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c-Myc Amplification Altered the Gene Expression of ABC- and SLC-Transporters in Human Breast Epithelial Cells

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Abstract: The present study aimed to investigate the effect of c-Myc overexpression on the gene expression and functional activity of major drug transporters in breast epithelial cells. In order to reflect c-Myc overexpressed epithelial cells, MCF10A cells (human mammary epithelial cells) stably expressing c-Myc (c-Myc-MCF10A cells) have been established by retroviral infection and then the effect of c-Myc activation on the gene expression and functional activity of 16 influx and efflux transporters was examined in c-Myc-MCF10A cells. In RT-PCR analysis, the quantitative difference in transporters' gene expression profiles was observed after c-Myc activation. Western blot analysis also indicated that the protein expression of some transporters (MCT1, OCT1, MRP1, MRP2 and BCRP) increased by c-Myc overexpression. In parallel to the alteration in gene expression, functional activity studies indicated that the cellular uptake of benzoic acid, MPP^+, calcein-AM, H33342, representative substrates of MCT1, OCT1, MRPs and BCRP, respectively, was significantly ($p < 0.05$) altered by c-Myc overexpression. In conclusion, c-Myc amplification appeared to be coupled with the modulation of gene expression of certain drug transporters in human breast epithelial cells.

Keywords: c-Myc; transporters; gene expression; regulation; human breast epithelial cells

Introduction

Drug transporters play an important role in the cellular influx and efflux of various chemotherapeutic agents in cancer cells.[1−4] As a consequence, the degree of expression and functionality of drug transporters may affect the therapeutic effectiveness, safety and target specificity of anticancer drugs.[2−4] In addition, given that the gene expression of drug transporters can be changed during pathogenesis, understanding the regulation change of drug transporters at diseases state is critical toward the success of drug therapy.

c-Myc gene is a proto-oncogene and yields three major proteins named c-Myc1, c-Myc2 and c-MycS.[5,6] Among c-Myc proteins, c-Myc2 is the major form of c-Myc proteins and referred to as "c-Myc" in most studies.[5] Although the accurate function of c-Myc is not clearly defined yet, many studies have demonstrated that c-Myc might play a crucial role in cell cycle progression including proliferation, cellular transformation and apoptosis.[7−9] Furthermore, c-Myc oncogene is commonly amplified and overexpressed in many

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Table 1. Primer sequences and PCR conditions for RT-PCR analysis

gene	primer pairs (F/R)	PCR condition			
		denaturation	annealing	elongation	no. of cycles
MDR1[a]/ABCB1[b]	GCCAAAGCCAAAATATCAGC TTCCAATGTGTTCGGCAT	95 °C, 30 s	49 °C, 30 s	72 °C, 18 s	39
BCRP/ABCG2	TGCAACATGTACTGGCGAAGA TCTTCCACAAGCCCCAGG	95 °C, 30 s	51 °C, 30 s	72 °C, 18 s	37
MCT1/SLC16A1	CACCGTACAGCAACTATACG CAATGGTCGCCTCTTGTAGA	95 °C, 30 s	54 °C, 30 s	72 °C, 18 s	20
PEPT1/SLC15A1	AATGTTCTGGGCTTGTTTG CATCTGATCGGGCTGAATTT	95 °C, 30 s	48 °C, 30 s	72 °C, 18 s	39
MRP1/ABCC1	GGGCTGCGGAAAGTCGT AGCCCTTGATAGCCACGTG	95 °C, 30 s	50 °C, 30 s	72 °C, 18 s	25
MRP2/ABCC2	TGAGCAAGTTTGAAACGCACAT AGCTCTTCTCCTGCCGTCTCT	95 °C, 30 s	50 °C, 30 s	72 °C, 18 s	39
MRP3/ABCC3	TCACCACTTGGGGATCATTT TCACCACTTGGGGATCATTT	95 °C, 30 s	50 °C, 30 s	72 °C, 18 s	22
MRP4/ABCC4	GCTCAGGTTGCCTATGTGCT CGGTTACATTTCCTCCTCCA	95 °C, 30 s	49 °C, 30 s	72 °C, 18 s	28
MRP5/ABCC5	CGAAGGGTTGTGTGGATCTT GTTTCACCATGAAGGCTGGT	95 °C, 30 s	49 °C, 30 s	72 °C, 18 s	35
MRP6/ABCC6	TGTCGCTCTTTGGAAAATCC AGGAACACTGCGAAGCTCAT	95 °C, 30 s	49 °C, 30 s	72 °C, 18 s	35
OCT1/SLC22A1	TAATGGACCACATCGCTCAA AGCCCCTGATAGAGCACAGA	95 °C, 30 s	49 °C, 30 s	72 °C, 18 s	35
OCT2/SLC22A2	AAGAATGGGGATCACAATGG AGATGTGGACGCCAAGATTC	95 °C, 30 s	49 °C, 30 s	72 °C, 18 s	35
OCT3/SLC22A3	CCCTGGAATTGCCTACTTCA GACTCAGGGACCACCCAGTA	95 °C, 30 s	49 °C, 30 s	72 °C, 18 s	28
OAT1/SLC22A6	GCGCCTTTTTTTGCCTTCT TTCCCGCTTCCCATTGATC	95 °C, 30 s	51 °C, 30 s	72 °C, 18 s	38
OAT2/SLC22A7	CCATCCAGGACGTGGAGAGA CCCACTTAGTTCTGGACCTGCTT	95 °C, 30 s	51 °C, 30 s	72 °C, 18 s	38
OAT3/SLC22A8	CACCATCCTCTCCTTAAGCTACCT ACTGTCTCCACGGTCTGCAAGT	95 °C, 30 s	51 °C, 30 s	72 °C, 18 s	38
S16 ribosomal protein/RPS16	TCCAAGGGTCCGCTGCAGTC CGTTCACCTTGATGAGCCCATT	95 °C, 30 s	51 °C, 30 s	72 °C, 60 s	20

