Expression of plasma membrane Ca^{2+}ATPases during gastric and colon cancer cell differentiation

Ph.D. Thesis

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Introduction

Cytoplasmic Ca^{2+} concentration of eukaryotic cells is approximately 10^4 times lower compared to that of the extracellular space. This low cytoplasmic Ca^{2+} concentration is generated and maintained by Ca^{2+} transport proteins located in the membranes of various cellular organelles and in the plasma membrane. Plasma membrane Ca^{2+}ATPases (PMCAs) transport Ca^{2+} from the cytoplasm to the extracellular space using the energy of ATP hydrolysis, therefore PMCAs participate in maintaining of low cytoplasmic Ca^{2+} concentration. Changes of cytoplasmic Ca^{2+} concentration triggered by physiological stimuli initiate signal transduction cascades that control cellular events, such as gene expression, proliferation, differentiation or apoptosis. Cells are gaining special information from spatial and temporal changes of cytoplasmic free Ca^{2+} concentration. As PMCAs play a key role in the regulation of global and local Ca^{2+} signalling, their expression, subcellular localization and activity should be tightly regulated.

PMCA proteins are encoded by 4 genes (*ATP2B1-4*), and alternative splicing of the primary transcripts of these genes generates more than 20 PMCA isoforms. As the PMCA isoforms display distinct biochemical characteristics, tissue/cell-specific expression, subcellular localization and protein-protein interactions, various PMCA isoforms can regulate diverse Ca^{2+}-dependent cell functions.

Recent studies demonstrated that changes of cytoplasmic Ca^{2+} concentration and function of Ca^{2+} transport proteins could regulate cell differentiation. Modulated expression of Ca^{2+} transport proteins were also demonstrated during the differentiation of various cell types. Several studies showed that the expression and activity of Ca^{2+} transport proteins that are key regulators of cellular Ca^{2+} signals were often altered in cancer cells characterized by less differentiated phenotype. These data taken together led to the proposal that Ca^{2+} transport proteins could become important targets in tumor therapies in the near future.

Aims of the study

1. Analysis of PMCA expression during gastric and colon cancer cell differentiation

As changes in Ca^{2+} homeostasis and in the expression of Ca^{2+} transport proteins during gastric and colon epithelial and carcinoma cell differentiation are largely unknown, we set the following aims:

a) Mapping of PMCA isoforms expressed in gastric and colon cancer cell lines.
b) Investigation of changes in PMCA isoform pattern during enterocyte-like and goblet cell-like colon cancer cell differentiation initiated by post-confluent culturing. Study of subcellular localization of PMCA proteins in differentiated enterocyte-like Caco-2 cells.
c) Study of changes in PMCA expressions during short chain fatty acid- (Na^+-butirate, Na^+-valerate) induced differentiation and trichostatin A (specific histone deacetylase inhibitor) treatment of gastric and colon cancer cell lines.
d) Comparison of PMCA-specific Ca^{2+} transport activities of microsomal membrane vesicles prepared from Na^+-butirate- or Na^+-valerate-treated and from untreated colon and gastric cancer cells.
e) Investigation of PMCA expression during growth inhibition without differentiation induction in gastric cancer cells.
f) As 1,25-$(OH)_2$-D_3 is a key regulator of Ca^{2+} homeostasis and is known to potentiate the differentiation of various tumor cell types, our aim was to study the effect of this vitamin on the maturation of colon cancer cells, and on the expression and subcellular localization of PMCA proteins expressed in colon cancer cells.

2. Development and validation of a novel quantitative RT-PCR method that provides more reliable data than those obtained by normalization to frequently used internal standard genes

Several studies reported that the expression level of commonly used internal standards in quantitative RT-PCR measurements may change considerably in response

to various factors, which can bias the quantification of the expression of target mRNAs in various experimental conditions. Therefore, we aimed to develop and validate a novel quantitative RT-PCR method that overcomes the incertitude of normalization to internal standard genes.

