Norwegian University of Life Sciences

Faculty of Veterinary Medicine and Bioscience

Department of Chemistry, Biotechnology and Food Science

Master Thesis 2014
60 credits

A study of the influence of hormones and growth factors to the accessible chromatin landscape in human breast cancer

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Acknowledgments

This work was performed at the Breast Cancer Research group of dr. Toni Hurtado at Center for Molecular Medicine Norway (NCMM) at the University of Oslo in collaboration with the University of Life Science, Ås, Norway, from june 2013 to may 2014.

I would like to thank my main supervisor, Toni Hurtado, for the opportunity to come work in your lab. I would also like to thank Elisa Fiorito for all the help with the project, in the lab and with theory. I have learned so much from you, thank you for your patience and sharing all your knowlegde. I would also like to thank the rest of my group, Siv Gilfillan, Madhu Ratika, Elena Gonzales, Baoyan Bai and Yogita Sharma.

My supervisor at University of Life Science, Ås, Charlotte Kleiveland, for helping me when I was stuck in my writing process.

And to my husband, Gard, for his patience and understanding of my frustration during this time, and my children for accepting that I'm not always there to tuck them in at night. Also a big thank to my parents and parents-in-law for taking care of our children when needed.

Siri Nordhagen,
May, 2014

1 Abstract

1.1 English

Mitogens are substances capable of inducing cell proliferation. Upon binding to their receptors a signal pathway is activated resulting in gene transcription and cell proliferation. These signals are usually carefully regulated, but cancer cells lack this regulation and the result is uncontrolled cell division. Breast cancer is the most common cancer among women in the western world. Several subtypes are involved depending on which receptor is overexpressed, each type with its own expression, treatment and prognosis. FoxA1 is a protein functioning as a pioneer factor due to its chromatin remodeling ability, so receptors like estrogen receptor can bind to estrogen responsive elements (ERE) on the DNA and start transcription of estrogen target genes. The treatment of breast cancer depends on the subtype, but the women do not always respond to treatment without a clear reason. Due to this, it is interesting to explore if the hormones, like estrogen and androgen, or growth factors, like heregulin and epidermal growth factor, are independently able to remodel the chromatin or if the presence of FoxA1 is necessary. This project was conducted with a method called FAIRE, which isolates the nucleosome free DNA, the part of DNA supposedly active during transcription. When the DNA is not being transcribed it is densely packed in nucleosomes. The results show that growth factors are able to open several regions while the hormones are more active in closing them. All the hormones and growth factors are more or less affected by FoxA1.

1.2 Norwegian

Mitogener er substanser som har evne til å indusere celledeling. Når disse bindes sine respektive reseptorer starter en kaskade av cellulære signaler som kan ende i nukleus med transkripsjon av gener og celledeling. Disse signalene er vanligvis nøye regulert, men i kreftceller virker ikke denne reguleringen og resultatet er ukontrollert cellevekst. Brystkreft er den vanligste kreftformen blant kvinner i den vestlige verden. Det finnes flere ulike subtyper

brystkreft, avhengig av hvilke reseptorer som er uttrykt, hver med sitt sykdomsbilde, behandling og prognose. FoxA1 er et protein som fungerer som en pionerfaktor, med sin evne til å åpne kromatinet slik at eksempelvis østrogenreseptor kan binde seg til østrogen responsivt element (ERE) på DNA'et og starte transkripsjon av østrogen mål-gener. Det er ulike måter å behandle brystkreft på, avhengig av typen, men det er ikke alltid behandlingen virker uten at man forstår hvorfor. Det er derfor spennende å se om hormoner som østrogen eller androgen, eller vekstfaktorer som heregulin eller epidermale vekst faktorer kan åpne kromatinet på egenhånd eller om det er avhengig av FoxA1. Dette prosjektet ble utført med en metode som heter FAIRE, som isolerer nukleosomfritt DNA, den delen av DNA som antas å være i aktiv transkripsjon. Når DNA ikke er i transkripsjon er det pakket tett i nukleosomer. Resultatene viser at vekstfaktorer kan åpne flere regioner, mens hormonene er mer aktive i å lukke dem. Alle hormoner og vekstfaktorer viser seg å affekteres av FoxA1 i større eller mindre grad.

2 Abbreviations

DMEM - dulbeccos modified eagel medium

FBS - fetal bovine serum

SFBS - stripped fetal bovine serum

ER - estrogen receptor

AR - androgen receptor

EGF - epidermal growth factor

EGFR - epidermal growth factor receptor

Her2 - Heregulin

Her2R - Heregulin receptor

PCR - polymerase chain reaction

FAIRE - formaldehyde assisted isolation of regulatory elements

EtOH - Ethanol

NaCl - sodium chloride

SERM - selectively estrogen receptor modulators

AI - aromatase inhibitor

3 Introduction

3.1 The normal female breast

The female breast consists of mammary gland, a specific type of apocrine gland. It consists of about 20 compartments, alveoli. The alveoli are lined with milk-secreting cells called cuboidal cells, a type of epithelial cells formed as cubes. Myoepithelial cells surrounds the cuboidal cells and together these cells are called the lobular cells, creating the lobular gland (Sopel 2010), visualized in fig 1. Myoepithelial cells contracts by stimulation of the hormone oxytocin leading to lactation through milk ducts to the nipples. The milk duct is called lactiferous duct and is lined with epithelial cells surrounded by myoepithelial cells (Sopel 2010). Adipose tissue and connective tissue is surrounding the lobules and the ducts, and also blood vessels and lymphatic vessels.

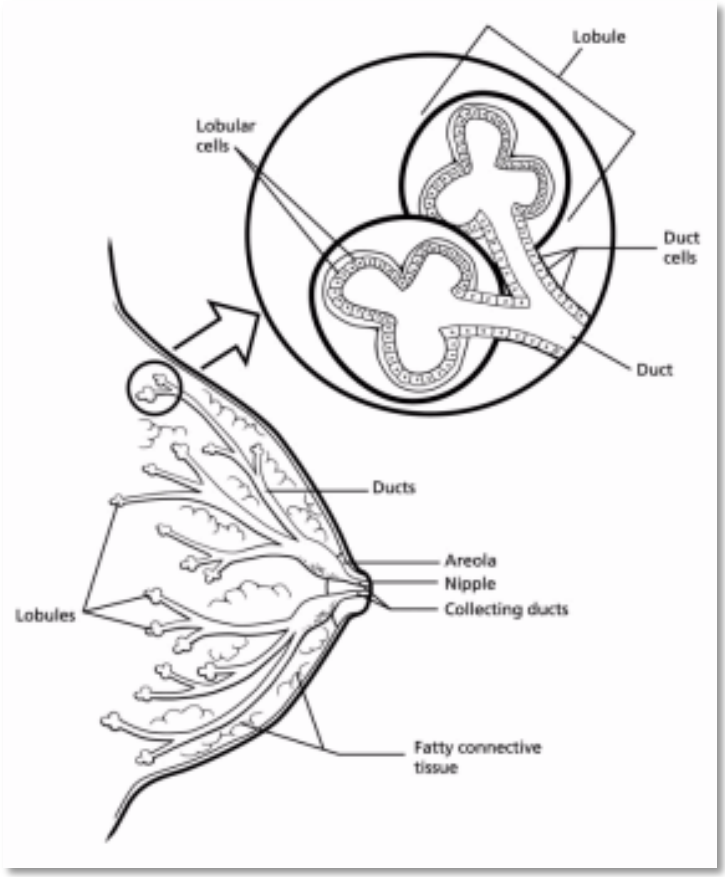

Fig 1: The anatomy of a normal breast, visualizing the lobulis and the ducts. (American Cancer Society 2014)

3.2 Gene transcription

Housekeeping genes are expressed at all times, but a gene that is just transcribed when needed is called regulated transcription. Transcription is closely regulated by intracellular signals and is a result of extracellular signaling.

3.3 Mitogens

A mitogen is a substance, like a hormone or a growth factor, which induces cell proliferation. Every cell uses receptors to mediate signals from the extracellular space to the intracellular space. The specific substance binding to their receptor is called a ligand and can be, among others, hormones and growth factors. This binding starts a pathway of signal transduction in which leads to different results depending on the ligand and receptor. In many cases the pathway leads to gene transcription and cell proliferation.

3.4 The Ras-MAP kinase pathway and PI3K/Akt/mTOR pathway

The two pathways are both activated by mitogens that controls the cell cycle. In a normal cell these signaling pathways are strictly controlled and loss of normal signaling is present in all human tumors. Binding of ligand to the receptor activates the receptors by cross-phosphorylation and thereby activating either MAPK pathway or PI3K pathway. This activation initializes a signal transduction pathway leading to the nucleus. Upon signal arrival in nucleus the transcription factors are activated and target genes are transcribed. In a tumor cells this transcription can imply replication of tumor DNA and cell division with an exponential growth potential.

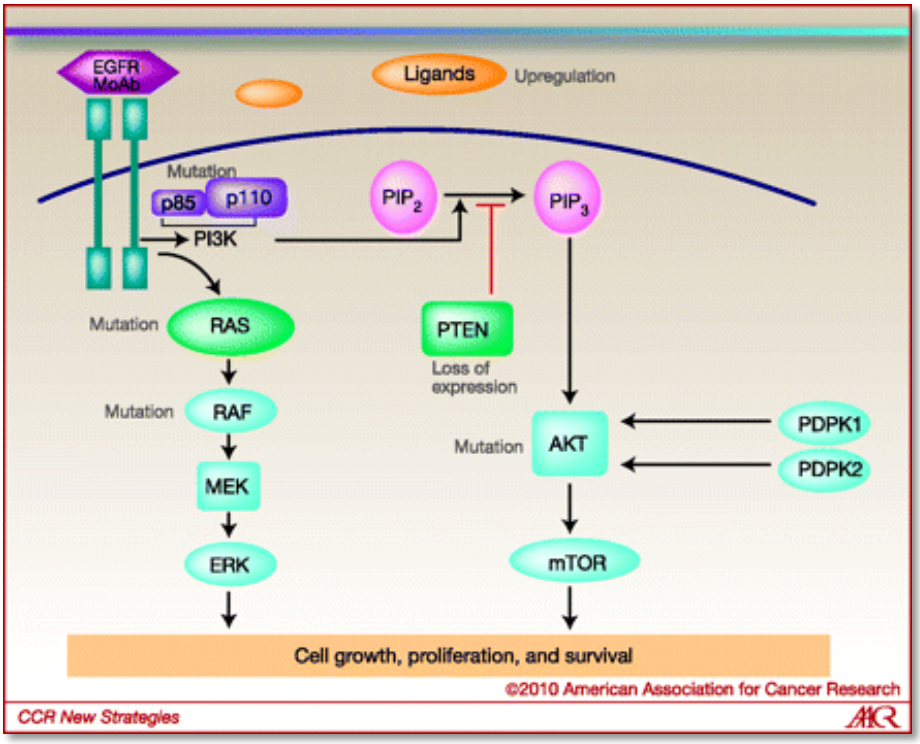

Fig 2: An overview of PI3K/MAPK pathway (Clinical Cancer Research 2014).