[a] Trivial name. [b] Gene symbol.

types of human cancers including breast cancer.[5,6,10,11] To induce a tumor, c-Myc may promote cell proliferation and also simultaneously inhibit cellular apoptosis, so as to increase the cell number to form a tumor mass.[7−9] However, although c-Myc may induce cell proliferation, the proliferating cells are usually more sensitive to chemotherapy and thus higher c-Myc expression levels are correlated with a better survival although on the other hand they are correlated with larger sizes of tumors.[12,13] For instance, Augenlicht et al.[12]

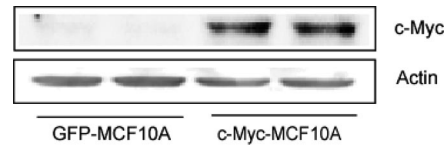

Figure 1. Western blot analysis of c-Myc expression in the infected MCF10A cells.

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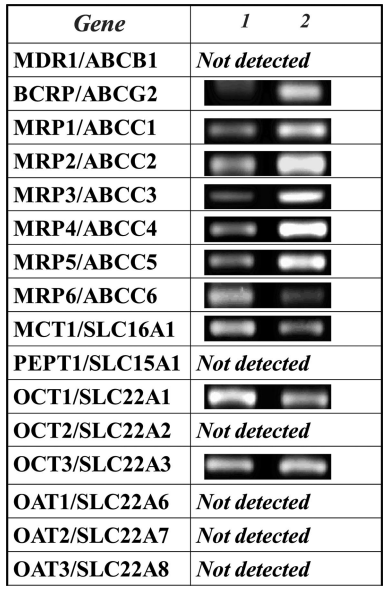

Figure 2. Comparison of transporter expression profiles analyzed by RT-PCR. 1: GFP-MCF10A cells. 2: c-Myc-MCF10A cells.

have reported that colonic cancer with c-Myc gene amplification responded to adjuvant chemotherapy much better than that without the gene amplification. For the explanation on this sensitivity change, we hypothesized that the gene expression of drug transporters may be altered by c-Myc overexpression and the subsequent change in the cellular transport of chemotherapeutic agents may affect the sensitivity to the chemotherapy. Therefore, the present study aimed to investigate the effect of c-Myc overexpression on the gene expression as well as functional activity of major drug transporters using MCF10A cells stably overexpressing c-Myc (c-Myc-MCF10A cells).