Methods

1. Culturing of mammalian and *Drosophila* cells

The human gastric (KATO-III) and colon (Caco-2, DLD-1, LS-174T, HT-29, HT29-5M12, HT29-5M21) carcinoma, and the mouse erythroid leukemia (MEL F4-6) cells were maintained at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. *Drosophila* Schneider Line 2 cells were cultured at 22 ^{o}C.

2. Induction of colon cancer cell differentiation in post-confluent cultures, and treatment of gastric and colon cancer cells

The differentiation of human colon carcinoma cells (Caco-2, DLD-1, HT-29, HT29-5M12 and HT29-5M21) was induced in post-confluent cultures. Exponentially growing colon tumor cells were cultured in Petri dishes until they reached confluency and further cultured for additional 22-32 days. Culture medium was renewed every second day.

The differentiation of gastric (KATO-III) and colon (DLD-1, HT-29, LS-174T) carcinoma cells was induced by treatments with Na^+-butyrate or Na^+-valerate. Gastric (KATO-III) and colon (DLD-1, LS-174T) cancer cells were also treated with the specific histone deacetylase inhibitor trichostatin A. Colon cancer cells were treated with the potential differentiation inducing 1,25-$(OH)_2$-D_3, as well. Exponentially growing cells were seeded into Petri dishes. At day 1-3 post-plating, medium was renewed and drugs were added from the concentrated stock solutions. In contrast to Na^+-butyrate, Na^+-valerate or trichostatin A treatments, 1,25-$(OH)_2$-D_3 was added repeatedly at second and fourth days of treatment, as well. 2 and 20 days post-confluent Caco-2 and 1 day post-confluent DLD-1 cells were also treated with 1,25-$(OH)_2$-D_3 every second day for 6 days.

3. Western blot analysis

Equal amounts of total cellular proteins obtained from gastric/colon cancer cell lines were analyzed by Western blotting for the expression of PMCAs (using the mAb

5F10: pan-anti-PMCA, the pAb NR1: anti-PMCA1, the mAb JA9: anti-PMCA4, the mAb JA3: anti-PMCA4b antibodies), the differentiation markers: CEA, DPP-IV, SERCA3 (using the mAb anti-CEA, the mAb anti-DPP-IV, the mAb PL/IM430: anti-SERCA3 antibodies), and the SERCA2 or Na/K-ATPase (as controls) (using the mAb IID8: anti-SERCA2, the mAb anti-Na/K-ATPase antibodies). The protein concentration of the samples was determined by the modified Lowry method.

4. Quantitative evaluation of the changes in PMCA expressions using Western blot analysis

To estimate the fold increases in PMCA protein expressions, the following protocol was established. Increasing amounts of total cellular proteins obtained from the samples to be compared were loaded onto SDS-polyacrylamide gels. After gel electrophoresis and electrotransfer onto PVDF-membranes, blots were immunostained with the mAb 5F10 pan-anti-PMCA (for PMCA1b), or with the mAb JA3 anti-PMCA4b (for PMCA4b) antibodies. Luminograms were scanned and densitometrically analyzed. Lines were fitted to the values obtained for each particular sample. The fold increases in PMCA protein expressions were calculated from the analysis of the corresponding lines.

5. Isolation of mixed microsomal membranes and Ca^{2+} transport assay

Following the Na^{+}-butyrate or Na^{+}-valerate treatments of the KATO-III gastric cancer or DLD-1 colon cancer cells, microsomal membranes were prepared. PMCA-driven Ca^{2+} uptake by microsomal membrane vesicles prepared from control and treated cells was measured in the presence of calmodulin (1174 nM), thapsigargin (to block SERCA-driven Ca^{2+} uptake) and oligomycin (to block mitochondrial ATP synthesis) at saturating Ca^{2+} concentration.