3.4.1 The Ras-MAP kinase pathway

The Ras-MAP kinase pathway is the most studied pathway in biology. Ras is a small GTPase located in the intracellular side of the plasma membrane, anchored by a lipid and relays signals regarding cell survival, proliferation and differentiation from receptor tyrosin kinases at the cell surface in to the nucleus (Lin et al. 2014). When Ras is activated Raf is recruited to the plasma membrane and activated. This activation activates Mek, which activates Erk. Activated Erk translocate to the nucleus where it phosphorylate several targets proteins, many of them transcription factors. Myc is a gene regulatory protein and one of the early genes targeted by Ras. Myc increases the expression of several delayed response genes, such as cyclin D1which leads to increased G1-Cdk-activity. Activated G1-Cdk will phosphorylate and inactivate proteins in the Rb-family, releasing the gene regulatory protein E2F. The E2F protein activates transcription of G1/S-genes, including the gene for G1/S-cyclin and S-cyclin. Due to this activation, G1/S-Cdk (E-cyclin) and S-

Cdk (A-cyclin) is produced and promotes additional phosphorylation of Rb-proteins, making a positive feedback loop. E2F stimulates transcription of its own gene, making another positive feedback loop. Activation of cyclin E and cyclin A will lead to both DNA synthesis and cell proliferation.

Studies show that Ras is hyperactivated in over 50% of cases of cancer. Several signal cascade pathways is dependent of Ras, making Ras an oncogene when not regulated properly (Christoffersen 2013; Kleiveland 2013).

3.4.2 The PI3K/AKT/mTOR pathway

Phosphoinositide 3-kinase (PI3K) binds activated receptor tyrosin kinase through Src homology 2 (SH2) interaction domain, activating the kinase. PI3K phosphorylates the inositol 3-position of phosphpoinositol resulting in $PI(3,4,5)P_3$. PIP_3 recruit signaling proteins with a pleckstrin homology (PH) domain to the membrane. The signaling proteins are the serine/threonine-kinases Akt and PDK1. They are both recruited to PIP_3 domains through their PH-domain. Akt is activated by phosphorylation by PDK1 and mTOR. Akt then dissosiates from the membrane and phosphorylates the target protein (Christoffersen 2013).

3.5 Breast cancer

Cells usually undergo apoptosis or cell death after some time, but cancer cells avoid this mechanism and keeps reproduce in defiance of the normal restraint on cell growth and division creating a tumor. A tumor can be benign, non cancerous, or malign, cancerous. Malign tumors can invade nearby tissue and metastasize to other parts of the body through lymphatic vessels and the blood stream (National Cancer Institute).

Breast cancer is a rapidly growing disease in the western world and is ranked as the most common cancer in females (Jemal et al. 2011). It is a

heterogenouse disease, aggression and proliferation varying from woman to woman (Rouzier et al. 2005) and a genetic abnormality is always the cause (Vogelstein & Kinzler 2004). These abnormalities can be inherited (5-10%), but most common they arise due to age and environmental factors (85-90%) (Breastcancer.org 2013). Breast cancer normally starts in the lobules of the breast, the glands producing milk, or the duct (Brisken 2013). Tumors arising from the ducts are called ductal carcinomas and are the most common (Keller et al. 2012), while tumors arising from the lobules are called lobular carcinomas. The first symptoms of breast cancer may be a lump in the breast or a change in the nipple shape or position. The smallest tumors, however, are only noticeable with mammography, but the screening effect of mammography is still debatable. Breast tumors arise from epithelial cells and are called carcinomas. The carcinomas are divided according to localization and stages. About 75% of breast carcinomas start in the ducts, while approximately 25% starts in the lobules. Before the tumor spreads to the tissue, it is described as "in situ", meaning it has not invaded the surrounding tissue, as shown in fig 3.

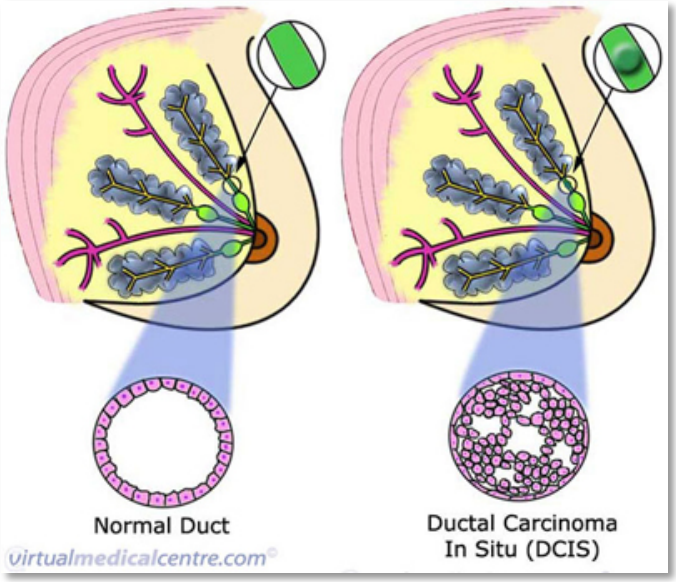

Fig 3: The development of ductal carcinoma in situ (Justoncology.wordpress.com 2011)

When the tumor invades the surrounding tissue, the carcinoma is describes as "invasive", as shown in fig 4.

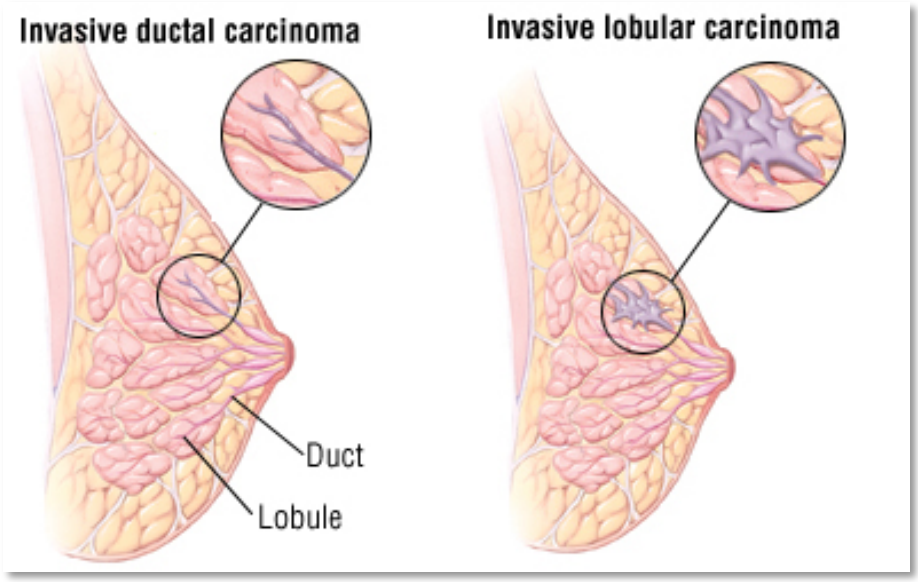

Fig 4. About 75% of breast cancers start as ductal carcinomas, while about 25% starts as lobular carcinomas. (Community Connect to Research 2012)

Types of breast cancer are defined according to which receptors that are over-expressed by tumor cells. The tumor cells are classified as positive if the specific receptor is over-expressed and negative if the tumor cells are not over-expressed for a specific receptor. The tumor cell is either endocrine receptor (estrogen or progesterone receptor) positive, heregulin (HER2) positive, negative for all receptors above (triple negative) or positive for all receptors above (triple positive) (Carey et al. 2007).

3.6 ER - Estrogen receptor

Estrogen is a steroid hormone and the primary sex hormone in women. It is mainly produced in the ovaries and, in small amounts, other parts of the body, like fat cells, liver cells adrenal glands and breasts. Estrogen is responsible for the normal female development. It is synthesized from cholesterol via androstenediol to estradiol, the most potent estrogen, by the enzyme

aromatase.

Estrogen is the ligand for the nuclear receptor, estrogen receptor (ER) which acts as a transcription factor to regulate cell division (Hurtado et al. 2011). Estrogen binds to estrogen receptor within the cell and the receptors dimerize and translocate into the nucleus where the complex binds to estrogen-responsive elements (ERE) in the DNA. ERE are specific regions in the promoters for estrogen target genes. About 75% of breast cancer overexpress ER (Dowsett 2001). ER mediates the response of estrogen in mammalian cells and starts transcription of target genes (Khan et al. 1998). Overexpression of ER leads to increased induction of cell proliferation and the most effective treatment is to inhibit the signaling transduction by this nuclear receptor.

Patients with an overexpression of ER have a better outcome with lower rate of recurrence within the first several years of treatment (Platet et al. 2004). ER is not only correlated to breast cancer, it is also found in normal breast epithelial with expression varying throughout the menstrual cycle, but then expressed at a normal level (Khan et al. 1998). There are two types of ER, α and β. These two are similar in structure and function, but only ERα significance in breast cancer is known (Sommer & Fuqua 2001). Estrogen can also bind ERβ in which together with GPR30 give a nongenomic responce, also shown in fig 2.

3.7 AR - Androgen receptor

Androgen is a collection of steroid hormones and the primary sex hormone in men and is produced in the testis. Women produce testosterone in the ovaries.

Like ER the androgen receptor is a ligand dependent transcriptional activator, a nuclear receptor, shown in fig 3, (Mehdipour et al. 2011). Its target genes is a protein functioning as a steroid-hormone transcriptional factor (NCBI 2014).

AR can be present in breast cancer independent of subtype-definition by receptors, and is expressed in up to 90% of ER-positive and 55% ER-negative tumors (Lakis et al. 2014). The prognosis value of AR in ER-negative tumors is less clear, but in ER positive cells AR is reported to have an inhibitory effect on proliferation (Hickey et al. 2012). It is suggested that AR occupies similar cis-regulatory elements as ER, thereby inhibiting ER transcription, and data analysis support these thesis (Robinson et al. 2011).

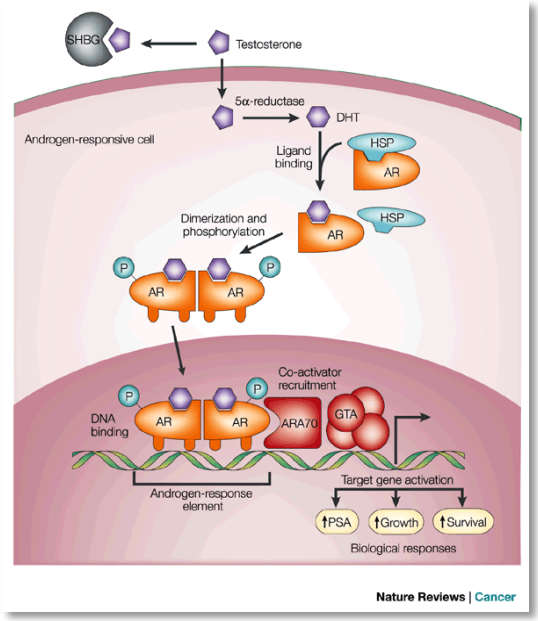

Fig 5: The actions of AR (Nature.com 2001).

