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The Electronics of HER2/neu Positive Breast Cancer Cells

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1. Introduction

Approximately 20 to 30 % of all breast cancer cases are classified as HER2/neu-positive (McCann et al. 1991). The overexpression the HER2 receptor tyrosine kinase is most commonly caused by a gene amplification and is associated with aggressive tumor behavior (Hynes & Stern 1994). Patients with HER2/neu overexpressing tumors have a significantly shorter time to relapse as well as a reduced overall survival rate compared to patients with normal HER2/neu expression levels (Berchuck et al. 1990; Slamon et al. 1989; Slamon et al. 1987). The oncogenic properties of HER2/neu were first discovered in rat neuroblastoma ("neu") as a tumor antigen related to epidermal growth-factor receptor (EGFR) (Schechter et al. 1984). The human homologue to *neu* was mapped to chromosome 17q21 and, because of its similarity to the human EGF receptor, named human EGF receptor 2 (HER2) (Coussens et al. 1985; King et al. 1985). Two more members of the HER family, namely HER3 and HER4, were subsequently identified (Kraus et al. 1989; Plowman et al. 1993). In this review we will discuss the altered metabolism and potential downstream effects found in breast cancer cells with increased HER2/neu expression.

1.1 The HER family of receptor tyrosine kinases

The HER family of receptors are found in a variety of tissues and interact with several EGF-like ligands (Harris et al. 2003). HER2 plays an important role in human development, it has been detected in the nervous system, bone, muscle, skin, heart, lungs and intestinal epithelium (Coussens et al. 1985; Quirke et al. 1989). Deletion of HER2 in mice leads to embryonic lethality (Meyer & Birchmeier 1995). Upon ligand binding, the receptors either homo- or heterodimerize and transphosphorylate each other. This initiates downstream signaling cascades through a variety of adaptor proteins and second messengers resulting in cell cycle progression, proliferation and survival (Bazley & Gullick 2005; Alroy & Yarden 1997). Even though HER2 does not bind any ligand by itself, it has been reported to display low affinity interactions with many, if not all, ligands in any given receptor heterodimer (Tzahar & Yarden 1998). It has been suggested that HER2 is the preferred dimerization partner for all the ligand-binding members of the HER family and that HER2 heterodimers are more active than their homodimeric counterparts (Yarden 2001), which might be due to enhanced recycling of HER2 receptor heterodimers to the cell surface (Lenferink et al. 1998).

This indicates that a dysregulation of HER2 levels will lead to increased receptor dimerization and thus increased signaling.

The expression levels of HER2 in malignant cells can be increased up to 100-fold compared to normal cells, resulting in as many as two million HER2 molecules per cell (Park et al. 2006; Liu et al. 1992; Venter et al. 1987). This overexpression is most commonly caused by an amplification of the HER2 gene, which leads to increased transcription and protein synthesis. Experiments utilizing fluorescence *in situ* hybridization suggest that only about 3 % of HER2 overexpressing breast cancers do not carry a corresponding gene amplification (Pauletti et al. 1996). The majority of HER2 overexpressing breast cancer cells have been shown to have 25-100 copies of the HER2 gene (Kallioniemi et al. 1992). This gene amplification and overexpression of the HER2 protein identifies a subset of the breast cancer disease which is found independent of disease stage. In fact, gene expression studies show that HER2 overexpressing tumors display a characteristic molecular pattern that is maintained as the cancer progresses, indicating that HER2 amplification is an early event in carcinogenesis (Weigelt et al. 2005; Perou et al. 2000). HER2 overexpression is found in almost half of all ductal carcinoma *in situ* (DCIS) that show no evidence of invasion but does not occur in benign breast disease (Allred et al. 1992; Park et al. 2006; Liu et al. 1992).

1.2 Treatment of HER2/neu-positive breast cancer

These advances in basic breast cancer research lead to the development of the first truly targeted cancer therapy agent, the humanized monoclonal antibody trastuzumab (Herceptin®, Genentech) (Carter et al. 1992). Herceptin was FDA approved in 1998 for the treatment of advanced metastatic breast cancer (Hortobagyi 2001). Possible mechanisms of action and the safety profile of trastuzumab are reviewed in Hudis et al. (2007). After initial success of using Herceptin in the clinic (Vogel et al. 2002), more and more reports about tumors developing Herceptin resistance were published (recently reviewed in Mukohara 2011). Truncated HER2 receptors (p95HER2) lacking the extracellular domain, which is targeted by Herceptin, were identified in human breast tumors (Molina et al. 2002). This prompted the development of new, second generation targeted therapies like tyrosine kinase inhibitors, HSP90 inhibitors, inhibitors of PI3 kinase, new anti-HER2 antibodies as well as HER2-based vaccination strategies. These novel anti-HER2 strategies are reviewed in Mukohara (2011).

Even though it has been almost 25 years since the discovery of HER2 as an identifier of a unique subset of breast cancers, we still have not conquered the disease with therapies directed against HER2. The ability of HER2 driven breast tumors to consistently develop resistance against therapies targeting HER2 underlines the importance of further research in this area. The underlying genetics associated with HER2 overexpression seem to be responsible for a more complex picture that is only hinted at by HER2 diagnostics.