6. RNA isolation and real-time quantitative RT-PCR measurements

Pre- and post-confluent Caco-2 colon cancer cells, DLD-1 colon tumor cells mixed with *Drosophila* S2 cells, or KATO-III gastric tumor cells mixed with *Drosophila* S2 or mouse MEL F4-6 cells were lysed in Trizol reagent. Following the

total RNA isolation, total cDNA was prepared by reverse transcription from equal amounts of RNAs. PMCA4b/SERCA3/IAP/E-cadherin/GAPDH/β2-microglobulin/ Pu.1/ddd4 cDNS concentrations were determined by quantitative PCR using a Light Cycler instrument.

During the post-confluent differentiation of Caco-2 cells, the mRNA expressions of PMCA4b, furthermore, in 1,25-$(OH)_2$-D_3-/vehicle (DMSO)-treated early post-confluent Caco-2 cells, the mRNA expressions of two well-established differentiation markers (the IAP and E-cadherin) were quantified by normalizing to the β2-mikroglobulin and/or GAPDH mRNA expressions.

The SERCA3 and/or PMCA4b mRNA expression levels were measured in control and Na^+-butyrate-treated KATO-III and DLD-1 cells using our novel eccPCR method. In the eccPCR measurements *Drosophila* S2 or mouse MEL F4-6 cells were used as control cells. Target mRNA expression levels were determined by normalization to the *Drosophila* ddd4 or mouse Pu.1 mRNA standards.

7. Immunocytochemical studies

Subcellular localization of PMCA proteins and the effect of 1,25-$(OH)_2$-D_3 on PMCA localization were analyzed in differentiated Caco-2 cells. PMCA-specific immunofluorescent stainings were performed using the mAb 5F10 (pan-anti-PMCA), the pAb NR1 (anti-PMCA1) and the mAb JA3 (anti-PMCA4b) antibodies. Subcellular localization of the basolateral plasma membrane marker Na/K-ATPase and of the mainly apical plasma membrane located DPP-IV was also detected.

Results

1. Development and validation of the eccPCR (external cell control quantitative RT-PCR) method

We developed external cell control quantitative RT-PCR, a novel method that overcomes the incertitude of normalization to internal standard genes generally used in quantitative RT-PCR methods. The main step of the eccPCR method is the addition of fixed amount of control cells to fixed amount of cells of interest. The subsequent steps of this method, i.e. sample preparation and analysis (RNA isolation, reverse transcription and real time quantification) are performed on these cell mixtures. In eccPCR the calculated relative concentration of the target mRNA is normalized to the relative concentration of a control mRNA exclusively expressed in the control cells. This way the amount of control mRNA is certainly not changed by any effect happening prior to cell mixing (e.g. treatments of studied cells). At the same time, this technique also controls all steps of sample preparation and analysis, similarly to quantitative RT-PCR methods that use internal standard genes. The control cells and the primers designed for the reference and the target genes should be tested for their inability to amplify from the target and control cells, respectively.

Subsequently, we validated this novel eccPCR method. The expression levels of the SERCA3 mRNA in untreated and Na^+-butyrate-treated KATO-III gastric cancer cells were detected both by eccPCR method and also by normalizing to the widely used GAPDH mRNA standard. Our measurements showed that the expression of the SERCA3 mRNA was highly elevated in Na^+-butyrate-treated KATO-III cells as compared to untreated cells, and this change in SERCA3 mRNA expression could be detected by both methods. However, the fold increases in SERCA3 mRNA level determined with the eccPCR or the internal standard based methods differed significantly. We could confirm using the eccPCR method that this difference was due to a 50% decrease in the expression of the GAPDH mRNA provoked by the Na^+-butyrate treatment. Our results demonstrate that the eccPCR method can be used to detect either great or small mRNA expression level differences.

