Published in final edited form as:

Clin Transl Oncol. 2013 January ; 15(1): 55–64. doi:10.1007/s12094-012-0882-3.

Progressive increase of glucose transporter-3 (GLUT-3) expression in estrogen-induced breast carcinogenesis

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Abstract

Introduction—Increased glucose uptake and glycolysis are main metabolic characteristics of malignant cells. A family of glucose transporters (GLUTs) facilitates glucose movement across the plasma membranes in a tumor-specific manner. Glucose transporter-1 (GLUT-1), GLUT-3 and recently GLUT-12, have been previously shown in breast cancer cells and are found to be associated with poor prognosis. In addition, it has been shown that estrogen plays critical roles in GLUT regulation, however, the stage-specific GLUT regulation of mammary carcinogenesis is unclear.

Methods—GLUT expression patterns were investigated in an in vitro–in vivo progressive, estrogen-induced, mammary carcinogenesis model which consisted of four cell lines, with same genetic background. In this model, different stages of tumor initiation and progression are represented, MCF-10F being the normal stage, E2 cells the transformed stage by estrogen, C5 cells, the invasive stage, and T4 cells the tumorigenic stage. In addition, loss of ductulogenesis and solid mass formation in collagen matrix and invasiveness of the cells were counted.

Results—Real time PCR showed that GLUT1 expression was downregulated in MCF10F after treatment with 17β-estradiol (E2), and in the invasive cell type (C5), but not in the tumor cells (T4), which had no changes compared to MCF10F. C5 and T4 cells showed the highest rate of GLUT-3 expression. These cells were also found to be associated with loss of ductulogenesis, solid mass formation and higher invasive capacity, whereas, GLUT-12 was downregulated in C5 and T4 cells.

Conclusion—Estrogen-induced malignant transformation is associated with remarkable and progressive GLUT-3 expression, GLUT-1 re-expression at further stages, as well as GLUT-12 downregulation.

Keywords

GLUT; Estrogen; Breast Cancer

Introduction

Mammalian cells use a wide variety of plasma-membrane transport systems in order to translocate several substrates into the cell. Apart from the Na⁺-dependent symport system

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Conflict of interest

The authors have nothing to disclose.

commonly used by intestinal and renal tubular cells, glucose and the other energy substrates are translocated by passive transport from extracellular space in all the tissues [1]. A family of glucose transporters (GLUTs) facilitates the movement of glucose across the plasma membranes in a tissue-specific manner, dependent on concentration gradient. The properties of glucose transporters differ between tissues, reflecting the function of their monosaccharide metabolism [2]. For example, Glut1 is a main isoform on erythrocytes and brain cell membranes, whereas Glut5 of sarcolemmal membranes are used mainly for fructose transport into skeletal muscle cells [1, 2]. Additionally, liver cells commonly use Glut2 for glucose transportation. So far, 14 different GLUT isoforms are established and classified.

Cancer cells, similar to non-tumoral mammalian cells, utilize glucose as main energy substrate using only these facilitative transport systems (GLUTs) depending on their increased metabolic activity and rapid proliferation [3, 4]. Cellular transformation under several oncogenic stimuli and carcinogenic progression lead to altered substrate metabolism, increased aerobic glycolysis and excessive glucose consumption [5, 6]. This phenomenon was observed for the first time by biochemist Otto Warburg in 1924 [3, 7]. He hypothesized that cancer cells may be caused by increased glycolysis and impaired respiration due to defective mitochondrial function and thus, cancer cells tend to convert most glucose to lactate even in the normoxic conditions. The glycolytic phenotype (or Warburg effect) is not only associated with excessive glucose consumption by overexpression of different Glut isoforms, but also increased activity of enzymes involved in glycolysis [5, 8]. It has been also observed that more advanced tumor stages are associated with more increased glucose uptake. For example, hexokinase and other glycolytic enzymes were found to be higher in metastasis than in primary breast tumors [9].

