Original Article

Regulation of cellular communication factor 2 (CCN2) in breast cancer cells via the cell-type dependent interplay between CCN2 and glycolysis

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Abstract

[***Objectives***] Anti-osteoclastic treatments for breast cancer occasionally cause medication-related jaw osteonecrosis. Moreover, elevated glycolytic activity, which is known as the Warburg effect, is usually observed in these breast cancer cells. Previously, we found that cellular communication network factor 2 (CCN2) production and glycolysis enhanced each other in chondrocytes. Here, we evaluated the interplay between CCN2 and glycolysis in breast cancer cells, as we suspected a possible involvement of CCN2 in the Warburg effect in highly invasive breast cancer cells.

[***Methods***] Two human breast cancer cell lines with a distinct phenotype were used. Glycolysis was inhibited by using 2 distinct compounds and gene silencing was performed using siRNA. Glycolysis and the expression of relevant genes were monitored via colorimetric assays and quantitative RT-PCR, respectively.

[***Results***] Although *CCN2* expression was almost completely silenced when treating invasive breast cancer cells with a siRNA cocktail against *CCN2,* glycolytic activity was not affected. Notably, the expression of glycolytic enzyme genes, which was repressed by CCN2 deficiency in chondrocytes, tended to increase upon *CCN2* silencing in breast cancer cells. Inhibition of glycolysis, which resulted in the repression of *CCN2* expression in chondrocytic cells, did not alter or strongly enhanced *CCN2* expression in the invasive and non-invasive breast cancer cells, respectively.

[***Conclusions***] High *CCN2* expression levels play a critical role in the invasion and metastasis of breast cancer. Thus, a collapse in the intrinsic repressive machinery of *CCN2* due to glycolysis may induce the acquisition of an invasive phenotype in breast cancer cells.

Keywords**:** CCN2; glycolysis; Warburg effect; breast cancer; bone metastasis

Abbreviations**:**

RT-PCR, reverse transcription-polymerase chain reaction; MIA, monoiodoacetic acid; MRONJ, medication-related osteonecrosis of the jaw; CCN2, cellular communication network factor 2

1. **Introduction**

It is widely recognized that malignant tumors form metastatic lesions in preferred tissues and organs, which depend upon the tumor origin [1]. For example, the liver is the most common metastasis target of esophageal, gastric, colorectal, and pancreatic cancer cells. However, breast and prostate cancers form metastases preferentially in the bone tissues, including those in the orofacial region. Bone metastasis of these cancers in the orofacial region is a life-threatening complication that severely affects the quality of life and orofacial functions of patients. Moreover, even the chemotherapeutics used to control these severe complications are known to impair the integrity of orofacial bones, particularly the mandibular bone. In order to develop a secondary tumor in the bone tissue, metastasizing tumor cells have to remove the calcified bone matrix. Although they can degrade extracellular matrix proteins by producing proteases, these cells are not able to resorb the calcium phosphate precipitate. Therefore, in order to achieve and progress bone metastasis, tumor cells need the cooperation of osteoclasts, as they are the only cells that can resorb calcified bone. Thus, the repression of osteoclast activity is one of the major strategies employed for the treatment of bone metastases caused by highly metastatic breast cancers. Currently, bisphosphonates and neutralizing antibodies against receptor activator of NF-κB ligand (RANKL), which is required for osteoclastogenesis, are widely used in clinical practice [2,3]. Indeed, these agents have been found to be effective in preventing and delaying skeletal-related events, which include bone pain, pathologic fractures, spinal cord compression, and hypercalcemia. Particularly in estrogen receptor-positive breast cancers, these therapeutics are also useful in ameliorating therapy-induced bone loss. However, it is now clear that these anti-osteoclastogenic agents occasionally

cause an irreversible complication called medication-related osteonecrosis of the jaw (MRONJ) [4]. As such, an alternative strategy to repress bone metastasis is necessary, in order to protect the oral function of the patients. However, as long as the therapeutic targets are limited to osteoclasts, MRONJ may be unavoidable. Thus, a novel strategy which targets the tumor cells themselves in order to repress bone metastasis is more feasible. For this purpose, more knowledge on the molecular mechanisms that underlie the development of a malignant phenotype in breast and prostate cancer cells is required.