2. The genetics of HER2/neu-positive breast cancer

Several studies in the past decade show that HER2 protein overexpression or gene amplification is not the only alteration in the breast cancer subtype denoted HER2 positive. Transcriptional profiling (meta-) analyses have demonstrated that a number of genes are commonly overexpressed along with HER2. At the beginning of this century, a specific gene expression signature was reported for HER2/neu-positive tumors and cell lines using DNA

microarrays as well as comparative genomic hybridization techniques (Pollack et al. 1999; Perou et al. 2000). Co-amplification of individual genes was reported as early as 1993 (Keith et al. 1993). A microarray based screen (probes for 217 ESTs on chromosome 17) by Kauraniemi et al. (2001) using 14 breast cancer cell lines identified seven transcripts as the minimal region of co-amplification with HER2.

In a more extensive study, Bertucci et al. (2004) used a microarray platform with ~9000 cDNA probes. The analysis of 213 different tumor samples as well as 16 breast cancer cell lines yielded a characteristic gene expression signature of 37 differentially expressed genes for HER2/neu-positive tumors/cell lines. According to this "HER2-Signature", 29 genes were up- and 8 downregulated. Using this expression signature the group was able to predict the HER2 status of tumors with remarkable accuracy compared to the classical histologic classification methods (92.2% accurate compared to 85.9% for FISH and IHC). Seven of those 29 upregulated genes are located on chromosome 17q12, close enough to the HER2 gene locus to be candidates for co-amplification. Indeed, of these seven genes four were also part of the minimal region of co-amplification identified by Kauraniemi et al. (2001). The most striking targets in this "HER2 gene expression signature", apart from HER2, are the peroxisome proliferator receptor binding protein (PBP) and NR1D1, a nuclear receptor for heme and regulator of adipogenesis as well as circadian rhythm. NR1D1 is also known as Rev-erb-alpha. Later studies confirmed the overexpression and co-amplification of these genes in HER2/neu-positive breast cancer (Arriola et al. 2008; Chin et al. 2006).

Recently, an extensive RNAi-based screen by Kourtidis et al. (2010) evaluated 141 genes that were previously reported to be overexpressed in HER2/neu-positive breast cancers. Kourtidis et al. used shRNA constructs derived from a genome-wide shRNA library to transfect the well established HER2/neu-positive breast cancer cell line model BT474 and subsequently monitored changes in cell proliferation. The shRNA targets that resulted in the highest decrease of cell proliferation were confirmed by a second round of transfections. The most significant reduction in proliferation occurred after knockdown of HER2, indicating that the experimental approach was valid. Interestingly, knockdown of NR1D1 and PBP resulted in the third and fourth most significant reduction of cell proliferation which was found to be due to decreased viability. To confirm these results, SKBR3 and MDA-MB-361, both HER2/neu-positive cell lines, were transfected with the same constructs, resulting in a severe decrease of viability compared to control. Knockdown of these two targets in cell lines that do not carry the HER2 amplicon (breast cancer MCF7, MDA-MB-453, MDA-MB-468, normal human mammary epithelial cells - HMEC - and human kidney HEK293 cells) was without effect.

NR1D1 was first identified as an orphan nuclear receptor in 1989 (Miyajima et al. 1989). Since then it has been discovered that NR1D1 is a constitutive transcriptional repressor (Harding & M A Lazar 1995) and a nuclear receptor for heme, the binding of which actually enhances repression (Yin et al. 2007; Raghuram et al. 2007). NR1D1 is also an important regulator of the circadian rhythm. Circadian oscillations in gene expression are based on a 24 hour time frame and are present throughout the animal kingdom (Panda et al. 2002). They allow the organism to anticipate changes in metabolic activity and food availability. The master clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus which is responsive to light. This master clock synchronizes peripheral clocks that have an important role in organ homeostasis. The circadian regulation allows for metabolic enzymes to be expressed at the appropriate times to avoid, for example, the simultaneous expression

of glycolytic and gluconeogenic enzymes (Duez & Staels 2009). Disruptions of circadian cycles have been linked to various diseases such as mental illness, metabolic syndrome and cancer (Gachon et al. 2004). The cycle of a peripheral clock starts when the two positive regulators BMAL1 and CLOCK heterodimerize and initiate transcription of their target genes. Among those targets are also negative regulators of BMAL1 and CLOCK transcription like NR1D1, which binds to their respective promoters and, by recruiting NCoR and HDAC3, represses transcription of BMAL1 and CLOCK (Yin and Lazar 2005). This creates a negative feedback loop resulting in oscillating levels of circadian rhythm regulatory proteins. NR1D1 also binds to its own promoter, thus repressing NR1D1 transcription once a critical protein concentration is reached (Adelmant et al. 1996). NR1D1 levels are high in metabolically active tissues including adipose tissue and liver (Lazar et al. 1989). NR1D1 is required for adipocyte differentiation (Wang & Lazar 2008) and its overexpression enhances adipogenesis in 3T3-L1 adipocytes (Fontaine et al. 2003). This is particularly intriguing as Kourtidis et al. observed significant differences in the amount of stored neutral fats in HER2 overexpressing breast cancer cells compared to other breast cancer cells with normal HER2 expression levels as well as human mammary epithelial cells. HER2 overexpressors consistently showed higher fat content than other breast cancer cells. The peroxisome proliferator receptor-gamma binding protein (PBP) is a nuclear receptor co-activator also called mediator complex subunit 1 (MED1). Other names include CRSP1, RB18A, TRIP2, CRSP200, DRIP205, DRIP230, TRAP220 and MGC71488. Throughout this text we will refer to it as PBP. PBP was first identified through a yeast-2-hybrid screen using PPAR γ as bait. It was capable of enhancing PPAR γ dependent transcription (Zhu et al. 1997). Subsequently, PBP has been shown to be a critical component of the mediator complex, which is required for polymerase II mediated transcription (Kornberg 2005; Malik & Roeder 2005). Apart from binding to PPAR γ , PBP also interacts with various other nuclear receptors like PPAR α , TR β , VDR, ER α , RAR α , RXR and GR and is strongly expressed in the developing mouse embryo, suggesting an important role in cellular proliferation and differentiation (Viswakarma et al. 2010; Zhu et al. 1997). Indeed, a complete knockout of PBP in mice leads to embryonic lethality on day 11.5 (Zhu et al. 2000). In a carcinogenesis study using diethylnitrosamine followed by phenobarbital promotion, PBP null liver cells fail to undergo proliferation. All tumors that arise in mice carrying a conditional liver PBP knockout develop from cells that have retained PBP expression. This indicates that PBP is required for the development of hepatocellular carcinoma in mice (Matsumoto et al. 2010). Moreover, it has been shown that PBP is required for mammary gland development (Yuzhi Jia et al. 2005).