Especially within the last decade, expression patterns of GLUT isoforms (mostly Glut1–5) have been studied in several tumor cell lines and tumor tissue specimens [4]. Glut1 overexpression has been widely observed in many cancers including breast, lung, renal cell (RCC), colorectal and pancreas carcinoma specimens; some, (mostly Glut1, 3) are being found to be related to metastasis and poor prognosis [4, 10]. Although complete expression patterns of GLUT isoforms have not been evaluated in most cases, diversity of expression types have been appearing in “tumor-specific” manners. For example, Sukanuma et al. [9] have studied complete expression patterns in normal and renal tumor tissues and found that normal kidney tissues express all GLUT isoforms, however, in clear cell RCC, Glut1 expression remarkably increased while Glut4, 9, 12 decreased, whereas in chromophobe RCC the expression of Glut4 increased and Glut2, 5 decreased. On an in vitro basis, GLUT expression patterns have been most extensively studied in breast cancer cell lines [4]. Glut1 expression is being consistently observed in all breast tumors, but the expressions of Glut3, 5 and more recently Glut12 have been reported in several cultured breast cancer cells [4, 10–12].

Switching of preinvasive characteristics into invasive tumors or gaining of “invasive” (glycolytic) phenotype have been found to be related to GLUT expressions and alterations during the malignant transformation [6, 13–15]. Tumor hypoxia, another hallmark of malignant progression, up-regulates glucose transporter proteins. There is growing evidence that hypoxia inducible factor (HIF) system plays a critical role in the gain of GLUT expression characteristics. Under the hypoxic conditions, oncogenic signals activate a transcription factor (HIF-1 complex) that controls the cellular adaptation of transformed cells to low oxygen. The stabilized HIF-1 α subunit binds to hypoxia responsive elements (HRE) in promoters of target genes and this transcriptional regulation results in the activation of some glycolytic genes (i.e., Glut1, Glut3 expressions, several glycolytic enzymes including pyruvate kinase, phosphofructokinase, aldolase, LDHA) as well as

angiogenic gene activation [13, 16, 17]. Some oncogenes c-Myc, Ras and Src have been shown to activate HIF-1 system as well as glucose transporters [18, 19]. Also, Glut1 expression and at least one isoform of glycolytic enzymes are transcriptionally increased by HIF-1 following hypoxic conditions. In concordance with these findings, HIF-1 proteins and Glut1 expression have been extensively observed in aggressive phenotypes and metastatic breast tumors [16, 20–22]. In the ER(+) breast cancer cell MCF-7, it has been shown that knockdown of HIF-1 α results in reduced tumor growth and increased sensitivity to chemotherapeutics as well as attenuated Glut1 expression and phosphoglycerate kinase enzyme activity [23]. In addition, the same cell line exhibits increased 3H-FDG uptake and Glut1/Glut3 overexpression when exposed to acute hypoxia [24]. Reduced tumorigenicity and metastasis have also been observed in HIF-1 α knockout MMTV–PyMY transgenic mice [25]. Therefore, specific Glut overexpression and HIF-1 activation seems to be responsible for more aggressive phenotypes in mammary carcinogenesis.

It has been well known that prolonged estrogenic exposure, such as early menarche, late menopause or hormone replacement therapy, have been considered main risk factors for the development of human breast cancer [26, 27]. In our previous studies, we have demonstrated that estrogen and its metabolites initiate malignant transformation in normal breast epithelial cells (MCF10F) showing early hallmarks of carcinogenesis, i.e., anchorage-independent cellular growth in agar methocel, loss of ductulogenesis as well as solid mass formation in collagen matrix and epithelial mesenchymal transition (EMT) [28–30]. This hormone is also particularly effective in further stages, for example acceleration of the cellular growth and progression of the premalignant xenografts [31]. In addition, tumoral tissue continues its own estrogen production by increasing intra-tumoral aromatase activity [32]. Therefore, estrogenic dependence is obvious in almost all steps of mammary carcinogenesis. On the other hand, estrogenic action on breast cancer progression and malignant transformation have been found to be related to the regulation of glucose transporter proteins [4]. For example, treatment of breast cancer cell lines with 17 β -estradiol (E2) stimulates glucose metabolism via increased glycolysis, tricarboxylic acid cycle as well as increased Glut1 expression [33, 34]. Significant Glut12 expression has been observed after E2 treatment in several ER(+) cell lines [4]. Similarly, Glut3 expression is observed in the ZR-75 (ER+) cell line [35]. In addition, it has been shown that intermittent hypoxia and hypoxic environmental conditions induce down-regulation of estrogen receptor α and reduced hormone responsiveness in several breast cancer cell lines [36, 37].