Materials and Methods

Materials. Calcein-AM, [^{14}C]-benzoic acid, MPP^+ (1-methyl-4-phenylpyridinium) and H33342 were purchased from Sigma Chemical Co. (St Louis, MO). TRIzol reagent was purchased from Invitrogen Co. (Carlsbad, CA) and oligo(dT) 18mer, specific primers and M-MLV reverse transcriptase were purchased from Bioneer (Eumsung, Korea). The anti-hMCT1 antibody, anti-hOCT1, and anti-hBCRP antibody were supplied from Chemicon (Temecula, CA), Abcam (Cambridge, U.K.) and Calbiochem (San Diego, CA). Anti-hMRP1 antibody and anti-hMRP2 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Alkaline phosphatase donkey antimouse IgG, horseradish peroxidase-conjugated donkey antirabbit IgG and horseradish peroxidase-conjugated donkey antimouse IgG were purchased from Jackson ImmunoResearch (West Grove, PA). Antiactin antibody and the other reagents in the molecular studies were obtained from Sigma Chemical Co. (St Louis, MO). All other chemicals were commercial products of reagent grade.

Construction of c-Myc Retroviral Plasmid and the Infection of MCF10A Cells. c-Myc was stably expressed in MCF10A cells using an MSCV-GFP retrovirus system. Human c-Myc cDNA was subcloned into MSCV-GFP retroviral vector and phoenix cells (a packaging cell line) were transfected with MSCV-GFP (Control) or MSCV-c-Myc-GFP (c-Myc overexpressed) plasmid. Supernatants containing amphotrophic replication-incompetent retroviruses were collected and then stored at −80 °C until required. 20% confluent MCF10A cells were infected multiple times (12 times) with retrovirus particles. Intensities of infection were monitored by GFP-fluorescence and Western blot analysis using c-Myc specific antibody. Established c-Myc-MCF10A and GFP-MCF10A cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 1% horse serum, insulin (10 mg/mL), hydrocortisone (250ug/mL), human EGF (50ug/mL) and 1% antimycotic solution at 37 °C in a 5% CO_2 humidified atmosphere.

Reverse Transcription Polymerase Chain Reaction (RT-PCR). Sixteen drug transporters were selected and their mRNA levels were determined by RT-PCR analysis. The total RNA was isolated from the cells using total RNA isolation kit (RNAgents, Promega, Madison, WI). The total RNA (1.0 g) obtained from the cells was reverse-transcribed using an oligo(dT) 18mer as a primer and M-MLV reverse transcriptase to produce the cDNAs. PCR was performed using the selective primers for various transporters and S16 ribosomal protein gene. Primer sequences and PCR conditions were summarized in Table 1. The band intensities of the amplified DNAs were compared after visualization on a UV transilluminator. For relative quantification of target genes, the expression level of the target gene was normalized by endogenous control (S16 rRNA protein) in each sample.

Western-Blot Analysis. Protein expression levels of MCT1, OCT1, MRP1, MRP2 and BCRP in c-Myc-MCF10A and GFP-MCF10A cells were determined by Western-blot analysis. Briefly, cells were washed with sterile PBS and were lysed in buffer containing 25 mM MES (pH 6.5), 150 mM NaCl and a combination of protease inhibitors (1 mg/mL Pefabloc, 5 mg/mL leupeptin, 1 mg/mL pepstatin and 1 mg/mL aprotinin). Cell lysates were centrifuged at 10000*g* for 10 min to remove debris, and proteins were fractionated using a 10% separating gel. Fractionated proteins were then electrophoretically transferred to nitrocellulose paper, and proteins were immunoblotted with specific antibodies (MCT1 (1:1000), OCT1 (1:800), BCRP (1:50), MRP1 (1:1000), MRP2 (1:1000)). The secondary antibodies used were horseradish peroxidase- or alkaline phosphatase-conjugated anti-IgG antibodies. Nitrocellulose papers were developed using an ECL chemiluminescence system.

Cellular Uptake Studies. Cells were seeded into 24-well plates at a density of 1×10^5 cells/well. Two days postseeding, cells were incubated with each drug solution (calcein-AM (2.5 μM), ^{14}C-benzoic acid (0.5 μCi/mL), H33342(10 μM), or MPP^+ (20 μM)) for 30 min. At the end of incubation, drug solution was removed and the cells were washed three times with ice-cold phosphate-buffered saline.

