breast cancer. The effect of FOXA1 on FOXC2 protein expression is studied with Western blotting. Additionally, the binding kinetics of FOXC2 protein with FOXA1 gene are investigated using Scanning Tunnelling Microscopy at the single-molecule level. The results of the project will extend the understanding in the role of FOXA1 in gene regulation, providing important information for the use of FOXA1 as a biomarker for breast cancer as well as the development of new treatment techniques.

Keywords: electrochemical STM, protein-DNA interaction, transcription regulation

1 INTRODUCTION

1.1 The role of FOXA1 in cancer

Breast cancer is the most common female malignancy and second most common cause of death in woman in the Western word. Cancer can result from an accumulation of genetic mutations leading to dysfunction of critical genes, including tumour suppressor genes [1]. The FOXA1 protein belongs to a superfamily of transcription regulators, the Forkhead Box (Fox) proteins. Current evidence suggests a crucial role for FOXA1 in the development of breast cancer. Consequently, FOXA1 has generated considerable interest as a biomarker for predicting and monitoring cancer development. What is evident is that FOXA1 can keep a balance between cell proliferation and maintenance of a differentiated phenotype by expressing a specific subset of gene targets [2]. FOXA1 binds to target DNA sequence as a monomer, using a helix-turn-helix motif of 110 amino acids. Thus, the transcription factor FOXA1 is thought to contribute to gene regulation through its ability to act as a pioneer factor binding to nucleosomal DNA.

The family of Fox Proteins is defined by a common DNA-binding domain (DBD) termed the forkhead box. In humans, at least 41 forkhead genes have been identified so far and they cover a wide spectrum of biological processes, including metabolism, development, differentiation, proliferation, apoptosis, migration, invasion and longevity. Since Fox proteins control these fundamental developmental and homeostatic processes, it is anticipated that a loss or gain of Fox function can have serious implications on the cell cycle. Although our knowledge on Fox transcription factors is still limited, several Fox families such as FoxO, FoxM, FoxP, FoxC and FoxA have been linked to tumorigenesis and the progression of certain cancers. Deregulation of Fox factors can result in changes in both direct and indirect genes and may be associated with multiple or specific cancer types [2]. Therefore, characterisation of such molecules is critical for therapeutic purposes.

For example, the interaction of FOXA1 protein with the hereditary breast cancer predisposition gene BRCA1 has been studied [3]. BRCA1 is a tumour suppressor gene which is expressed in the cells of breast and other tissue, where it helps repair damaged DNA, or destroy cells if DNA cannot be repaired. If BRCA1 itself is damaged, i.e. it has a mutation, damaged DNA is not repaired properly and this increases the risk for breast cancer. The BRCA1 protein has been assigned a number of different functions including metabolism, apoptosis, cell cycle regulation and as a transcriptional factor which regulates expression of many genes. The BRCA1-responsive element was defined as a 35bp region and a bioinformatics analysis has suggested that the BRCA1-responsive element contains a potential binding site for the transcription factor FOXA1 [4].

FOXC2 is a protein which is believed to participate in the transcriptional regulation of FOXA1. The FoxC family plays a crucial role in the formation and maturation of the vasculature as well as in lymphatic sprouting. Upregulation of FOXC2 has been significantly correlated with the highly aggressive basal-like subtype of human breast cancers. These observations indicate that FOXC2 plays a central role in promoting invasion and metastasis. It is believed that deregulation of FOXC2 contributes to the aggressive clinical behaviour of carcinomas and that it may prove to be a highly specific molecular marker for human basal-like breast cancers [5].

1.2 Scanning Probe Microscopy

The development of the Scanning Tunnelling Microscope (STM) by G.Binnig and H.Rohrer in 1982, introduced an entirely new approach to visualisation [6]. The STM obtains images of the conductivity maps of samples by monitoring the tunnelling current flowing between a sharp metallic probe and the conductive sample

surface. This revolutionising technique provides information at atomic resolution on many electrically conducting structures. Since then, a large family of Scanning Probe Microscopes (SPMs), all capable to generate a map of surfaces with sub-nanometre resolution, has emerged. Their common feature is a sharp probe that examines the local surface properties as it raster scans over the sample. Depending on the physicochemical interactions responsible for the image formation, a whole range of material surface properties is accessible. Atomic Force Microscopy (AFM), for example, measures forces between the tip of a cantilever and the sample [7].