Taken together, Glut1, 3, and 12 seem to be relevant to estrogen-induced mammary carcinogenesis and malignant transformation. However, under the estrogenic exposure specific selection of GLUT isoforms depending on tumor stage as well as their metabolic importance during the mammary carcinogenesis have yet to be elucidated. In the present study, we aimed to investigate the expression patterns of three GLUT isoforms in the E2-induced in vitro mammary carcinogenesis model.

Materials and methods

The experimental model of transformation of MCF-10F cells by 17- β estradiol treatment

In this study, our previously defined cell lines, the spontaneously immortalized ER- α and progesterone receptor (PgR) negative human breast epithelial cell line MCF-10F (passage 136), its transformed sequence using by 17- β estradiol E2 (passage 25), its invasive sequence C5 (passage 21) and finally its tumorigenic phenotype T4 (passage 10) were used [29, 30].

Schematic presentation of the previous experimental protocol and genomic characteristics of the cell lines used are summarized in Fig. 1. Briefly, the MCF-10F cell line was cultured in

Dulbecco's modified Eagle medium (DMEM):F12 containing 1.05 Calcium, antibiotics, hormones, growth factors and equine serum, and treated with 70 nM 17- β -estradiol (E2) (Sigma Chemical Co., St. Louis, MO, USA) or DMSO (control) for 24-h periods twice a week for 2 weeks; 24 h after the last treatment the cells were plated and maintained in culture for ten additional passages. Transformed cells were seeded onto Matrigel invasion chambers and at the end of a 22-h incubation period. Cells which had crossed the membrane were collected and identified as C5. Thereafter, tumorigenic ability of MCF-10F, E2 and C5 cells was tested by mammary fat pad inoculation in 45-day-old SCID mice. Tumorigenic response was observed only in four C5 inoculated mice. T4-cancer cells were then isolated from these tumors [38].

Three-dimensional growth in type 1 collagen matrix: loss of ductulogenesis and solid mass formation

Ductulogenic capacity and solid mass formation were evaluated as previously described [28, 30]. Briefly, the cells were suspended in collagen matrix consisting of 2.68 mg/ml (89.3 %) type 1 collagen (PureCol, Inamed Biomaterials Co, Fremont CA), 8 % 12.5 X DMEM-F12 with antibiotics, 0.1 mg/ml insulin, 14 mM NaHCO₃ and 0.01 N NaOH. Thereafter, the mixture was plated into four wells of a 24-well chamber (3,000 cells per well) pre-coated with 89.3 % of collagen base. After the solidification of collagen, the cells were fed with DMEM medium with daily intervals for 8 days. The cells were examined under an inverted microscope (Nikon Eclipse, TS100, Japan).

Invasion assay

Invasion assay was performed using 24-well plate Matrigel invasion chambers (BD Biosciences, Bedford, MA) fitted with cell culture inserts (Falcon Cell Culture Inserts) closed with an 8 μ m pore-size PET membrane coated with a uniform Matrigel basement membrane matrix. Chambers were stored at 20 °C and brought to room temperature in a laminar flow hood for 2 h, and the insert chambers were hydrated by placing 500 μ l of culture medium containing 5 % horse serum at 37 °C for 2 h in a humidified tissue culture incubator. Then the medium was removed from the inserts and 500 μ l of 20 % horse serum was added to each well as chemoattractant. Then all cells were trypsinized, and each cell line was seeded in triplicate in the upper chamber at a concentration of $2, 5 \times 10^4$ cells/well and incubated at 37 °C in a 5 % carbon dioxide incubator for 22 h. At the end of this period, the membranes of each chamber were fixed with Diff-Quick fixative and stained with DiffQuick solutions I and II (Sigma), cut out with a sharp scalpel, and mounted onto glass slides. The total number of cells that invaded through the membrane was counted under a light microscope and the invasion index was expressed as the mean $\pm$ SEM.