3.8 Her2-receptor

20-30% of breast cancer tumors are Her2 positive (Sanpaolo et al. 2012). Her2 is a oncogene, also called ErbB2, and its receptor is a member of the epidermal growth factor receptor (EGFR) family, a receptor tyrosin kinase, located at the cell surface (Moasser 2007). Her2-receptor exists initially as monomers, but form receptor heterodimers, usually with Her3, upon binding of the ligand heregulin. Her2 cannot form homodimers, implying that there is no known ligand that directly binds Her2 (Rubin & Yarden 2001). Upon dimerization the MAP kinase pathway or PI3K pathway is activated by phosphorylation, as shown in figure 6, leading to transcription of genes regulating the cell cycle progression, resulting in cell prolifereation. Tumors that are positive for Her2 can be treated with monoclonal antibodies, like Trastuzumab. These antibodies reduce the growth of the tumor by inhibiting

Her2 function (Gajria & Chandarlapaty 2011; Sorensen et al. 2013). Although these treatments, patients with Her2 positive tumors still have lower overall survival rate (Chandarlapaty & Modi 2011; Weigelt et al. 2010).

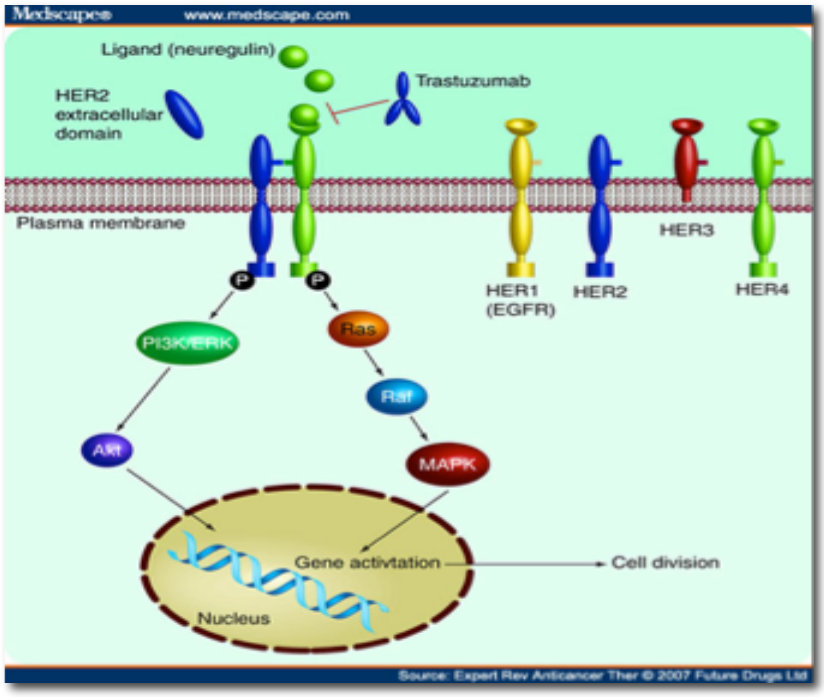

Fig 6: Her2R/EGFR and their cascades (Perik et al. 2007)

3.9 EGFR - EGF-receptor

EGF receptor is also caller Her1/ErbB1 receptor. The receptor is also a tyrosin kinase, located on the cell surface, like the Her2 receptor. EGF receptor belongs to the tyrosin kinase family. Unike Her2 receptor, Her1 receptor homodimerizes upon binding of the ligand EGF. Dimerization is necessary for the receptor tyrosin kinases to cross-phosphorylate each other by activated kinase domain. This activation starts a cascade of actions, phosphorylation-driven signal transduction ending in cell proliferation. EGFR is found in, amongst other, breast cells and regulates cell proliferation, differentiation and cell survival. Studies have revealed that EGFR is over-expressed in triple negative breast cancers, showing the importance of improving anti-EGFR therapy (Brand et al. 2014). There is also indications of

a link between tumor resistance to tamoxifen and increased EGFR expression (Moerkens et al. 2014).

3.10 Treatment of breast cancer in patients

There are several ways of treating breast cancer and it is often necessary to combine treatments. It is important for at treatment plan to reveal as much information as possible from the tumor. Triple diagnostics is therefore the first step. Triple diagnostic includes a clinical examination, imaging examination, mammography and ultrasound, and a needle biopsy (Schlichting 2009). The information gathered in these examinations is the background for the treatment plan. Sometimes it is desirable to start with radiation to reduce tumor size, prior to surgically removal. Depending on the size of the tumor, the surgeon will remove the whole breast or just the tumor with tissue around, called breast-conserving surgery. Analysis of the removed tissue will reveal if it is necessary to remove more of the surrounding tissue. After surgery, the treatment is radiation, chemotherapy or hormone therapy, depending on the tumor type. In this thesis I will only discuss the hormonal therapy.

The aim of hormonal treatment is to reduce the risk of a new tumor. The hormonal treatment of choice will depend on the tumor type, which receptors are present in the breast cancer cells. For patients with breast cancer cells who are positive for estrogen receptor (ER) the optimal treatment is inhibiting the action of estrogen and thereby inhibiting transcription of the genes in question. There are two ways of inhibiting estrogen action, beside surgical removal of the ovaries, selective estrogen receptor modulators (SERM) and aromatase inhibitors (AI). Aromatase is an enzyme that converts androgenic hormones to estradiol, as shown in fig 7, the main source of estrogen in breast cancer. The treatment depends on whether the woman is premenopausal or postmenopausal (Dowsett 2001). Estrogen deprivation in premenopausal women will give several unwanted side effects. SERM, like Tamoxifen, binds to ER with a higher affinity than estrogen, thereby inhibiting binding of estrogen and preventing estrogen response.

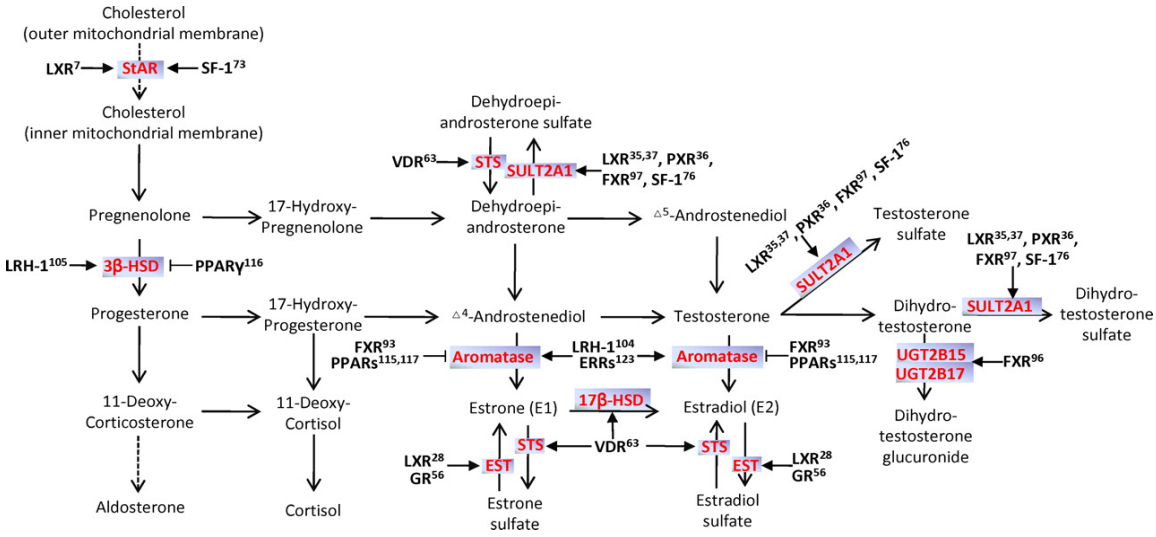

Fig 7: Cholesterol is converted throught several steps, included to progesterone, androstenediol and testosterone, until it is converted to estradiol by aromotase (Molecular endocrinology 2014).

As previously mentioned, the most effective way of treatment for ER positive breast cancer is to stop estrogen response. There are different ways of conducting this. Endocrine therapy drugs like tamoxifen and aromatase inhibitors are the most common. Patients with Her2 positive tumors can be treated with monoclonal antibodies which binds the Her2 receptors,

Cancer therapy can target several of the components of the signal transduction pathway. The ligands can be neutrialized before binding to receptors, the receptors can be inhibited by binding of antibody or inhibiting phosphorylation, intracellular second messengers can be bound by inhibitors and nuclear transcriptional factors initializing cell proliferation can be inhibited. However, several patients do not respond to these treatments, without an obvious reason (Hurtado et al. 2011), implying that it might be other factors contributing to these progression. An important discovery for in vitro studies is that the extra cellular matrix, and thereby 3D cultures, is necessary for the response of treatment for Her2 positive tumors (Weigelt et al. 2010).

3.11 MCF-7

The MCF-7 cell line is a breast cancer cell line derived from a 69-year old woman in Detroit. She had breast cancer that metastasized and the MCF-7 cells were taken from a pleural effusion revealing malignant adenocarcinoma (MCF7.com). MCF is abbreviation for Michigan Cancer Foundation and the cell line has proven useful for in vitro cancer studies because it still has the same properties as mammalian cells (Levenson & Jordan 1997). The MCF-7 cells are ER positive and AR positive (Osborne et al. 1987). Her2 is expressed, but not amplified, thereby characterized as Her2 negative.

3.12 FoxA1

FoxA1 is an abbreviation for forkhead box protein A1. The forkhead box proteins are transcription factors within DNA-transcription due to their "winged-helix" DNA-binding domain (Lam et al. 2013). They are known to open condensed chromatin upon binding, thereby probably functioning as pioneer factors (Hurtado et al. 2011). Many proteins associated with gene expression have a specific structural motif, the helix-turn-helix (HTH) motif, which is necessary for the protein to bind DNA (Alberts et al 2008). Between the to α-helices there is a short turn of amino acids and flanking this HTH-region are two loops ("wings") of polypeptide chains, shown in fig 8, making FoxA1 able to bind DNA with high affinity at specific sites, as a monomer (Cirillo & Zaret 2007). Cheung and Ruan proposed a model in Nature Genetics 43, 2011, supposedly of how FoxA1 interacts with the chromatin, presented in fig 9. The illustration shows how FoxA1 is supposedly necessary for the binding of estrogen receptor to the chromatin, and the end result in this illustration is how no target genes are transcribed if FoxA1 is not present. (Cheung & Ruan 2011)

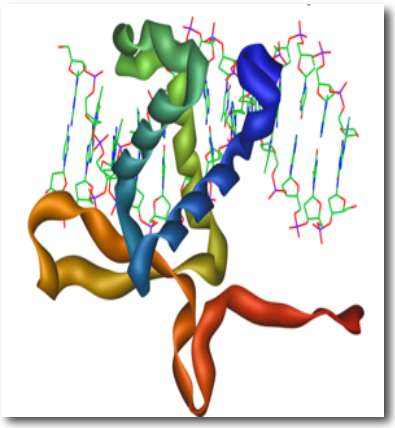

Fig 8: How FoxA1 is assumed to bind DNA through "winged shaped" helixes (Transcription Factor Encyclopedia 2011).