2. Expression of PMCA isoforms in gastric and colon cancer cells

Our studies demonstrated that PMCA1b is the dominant PMCA isoform expressed in gastric (KATO-III) and colon (DLD-1, Caco-2, LS-174T, HT-29, HT29-5M12, HT29-5M21) carcinoma cells, and that PMCA4b is also expressed, but at lower levels.

3. Expression of PMCA isoforms during the post-confluent differentiation of colon cancer cells

Several studies demonstrate that the differentiation of various colon tumor cell lines can be induced in post-confluent cell cultures. During the post-confluent maturation of Caco-2, DLD-1 and HT-29 cells we followed the expression of the PMCA1b and 4b isoforms. In all the three cell lines the post-confluency-induced differentiation resulted in marked upregulation of PMCA4b protein expression, while the amount of PMCA1b increased (Caco-2) or decreased (HT-29) to a smaller degree, or did not change (DLD-1). We also detected the upregulated PMCA4b mRNA expression during the differentiation of Caco-2 cells. It indicates that the upregulation of PMCA4b protein expression is mainly due to an increase in the PMCA4b mRNA expression, and is not a result of unknown translational or post-translational regulation mechanisms.

Furthermore, we investigated the expression of the PMCA1b and 4b proteins, and of well-established differentiation markers (SERCA3, CEA) in HT-29, and the HT-29-derived enterocyte-type (HT29-5M12) and goblet cell-type (HT29-5M21) colon cancer cells. Our results showed that the expression of PMCA4b, as well as that of the differentiation markers was elevated in the daughter cells compared to those of the parental HT-29 cell line. In contrast, PMCA1b expression was similar in all the three cell lines examined. In accordance with our previous data, these results also demonstrate that PMCA4b expression is in strict correlation with the differentiation state of colon cancer cells.

Changes of PMCA expressions were also monitored during the post-confluent differentiation of the enterocyte-type HT29-5M12 and the goblet cell-type HT29-5M21 cells. We found that the expression of PMCA4b increased during both types of

maturation, whereas the expression of PMCA1b did not change in these processes. These data suggest that the upregulation of PMCA4b expression is characteristic to either enterocyte-type or goblet cell-type colon cancer cell differentiation.

4. Changes in PMCA expressions during short chain fatty acid and trichostain A treatments of gastric and colon cancer cells

There are data demonstrating that the differentiation of gastric and colon cancer cells can be initiated by short chain fatty acid (e.g. Na^+-butyrate, Na^+-valerate) treatments. Therefore, we investigated the changes in the expression of PMCA proteins during the Na^+-butyrate- or Na^+-valerate-induced maturation of gastric (KATO-III) and colon (DLD-1, HT-29 and LS-174T) cancer cells. Our results showed marked upregulation of PMCA4b expression during the short chain fatty acid-induced differentiation of all tested cell lines, whereas the expression of the PMCA1b protein either did not change or upregulated only slightly, depending on the treatment and cell-type. Using the novel eccPCR method we confirmed that Na^+-butyrate treatment resulted in upregulated PMCA4b mRNA expression of the tested gastric (KATO-III) and colon (DLD-1) cancer cell lines. The expression of PMCA4b was also elevated in gastric (KATO-III) and colon (DLD-1, LS-174T) cancer cells treated with the specific histone deacetylase inhibitor trichostatin A as compared to untreated cells. As short chain fatty acid treatments are known to regulate gene expressions principally by inhibiting the activity of histone deacetylase enzymes, the Na^+-butyrate- or Na^+-valerate-induced upregulation of PMCA4b expression in gastric and colon cancer cells may be mainly due to their histone deacetylase inhibitor activity. However, trichostatin A treatment increased PMCA4b protein expression to smaller extent as compared to Na^+-butyrate treatment, in all cell lines examined. These results suggest that other short chain fatty acid-induced regulatory mechanisms may also participate in the stimulation of *PMCA4* expression.