Colony formation in agar-methocel

Control and treated cells were suspended at a density of 2×10^4 cells/ml in 2 ml of 0.8 % methocel (Sigma) dissolved in DMEM:F-12 (1:1) medium containing 20 % horse serum. Cells from each treatment group were plated in eight 24-well chambers pre-coated with 0.5 ml 5 % agar base in DMEM: F-12 medium. Cells were fed fresh medium twice a week. To evaluate colony efficiency, the total number of viable cells was counted at 10 $\times$ magnification in four wells that were stained with neutral red (1:300) after 24 h post-plating and in four additional wells after 21 days in culture. Three weeks after, each colony was measured using a graduated eyepiece fitted in a transmission light microscope at 10 $\times$ magnification. Colonies >50 μ m in diameter were counted and results of colony efficiency were expressed as a percentage of the original number of viable cells after 24 h of plating.

Gene expression

Total RNA from cultured cells was extracted using the RNeasy Mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The quality and quantity of each sample were verified by spectrophotometry using NanoDrop 3.3.0 (NanoDrop Technologies, Wilmington, DE) and by capillary electrophoresis using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Relative quantification was performed through Real time RT-PCR. The reactions were performed on the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the fluorescent Taqman methodology (TaqMan One Step RT-PCR Master Mix Reagents, Applied Biosystems), 100 ng of total RNA and the probes (Table 1) in a total volume of 50 μ l according to the manufacturer's protocol. The thermal cycling conditions comprised 30 min at 48 °C, 10 min at 95 °C, and 40 cycles of 15 s denaturation at 95 °C and 60 s annealing at 60 °C.

The end point used in the real time RT-PCR quantification were the C_T values, defined as the PCR cycle number in which the fluorescence generated by the amplification crosses the threshold. Each gene was normalized using 18S as an endogenous control gene (ΔC_T). The relative expression was calculated through the difference between the normalized expression of each cell type and MCF10F ($\Delta\Delta C_T$). The fold change for each cell type compared to the MCF10F was calculated using the formula $2^{-\Delta\Delta C_T}$.

Statistical analysis

Two-tailed unpaired t test was used for gene expression analysis. Other analyses were performed using statistical software and differences were further evaluated using Holm–Sidak test for multiple pair-wise comparison procedures (SigmaStat 3.5, Systat Software Inc., San Jose, CA) and $p < 0.05$ was considered statistically significant.

Results

Ductulogenesis, solid mass formation, colony formation and invasiveness

In collagen matrix, the mean numbers of ductules was significantly higher in the MCF-10F group than in the other cells. E2 cells showed a higher rate of ductus formation when compared to C5 cells ($p < 0.02$). However, solid mass formation was only observed in the transformed cells (E2, C5 and T4). Maximum number of solid mass formation was observed in E2 group. Invasion capacity was significantly higher in C5-cells group than the others (ANOVA followed by Holm–Sidak test). Anchorage-independent growth in agar-methocel revealed that colony formation less than 250 μ m is remarkable in E2 (transformed phenotype) cells, whereas, larger colony formation ($>250 \mu$ m) was observed only in invasive (C5) and tumorigenic cells (T4). The mean number of bigger colonies in agar-methocel, however, was found to be higher in the C5 group ($p = 0.008$; Student t test). Data are summarized in Table 2.

Gene expression

The real time technique showed that Glut1 expression was downregulated in MCF10F after treatment with 17 β -estradiol (E2) and in the invasive cell type (C5), but not in the tumor cells (T4), which had no changes compared to MCF10F. Glut3 had an increase in gene expression in all the cells derived from MCF10F. The expression of Glut3 in the E2 cell was 1.7-fold from those detected in the MCF10F cell. In C5 and T4 the expressions were 40 and 70-folds of Glut3 in the relation to the control. The expression of Glut12 was decreased in all the cell types, E2, and C5 demonstrated a reduction of 24, and 49 % of expression, respectively, while T4, had a down-regulation of 11 %, not statistically significant (Fig. 2).