Due to the capability to generate profiles of molecular structures with nanometre resolution, SPM has many applications in biosciences, including DNA analysis, imaging of peptides, protein-DNA complexes, chromosomes, cells and cellular membranes and many more. One of the features which have contributed to its huge success is the possibility of acquiring data both in ambient conditions as well as in a liquid environment (in-situ SPM). In-situ SPM systems hold a clue not only to structural mapping and visualization of adsorbed molecules with unprecedented resolution but also to single molecule function and spectroscopy [8]. To reproducibly achieve high-resolution data, however, sample preparation requires unprecedented attention to detail. The reactions have to take place on a surface which needs to be clean and atomically flat.

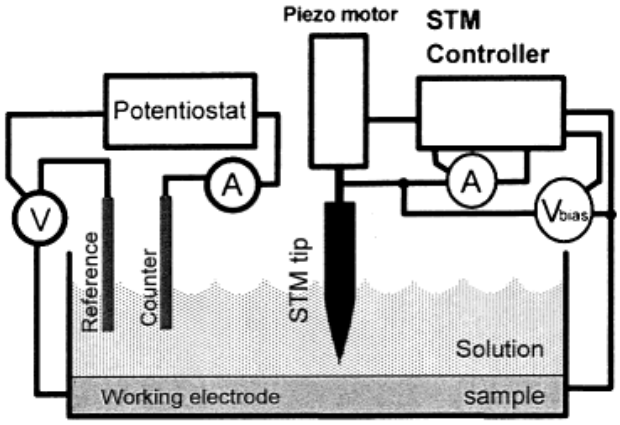

Figure 1: Schematic view of in-situ STM configuration. Both the sample-solution and the tip-sample potential differences are controlled. The tip is insulated except at the very end. [8]

1.3 Regulation of Transcription

Because of its essential role in biology, DNA has been studied extensively by SPM [9]. Studies of protein-DNA complexes have provided information about binding sites and stoichiometry of the proteins as well as ligand-induced bending of DNA [10,11]. By isolating a single molecule, the system under study is simplified in the absence of interactions between molecules, and details which otherwise would be lost due to ensemble averaging in a bulk experiment are obtained. The spatial arrangement of DNA upon protein binding has always been of great interest, for its important implications in a variety of issues such as transcription regulation.

Transcription is an essential biochemical process in gene expression but it is still not fully understood. During this process, genetic information from the DNA is copied to functional RNA molecules. These are subsequently translated into proteins or function directly in cellular metabolism. Transcription is subject to regulation by proteins, called transcription factors. These proteins bind to specific DNA sequences in order to control the movement of genetic information from DNA to RNA. This process has long been the subject of biochemical investigation. However, certain aspects of the molecular mechanisms underlying transcriptional regulation cannot be studied in sufficient detail with these techniques. The SPM has provided the means to address questions regarding the architecture and dynamics of protein-DNA complexes involved in the process of transcription [12].

This work focuses on the regulation of the FOXA1 gene by the FOXC2 transcription factor. Additionally, the role of the FOXA1 protein in the regulation of the BRCA1 gene will be investigated. The studies include both molecular biology methods, namely Western blotting, as well as SPM techniques.

2 METHODOLOGY AND MATERIALS

2.1 Cell Culture

The human breast carcinoma MCF-7, endocrine resitant MCF-7 clone (MLET-2), ZR-75-1 and HBL-100 cell lines originated from the American Type Culture Collection and were acquired from the Cell Culture Service, Cancer Research UK (London, UK). They were maintained in DMEM supplemented with 10% FCS, 2mM glutamine, and 100 units/ml penicillin/streptomycin. The cells were maintained in 10% CO2 humidified atmosphere at 37°C. The FOXA1 transfected cells (MCF-7-FOXA1, ZR-75-FOXA1) were selected by 300 mg/L of Geneticine (G418) antibiotic.