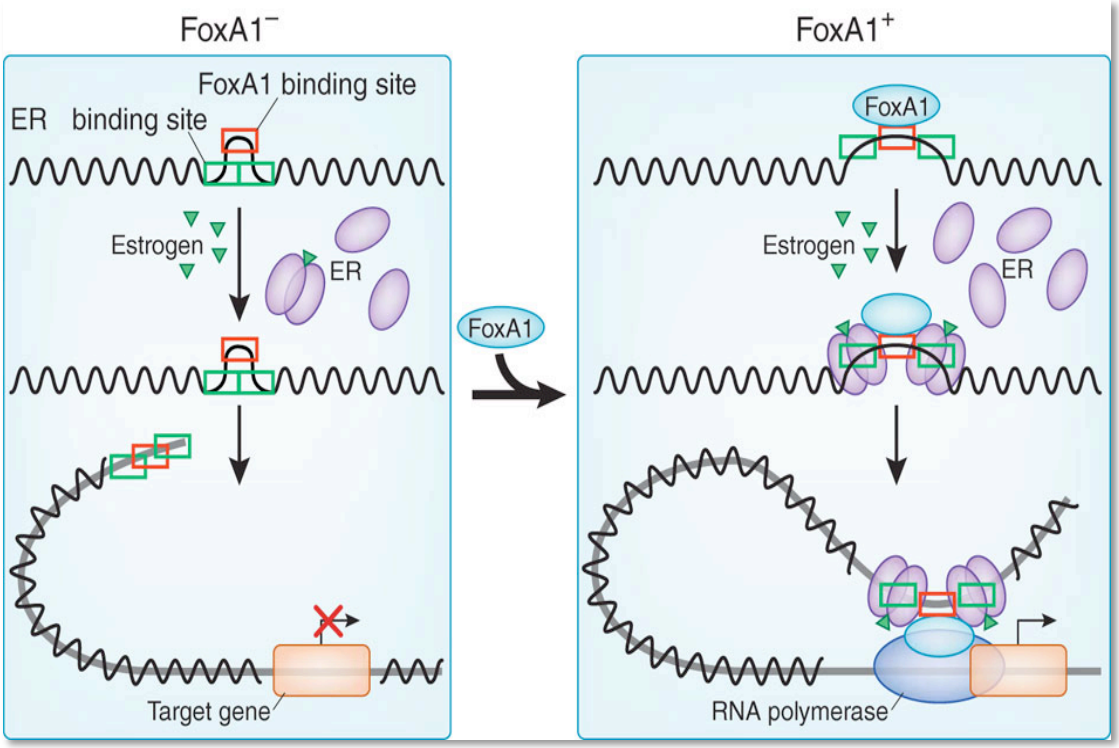

Fig 9: The proposed model for the role of FoxA1 in determining ERα-chromatin interactions. (Cheung & Ruan 2011)

3.13 Aim of the study

ER+ and AR+ positive tumors are widely represented in all breast cancer subtypes. It is necessary for ER/AR to gain access to the open chromatin before starting transcription, and it is assumed that FoxA1 is opening the chromatin. However, we don't know how different factors contribute to these functions, as shown in fig 10. There has been suggested that hormones or growth factors controls the expression of genes by influencing the opening of the chromatin or that this opening is regulated by a pioneer transcription factor.

Hypothesis:
Hormones or growth factors influence the opening of the chromatin.

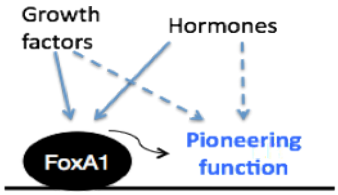

Fig 10: How are hormones/growth factors influencing FoxA1 in opening of the chromatin?

1. Is the opening of the chromatin regulated by growth factors and/or hormones?
2. Does FoxA1 open adjacent chromatin under different signals?

Due to these questions the design for the experiment were made. First, knock down of FoxA1 in half of the plates cultured, then treat the cells with different hormones or growth factors, as shown in fig 11, isolate the open chromatin with FAIRE technique, as shown in fig 12, and compare the results from sequencing.

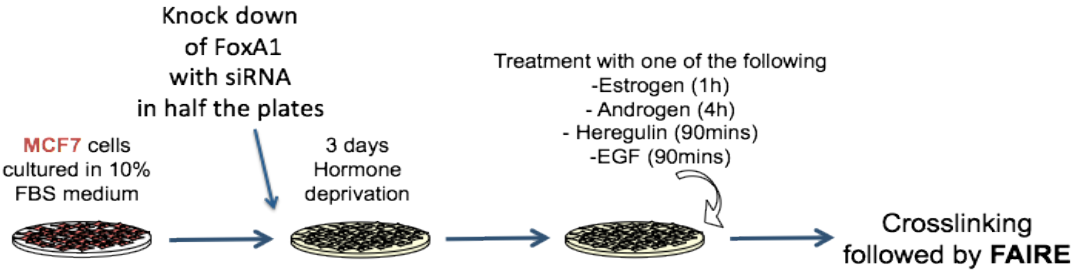

Fig 11: Experimental design step 1. Knock down of FoxA1 and treat the cells with hormones/ growth factors.

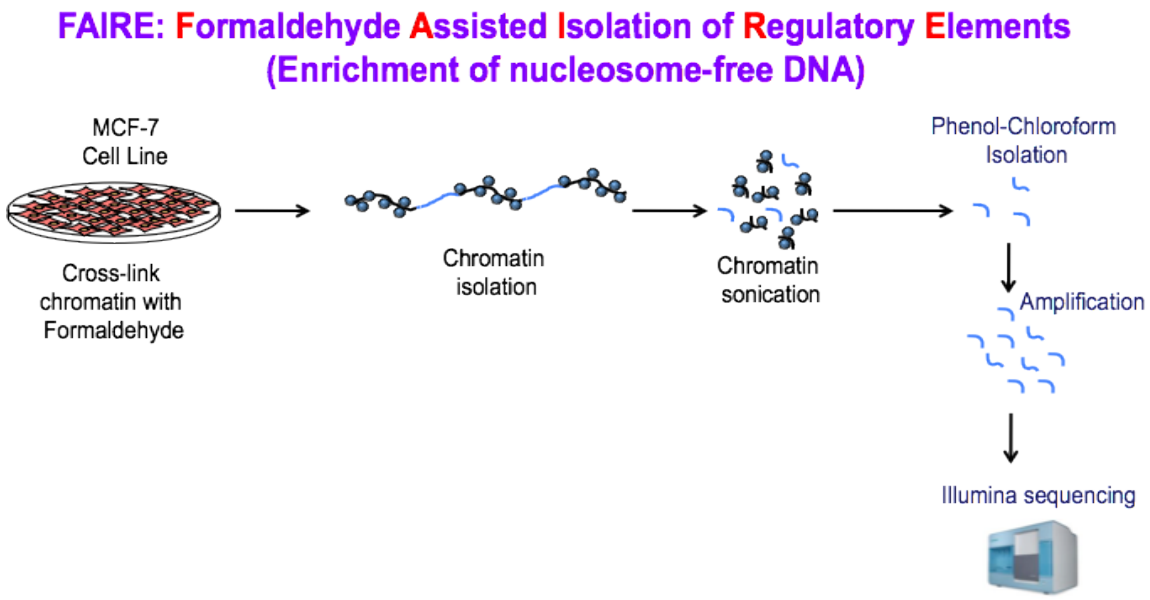

Fig 12: Experimental design step 2. FAIRE is a technique that isolates the nucleosome free regions, though of as open regions.

4 Materials and methods

4.1 Materials

4.1.1 MCF-7 cell line

The cells were taken fresh from a stock frozen at -170'C. The cells were thawed gently and distributed in a flask with 10ml 10% FBS red DMEM medium, and incubated at 37'C.

4.1.2 Growth medium

The medium used were phenol red DMEM with FBS to a total concentration of 10%. Phenol red DMEM is only used before transfection. When the cells were prepared for transfection, the medium was changed to clear DMEM added glutamax and pyruvat with charcoal stripped FBS to a concentration of 5%. When changing the medium 48h after transfection this concentration was changed to 2% for the plates supposed to be treated with growth factors and remained 5% for the plates supposed to be treated with hormones. The phenol red can interference with experiments involving estrogen treatment as it mimics estrogen (Berthois et al. 1986).

4.1.3 Treatments

The different treatments were diluted in clear DMEM. R1881 is a synthetic androgen, called metyltrienolone. It is the most potent synthetic androgen for binding to AR and was added to a total dilution of 1nM the plate for 4 hours. Estradiol (E2) is the most potent estrogen for binding to ER. Estradiol were added to the plate to a total dilution of 10nM for 1 hour. Vehicle is ethanol, added to a total concentration of 10nM to the plate, also for 1 hour. EGF was

added for a total dilution of 100ng/ml in the plate, for 90 minutes. Heregulin was added to a total concentration of 25ng/ml to the plate for 90 minutes. All incubations were in 37'C.

4.1.4 Reagents, buffers and chemicals

For a specific overview of buffers and treatment dilutions, see appendix.

Cell culture

clear DMEM (1X) - Dulbeccos Modified Eagel Medium, added glutamine and pyruvat - Life Technologies ™

clear DMEM (1X) - dulbeccos modified eagel medium - Life Technologies ™

red DMEM (1X) - dulbeccos modified eagel medium, with phenolred - Life Technologies ™

FBS - fetal bovine serum - Life Technologies ™

SFBS - charcoal stripped fetal bovine serum - Life Technologies ™

0,25%Trypsin-EDTA (1X) - Life Technologies ™

Optimem - redused serum medium, no phenol red - LifeTechnologies™

Lipofectamine RNAiMAX - invitrogen - Life Technologies™

siRNA - invitrogen - Life Technologies™

siNT - invitrogen - Life Technologies™

Formaldehyde, 37% - sigma aldrich

Glycine - invitrogen - Life Technologies™

protease and phosphatase inhibitor - Thermo scientific

PBS - phosphate buffered saline

Electrophoresis

DNAzapp 1 and 2 - ambion Life Technologies™

Tris Acetate-EDTA buffer - TAE (1X) - sigma aldrich

Agarose - sigma aldrich

SYBR® gold nucleic acid gel stain - invitrogen - Life Technologies™

1kb plus DNA ladder - invitrogen - Life Technologies™

Glygerol loading buffer - 50% glycerol and 50% Tris-HCl

Bio-rad mini-sub cell gt.

Bio-rad power pac basic sets current.