The PPARs belong to the superfamily of nuclear receptors and typically regulate the transcription of genes associated with lipid metabolism (Desvergne & Wahli 1999). In fact, PPAR γ is required for adipocyte differentiation and NR1D1 is a transcriptional target of PPAR γ (Tontonoz et al. 1994; Fontaine et al. 2003). Indeed, Kourtidis et al. showed that knockdown of PBP results in decreased mRNA levels of NR1D1. Co-transfection of BT474 cells with shRNAs targeting PBP and NR1D1 does not result in increased cell death, indicating that these two regulators work synergistically. Treatment of cells with a PPAR γ antagonist results in reduced message levels of NR1D1 and induces apoptosis in BT474 cells. The survival advantage caused by PBP and NR1D1 overexpression is independent of HER2 overexpression as HER2 message levels do not change after NR1D1 knockdown. Instead, NR1D1 and PBP indirectly induce a lipogenic phenotype which results in increased fat

synthesis and storage. Disruption of this synthetic pathway results in HER2/neu-positive breast cancer cell apoptosis but does not affect HER2 negative cells.

PPAR γ , PBP and NR1D1 are responsible for a markedly altered physiology in cells carrying the HER2 amplicon. These new co-amplified and overexpressed transcriptional regulators cooperatively change the metabolism of HER2/neu-positive breast cancer cells by inducing a unique, Warburg-like metabolism that is primed towards fat production. The next section will discuss this metabolism in detail.

3. The altered metabolism of HER2/neu-positive breast cancer

NR1D1, PBP and PPAR γ are required for adipogenesis and many of their transcriptional targets are related to lipid metabolism. Their unregulated overexpression in HER2/neu-positive breast cancer causes a unique metabolic phenotype that relies on aerobic glycolysis and fatty acid synthesis for energy production and survival.

Kourtidis et al. show that the amount of stored neutral fats is about 20-fold higher compared to human mammary epithelial cells and about 10-fold higher compared to HER2 negative breast cancer lines. Knockdown of PBP and NR1D1 results in a significant decrease of fat stores and overexpression of NR1D1 in immortalized, non-tumorigenic, MCF10A cells increases their fat content by about 4-fold compared to vector control. Further studies using fructose and galactose as alternative fuel sources indicate that it is not the amount of fat stores but the requirement for active fatty acid synthesis that is important for the survival of HER2/neu-positive breast cancer cells. While PBP and NR1D1 knockdown decreases fat stores and viability, similar decreases in fat stores through cell growth in fructose or galactose containing media did not alter viability in these cells.

Investigation of transcript levels of potential downstream targets of PBP and NR1D1 revealed significant changes in ATP-citrate lyase (ACLY), acetyl-CoA carboxylase α (ACACA), fatty acid synthase (FASN) and fatty acid desaturase 2 (FADS2) mRNA levels after knockdown of either PBP or NR1D1. Indeed, shRNA mediated knockdown of ACLY, ACACA and FASN resulted in decreased viability in HER2/neu-positive breast cancer cells. Other studies have already shown a tight link between FASN and HER2. Overexpression of FASN in immortalized, non-tumorigenic HBL100 and MCF10A cells induces oncogenic properties and results in upregulation and activation of HER1 and HER2 (Vazquez-Martin et al. 2008). There is evidence that HER2 phosphorylates FASN, which results in increased enzymatic activity. Blocking FASN phosphorylation and enzymatic activity by either lapatinib (HER2 specific tyrosine kinase inhibitor) or C75 (FASN inhibitor) suppressed invasion of SKBR3 and BT474 cells (Jin et al. 2010).