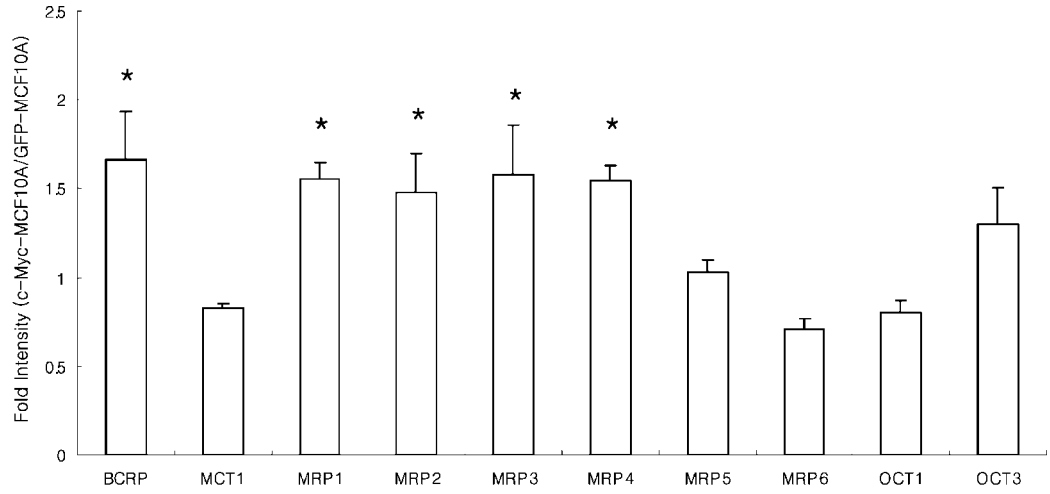

Figure 3. mRNA levels of drug transporters in breast epithelial cells. Data are the mean ± SD from two independent cultures, each being analyzed in triplicate; they are expressed as fold difference (a ratio of values from c-Myc-MCF10A and GFP-MCF10A cells).

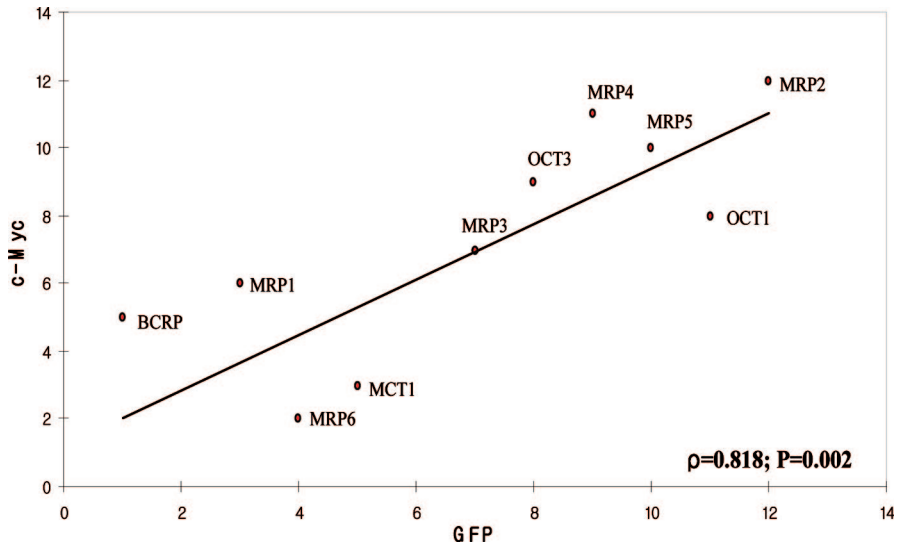

Figure 4. Rank correlation analysis of drug transporter expression in c-Myc-MCF10A and GFP-MCF10A cells. Correlations were analyzed using the Spearman's rank correlation method. Spearman's rank coefficients (ρ) and *P*-values are provided on the right bottom of the correlation graphs.

After the cell lysis, cells were harvested and the drug concentration of each sample was determined. Fluorescence intensity of calcein and H33342 was measured by a spectrofluorometer. The excitation and emission wavelengths were 496 and 516 nm for calcein and 355 and 460 nm for H33342, respectively. The concentration of ^{14}C-benzoic acid was measured by a scintillation counter. The concentration of MPP^+ was measured by a HPLC assay reported by Richardson et al.[14] with slight modification. The chromatographic system consisted of a pump (LC-10AD), an automatic injector (SIL-10A) and a UV detector (SPD-10A) (Shimadzu Scientific Instruments, Japan) set at 290 nm. An octadecylsilane column (Gemini C18, 4.6 × 250 mm, 5 μm; Phenomenex, Torrance, CA) was eluted with a mobile phase at a flow rate of 1.0 mL/min. The mobile phase consisted of 50 mM KH_2PO_4: acetonitrile (80:20, v/v %). The protein amount of each sample was determined with BCA protein assay kit following the manufacturer's instruction (Sigma Chemical Co., St. Louis, MO).