Discussion

In the present study, we attempted to identify the gene expression profiles of particular GLUTs in an isogenic group of cell lines in order to better understand the stages of specific alterations within estrogen-induced carcinogenesis. We used our in vitro–in vivo progressive mammary carcinogenesis model which consisted of 4 cell lines, from healthy (MCF-10F) to tumorigenic cells (E2, C5, T4), with the same genetic background (Fig. 1).

The immortalized ER α and PrR negative, but ER β -positive human breast epithelial cell line MCF-10F forms ductal-like structures under 3D cell culture conditions (Fig. 3). However, when this cell line is subjected to carcinogenic exposure (i.e., estrogen or chemical carcinogen) it starts gaining neoplastic phenotypes: i.e., loss of ductulogenic capacity but solid mass formation in collagen, invasiveness in a Matrigel basement membrane, as well as forming colonies in agar methocel [30, 39].

Our laboratory previously demonstrated that short-term (2 weeks) treatment of MCF-10F cells with estradiol and its catechol metabolites induce these phenotypes indicative of neoplastic transformation [30, 39]. In order to mimic the intermittent exposure of MCF10F to endogenous estrogens, cells were first treated with physiological doses of E2 at 72 and 120 h post-plating. Treatments were repeated during the second week, and cells were collected at the 14th day for phenotypic and genotypic analysis [39]. After treatment with E2, MCF-10F cells almost completely lost their ductulogenic capacity, while acquired solid mass formation in collagen. More importantly, the expression of these phenotypes induced by E2 were not abrogated by their simultaneous treatment with anti-estrogen ICI-182780, suggesting that the transformation of MCF-10F cells by these compounds did not require the presence of the ER α [28, 39]. In the present study, this phenotype was confirmed in all transformed cells (Fig. 3; Table 2).

In our previous studies, transforming potential of E2 on MCF10F was compared with chemical carcinogen benzo[a]pyrene (BP) by using the same in vitro model. Estradiol and BP showed similar phenotypical, but different genomic alterations [40]. For example, E2-transformed cells exhibited LOH in chromosomes 3 and 11, whereas BP did not induce genomic changes during the same period of time. In addition, E2-transformed MCF-10F cells were compared with ER(–) breast cancer cell lines BP1-Tras and MDA-MB-231 [30]. Similar to E2 cells, BP1-Tras and MDA-MB-231 exhibited a complete absence of ductule formation, forming instead solid masses in collagen whose values were not significantly different from those formed by E2-induced cells. However, when injected into SCID mice, neither MCF-10F nor E2-treated cells formed tumors after a 6 month follow-up period. Instead, BP1-Tras and MDA-MB-231 cells showed highly tumorigenic response with a short latency period [30]. In order to obtain more invasive cells among E2 transformed MCF-10F cells, cells that had crossed the reconstituted basement membrane in the Boyden Chamber were collected, expanded (C5 cells) and then injected to SCID mice (Fig. 1). From the nine tumors obtained from C5 cells, four tumoral cell lines (T4) were derived. Histopathologic analysis revealed that all C5 derived tumors were ER α (–), Progesterone receptor (–), and cErbB2 (–) poorly differentiated adenocarcinomas [30, 41]. In addition, fingerprint analysis using six different markers revealed that all the tumors and cell lines originated from MCF-10F cells and these data were also confirmed using variable number of tandem repeat (VNTR) analysis [30]. Comparative genomic hybridization (CGH) revealed cumulative genomic changes in all transformed cell lines. For example, loss of 9p11-13, LOH D13S893, Delp53 exon4, persisted in all transformed cells; complete loss of chromosome 4 was observed in C5 and T4 cells. Additionally, Gain1P, Gain5q-15qtr, Loss3-12.3-13, and Loss8p11.1-21 were observed in T4 cells [29, 30, 41].