Protein isolation

lysis buffer - see table for details

NaCl 5M - NaCl from Sigma Aldrich

DTT - Life Technologies ™

Bradford solution for protein detection - AppliChem

Tecan Infinite f200 pro and the software i-control 1.7

Western blot

PBS (1X)

Mini-PROTEAN® TGX™ Precast Gels

Precision plus protein TM dual color standards - ladder

Mini-PROTEAN® Tetra Cell for Mini Precast Gels

Bio-rad trans-blot turbo transfer system

Trans-blotR Turbo TM Mini PVDF Transfer packs

methanol - sigma aldrich

TBS-Tween 20 - Thermo Scientific

skimmed milk-powder - sigma aldrich

anti-FoxA1 - primary mouse antibody - Abcam

anti-RPL13a - primary rabbit antibody - Abcam

second mouse antibody and second rabbit antibody - Abcam

Film for exposure - GE healthcare amersham hyperfilm ™ EacL

SuperSignal West Femto - Chemiluminescent Substrate

SuperSignal West Pico - Chemiluminescent Substrate

FAIRE

Eppendorf tubes 1,5ml

PI - Life Technologies ™

DTT - Life Technologies ™

phenol:chloroform:isoamyl alcohol 25:24:1 - sigma aldrich

Phase lock gel light - 5 prime

ProteinaseK - ambion, Life Technologies ™

RNase - Quiagen

100% EtOH - sigma aldrich

Eppendorf thermo mixer c

Quantification

Quant-IT buffer - Invitrogen molecular probes - Qubit dsDNA HS buffer

Quant-IT dye - Invitrogen molecular probes - Qubit dsDNA HS reagent

Quant-IT standards 0 and 1 - Invitrogen molecular probes - Qubit dsDNA HS

Quant-IT assay tubes - Invitrogen molecular probes

Fluorometer - Invitrogen qubit fluorometer

Library preparation

MicroPlex Library Preparation^{TM} kit - Diagenode

DNA extraction after gel - MinElute Gel Extraction Kit from Qiagen with MinElute spin coloumn from Qiagen

Invitrogen dynal bead separator

PCR - bio-rad s1000 tm thermal cycler

Pipets

Eppendorf research plus 0,5-10ul, 2-20ul,10-100ul, 100-1000ul with VWR filtertips 10ul, 20ul, 100ul, 1000ul.

Pipetboy comfort with stripettes 5ml, 10ml, 25ml, 50ml - Sarstedt maskiner

Transferpipettes 1,5ml - Sarstedt

Other equipments

Eppendorf tubes 1,5 ml

BD Falcon flasks, 250ml and 600 ml

BD Falcon petri dishes, 10 cm.

Sarstedt tubes 15 ml and 50 ml

sonicator - Bioruptur R plus and Bioruptur watercooler from Diagenode

Suction - integra vacusafe

Illuminator - Transilluminator DR-195M

Waterbath - Grant GD100

Bio-rad molecular imager ChemiDoc XRS imaging system with the software Image Lab3.0.1

Agilent 2100 Bioanalyzer

Agilent technologies high sensitivity DNA reagents and chips

Labgard class II biological safety cabinet

Eppendorf centrifuge 5810

nuare autoflow ir direct het CO2 incubator

Stuart see-saw rocker SSL4

Stovall life science - the belly dancer

Leica dmil led microscope

Bürker chamber for counting cells.

4.2 Methods

4.2.1 Grow cells

The MCF-7 cells were grown in phenol red DMEM with 10% FBS for 3 days to a confluence of about 80%. After this period, the medium was removed and the cells were washed with 10ml PBS. The PBS was aspirated. To detach the cells from the flask surface 2ml Trypsin EDTA were added. After 5 minutes of incubation in 37'C the cell suspension were transferred to a tube, added 10ml phenol red DMEM 10%FBS and centrifuged for 5 minutes by 10.000g. The cells formed a pellet and the medium was aspirated. The pellet was resuspended in 10ml clear DMEM 10%SFBS and 7,5ul transferred to Bürker chamber for counting. A Bürker chamber consist of 9 big A squares with inner frame. In this experiment 3 A squares were counted and the average was used for calculating the number of cells pr ml.

In this experiment there were plated 1.500.000 cells in each plate, in clear DMEM 5%SFBS - charcoal stripped FBS. 1ml of cell suspension and 9ml of clear DMEM 5%SFBS were added each plate. It is important to avoid phenol red DMEM from this point, since the phenol red can mimic the action of estrogen, it binds to ER with an affinity of 0.001% of estrogen, and can interfere with the results (Berthois et al. 1986).

4.2.2 Reverse transfection - to knock down FoxA1

Transfection of MCF-7-cells is to implement new RNA to the cell, in this study short interference RNA - siRNA.

At the first experiment, the cells were transfected in a regular way according to common procedure. This turned out not to be a success since the MCF-7 cells are sticky and it seemed impossible to separate them down to singles. The transfection will not be successful if the cells are dimers or more. A

reverse transfection will not be so dependant of single cells, some dimers will not interfere with the process.

Knock down in 10 cm plates. 1.500.000 cells in each plate.
14 plates - 2 different transfections, 6 different conditions + 2 for knock down controls. Added 1ml Optimem to each plate. Added respectively 20ul siRNA - either siFoxA1 or siNT to each 7 plates. Added 43ul Lipofectamine to each plate on top of droplet. Mixed gently to hand and left for 40 minutes in roomtemperatur before adding 1ml cells in 10ml clear DMEM.

Lipofectamine is a lipidreagent and when mixing it with siRNA they form a RNA/lipid complex. When adding the cells to the mix the positively charged cation in the complex will bind the negative charged DNA in the cells. The cell will take in the complex by endocytic pathway. Within the cell, the complex is degraded and the nucleic acid binds the RISC complex resulting in degradation of the targeted mRNA (Hammond 2005).

2 days (48h) after transfection the medium was changed into 5% clear DMEM for plates that would be treated with hormones and 2% clear DMEM for plates that would be treated with growth factors, and knock down control.

4.2.3 Treatment of the cells

6 conditions
Hormones: Estrogen (E2), androgen (R1881) and control (EtOH)
Growth factors: epidermal growth factor (EGF), Heregulin and control (2% clear DMEM).

The estrogen plates were added estradiol to a final concentration of 10nM and incubated for 1 hour. Estradiol (E2) is the most potent estrogen for binding to ER. The androgen plates were added R1881 to a final concentration of 1nM and incubated for 4 hours. R1881 is a synthetic androgen, called

metyltrienolone and is the most potent synthetic androgen for binding to AR. The heregulin plates were added heregulin to a final concentration of 25ng/ml and incubated for 90 minutes. The epidermal growth factor plates were added epidermal growth factor to a final concentration of 100ng/ml and also incubated for 90 minutes. All the plates were incubated in 37'C.

After treatment the medium were aspirated and crosslinked with formaldehyde to a final concentration of 1%, for 7 minutes. The 2 plates for knock down control were not crosslinked. The formaldehyde induces DNA-protein- and protein-protein crosslinks and is an effective method to bind nucleosome rich DNA. This is the key element in isolation the naked DNA, thought of as the regulatory elements, which is the basis for the next step called FAIRE. FAIRE was performed on all the treated cells after Western Blot of the control-plates, to make sure knock down was successful. To quench formaldehyde, 375ul glycine 2.5M was added and left in roomtemperatur for at least 5 minutes. The fluids were then aspirated and washed with 600ul cold PBS on ice x 3. The cells were scraped with500ul cold PBS+PI and collected in tubes prior to freeze at -80'C.

4.2.4 Protein isolation for Western Blot - knock down control

This step was only executed for the 2 plates functioning as knock down control - to make sure knock down of FoxA1 was successful. The pellets were resuspended in 400ul lysis buffer and kept 5 minutes on ice. They were sentrifuged 2000g for 5 minutes and supernatant was discareded. The pellet were resuspended in 87,3ul lysisbuffer and 1,7ul NaCl 5M and the samples rotated at 4'C for 1hour. The samples were then sentrifuged at 14.000g for 15 minutes and the pellet discarded. The supernatant were pipetted into a fresh tube. When performing western blot it is important to load the same amount of protein in each lane to be able to see if there is a difference of expression. Due to this, the protein amount was quantified with Bradford assay at 450nm.

4.2.5 Western blot

It is important to denaturate the proteins before loading the samples on to the gel. If the proteins are in its nature state, they will not be able to move through the gel. To denaturate the sample it is necessary to boil the samples at 95'C for 5 minutes, just prior to loading. 20ug protein pr sample + dual color ladder were loaded and the gel ran at 100V for about 1hour, until the dual color marks started to move out at the bottom. The gel chamber was opened and the proteins in the gels was blotted to PVDF membrane, using a Bio-rad trans-blot turbo transfer system.

The PVDF membrane is very hydrophobic and needs to be activated with methanol to gain full binding capacity to proteins. After methanol activation the PVDF membrane were shaked in 2% skimmed milk for 1hour in room temperature. The skimmed milk is necessary for its protein contents. The PVDF membrane binds proteins unspecific, and as the antibodies used to detect the proteins we are looking for in itself is proteins, they will bind the membrane as well as the desired proteins. The skimmed milk proteins bind to the PVDF membrane, leaving no other room for the antibodies to bind but the desired proteins.

The membrane contains to different proteins of interest, the FoxA1-proteins and the loading control, RPL13a-proteins. It is necessary to cut the membrane in two in order to add the right antibody to the right part. The membrane was cut at 75 kD mark and 25 kD mark. FoxA1 is around 50 kD. The part of the membrane under 25 kD mark contain the ribosomal protein, RPL13a.
6ml of first antibody solution were made for each, in dilution 1:500. 12ul of antibody were added to 6ml of skimmed milk. The FoxA1 part of the membrane was added the mouse first antibody and the RPL13a rabbit

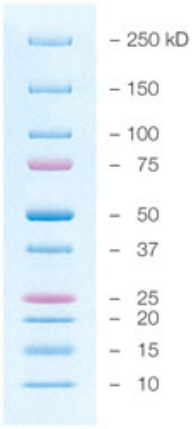

Fig 13: kD marks of precision plus protein dual color ladder.

first antibody. The membrane with the antibodies was left over night in 4'C on a tilter.

The antibodies can be used several times, so they were recovered by pipetting and transferred back to the Falcon tube. The membranes were washed 3 times with TBS tween, 10 minutes each wash, to remove excess antibodies. Secondary antibodies were added, in dilution 1:20.000 in 5ml skimmed milk, and left on shaker in room temperature for 1h. The secondary antibodies were removed and TBS tween were added for washing 4 times within an hour.

To see the bands a developing technique was used. In the dark room a solution of Femto were added to the FoxA1 membrane and a solution of Pico were added to the RPL13a membrane. The Femto is highly more sensitive than Pico, detecting low femtograms amounts of protein (Thermo Scientific). The Kodak R developing film was exposed to the membrane for different timepoints, to find the optimal time for exposure, 1 min, 30 sec, 20 sec, 10 sec, 5 sec and 2 sec. After development, the section containing the RPL13a will show if the same amount of protein is loaded in both lanes and if the knock down of FoxA1 was success.

4.2.6 FAIRE

FAIRE is abbreviation for formaldehyde-assisted isolation of regulatory elements, the part of the DNA that is active at the specific timepoint (Gilfillan et al. 2012). Studies confirm that DNA isolated with this approach corresponds to active regulatory regions (Giresi et al. 2007).

The pellets were resuspended in 250ul lysisbuffer and sonicated in waterbath sonicator for 15 mins. During the sonication the DNA ruptures and the naked DNA, though of as the regulatory elements, breaks away from the complexes

made under crosslinking. After sonication the samples were centrifuged at 14.000rpm for 15mins at 4 'C. The DNA is more stable at this temperature.