Statistical Analysis. All the means were presented with their standard deviation. The statistical significance of the difference in the parameters was determined using ANOVA

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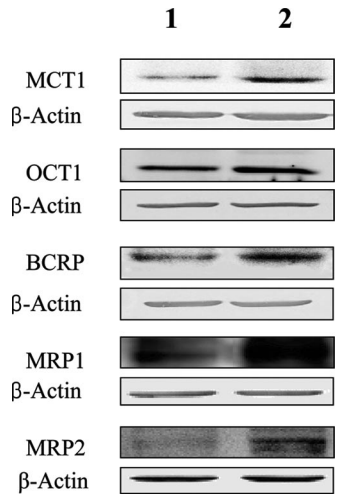

Figure 5. Western blot analysis on the protein expression of drug transporters in GFP-MCF10A (1) and c-Myc-MCF10A cells (2).

or a Student's *t* test. A *P* value <0.05 was considered statistically significant.

Results

Effect of c-Myc Amplification on the Gene Expression of Drug Transporters. To reflect the c-Myc overexpression in breast epithelial cells, MCF10A cells stably overexpressing c-Myc gene (c-Myc-MCF10A cells) were established by retroviral infection. As shown in Figure 1, c-Myc protein was highly expressed in c-Myc-MCF10A cells while it was not detectable in the control cells (GFP-MCF10A cells).

To examine whether c-Myc overexpression modulates the gene expression of drug transporters, sixteen drug transporters from ATP-binding cassette (ABC) transporter and solute carrier (SLC) transporter families were selected and RT-PCR analysis was performed using c-Myc-MCF10A cells as well as GFP-MCF10A cells. Among tested transporters, mRNA expression of 10 transporters was detected in both cells while mRNA levels of the other 6 transporters (MDR1, PETP1, OCT2, OAT1, OAT2, OAT3) were practically undetectable (Figure 2). The mRNA levels of ABC transporters including BCRP, MRP1, MRP2, MRP3, and MRP4 were significantly higher in c-Myc-MCF10A cells compared to those in GFP-MCF10A cells, while there was no significant change in mRNA levels of MRP5 and MRP6 after c-Myc overexpression. In the case of SLC transporter family, the mRNA level did not show statistically significant difference after c-Myc expression (Figure 3).

Western blot analysis was also performed with MCT1, OCT1, BCRP, MRP1 and MRP2. As illustrated in Figure 5, the protein expression of all tested transporters significantly increased after c-Myc overexpression.

Cellular Uptake Studies. To examine the effect of c-Myc amplification on the functional activity of drug transporters, the cellular uptake studies were performed using ^{14}C-benzoic acid, calcein-AM, H33342 and MPP^+ as a substrate of MCT1, MRPs, BCRP and OCT1, respectively in MCF10A cells with or without c-Myc expression. As shown in Figure 6, the cellular accumulation of fluoresecent calcein and H33342, substrates of efflux transporters, decreased significantly ($p < 0.05$) after c-Myc expression. However, the cellular uptake of ^{14}C-benzoic acid and MPP^+ increased approximately 60−71% in c-Myc-MCF10A cells compared to those in the control cells (Figure 6).

Discussion

Drug transporters are actively involved in the absorption, distribution, and elimination of various xenobiotics. Therefore, defects or alteration in transporter function can lead to a significant change in drug disposition, toxicity or pharmacological drug response. In many cases, altered transporter expression and variable drug response have been demonstrated in cancer, which can be attributed, at least in part, to changes in the expression of transporter genes.[2−4] Thus, regulating transporter expression and utilizing the knowledge of transporter functions are critical toward the success of anticancer chemotherapy. Approximately 50−100% of breast cancer cases show an increased level of c-Myc oncogene that could also be involved in the amplification of multiple other genes.[5,15−17] Although amplification, rearrangement, and overexpression of c-Myc are clearly present in a significant number of breast tumors, the predictive value and pathophysiological consequences of c-Myc amplification are not clearly defined yet. Furthermore, c-Myc amplification seems to be associated with better response to the chemotherapy in certain cancer cells,[12] which may be due to altered transporter function. Therefore, the present study constructed an in vitro model overexpressing c-Myc in breast epithelial cells and investigated the impact of c-Myc amplification on the gene expression and functional activity of 16 major influx and efflux transporters during the transition to the breast cancer.