Similar model systems that originate from the same immortal cell line (MCF10) including transformed, in situ, invasive and metastatic subgroups (named as MCF10A model) have been developed by Miller and co-workers [42, 43]. In this model, malignant transformation was initiated by H-ras oncogene transfection in non-malignant breast epithelial cells (MCF10) and estrogenic compounds were not used in any other cell lines representing further stages, whereas in our model system, malignant transformation was initiated with 17- β Estradiol. Therefore, we believe that the present model is more appropriate with regard to testing stage-specific gene expression profiles under the influence of estrogenic action. On the other hand, both carcinogenic initiators may give rise to different types of GLUT expressions. For example, malignant transformation of Rat1 fibroblasts with H-ras results in a substantial increase in glut1 mRNA levels in hypoxic conditions and thus, H-ras may specifically up-regulate Glut1 promoter [44]. In addition, it has been shown that transfection of the MCF10A cell line with V12-ras causes HIF-1 α and VEGF overexpression (a hallmark of glycolytic phenotype) under hypoxic conditions [45], whereas ovarian hormones, particularly estrogen, seem to be effective in GLUT regulation [4, 10]. Although, estrogenic regulation of glucose transporters on mammary epithelium is unclear. The mouse mammary epithelial cell line, COMMA-D, isolated from pregnant Balb/c mice shows Glut-1 expression, but not Glut3 or Glut-4. During pregnancy, the expression of Glut4 decreased progressively, whereas that of Glut1 increased [46]. In malignant conditions, it has also been shown that 17- β estradiol (E2) treatment stimulates glucose utilization [33]. In addition, E2 and progesterone differentially regulate Glut1-4 expression correlated with glucose uptake in ER(+) ZR-75 cell line [15]. Similarly, in a study, long-term treatment with E2 showed increased Glut12 levels in MCF-7 cells [4].

In the present study, Glut-1 and Glut-12 overexpression have not been observed in more-progressed (tumorigenic-T4) cells (Fig. 2). This result may be related to lack of a metastatic cell line in our model. Increased expression levels, particularly for Glut1, could be observed in metastatic stages. For example, Alo and co-workers [47] reported that Glut-1 staining has been identified in 36 % of hyperplastic breast lesions and 74 % of invasive breast carcinoma specimens. They observed progressive increase of Glut-1 positive cell content in immunohistochemical sections according to tumor grade and stage. Recently, high correlations between the primary tumors and metastatic lymph nodes with respect to Glut1 expression have been observed in several invasive tumors [48, 49]. Similar results have been observed in normal and malignant breast tissues with respect to Glut12 expression. For example, Rogers and co-workers [50], reported that Glut12 staining has been found in 90 % of invasive tumors and 60 % of DCIS sections. They also found very weak Glut12 staining in 60 % of normal breast epithelium.

On the other hand, down-regulation of Glut-1 in E2 and C5 cells in comparison with normal breast epithelial cells may also be related to tissue-specific endothelial differentiation response to 17- β Estradiol. For example, Glut-1 is extensively expressed in brain tissues under the normal circumstances [10]. In vivo treatment of blood-brain-barrier (BBB) endothelium with E2 resulted in Glut-1 mRNA over-expression [51]. Whereas, it has been shown that during the development and differentiation of the mammary gland, from the virgin rat to pregnancy period, the amount of Glut4 mRNA is decreased by 90 %, but the amount of Glut1 mRNA is increased by eightfold [46]. Interestingly, Glut12 co-expression has been observed as a second transporter with Glut1 in mammary epithelial cells during pregnancy and lactation [52]. We have previously postulated that differentiation of mammary gland determines the susceptibility to carcinogenesis [53]. For example, following early-full term pregnancy and lactation periods, terminally differentiated breast epithelium becomes resistant to E2-induced carcinogenesis [30, 54]. Therefore, differentiation of mammary epithelium may be reversely associated with Glut1 and Glut12 expression. Concordant with this suggestion, Rivenzon-Segal and co-workers [55] have

found that differentiation of the breast cancer cell line T47D with retinoic acid led to a reduction in Glut-1 and to an increase in cytoplasmic Glut-3 as well as decreased glycolysis.