An input control of 5ul was taken out before the phenol chlorophorm extraction. The same amount of phenol chloroform as sample were added, the solution vortexed and transferred to a phase lock tube before centrifugation for 5 minutes at 11.000rpm room temperature. The phase lock tube separates the organic phase from the aqueous phase with a gel and thereby eliminates contamination of interphase proteins (5 PRIME). The phenol chloroform extraction and centrifugation were repeated 3 times and the product of the extraction, the naked DNA, was put on ice. To remove any residues of RNA, the samples were treated with 1ul RNase, ribonuclease, to every 100ul sample for 40 minutes in 37'C. RNase is an enzyme that degrades RNA. After treatment with RNase, the samples were added proteinase K, 1ul to every 100ul sample and put in waterbath for 16 hours to decrosslink. The proteinase K inactivates the RNase and degrades any left proteins, while decrosslinking is to remove any covalent interactions still present after the crosslinking.

The sample was then spun down and DNA precipitated with NaCl to a final concentration of 200mM and 100% ethanol. 1ul glycogen was added to the sample as well. Glycogen binds DNA and makes the DNA pellet visible. The DNA amount is so low that no pellet is visible without glycogen. Cold EtOh 100% was added as last, double amount as sample before the samples was left for 30 minutes in -80'C for precipitation. DNA precipitates in the combination of NaCl and ethanol. After precipitation the NaCl is removed. The samples are first centrifuged, supernatant discarded and new cold EtOH is added. After a new round of centrifugation, the supernatant is removed and the pellet air-dried. The NaCl is now removed and the DNA is resuspended in warm Tris-HCl, a weak buffer. The samples are kept on ice and frozen in - 20'C.

4.2.7 Quantification

The quantification of DNA is done with Qubit and will tell how successful the experiment was by measuring the difference between the input control and the FAIRE product. The measurement with Qubit is calculated from a standard curve which is made with the standards 0 and 1 prior to measurements with the samples. The measure is photometric. The measuring mix is made with 199ul Quant-IT buffer and 1ul Quant-IT dye. The samples were diluted in the mix, 199ul of mix and 1ul sample. The standards, 0 and 1, were diluted in 190ul mix and 10ul standard.

4.2.8 Electrophoresis

After confirming successful FAIRE experiment the DNA were separated by bandsize using electrophoresis. The agarosegel was made as high as 2% to be able to distinguish between the smaller molecules. SYBR gold were used as fluorescent stainer as it is far more sensitive than Ethidium Bromide and proved to be a good choice as the DNA product was very small. The ladder used was 1kb. To avoid smear, and then misinterpretation of the bands, from the regular blue loading dye, a loading buffer with 50% glycerol and 50% Tris-HCl was made. 12ul of sample was mixed with 3.58ul of the glycerol loading buffer. During loading it was only possible to see a change of light fraction where the samples were loaded. The gel was run at 30V for 3 hours as a lower voltage separates the bands better. After the run the 200kb bands were cut, approximately at 150kb and 250kb, on a trans illuminator, and purified with Gel Extraction kit. The eluted DNA was quantified again.

4.2.9 Library preparation

DNA library were made with the use of a library preparation kit and PCR. The steps in library preparation are template preparation, library synthesis, library

amplification with indexing and library purification. The libraries were quantified and the bands controlled in Bioanalyzer, making sure there was only 1 peak in each lane.

4.2.10 Sequencing

The samples were prepared for sending for high throughput sequencing at Beckman in Chicago, USA, and was made as 20ul containing 10ug DNA/ul. The sequencing was performed with Illuma HiSeq. 2000.

4.2.11 Data analysis

The data analysis is based on the idea that the open regions are transcriptional regions and the more open regions at the same location, in all the samples submitted, the higher the level of transcription. By comparing these regions in different conditions/treatments it will be visible which condition/treatment results in a higher rate of transcription.

The results from sequencing were analyzed using scripts made by the University of Oslo. The sequencing file from the control and the sequencing file from the treatment were analyzed the same way until they were compared in Galaxy. The files were aligned with hg19, which is the most updated database for the humane genome, using Bowtie and then sorted before removal of duplicates. The numbers of duplicates in the samples were low, ranging from 9,4% to 22,3% with the average at 15,4%. Then, peak calling with MACS and normalizing with a MCF-7-file. When normalizing with MCF-7-input file, the peaks become visible. These peak-files were sorted by "lost" (A), "common" (B) and "gained" (C) regions using Galaxy, an "open source, web-based platform for data intensive biomedical research." (Galaxy). Galaxy made it possible to find overlapping regions to calculate numbers of each

region, and to sort them into common, lost and gained regions. First step was to find the overlapping region of at least 1 basepair between two individual treatments. This is the common region (B). Then, subtract the common region from the control. This is the region lost by treatment (A). At last, subtract the common region from the treatment. This is the regions gained by treatment (C).

The files were also compared to its wild type, meaning the knock down (siFoxA1) file in one treatment was compared to the control (siNT) file in the same treatment. By these comparisons it was possible to see the difference between conditions, and treatments.

5 Results

The first interesting result was to see if knock down of FoxA1 was a success. As seen in fig 14 the lane loaded with siNT sample have much stronger bands than the lane loaded with siFoxA1. The amount of proteins loaded was the same in both lanes, as seen in the loading control RPL13a, so any difference in the result is due to different expression.

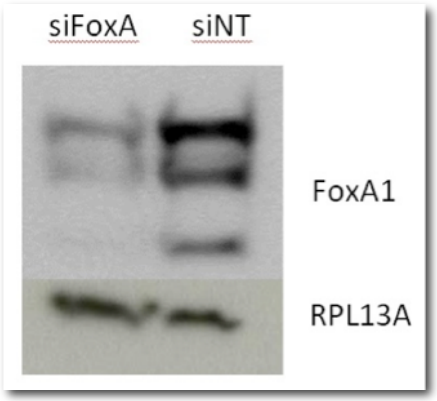

Fig 14: Knock down of FoxA1 was successful.

Results by treatment:

Estrogen (estradiol, E2)

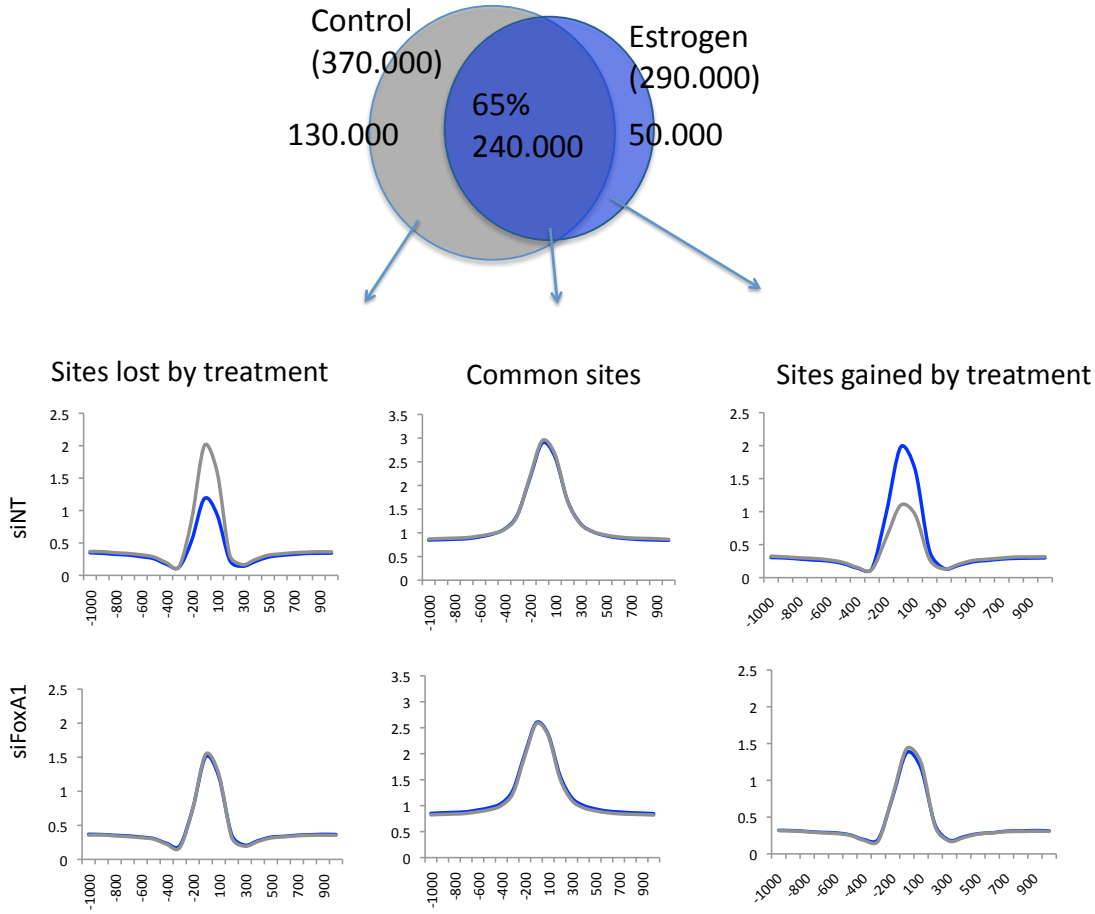

Fig 15: Comparison between which sites are lost by treatment, which are common with treatment and which are gained. The samples displayed as a venn diagram are the open regions with treatment and without (control). FoxA1 has not been knocked down.

The cells treated with estrogen were grown in clear DMEM with 5%SFBS after the medium was changed, 2 days after transfection. The stimulation time for the treatment was 60 minutes.

As seen in the venn diagram where FoxA1 is not knocked down, there is a small opening of about 50.000 sites when the cells are treated with estrogen, compared to the control. 50.000 sites are only 18% of all the treated regions, not enough to be sure its not background.

As seen on the venn diagram the sites closing are 130.000 which is 35% of the original sites. It seems like estrogen have an stronger action for closing regions, than opening new.

The common sites are 240.000, 65% of the original sites.

When comparing the siNT to the siFoxA1 we see that less sites are closing in the control when knocking down FoxA1, implying that FoxA1 is necessary to keep the regions open. For the opening sites it seems like the activity is dependant of FoxA1, event thought it is a small number.