In order to sustain this lipogenic phenotype, the cells are in constant demand of cofactors that are essential for glycolysis and fatty acid synthesis. Nicotinamide adenine dinucleotide (NAD $^{+}$) is required to take up electrons from glucose and coenzyme A is required to transfer carbons from glucose to fatty acids. Once NAD $^{+}$ is reduced to NADH the electrons need to be shuttled to NADPH so that they can be used in fatty acid synthesis. Two cytoplasmic enzymes are required for the formation of cytoplasmic NADPH. Malate dehydrogenase (MDH1) produces malate from oxaloacetate using NADH as a cofactor, whereas malic enzyme (ME1) cleaves malate to pyruvate and CO $_2$ while reducing NADP $^{+}$ to NADPH. ME1 is the primary producer of cytoplasmic NADPH which can then be used for fatty acid synthesis. Knockdown of MDH1 and ME1 with shRNAs significantly reduced mRNA levels and cell viability in BT474 cells (Kourtidis et al. 2010). This represents a new physiological

alteration that is transcriptionally independent from the NR1D1 and PBP axis since knockdown of PBP does not change mRNA levels of MDH1 or ME1. The importance of fatty acid synthesis in this type of cancer is underscored by the finding that FASN inhibition reverses Herceptin resistance in SKBR3 cells (Vazquez-Martin et al. 2007).

To date, there is not much known about the contribution of coenzyme A (CoA) metabolism to this lipogenic phenotype. One study reported that a PPAR α agonist regulates CoA levels through the induction of pantothenate kinase 1 α (Pank1 α), which catalyzes the rate-limiting step in CoA biosynthesis (Ramaswamy et al. 2004). The agonist used in this study is actually a pan-PPAR agonist (Krey et al. 1997), so there is a plausible connection between PPAR action and coenzyme A. Interestingly, overexpression of NR1D1 in MCF10A cells results in a 50% decrease of pantothenate levels compared to vector control, indicating increased substrate flux through pantothenate kinase (Baumann et al, unpublished data). Further studies are under way in our lab to determine how PBP and NR1D1 influence the metabolism of coenzyme A and vice versa.

The amount of palmitate produced by HER2/neu-positive breast cancer cells would normally result in cytotoxicity and apoptosis but the overexpression of PBP and NR1D1 allows for the neutralization of these toxic products by generating neutral triglycerides in a PPAR γ dependent fashion. This system is operating close to the limit, as addition of free palmitic acid results in ROS mediated cell death of HER2/neu-positive but not HER2 negative breast cancer cells (Kourtidis et al. 2009). Metabolomic analysis of NR1D1 overexpressing MCF10A cells shows a drastically altered metabolite profile (Baumann et al, unpublished data). Levels of pathway intermediates in glycolysis and TCA are decreased, whereas lipids and lipid progenitor molecules are markedly increased. Overall energy levels seem to decrease with NR1D1 overexpression as nucleotide triphosphate levels are low. Nucleotide precursors from the pentose phosphate shunt are increased, while NADPH levels are low. These data indicate that NR1D1 overexpression results in increased metabolic flux through glycolysis and the pentose phosphate shunt towards nucleotide precursors and lipids. Of course this type of metabolism has to be balanced by appropriate anaplerotic reactions or the flux towards fatty acids would quickly deplete the TCA cycle of oxaloacetate, the “acceptor”-molecule for acetyl-CoA generated from pyruvate. Many cancer cells in culture have been shown to utilize glutaminolysis to achieve this goal (Weinberg & Chandel 2009). While BT474 cells also require glutamine in the culture medium, metabolomic analysis shows an unusual accumulation of glutamine in the cells, indicating that uptake rates far surpass any flux through glutamine consuming pathways (Kourtidis and Conklin, unpublished data). It is possible that glutamine is used to take up or excrete other compounds through sym- or anti-port transporters, respectively. The exact nature of the anaplerotic and glutamine utilization pathways in HER2 positive breast cancer are currently under investigation in our lab.

The synergistic action of HER2, PBP and NR1D1 allows cells carrying the HER2 amplicon to maintain a high flux rate through glycolysis and accumulate building blocks to sustain a high rate of cell proliferation. This might explain why therapies that target only HER2 are initially effective and result in the development of resistance. NR1D1 and PBP might be sufficient to drive the oncogenic metabolism once it is established. This is evidenced by the reversal of Herceptin resistance by FASN inhibition.

This metabolic phenotype is reminiscent of Warburg’s original observation of aerobic glycolysis in cancer cells (Warburg 1956), except that the electrons do not end up in lactate. Metabolomic analysis indicates that overexpression of NR1D1 in MCF10A cells reduces

lactate levels by approximately 56 % (Baumann et al, unpublished data). The end result for the cancer cells are the same, if not more beneficial in the case of the “Warburg-like” metabolism we observed in HER2/neu-positive breast cancer cells. In the canonical Warburg effect lactate serves as an electron sink and as a means to regenerate NAD^+ , which allows for continued flux through glycolysis, but lactate is then excreted from the cells as a waste product. HER2/neu-positive breast cancer cells use fatty acids as an electron sink to regenerate electron acceptors while simultaneously generating building blocks needed for cell proliferation.

Considering the movement of electrons in these cells provides a valuable frame of reference for understanding the metabolism of HER2/neu-positive breast cancer. All living organisms rely on the oxidation of energy rich compounds like glucose to fuel cellular processes like growth and proliferation. This is achieved by directing electrons from glucose through various steps toward a terminal electron acceptor, often oxygen, to create water. During this process the potential energy of these electrons can be used to fuel endergonic reactions. The carbon backbone of glucose is terminally oxidized to CO_2 and excreted. Plants use water, CO_2 and energy to produce glucose, completing the cycle. Of course this is an oversimplified version of the real picture but it helps to illustrate a point. The problem for any metabolizing cell can be described as one of electrons and their corresponding energy levels, the nature of the compound they are part of in any system at any given time is only important in terms of its reactivity and potential inhibitory effects on enzymes. In a complex organism each cell takes up electrons at a high energy level which need to be shuttled towards stable, lower energy compounds for the cell to be able to utilize their potential energy. In a homeostatic, non-proliferative setting, when oxygen can function as the terminal electron acceptor, this process is highly regulated and very efficient. One mole of glucose generates 36 moles of ATP, 6 moles of CO_2 and 6 moles of H_2O . All electrons are bound in stable non-reactive products that can freely diffuse out of the cell. In anaerobic conditions, for example in muscle tissue, the terminal electron acceptor can be pyruvate, which generates lactate. Lactate is then excreted from the cell and further metabolized in other organs. In a proliferative setting a growth signal will instruct certain cells to deposit electrons in an intermediate stable acceptor like fatty acids and other compounds needed to create a new cell. This is again a tightly controlled and highly efficient process that will eventually return to oxygen reduction once the growth signal is removed.