2.2 Western Blotting

Western blotting was performed on whole cell extracts. Cells were washed twice with ice-cold PBS, scraped and centrifuged at 2000 rpm for 2min at 4°C. Lysis buffer 20 mM Tris-HCl pH 7-4, 20 mM dithiothreitol (DTT), 2 mM EDTA (sodium salt), 1% Triton X-100, 1% NP40, 1% sodium deoxycholate, 1 mM sodium pyrophosphate, 1 mM sodium orthovandate (prepared in Tris buffer) and 1 mM phenylmethylsulfonyl-fluoride) was added directly to the cell palette. Cells were re-suspended in lysis buffer and centrifuged at 13000 rpm for 10 min at 4°C. Proteins are in resulting supernatant and determined by Bradford assay.

Once extracted, they were boiled at 100°C for 5min in 2xSDS buffer.

Antibodies used were FOXA1 (ab23738, Abcam), FOXC2 (N-20, Santa Cruz Biotechnology) and β-tubulin (H-235, Santa Cruz Biotechnology). Primary antibodies were detected using horseradish peroxidase linked anti-rabbit conjugates and visualized using the Enhanced Chemiluminescence (ECL) detection system.

2.3 In-Situ STM

A STM from Agilent Technologies with a bi-potentiostatic three electrode system, for independent potential control of both substrate and tip, is used for in-situ STM. The in-situ cell is made of chemical-resistant polycarbonate and can be used with a wide variety of liquids. Two Pt wires serve as counter and reference electrodes. A Au (111) disk (Mateck, Germany) is used as a working electrode. The electrode is annealed in a hydrogen flame prior to use. Imaging is performed at the constant-current mode at room temperature. Tungsten tips are prepared by electrochemical etching in 2M KOH and coated with apiezon wax, as described elsewhere [13].

3 RESULTS AND DISCUSSION

3.1 FOXA1 modulates the expression of FOXC2 protein

To investigate whether FOXC2 protein is regulated by FOXA1 and is a downstream target of FOXA1, human breast cancer carcinoma cell lines MCF-7 and ZR-75-1 are transfected with FOXA1. An aminoglycoside antibiotic is used a dominant selective agent to select genetically engineered cells. Western blotting is used to compare the protein levels in transfected cell lines ZR-75-1-FOXA1 and MCF-7-FOXA1 with wild type ones.

Figure 2 shows a double-band for FOXA1 protein and a single-band for FOXC2 protein. FOXA1 transfected cells enhanced the expression of FOXA1 protein and results in slight increase of FOXC2 protein level in these cell lines. Beta-tubulin is used as a loading control during experiments which shows the same level of expression in different samples (Figure 2).

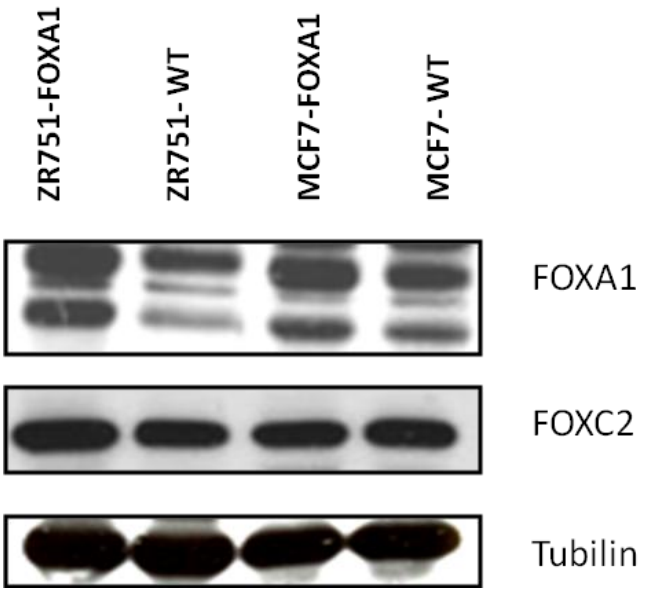

Figure 2: Expression of FOXA1 and FOXC2 in FOXA1 transfected and wild type MCF-7 and ZR-7-51 breast cancer cell lines. All the cell lines were cultured in phenol red medium supplemented with 10% Fetal Calf Serum (FCS). FOXA1 transfected cell lines (MCF-7-FOXA1 and ZR-7-51-FOXA1) were selected by 300 mg/L of Geneticine (G418) antibiotic. The western blot membrane was probed with FOXA1 (49 KDa) and FOXC2 (57 KDa) antibody and finally beta-tubilin antibody (55 KDa) is used as a positive loading control. The western blot shows higher expression of FOXA1 in transfected cells and slight increase in expression of FOXC2 protein as a result.