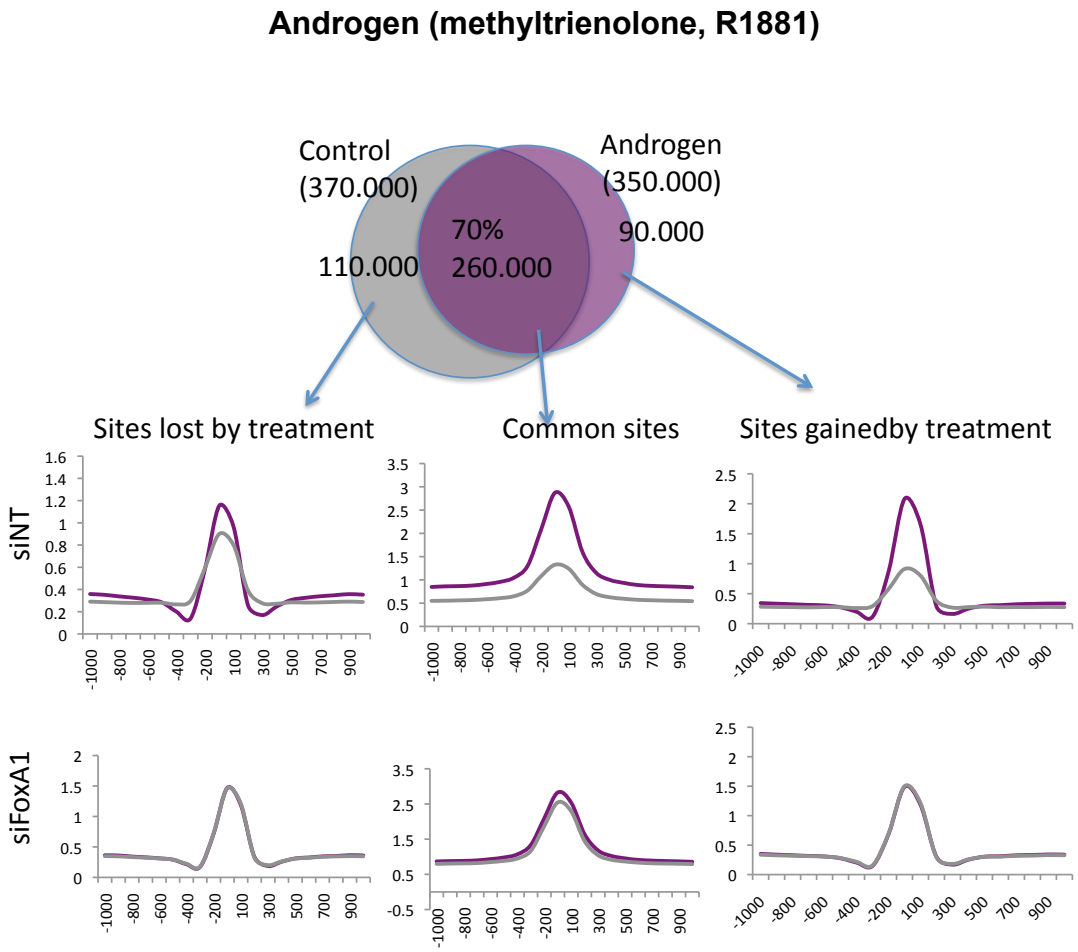

Fig 16: Comparison between which sites are lost by treatment, which are common with treatment and which are gained. The samples displayed as a venn diagram are the open regions with treatment and without (control). FoxA1 has not been knocked down.

The cells treated with androgen were grown in clear DMEM with 5%SFBS after the medium was changed, 2 days after transfection. The stimulation time for the treatment was 4 hours.

The venn diagram shows that when the cells were treated with synthetic androgen, methyltrienolone (R1881), there was an opening of about 90.000 new sites with 25% of the treated area was unique for androgen.

Of the control sites 70% of the sites were common with the treated sites.

110.000 sites were lost compared to the cells treated with androgen.

When comparing the siNT to the siFoxA1 the activity of androgen in gained and lost sites are dependant of FoxA1.

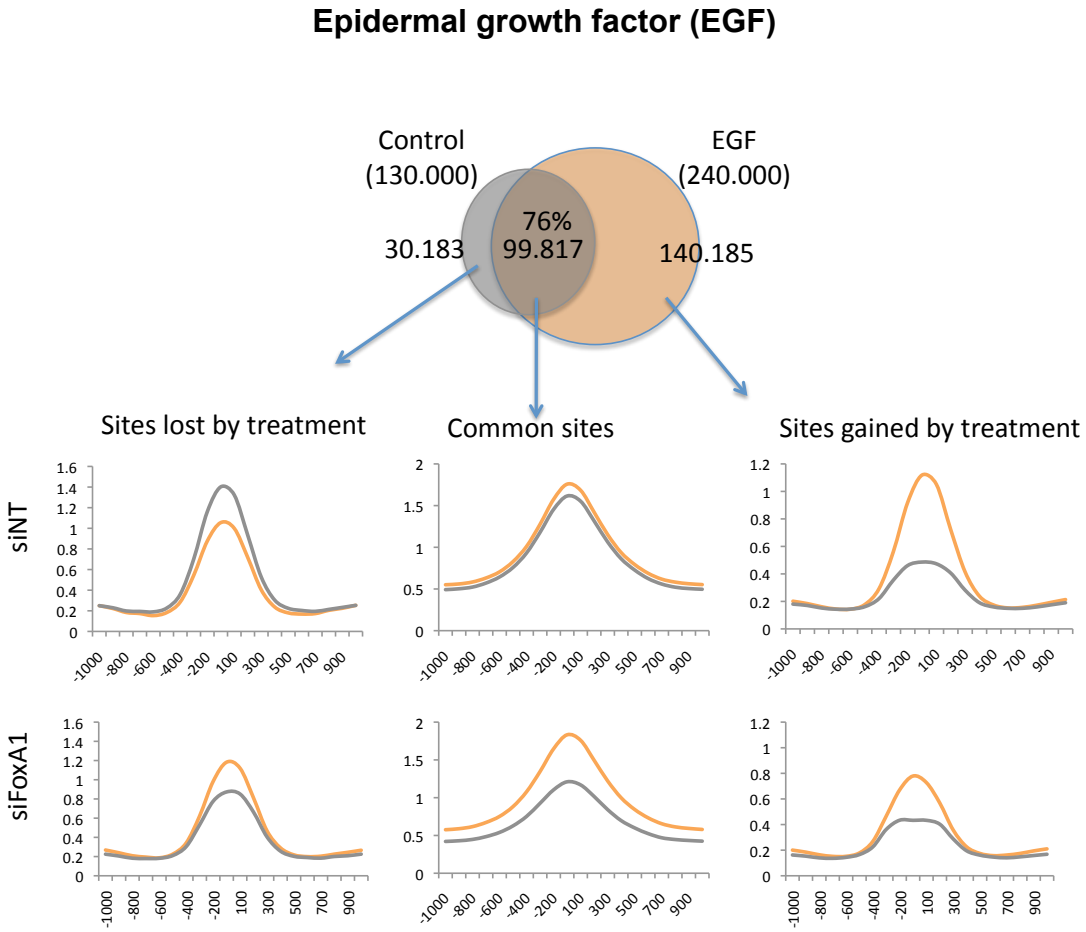

Fig 17: Comparison between which sites are lost by treatment, which are common with treatment and which are gained. The samples displayed as a venn diagram are the open regions with treatment and without (control). FoxA1 has not been knocked down.

The cells treated with EGF were grown in clear DMEM with 2%SFBS after the medium was changed, 2 days after transfection. The stimulation time for the treatment was 90 minutes.

As seen in the venn diagram EGF is able to open several new regions the control is not able to. When comparing the control to the EGF-treated regions EGF opens 140.185 new sites to a total of 240.000, with 41% of the sites common. 76% of the original sites were common. EGF is able to close more regions when FoxA1 is absence, than when FoxA1 is present.

When comparing the siNT to the siFoxA1 the amount of active regions are higher in the gained regions treated with siNT, than in the regions where FoxA1 were knocked down. The activity of EGF is not fully dependant of FoxA1, but seem to be affected in some way. The activity is lower when FoxA1 is knocked down, but not as low as for the control. For the common sites, the control is affected by FoxA1, but not EGF.

The control sample seems to be dependant of FoxA1 to keep the regions opened.

Heregulin (Her2)

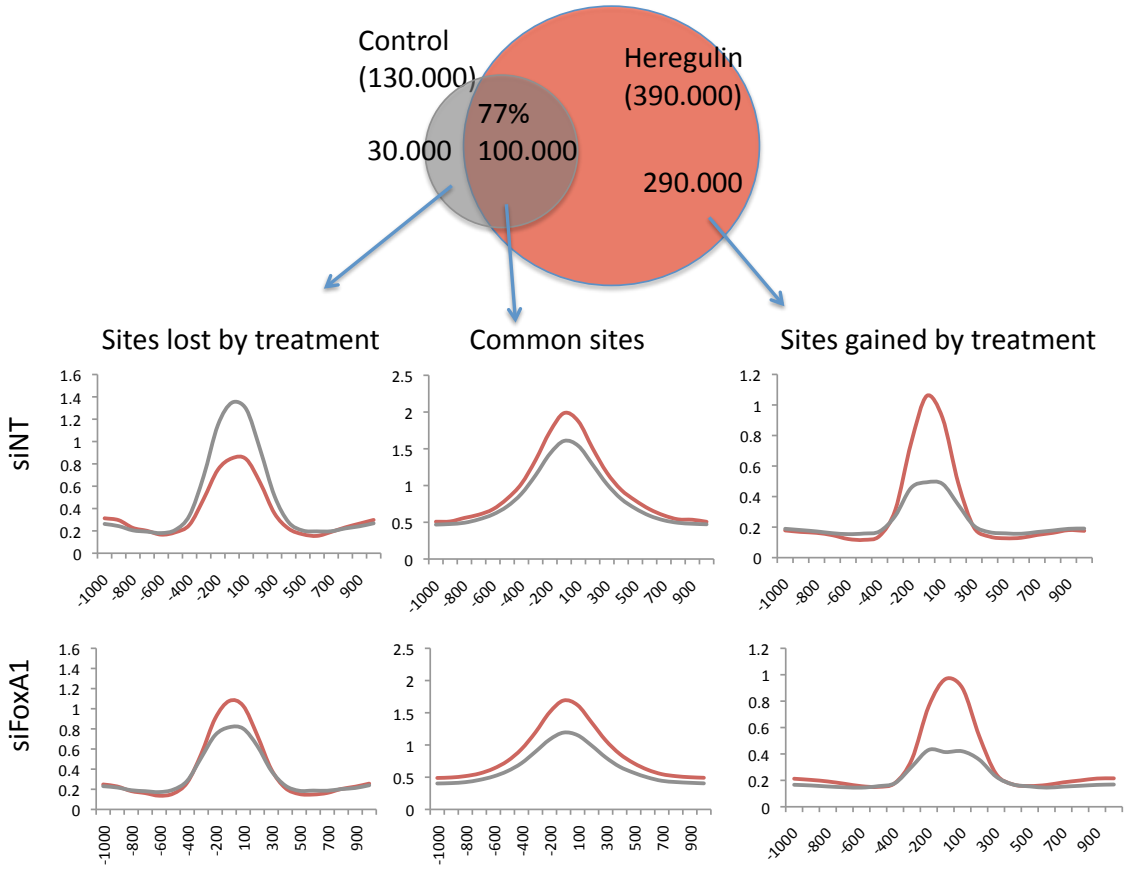

Fig 18: Comparison between which sites are lost by treatment, which are common with treatment and which are gained. The samples displayed as a venn diagram are the open regions with treatment and without (control). FoxA1 has not been knocked down.

The cells treated with heregulin were grown in clear DMEM with 2%SFBS after the medium was changed, 2 days after transfection. The stimulation time for the treatment was 90 minutes.

As the venn diagram shows heregulin is able to open several new regions, even more than EGF. Heregulin opens 290.000 unique new sites to a total of 390.000. The common sites are only 25% of the total treated sites, while the common sites compared to the original sites are 77%.