In a situation where uncontrolled proliferation takes place because of chromosomal aberrations the cell needs to substantially increase its uptake of high energy electrons in the form of glucose. Most of them will end up in new cellular matter, which leaves little energy to be used by the cell for other processes. This creates a disconnection between electron uptake, energy usage and deposition of electrons in stable end products. One round of glycolysis will generate 4 electrons, 2 molecules of ATP and 2 molecules of pyruvate per molecule of glucose. The deposition of the electrons into pyruvate will generate a stable end product that can be excreted but leaves no carbons to accumulate new biomass for proliferation. Increasing the flux through glycolysis will yield an increase in usable energy and carbons that can be used by the cell but it also requires fast regeneration of electron acceptors. The best solution for this problem is to uncouple the terminal electron acceptor from the primary anabolic pathways that need to be carried out in a regulated concerted way. Increased flux through glycolysis to increase energy uptake is only feasible if electron acceptors can be regenerated quickly and if end products can be excreted or shuttled into other pathways to avoid negative feedback through product inhibition. The accumulation of one or more metabolites in cancer cells might

simply be a result of an energetic necessity: the transfer of electrons into stable intermediates that have only a minor inhibiting effect on their corresponding biosynthetic pathways. HER2/neu-positive breast cancer cells produce lots of fatty acids because their genetic program enables them to further shuttle the toxic fatty acids towards non-toxic, basically inert, triacylglycerides that are then stored in the cell. Palmitic acid, which has a strong inhibitory effect on acetyl-CoA carboxylase α (ACACA), the rate-limiting step of fatty acid synthesis, never accumulate in these cells. The terminal electron acceptor in these cells is, for all practical purposes, acetyl-CoA.

The stoichiometry of these processes does not come out even and it does not have to. Stoichiometry only works if we look at a well-defined chemical reaction with a starting point and an end point, which is not necessary if we consider metabolic flux as an ongoing process. If one particular product cannot be formed any more, the preceding substrate will accumulate and be diverted into other metabolic pathways. Depending on all the different reactions going on in the cell that require either energy or carbons, any excess electrons can just flow towards other lower energy states in compounds that can serve as a substitute for oxygen, for example, pyruvate. Despite the fact that in HER2/neu-positive breast cancer cells the majority of electrons are deposited in triglycerides, these cells still produce lactate. Lactate can be excreted from the cells and will then feed into the Cori Cycle, which will result in glucose production in the liver and subsequent glucose export into the bloodstream. This type of metabolism is hardly efficient in terms of energy usage but it is very resilient and robust and allows for consistent production of energy and cellular matter. This is reflected in the slow growth rate of HER2/neu-positive cancer cell lines *in vitro*. The HER2/neu-positive cell line BT474 has a doubling time of approximately 100 hours whereas the HER2 negative line MCF7 takes about 29 hours for a population doubling. This seems counter-intuitive considering that HER2/neu-positive breast cancer has a worse prognosis and decreased survival time, but it is intriguing to speculate that in the complex environment of a human body growth rate might be less important than robust survival and growth in all conditions.

The accumulation of fat in HER2/neu-positive breast cancer cells raises other issues relating to the microenvironment, tumor growth and metastasis. By storing triglycerides these cancer cells store a vast amount of energy that is at the cells disposal if the environment of the cell changes. It is possible that these energy stores provide an advantage in case the cell enters a quiescent state or metastasizes to a new site as it will be less dependent on external energy sources if changes in metabolic regulation occur that allows the cells to switch to beta-oxidation for energy production. Tumors that produce a lot of lactate and excrete it into the tumor microenvironment will have a severe impact on the physiology of the surrounding cells. Excess lactate accumulation acidifies the tumor microenvironment and results in NF- κ B and Hif1 α activation which in turn results in angiogenesis and inflammation (reviewed in Allen & Jones 2011). This is likely to coincide with an increased influx of immune cells that will result in tumoricidal activity until the tumor is able to evade the immune response. In the case of HER2/neu-positive cells the efflux of lactate is decreased resulting in a less acidic tumor microenvironment, which might promote tumor immune evasion.

4. A potential role for metabolism in the epigenetics of Her2/neu-positive breast cancer

Epigenetic dysregulation is a well accepted contributing factor to tumorigenesis (Esteller 2007). Many of the cofactors that are required for the establishment of epigenetic marks are

intermediates in the cellular metabolism. These include S-adenosylmethionine (SAM), NAD^+ , and acetyl-CoA. Changes in the concentrations as well as in the intracellular spatial distributions of these molecules can have a profound impact on the epigenetic status of the cells. Alterations in how these molecules interact with each other can also influence the epigenetic modifications. By taking what has been learned about changes in cancer metabolism we can generate new ideas that will lead to a better understanding of how the flux of electrons in HER2/neu-positive breast cancer ultimately affects its epigenome and thus gene expression (Figure 1). More on the basics of epigenetics and cancer can be found in many recent reviews (Portela & Esteller 2010; Jovanovic et al. 2010; Sharma et al. 2010).