Here, we have demonstrated for the first time that estrogen-induced malignant progression is associated with progressive Glut3 over-expression. This isoform is commonly expressed in neuronal tissues under normal physiological conditions and therefore, known as a neuron-specific glucose transporter [56]. In a reported series exploring Glut1-6,9 immunostaining in normal and tumoral tissues, Glut3 expression was not encountered in any of the normal breast tissue, whereas it was found in 33 % of invasive breast tumor specimens [57]. In other series, Glut3 expression is relatively high in aggressive tumors including breast, choriocarcinoma and lung tumors [4, 10]. However, the expression pattern of Glut3, particularly in preneoplastic and proliferative lesions, are unknown. On the other hand, quite a few data have been published about the role of estrogens on Glut3 expression in tumoral cell lines. For example, Medina and co-workers [58] investigated the effects of estrogen and progesterone (P) Glut1-4 expression and glucose transport in ER(+) breast cancer cell line ZR-75-1. They found that there was no induction of Glut1-4 mRNA levels in the presence of 17- β estradiol (E2), whereas P and the combination E2 caused an increment in Glut1 and weakly Glut3 mRNA levels. A similar study design was applied into the Ishikawa endometrial cancer cell line and the Glut1 over-expression pattern was observed after estrogen and progesterone treatment individually [59]. Recently, Meneses and co-workers [35] demonstrated that 8-br-cAMP, an analog of cAMP is capable of activating both cAMP-dependent protein kinase Type I and Type II (PKA), in particular transcriptional Glut3 regulation, in breast cancer cell line ZR-75. This signal transduction pathway can also be initiated by Epithelial growth factor receptor activation (EGFR/PKA) [60]. It is well known that the members of EGFR family play important role in regulating a number of cellular processes, differentiation and malignant transformation [61]. This system is commonly activated by several growth factor ligands including TGF- α , EGF and hepatocyte growth factor (HGF) and responsible for the generation of more aggressive and more invasive phenotypes which are resistant to anti-estrogen treatment [62]. For example, TGF α -induced activation of EGFR signaling pathway greatly enhances invasion and motility of tamoxifen-resistant cells [63]. Also, estrogen itself, and homo/heterodimerization of the members of EGFR family initiates several well-known protein kinase-mediated signal transduction cascades including Ras-Raf-MAPK, PI-3K-Akt-GSK, PLC- γ -CaMK/PKC and STAT pathways [64–66]. Recently, cross-talk between the EGFR and HIF- α signal pathways has been defined on apoptotic resistance by up-regulating survivin gene expression in several breast cancer cell lines including MCF-7, SK-BR-3, MDA-MB-231, but not in MCF10A [67]. Interestingly, it has been shown that EGF and TGF- α were able to induce PKA expression in MCF10A cells [68].

Although increased glucose metabolism and overexpression of Gluts in cancer are well known, reasons for tumor and stage-specific alterations (as seen at Fig. 2) on Glut isoform expressions and their importance in cancer biology have not been completely understood. These alterations are appearing related to metabolic rates of transformed cells or tumors and their aggressive behavior. For example, it has been shown that differentiation to less aggressive phenotype led to a 33 % decrease of Glut1 expression and a 35–40 % reduction in the glucose consumption [55].

Breast tumor tissue specimens and cultured breast carcinoma cells are often associated with the overexpression of Gluts, particularly Glut1 and less frequently Glut3 [4, 10]. Glut12 was recently identified in MCF7 cells and detected in eight of ten invasive breast tumors [50]. Therefore, we selected and studied 3 isoforms (Glut1, 3, 12) among the identified members of facilitative glucose transporter family. Expression analyses of the other isoforms in tumor tissues appeared less consistent. For example, in a reported series of 30 patients, 57 % of the

breast cancer tissues showed positivity for Glut1, 43 % for Glut4 but Glut2 activity could not be detected [69]. However, Godoy et al. [57] have shown moderate immunoreactivity for Glut2 and Glut5(90 %), weak immunostaining for Glut3 (9 %) and Glut4 (6 %).