The cells treated with the growth factor heregulin were able to open more regions independent of FoxA1. Heregulin is not affected by the absence of FoxA1, as EGF is. When FoxA1 is knocked down, heregulin is able to close more regions. It seems that the control sample is dependent of FoxA1 to keep the regions opened, like for EGF.

It is important to remember, when comparing the number of regions for growth factors to the control the control plates for the growth factors are grown in 2%, not 5% like for the hormone control. This will influence the growth of the control cells and might give a false low amount of regions. It should not influence as much, since the cells treated with growth factors are grown in the same percentage medium, but the difference in the overall picture, where the hormones are compared to the growth factors in opening and closing of the regions, might be affected.

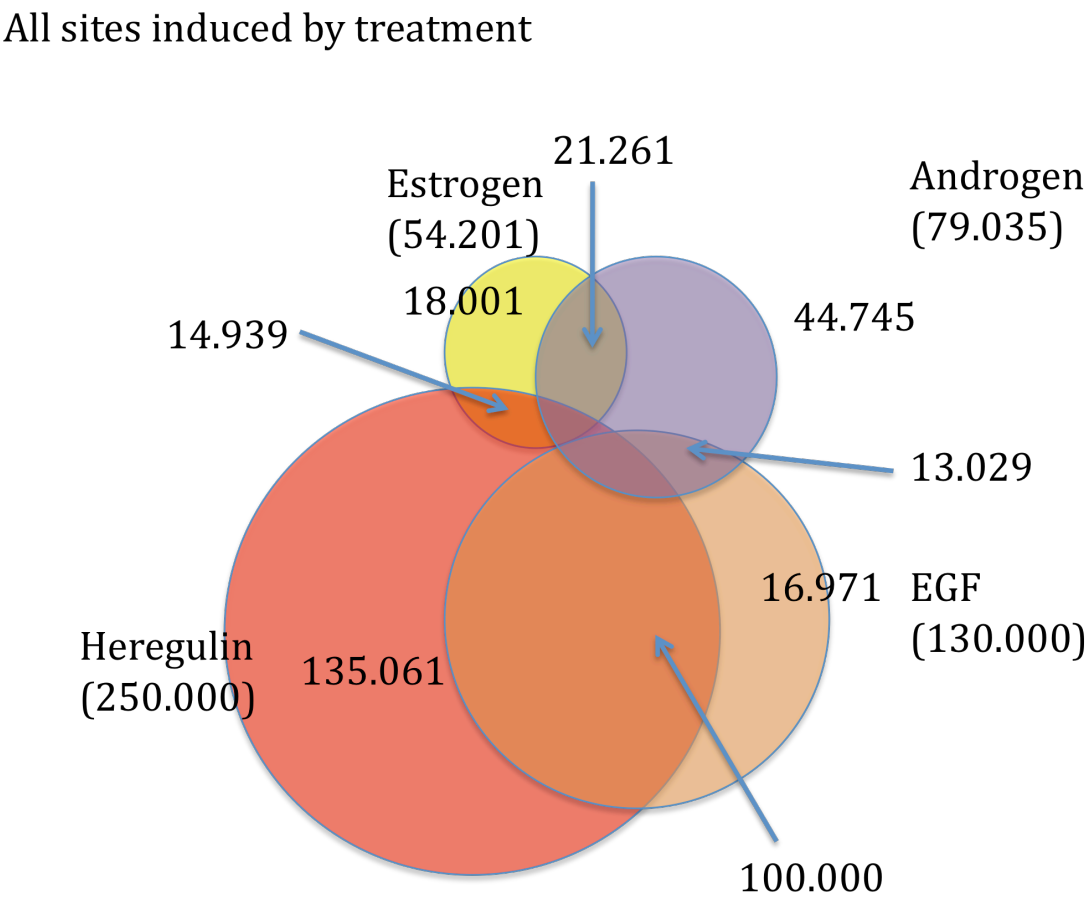

Fig 19: An overview of all the open regions in cells transfectet with siNT, FoxA1 has not been knocked down. The figure shows the result of the treatment with hormones and growth factors, comparing how many regions are open and how many are common.

The overview off all the sites opening under treatment shows that the growth factors are more active in opening the chromatin than the hormones. This observation corresponds to the individual results, the growth factors EGF and heregulin are able to open several more sites than the hormones, with more unique sites for the growth factors, only 13.000 sites common between EGF and androgen, and 15.000 common sites between heregulin and estrogen. In total, the growth factors opens 252.000 sites unique for growth factors, while the hormones opens 84.000 sites unique for hormones.

All sites lost by treatment

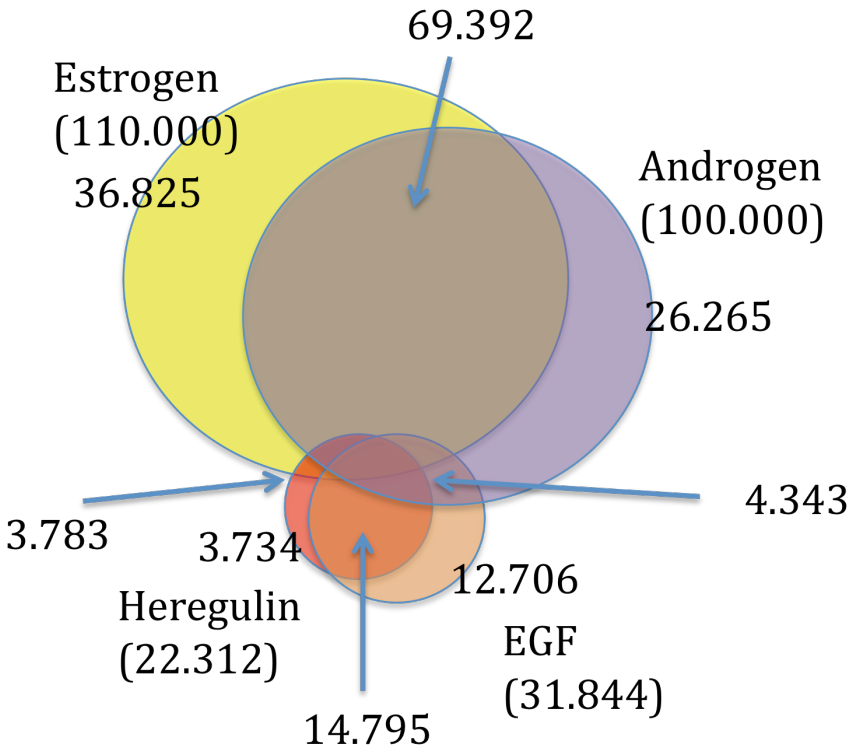

Fig 20: An overview of all the lost regions in cells transfectet with siNT, FoxA1 has not been knocked down. The figure shows the result of the treatment with hormones and growth factors, comparing how many regions are gained and how many are common.

The overview of the all the lost sites under treatment shows that the hormones are more active in closing the chromatin than the growth factors. This observation also corresponds to the result displayed by individual treatments showing that the hormones are more active in closing the regions than the growth factors. Together, the hormones closes 132.500 sites unique to the hormones, while the growth factors closes 31.000 sites unique to the growth factors.

6 Discussion

Studies have shown that MCF-7 cells with overexpression of Her2 are hyperactivated in the MAPK pathway and also resistant to antiestrogen treatment (Kurokawa & Arteaga 2003; Lupien et al. 2010) and this would explain why as much as 30% of breast cancer patients have a poor response to endocrine treatment. It is not uncommon to develop resistance to SERM after some time of treatment (Schiff et al. 2003). In addition, a study of long term estrogen depletion of MCF-7 cells (LTED) show that these cells have a higher level of ERα and a higher level of type I tyrosin kinase growth factor signalling in vitro (Martin et al. 2005). It might be due to adaptation to environmental factors and could have the same effect in vivo. These cells also show increased MAPK activity (Kurokawa & Arteaga 2003). It is possible that this is the reason why patients develop resistance to anti-estrogen therapy over time. Martin et al also have results that implies that the cell can adapt to different pathways to counter deprivation of a substance (Martin et al. 2005)

For the hormone treated cells in this study there is a small difference in opening of unique regions between androgen and estrogen, but how big this difference is can be debatable. The opening is affected by FoxA1 in both treatments. The overall comparison between the siNT hormones shows that most of the regions are overlapping, which means that most of the regions induces by estrogen or androgen is shared.

The growth factors have a strong influence in opening the chromatin, but not in closing. Heregulin have a much stronger action than EGF in opening, and they are both independent by FoxA1, EGF more than heregulin. Heregulin seems to open the chromatin and allow the function of Foxa1. This independence by the growth factors together with the fact that the cell can alter which pathway to use due to environmental factors might be the reason why patients with tumors positive for her2 have such bad prognosis.

Conclusion

Both experiment for growth factors shows a strong action in opening the chromatin, heregulin stronger than EGF. This opening is affected by FoxA1 to some extend, but not dependent. Heregulin seems to open the chromatin and allow the function of Foxa1. FoxA1 seems to be necessary to keep the chromatin open in cells treated with growth factors, as the closing of the chromatin is higher among the controls when FoxA1 is knocked down. The hormon treatment experiment shows that the opening of regions are low and more affected by FoxA1. The closing of the original regions however is higher in both hormon experiment and also strongly affected by FoxA1. This implies that the hormones have a strong action in closing regions, while the growth factors have a strong action in opening regions. From my result, it is not possible to conlcude that these actions are dependent of FoxA1 as there still is a level of transcription when FoxA1 is knocked down. From the western blot-result some small parts of the protein is still visible, and maybe it is enough to have a function? What I can conclude is that growth factors have the ability to open chromatin and that these chromatin remodelling actions are affected by FoxA1, the hormones more than the growth factors.

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Appendix 1 - table for treatments and solutions

Treatment of the cells

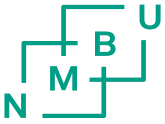
Treatment	Dilution		Apply to sample	Incubation time
Androgen (R1881)	1:1000	999ul medium + 1ul R1881 Final conc: 1nM	1ul	4h
Estradiol (E2)	1:100	99ul medium + 1ul E2 Final conc: 10nM	10ul	1h
Vehicle (ethanol)	1:100	99ul medium + 1ul ethanol	10ul	1h
EGF	1:100	297ul medium + 3ul EGF Final conc: 100ng/ml	100ul	90 mins
Heregulin	1:20	228ul medium + 12ul Heregulin Final conc: 25ng/ml	100ul	90 mins

Lysisbuffer for western blot

Lysis buffer for enrichment of Nuclear fraction		
Final cons	Stock	Making 10ml
20mM Hepes	1m Hepes pH 7,7	200ul
10mM NaCl	5M	20ul
1,5mM MgCl	1M	15ul
20% Glycerol	40 %	5ml
0,1%TritonX-100	10 %	100ul
1mM DTT	1M	For 4ml: 4ul
Thermo Scientific Proteinase & Phosphatase for 4ml: 40ul		

PBS

Salt	Concentration (mmol/L)	Concentration (g/L)
NaCl	137	8.0
KCl	2.7	0.2
Na_2HPO_4	10	1.44
KH_2PO_4	1.8	0.24



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