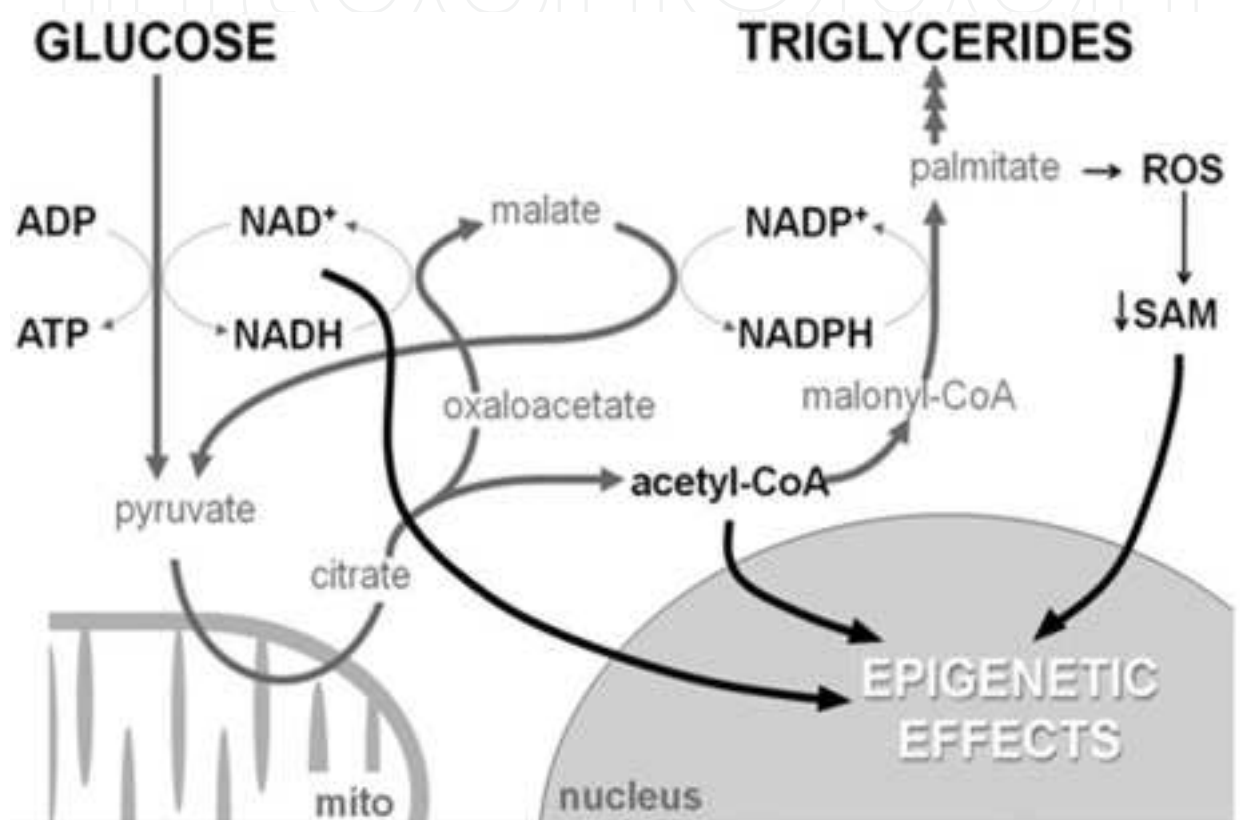


Fig. 1. The altered metabolism of HER-2/neu-positive breast cancer cells allows electrons from glucose to be deposited in triglycerides. Changes in levels of cofactors required for or affected by this process may have effects on epigenetic regulation. See text for details.

4.1 Methylation

Breast cancer, like all human cancers, is known to have differences in DNA methylation patterns (Esteller 2007; Ruike et al. 2010). During the development of the disease there is a global decrease in DNA methylation, also known as hypomethylation. However, concurrently there is an increase in CpG island (CGI) methylation at the promoters of tumor suppressor genes. Generally, genes important in apoptosis, metastasis, cell cycle regulation, angiogenesis and genes that encode non-coding RNAs (ncRNAs) are differentially methylated in breast cancer (Jovanovic et al. 2010). This improper methylation pattern begins in the primary tumor and increases upon metastasis, leading to alterations in gene expression (Feng et al. 2010). This change occurs in concert with an alteration in histone methylation, leading to a decrease in the expression of tumor suppressor genes in breast

cancer (Sharma et al. 2010). It appears that histone methylation levels are decreased in HER2/neu-positive breast cancer compared to other subclasses of breast cancer and may play a role in the poor prognosis of HER2/neu-positive tumors (Elsheikh et al. 2009).