Undoubtedly, besides Glut1, 3 and 12, other isoforms need to be analyzed using our model system in further studies for better understanding of estrogen induced tumor biology and defining novel molecular targets. The overexpressed Glut isoforms can be considered as molecular target for personalized cancer treatment. For example, Young and co-workers have shown that modulation of Glut1 expression levels alters mouse mammary tumor cell growth in the in vivo and in vitro conditions [70]. In addition, monoclonal Glut-1 antibody treatment has been found to be effective against ER-positive breast carcinoma cells MCF-7 and T47D [71]. Similar results can be obtained using Glut-3 antibodies or Glut-3 modulation methods in our model system.

Consequently, increased intracellular cAMP production due to glycolytic phenotype may involve cAMP-dependent PKA activation and transcriptional regulation of Glut3. Thus, under the estrogenic exposure, progressive Glut3 expression may be a result of activated EGFR/PKA signal transduction pathway in our study.

Acknowledgments

This work was supported by grant R21 ES015894 from the NIEHS.

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Figure 1.
Experimental protocol and harvesting of transformed cells

Figure 2.
Graphics showing the expression of each gene (Glut3, Glut1 and Glut12), for each cell type (E2, C5, T4). The expression is shown in fold change related to MCF10F cells

Figure 3. Growth patterns in 3-D conditions as indicators of malignant transformation. The transformed cells (E2, C5, T4) lost their ductulogenic capacity in collagen matrix and showed mass formation (left column). Colony formation of represented cells in Agar methocel (right column)

Table 1

Probes used to measure the gene expression through real time RT-PCR

Gene symbol	ABI assay ID	Alias	Gene name
18S	Hs99999901_s1		Eukaryotic 18S rRNA
SLC2A1	Hs00197884_m1	GLUT1	Solute carrier family 2 (facilitated glucose transporter), member 1
SLC2A3	Hs00359840_m1	GLUT3	Solute carrier family 2 (facilitated glucose transporter), member 3
SLC2A12	Hs00376943_m1	GLUT12/GLUT8	Solute carrier family 2 (facilitated glucose transporter), member 12

Table 2

	MCF-10F	E2	C5	T4	p value
Invasiveness	162 ± 11	683 ± 48	1322 ± 145	503 ± 231	0.001 ^a
Ductulogenesis in collagen matrix	31.8 ± 1.4	5.1 ± 0.7	0.7 ± 0.4	0.7 ± 0.2	0.001 ^b
Mass formation in collagen					
<250 nm	–	334 ± 9.4	12 ± 2	41.7 ± 4	0.001 ^c
>250 nm	–	24 ± 8.6	1 ± 0.4	13.1 ± 1.9	0.044 ^d
Colony forming in agar					
<250 µm	191.5 ± 8.5	619.2 ± 53.7	281.7 ± 29	113 ± 20	0.01 ^e
>250 µm	–	–	175 ± 20	91 ± 6.6	0.008 ^f

ANOVA followed by Holm–Sidak test was used for the comparisons of above-mentioned values except colony formation (bottom line) and results were expressed as ±SEM (standard error of the mean)

^aC5 cells showed higher invasion rates than the others

^bMCF-10F cells showed higher rate of ductal formation in collagen matrix when compared to the other groups. The comparison between E2 and C5 was also significant. No mass formation was observed in cultured MCF-10F cells

^cThe highest values observed in E2 cells. The all comparisons were significant

^dE2 values were higher than C5. Other comparisons were insignificant

^eMean colony numbers in E2 group were significantly higher than other groups. In addition, the comparison between C5 and T4 groups was statistically significant. However, other comparisons were insignificant (ANOVA followed by Holm–Sidak test). Other group comparisons in the aspect of colony formation in agar-methocel were insignificant

^fColony formation bigger than 250 µm was observed in C5 and T4 groups. Mean colony number in C5 group was statistically higher than T4 (Student *t* test)