Parallel to their effect on PMCA4b expression, Na^+-butyrate and Na^+-valerate treatments also inhibit growth and induce differentiation in gastric and colon cancer cells. Therefore, we investigated whether the inhibition of proliferation *per se* had any effect on PMCA4b expression. Our results showed that growth inhibition by serum withdrawal did not affect PMCA4b expression, so the differentiation inducing activity

of short chain fatty acids is likely to be necessary for the upregulation of PMCA4b expression.

We also studied differences in PMCA-driven Ca^{2+}-transport activities of microsomal membranes prepared from Na^{+}-butyrate- or Na^{+}-valerate-treated *v.s* untreated KATO-III gastric and DLD-1 colon cancer cells. PMCA-specific Ca^{2+}-transport activities were measured in the presence of calmodulin (1174 nM), thapsigargin (to block SERCA-driven Ca^{2+} uptake) and oligomycin (to block mitochondrial ATP synthesis) at saturating Ca^{2+} concentration. The treatment of both cell types resulted in upregulated PMCA-specific Ca^{2+}-transport activities of microsomal membrane vesicles. Our data suggest that the differentiation-dependent upregulation of PMCA expression results in increased PMCA-specific Ca^{2+}-transport activity in these tumor cell types. On the basis of these data we assume that upregulated PMCA4b expression together with a major switch in PMCA isoform pattern may significantly contribute to reorganization of cellular Ca^{2+} homeostasis and Ca^{2+}-dependent cell functions during the differentiation of gastric/colon cancer cells.

5. Changes in PMCA expressions during 1,25-$(OH)_2$-D_3-vitamin treatments of colon cancer cells

There is increasing evidence that the 1,25-$(OH)_2$-D_3-vitamin has antiproliferative, differentiation inducing and apoptosis inducing effects on various tumor cell types. In the case of some colon carcinoma cell lines the differentiation inducing effect of the 1,25-$(OH)_2$-D_3 was confirmed, while in others this vitamin had no effect on cell maturation. As our previous results demonstrated the upregulation of PMCA4b expression during colon cancer cell differentiation, we also investigated the effect of the potential differentiation inducing agent 1,25-$(OH)_2$-D_3 on the PMCA4b expression of some colon tumor cell lines.

First we tested whether the 1,25-$(OH)_2$-D_3 can induce the differentiation of pre-confluent or potentiate the maturation of differentiating early post-confluent Caco-2 colon cancer cells. As 1,25-$(OH)_2$-D_3 had no effect on the expression of well-established differentiation markers, DPP-IV, CEA and SERCA3, in pre-confluent Caco-2 cells, we concluded, that this vitamin could not induce the differentiation of Caco-2 cells. The post-confluency induced upregulation of the DPP-IV and SERCA3

protein expressions, and of the IAP and E-cadherin mRNA expressions were also not modified by the 1,25-$(OH)_2$-D_3 treatment of early post-confluent differentiating Caco-2 cells. This vitamin could only stimulate the post-confluency-induced upregulation of the CEA protein expression. Therefore, we concluded that 1,25-$(OH)_2$-D_3 could only potentiate the post-confluent differentiation of Caco-2 cells by regulating the expression of certain genes, but it had no effect on the majority of differentiation-related gene expressions. As our previous results demonstrated that PMCA4b expression is upregulated during colon cancer cell differentiation, we also monitored its expression during the 1,25-$(OH)_2$-D_3 treatment of pre-confluent and early post-confluent Caco-2 cells. These experiments showed that 1,25-$(OH)_2$-D_3 had effect neither on PMCA4b expression of pre-confluent Caco-2 cells, nor on the differentiation-induced upregulation of PMCA4b expression of early post-confluent Caco-2 cells.