Alterations to one-carbon metabolism, specifically the methionine cycle, play a major role in all methylation reactions that occur in epigenetics. During the methionine cycle, dietary methionine is converted into SAM. SAM is then used as the methyl donor in various reactions inside the cell leaving S-adenosylhomocysteine (SAH) as a byproduct, which can be reconverted to methionine in a folate (vitamin B-12) dependent reaction (Mato et al. 1997). Dietary reductions in folate have been linked to hypomethylation of both DNA and histones in liver diseases, the neurological development of embryos, as well as the development of both liver and colorectal cancer (Mato and Lu 2007; Greene, Stanier, and Copp 2009; Pogribny et al. 2004; van Engeland et al. 2003; Davis and Uthus 2003). Experimental evidence suggests that the tumor suppressor gene p53 is hypomethylated in mice with a folate deficiency, which results in decreased protein function as well as in an increased mutation rate (Kim et al. 1997; Liu et al. 2008). Work examining the direct role of SAM on the growth of cancer cells has also shown changes in methylation patterns. In rat models of hepatocarcinogenesis and in human prostate cancer xenografts, SAM treatment slows the growth of tumors and prevents new tumor growth (Pascale et al. 2002; Shukeir et al. 2006). It is believed that this effect occurs through increased methylation at the promoters of the protooncogenes, c-myc, c-Ha-ras, and c-K-ras (Simile et al. 1994). Taken together, these results from nutritional studies and from SAM treatment studies support the hypothesis that the availability of SAM can affect the epigenetic modifications of cancer cells.

Methylation is also directly correlated with the redox potential inside the cell. Normal cells maintain a reducing environment inside the cytosol, whereas cancer cells develop a pro-oxidant cytosolic environment (Cerutti 1985). Most of the intracellular reduction potential is due to the production of glutathione (GSH) which functions as the main cellular redox buffer. The ratio of GSH to its reduced form, glutathione disulfide (GSSG), is an important indicator of the intracellular redox state. In non-pathological states, GSSG levels approach almost zero (Schafer & Buettner 2001). Results from our lab indicate that there are alterations in the redox state in a HER2/neu-positive cell line compared to a normal breast epithelial line (Kourtidis & Conklin, unpublished data). Enzymes that contain redox sensitive amino acids such as cysteine are at particular risk of losing their catalytic activity in this environment. In fact many of the enzymes important in epigenetics are altered by the pro-oxidant state of the cancer cell (Hitchler & Domann 2009).

The decreased levels of GSH found in many cancer cells can alter epigenetic patterns by affecting SAM levels. After SAM has donated its methyl group, SAH is converted into homocysteine. Homocysteine can then reenter the methionine cycle, or, as has been shown in a pro-oxidant environment, be shuttled into GSH synthesis (Mosharov et al. 2000). As homocysteine is shuttled away from the methionine cycle less SAM is generated, which results in a reduced supply of methyl donors for DNA methyltransferases (DNMTs) and histone methyltransferases (HMTs). This finding is confirmed when GSH is chemically depleted (Lertratanangkoon et al. 1997). This scenario is further exacerbated because the enzymes in the methionine cycle are redox sensitive and inactivated by oxidation (Hitchler & Domann 2009). DNMT and HMT also have conserved catalytic cysteine residues present in their active sites (Chen et al. 1991; Zhang et al. 2003). Oxidation of these residues impairs the function of the both the DNMT as well as the HMT, which will also influence epigenetic

methylation patterns (Hitchler & Domann 2009). Taken together, all of these factors illustrate how the altered metabolism in HER2/neu-positive breast cancer cells can influence epigenetic modifications of both DNA and histones and thus influence gene expression.

4.2 Acetylation

The importance of histone modifying enzymes like histone deacetylases (HDACs) in cancer is also becoming clearer (Jovanovic et al. 2010). Breast cancer is no different. As with histone methylation, there is a global decrease in acetylation in HER2/neu-positive breast cancer in comparison with other classes of breast cancer (Elsheikh et al. 2009). It is believed that the hypermethylated CGI of tumor suppressor promoters attract HDACs either directly, or indirectly through methyl binding proteins (MBPs) increasing the repressed state of these genes (Dalvai & Bystricky 2010). Very little is known about HDAC expression in HER2/neu-positive breast cancer, despite the fact that HDAC 1 and 3 are overexpressed in most breast cancers and the expression levels of HDACs 2,4,6 decrease as the cancer develops (Dalvai & Bystricky 2010). The significance of these changes of acetylation has not yet been established, but it is intriguing to speculate that the altered metabolism in HER2/neu-positive breast cancer cells changes the availability of important co-factors that are required for histone acetylation and deacetylation.

The cofactor NAD^+ and its reduced counterpart NADH play a major role in the movement of electrons in cells. The overall cellular ratio of NAD^+/NADH effects the overall cellular redox environment, and alters the activity of various NAD^+ dependent enzymes. When cells use aerobic glycolysis as their main energy generation pathway, there is an overall decrease in the NAD^+/NADH ratio (Vander Heiden et al. 2009). We have noted that this phenomenon occurs in comparing NAD^+/NADH ratios of HER2/neu overexpressing breast cancer cell lines to normal breast epithelial cells lines (Kourtidis & Conklin, unpublished data).

Examining the effect of the decreased NAD^+/NADH ratio in relation to cancer cells is in its infancy, however, its role in lifespan extension has been studied for many years in organisms from yeast to human (Imai et al. 2000; Anderson et al. 2003; Guarente 2005). Caloric restriction, specifically a glucose reduction, leads to an increase in life span in yeast and mammals (Guarente 2005). The extension in longevity occurs because of the increase in ratio of NAD^+/NADH (Zhang & Kraus 2010). This shift is thought to inhibit the function of the sirtuins, a NAD^+ dependent class of HDAC, leading to a decrease in their functional ability to deacetylate histones, as well as other protein targets such as transcription factors (Imai et al. 2000; Zhang & Kraus 2010). These two facts together potentially allow for some of the aberrant gene expression that occurs in HER2/neu-positive breast cancer cells displaying a Warburg-like physiology.