Comparison of mRNA expression in MCF10A cells with/without c-Myc expression indicated qualitative similarities in expression profiles of transporters present at substantial levels or absent (MDR1, PETP1, OCT2, OAT1, OAT2, OAT3) in both cells. Transporter rank order according to mRNA levels and Spearman's rank correlation method ($\rho = 0.818$, $p = 0.002$) also supported qualitative similarities in mRNA expression profiles of major transporters after c-Myc overexpression (Figure 4). However, it is noteworthy that some quantitative differences were also observed after c-Myc expression. Particularly, the Western blot analysis and functional activity studies revealed that the gene expression

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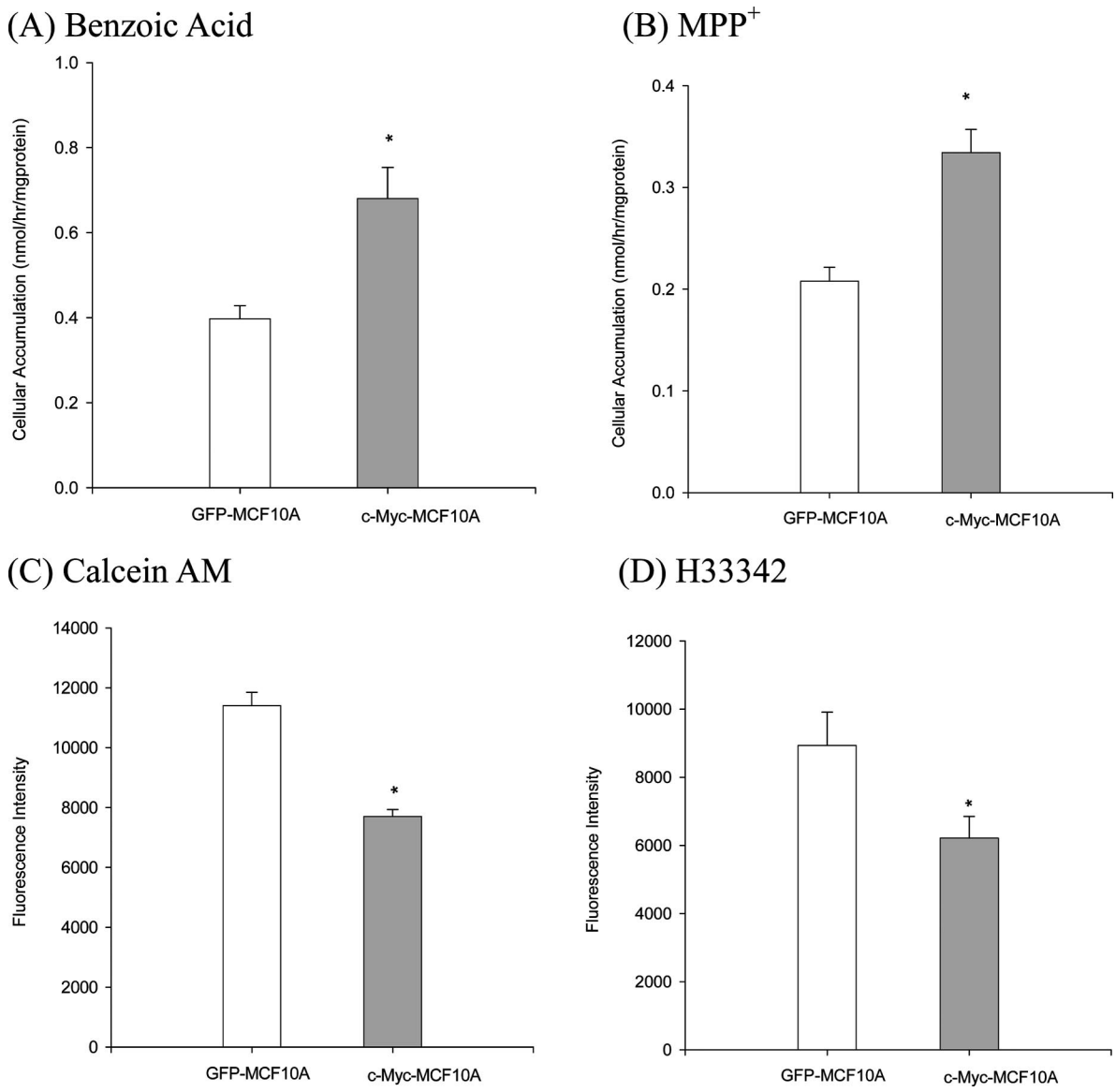