It is well-documented that 1,25-$(OH)_2$-D_3-vitamin plays an essential role in Ca^{2+} homeostasis. This vitamin can potentiate the efficiency of dietary Ca^{2+} absorption by the small intestinal enterocytes, by stimulating paracellular and transcellular intestinal Ca^{2+} transport mechanisms. PMCA proteins located in the plasma membrane of small intestinal enterocytes participate in transcellular Ca^{2+} absorption of the intestine. There is increasing evidence that 1,25-$(OH)_2$-D_3 can stimulate intestinal PMCA1b mRNA expression. Based on these observations it is presumed that 1,25-$(OH)_2$-D_3-modulated upregulation of PMCA1b expression is an important step of 1,25-$(OH)_2$-D_3-induced intestinal Ca^{2+} absorption. However, the upregulation of PMCA1b protein expression in human enterocytes has never been published. As differentiated Caco-2 cells are widely used as an *in vitro* model system of intestinal enterocytes, we investigated the effect of 1,25-$(OH)_2$-D_3 on PMCA1b protein expression using this cell line. Pre-confluent and early post-confluent Caco-2 cells were treated with physiologically relevant concentrations of 1,25-$(OH)_2$-D_3 and expression of PMCA1b protein was monitored. The 1,25-$(OH)_2$-D_3-vitamin stimulated the expression of PMCA1b protein both in pre-confluent and early post-confluent Caco-2 cells. The regulatory effect of 1,25-$(OH)_2$-D_3 on PMCA1b expression was also demonstrated in pre-confluent and early post-confluent DLD-1, the enterocyte-type colon cancer cells. In accordance with previous data our results also suggest that the stimulatory effect of 1,25-$(OH)_2$-D_3-vitamin on

intestinal PMCA1b expression could contribute to the vitamin-induced intestinal Ca^{2+} absorption.

6. Subcellular localization of PMCA1b and PMCA4b proteins in differentiated Caco-2 cells

We investigated the subcellular localization of PMCA proteins in differentiated Caco-2 cells for the following reasons. ***i.*** Differentiation-induced changes in PMCA expression can only influence resting cytoplasmic Ca^{2+} concentration and the cellular Ca^{2+} signalling if PMCA proteins are properly targeted to the plasma membrane. Therefore, we tested the plasma membrane localization of upregulated PMCA proteins in differentiated Caco-2 colon cancer cells. ***ii.*** It is well-documented that PMCA proteins located in the basolateral plasma membrane of small intestinal enterocytes participate in the transcellular pathway of intestinal Ca^{2+} absorption. Our previous results demonstrated that the PMCA1b and PMCA4b proteins are expressed in the differentiated Caco-2 cells, often used as *in vitro* model system of the small intestinal enterocytes. We can only preview the potential role of these PMCA isoforms in intestinal Ca^{2+} absorption, if we determine their exact subcellular localization.

The subcellular localization of PMCA1b and 4b proteins expressed in differentiated Caco-2 cells was examined using laser scanning confocal microscopy. The co-localization of the two PMCA isoforms and the plasma membrane marker Na/K-ATPase was confirmed, so we concluded that PMCA pumps localized mainly to the plasma membrane of differentiated Caco-2 cells. These results also suggest that the modulated PMCA expression observed during colon cancer cell differentiation may significantly influence cellular Ca^{2+} homeostasis and Ca^{2+}-dependent cell functions.

We also studied the localization of the PMCA1b and PMCA4b proteins within the plasma membrane of polarized, differentiated Caco-2 cells. Using the basolateral plasma membrane-located Na/K-ATPase and the mainly apical plasma membrane-located DPP-IV proteins as localization markers, we confirmed that the PMCA1b and 4b isoforms were expressed exclusively in the basolateral plasma membrane of differentiated Caco-2 cells. Based on these results it is likely that both PMCA1b and PMCA4b proteins assist intestinal Ca^{2+} absorption.