Work in our lab has shown that the nuclear receptor NR1D1 plays a role in the proliferation and lipid production in HER2/neu-positive breast cancer cells (Kourtidis et al. 2010). NR1D1, which is overexpressed in HER2/neu breast cancer, functions as a transcriptional repressor by recruiting HDAC3 to its target genes. Recent Chip-seq data from liver cells echoes this concept indicating that NR1D1 binds genes important in lipid synthesis and other metabolic pathways leading to a recruitment of HDAC3 (Feng et al. 2011). The exact effects of NR1D1 mediated transcriptional repression are likely to be tissue specific since gene expression data from HER2/neu-positive breast cancer cells shows NR1D1 dependent

upregulation of lipid synthesis genes. The mechanism of this phenomenon is likely to be indirect. These findings could indicate that the overall decrease in histone acetylation in breast cancer may be caused by overexpression of NR1D1 and widespread HDAC3 recruitment.

However, some genes like HER2/neu itself display increased acetylation which results in upregulated gene expression (Mishra et al. 2001). This leads to the conclusion that while there is a global decrease in histone acetylation, differential acetylation occurs in breast cancer cells compared to normal breast cells. The Warburg-like metabolism of HER2/neu-positive breast cancer cells is likely to alter the availability and consumption of acetyl-CoA as a cofactor for acetylation and thus may influence acetylation patterns. There is not just a global decrease in histone acetylation, but also an alteration of histone modifications that lead to the expression of genes necessary for the survival and proliferation of the HER2/neu-positive breast cancer. The availability of acetyl-CoA can also have implications for other proteins that are acetylated, for example p53. The differential acetylation of transcription factors is another mechanism apart from histone acetylation, which illustrates how acetylation patterns can alter gene expression.

5. Conclusion

In HER2/neu-positive breast cancer, several genes are frequently co-amplified along with HER2. Recent evidence has shown that the co-amplified genes, NR1D1 and PBP, are required for HER2/neu-positive breast cancer cell survival. NR1D1 and PBP are important regulators of adipogenesis and their overexpression, functionally associated with PPAR γ , induces a Warburg-like metabolism that uniquely primes these cells for fat production and fat storage. HER2/neu-positive breast cancer cells store significantly more fats compared to HER2 negative breast cancer cells and normal human mammary epithelial cells. Since it is the synthetic process that is required for cell survival and not the amount of stored fats, disruption of fat synthesis induces apoptosis, whereas a similar decrease in fat stores through growth on alternative carbon sources does not. NR1D1 and PBP do not act through HER2 as knockdown of NR1D1 does not change HER2 transcript levels. HER2/neu-positive breast cancer cells are dependent on this type of metabolism as disruption of other pathways required for continued fatty acid synthesis results in apoptosis. This altered metabolism allows HER2/neu-positive breast cancer cells to shuttle electrons from glucose to neutral fat stores. The constant production of fatty acids allows the regeneration of NAD $^{+}$, which in turn enables the cells to maintain a high flux rate through glycolysis. By storing those fatty acids in lipid droplets the cell avoids palmitate-induced cytotoxicity as well as feed-back inhibition of fatty acid synthase.

The accumulation of fatty acids might confer an advantage to HER2/neu-positive cells in a state of quiescence or during metastasis, however, these possibilities warrant further investigation. It is however possible that cancer cells in general might just accumulate those metabolic intermediates that their genetic program allows them to synthesize as a means of regenerating NAD $^{+}$ for continued energy production. For example, glioma cells have been reported to frequently express isocitrate dehydrogenase (IDH) mutations which result in the production of 2-hydroxy-glutarate from 2-oxo-glutarate. It is possible that this mutation poses an advantage simply because it enables the cells to use 2-hydroxy-glutarate as an electron sink, since the reaction consumes NADH, providing another parallel example of a potential Warburg-like physiology.

6. References

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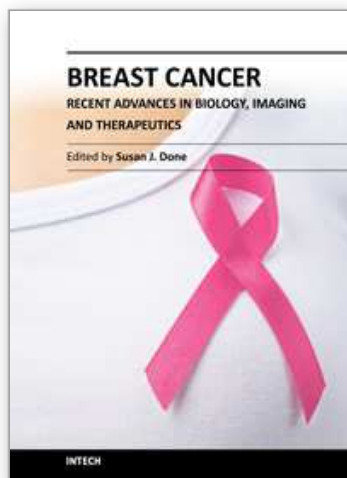
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In recent years it has become clear that breast cancer is not a single disease but rather that the term encompasses a number of molecularly distinct tumors arising from the epithelial cells of the breast. There is an urgent need to better understand these distinct subtypes and develop tailored diagnostic approaches and treatments appropriate to each. This book considers breast cancer from many novel and exciting perspectives. New insights into the basic biology of breast cancer are discussed together with high throughput approaches to molecular profiling. Innovative strategies for diagnosis and imaging are presented as well as emerging perspectives on breast cancer treatment. Each of the topics in this volume is addressed by respected experts in their fields and it is hoped that readers will be stimulated and challenged by the contents.

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