Figure 6. The effect of c-Myc overexpression on the cellular uptake of [^{14}C]-benzoic acid, MPP$^+$, calcein AM and H33342 in breast epithelial cells (mean ± SD, $n = 6$). * $p < 0.05$.

of selected uptake and efflux transporters were significantly increased after c-Myc amplification. For example, commonly overexpressed transporters in cancer such as MRP1, MRP2 and BCRP[18−20] were overexpressed in c-Myc-MCF10A cells, implying that c-Myc amplification may be associated with the upregulation of those efflux transporters. In addition to c-Myc, previous studies have suggested the role of several nuclear receptors including pregnane X receptor (PXR), farnesoid receptor (FXR), peroxisome proliferator-activated receptor (PPAR)α in the regulation of MRPs and BCRP gene.[21−24] Therefore, multiple signaling pathways seem to be involved in the regulation of gene expression of those efflux transporters and the mechanistic links among them

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need to be clearly defined in order that our knowledge of regulatory mechanisms aids in identifying and developing novel therapeutic strategies to modulate multidrug resistance gene expression.

In addition to efflux transporters, protein expression of uptake transporters such as MCT1 and OCT1 appeared to be upregulated by c-Myc overexpression although their mRNA levels were slightly decreased. The discrepancy between mRNA and protein expression of MCT1 and OCT1 is not uncommon. Reasons for the absence of correlation may be (i) there are many complicated and varied post-transcriptional mechanisms involved in turning mRNA into protein; (ii) proteins may differ substantially in their in vivo stability.[25] Jackson et al.[26] have also reported that there was little correlation between the mRNA levels and the protein expression of MCT1, suggesting that post-transcriptional mechanisms may be involved in regulating MCT1 expression. Furthermore, the enhanced protein levels of OCT1 and MCT1 in c-Myc-MCF10A cells may result from the increased protein stability.

As illustrated in Figures 5 and 6, the altered gene expression of transporters after c-Myc expression was well correlated with the altered transporter function. The cellular uptake of calcein AM, H33342, benzoic acid or MPP^+ was significantly changed in parallel to the enhanced gene expression of MRPs, BCRP, MCT1 and OCT1, respectively. While MRPs and BCRP efflux transporters confer resistance to a variety of anticancer drugs, the upregulation of uptake transporters such as MCT1 and OCT1 may be beneficial to increase the therapeutic effect of anticancer drugs. For example, among the major types of pH regulator for cancer cells, MCT1 is a proton-linked membrane carrier involved in the transport of monocarboxylates and plays an important role in preventing the apoptosis by lactic acidosis in tumor cells.[27,28] Therefore, c-Myc overexpression may contribute to prevent cellular acidosis, at least in part, via the upregulation of MCT1. Zhang et al.[29] have reported that the expression of OCT1, even at low levels, may play a significant role in the cytotoxicity of platinum-based anticancer drugs such as oxaliplatin. Therefore, c-Myc amplification may lead to greater tumor-sensitivity of oxaliplatin by up-regulating OCT1. Augenlicht et al.[12] have reported that the proliferating cells with higher c-Myc expression levels were more sensitive to chemotherapy. The reason that c-Myc amplification may predispose patients to better treatment response is unknown yet. Based on the results from our present study, a hypothesis is that this sensitivity change to chemotherapy in c-Myc overexpressed cancer cells might be, at least in part, due to the alteration of transporters' gene expression and the subsequent change in the cellular transport of chemotherapeutic agents. Our present study indicated that there was up-regulation of both uptake and efflux transporters with c-Myc amplification. However, if the functional activity of uptake transporters could overwhelm the effect of efflux transporters on the cellular accumulation, the overall sensitivity to certain anticancer drugs might be better in tumors with c-Myc amplification compared to tumors without c-Myc amplification. Further characterization should be warranted for more clarification.

Taken all together, c-Myc amplification could modulate the gene expression of certain drug transporters, which might influence the cellular delivery of anticancer drugs and chemosensitivity during the transition from normality to breast cancer.

Conclusion

c-Myc overexpression appeared to be coupled with the modulation of gene expression of certain drug transporters in human breast epithelial cells.

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