Recent results showed that the localization of PMCA proteins expressed in kidney cells could be affected by 1,25-$(OH)_2$-D_3. Therefore, we tested whether 1,25-$(OH)_2$-D_3 had any effect on the subcellular localization of PMCA proteins in differentiated enterocytic Caco-2 cells. The subcellular localization of PMCA proteins was unaltered by the 1,25-$(OH)_2$-D_3 treatment of late post-confluent Caco-2 cells. In this experimental setup, however, we observed that the PMCA-specific immunofluorescent signal intensity obtained from 1,25-$(OH)_2$-D_3-treated Caco-2 cells was significantly higher as compared to that of vehicle (DMSO)-treated cells. In accordance with our previous results, it also confirmed the regulatory effect of 1,25-$(OH)_2$-D_3 on PMCA (presumably on PMCA1b) protein expression.

Conclusions

1. Expression of PMCA isoforms during the differentiation or 1,25-$(OH)_2$-D_3 treatment of gastric and colon cancer cells

Our studies demonstrated that PMCA1b is the dominant PMCA isoform expressed in gastric and colon carcinoma cells, and that PMCA4b is also expressed, but at lower levels. Our results also showed that differentiation of gastric and colon cancer cells resulted in upregulated PMCA4b protein (and mRNA) expressions, while PMCA1b protein levels did not change or only moderately increased or decreased in these processes. If further studies confirm that PMCA4b expression is decreased in gastric and colon carcinoma cells compaired to normal epithelial cells in differentiation-dependent manner, the analysis of PMCA4b expression can provide a new diagnostic tool for monitoring tumor phenotypes. As our results demonstrated that PMCA-specific Ca^{2+}-transport activities of microsomal membranes prepared from Na^+-butyrate- or Na^+-valerate-treated gastric and colon cancer cells were significantly higher as compared to those of untreated cells, we suggest that differentiation-dependent changes in PMCA isoform pattern may influence cellular Ca^{2+} homeostasis and Ca^{2+}-dependent cell functions in gastric/colon carcinoma cells. If further studies show that the upregulation of PMCA4b expression contributes to the regulation of Ca^{2+}-dependent cellular events, such as proliferation, differentiation or apoptosis in gastric/colon cancer cells, PMCA4b may become a potential drug target in the therapy of human gastrointestinal malignancy.

As PMCA1b and PMCA4b proteins localize in the basolateral plasma membrane of the small intestinal enterocyte-like differentiated Caco-2 cells, we suggest that both PMCA isoforms may participate in the intestinal Ca^{2+} absorption. The 1,25-$(OH)_2$-D_3 treatments potentiated PMCA1b protein expression of Caco-2 cells, while PMCA4b expression level was not affected. Therefore, it is likely that the modulation of PMCA1b expression is important in 1,25-$(OH)_2$-D_3-induced intestinal Ca^{2+} absorption.

2. External cell control quantitative RT-PCR method

We developed and validated the novel eccPCR method, and demonstrated that it can be used to detect either great or small mRNA expression level differences. This technique overcomes the incertitude of normalization to internal standard genes generally used in quantitative RT-PCR methods. Similarly to internal standard-based qPCR methods, eccPCR also controls all steps of sample preparation and analysis. We conclude that the eccPCR method provides more reliable data than those obtained by normalization to frequently used internal standard genes, and we especially recommend it for the detection of physiologically relevant, but subtle mRNA expression level differences.

Publications related to the thesis

1. Ribiczey P, Tordai A, Andrikovics H, Filoteo AG, Penniston JT, Enouf J, Enyedi A, Papp B, Kovács T. (2007) Isoform-specific up-regulation of plasma membrane Ca^{2+}ATPase expression during colon and gastric cancer cell differentiation. Cell Calcium, 42(6): 590-605.
2. Bors A*, Ribiczey P*, Köblös G*, Brózik A, Ujfaludi Z, Magócsi M, Váradi A, Tordai A, Kovács T, Arányi T. (2008) External cell control polymerase chain reaction: replacing internal standards with an unbiased strategy for quantitative polymerase chain reaction normalization. Anal Biochem., 372(2): 261-263.
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