3.2 Positive correlation in FOXA1 and FOXC2 expression and other signaling transduction pathway components

To study the effect of FOXA1 on FOXC2 protein expression, protein lysates of a panel of breast cancer cell lines (MCF-7, MLET-2, ZR-75-1, HBL-100) were analyzed by Western blotting using specific antibodies. The analysis shows similar trend to figure 1. Western blot indicates that the expression of FOXA1 correlates with the expression of FOXC2 protein (Figure 3).

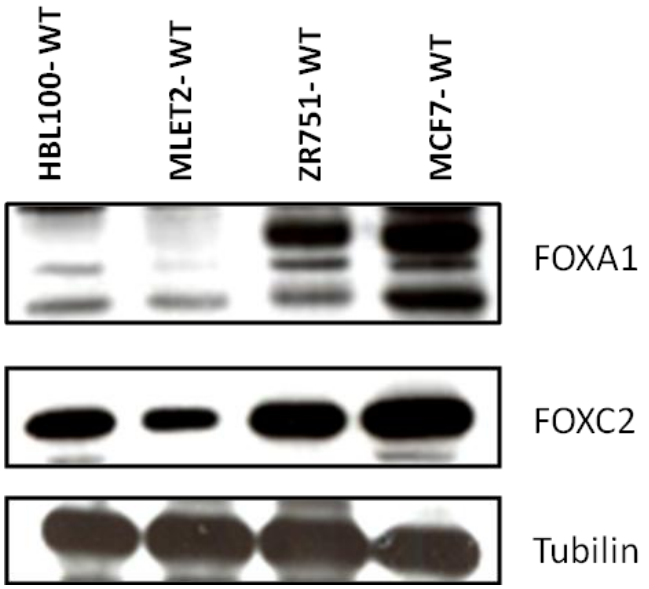

Figure 3: Expression of FOXA1 and FOXC2 proteins in a panel of breast cancer cell lines (MCF-7, MLET-2, ZR-75-1, HBL-100). All the cell lines were cultured in phenol red medium supplemented with 10% FCS. The western blot membrane was probed with FOXA1(49 KDa) and FOXC2 (57 KDa) antibody and finally beta-tubilin antibody (55 KDa) is used as a positive loading control. Western blot shows MCF-7 and ZR-75-1 express higher level of FOXA1 and FOXC2, whereas MLET-2 and HBL-100 shows lower expression of FOXA1 thus lower FOXC2 protein. This blot indicates expression of FOXA1 correlates with expression of FOXC2 protein, i.e. higher level of FOXA1 results in higher level of FOXC2 protein.

4 CONCLUSION

Preliminary molecular biology experiments suggest that the FOXC2 protein expression can be regulated by FOXA1. Western blot results indicate that a higher expression of FOXA1 protein results in an increase in the expression of FOXC2 protein.

The binding of FOXC2 protein to the promoter of FOXA1 gene is being studied with Scanning Tunnelling Microscopy. It is expected that the results will confirm the above results but also extend the understanding of the regulation of FOXA1 gene by FOXC2 protein

5 PROPOSED EXPERIMENTS

Future experiments will continue revolving around the regulation of FOXA1 by FOXC2. Other SPM methods, such as Atomic Force Microscopy (AFM) can also be employed. The application of SPM in the field of transcription can provide novel insights into transcriptional regulation and the dynamic aspects of this process. The structural effects of proteins on DNA, such as the induction of bends can be readily visualized and quantitatively analyzed.

Additionally, the role of FOXA1 protein in the regulation of BRCA1 gene will be investigated. Experiments will include both molecular biology methods as well as SPM techniques.

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