Highly malignant tumor cells, including breast cancer cells, are usually characterized by enhanced glycolytic activity, also known as the Warburg effect, which supports the aggressive behavior of these cells [5]. This effect was initially observed in the 1920s but the underlying mechanism is still unclear. Recently, we discovered that the glycolytic activity of chondrocytes, which survive in avascular cartilage tissues, was supported by the cellular communication network factor (CCN) 2 [6,7]. CCN2 is a member of the CCN family, which contains 6 proteins with multiple functions. The CCN protein family is involved in the physiological development of various tissues, as well as pathological processes which lead to fibrotic disorders or malignancies [8,9]. In relation to the energy metabolism, CCN2 and CCN3 were found to play significant roles, as *CCN2* expression was repressed, whereas *CCN3* expression was strongly induced by glycolytic inhibition in chondrocytic cells (Fig. 1A) [10]. Interestingly, strong *Ccn3* induction is also observed in *Ccn2*-null mouse chondrocytes [6]. Although the biological significance and cell-type specificity of this *CCN3* induction are still unclear, CCN2 was clearly shown to contribute to glycolysis in chondrocytes. In fact, glycolytic activity and ATP production were severely impaired in chondrocytes from *Ccn2*-null mice, which was also observed in *CCN2*-silenced human chondrocytic HCS-2/8 cells (Fig. 1A) [6]. However, since the

metabolic effect of CCN2 was not evaluated in other types of cells, it remains unclear whether this a chondrocyte-specific or a general cellular event. Notably, elevated CCN2 expression levels were observed in a variety of malignancies, including breast cancer [8,9,11,12]. Furthermore, CCN2 was reported to play a critical role in the bone-metastatic phenotype of breast cancer cells [13-15], suggesting that CCN2 plays a role in the aggressive phenotype of breast cancers.

Although their origin and phenotype are quite different, chondrocytic HCS-2/8 and breast cancer MDA-MB-231 cells are both characterized by high CCN2 expression levels. Thus, as we suspected that CCN2 may be a factor which drives the development of a malignant phenotype in breast cancer cells by inducing the Warburg effect, we investigated the interplay between CCN2 and glycolysis in the two cell lines mentioned and uncovered a novel regulatory system of CCN2 by glycolysis in breast cancer cells.

2. Materials and methods

2.1. Cell cultures

The MDA-MB-231 cell line is an estrogen-independent breast cancer cell line that is characterized by a highly malignant phenotype [16-18]. The other breast cancer cell line used, namely MCF7, is known to be less metastatic and retains estrogen dependence [16]. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 atmosphere.

2.2. Inhibition of glycolysis

In order to inhibit glycolysis in the cells, two distinct reagents were employed. One is monoiodoacetic acid (MIA), which is an inhibitor of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), while the other is sodium fluoride (NaF), which inhibits enolases [19]. MDA-MB-231 and MCF7 cells were seeded in 6-well plates and were cultured until confluence. Then, the cells were treated with the respective glycolysis inhibitors for 12 h. MIA (Sigma-Aldrich, St. Louis, MO, USA) was added at a final concentration of 0, 2 or 4 μg/mL, whereas NaF was used at a final concentration of 0, 1 or 5 mM. Afterwards, total RNA extraction was extracted from the cells as described below.

2.3. Lactate quantification

Lactate concentrations in the conditioned media from MDA-MB-231 cell cultures were quantified using Lactate Colorimetric/Fluorometric Assay Kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions. Briefly, the conditioned media was diluted in 50 μL of Lactate Assay Buffer in 96-well plates. Enzymatic color development was initiated by adding 50 μL of the reaction mixture containing the enzyme and colorimetric probe. The reaction was performed at room temperature for 30 min and the absorbance was measured at a wavelength of 570 nm using an automated microplate reader (Multiscan JX, Thermo Labsystems, Helsinki, Finland).

2.4. RNA extraction and quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA extraction and purification from cells were performed using an RNeasy® Mini Kit (Qiagen, Hilden, Germany) or ISOGEN (Nippongene, Tokyo, Japan), according

to the manufacturer's instructions. Equal amounts of total RNA were reverse-transcribed to cDNA using the avian myeloblastosis virus reverse transcriptase with an oligo dT primer in the PrimeScript™ RT reagent Kit (Takara Bio, Shiga, Japan). Subsequently, quantitative real-time PCR was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Basel, Switzerland) using the SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan).

The following primers were used for the specific amplification of the target cDNAs: 5′ - GCA GGC TAG AGA AGC AGA GC - 3′ (forward) and 5′ - ATG TCT TCA TGC TGG TGC AG - 3′ (reverse) for human *CCN2*; 5′ - GGA GCG CGC TAT AAA ACC TG - 3′ (forward) and 5′ - TCC CCT CTC GCT TTT ACC AA - 3′ (reverse) for human *CCN3*; 5′ - CCA AAT GGA ACA CAG AGG ATA AAG - 3′ (forward) and 5′ - AAC ACT AGG TTG ACT TAG GAG CAC - 3′ (reverse) for human *PGK*; 5′ - TCT GGA GAC GAT CTT ATG ATG TC - 3′ (forward) and 5′ - AGC TGG TCT TCA GTA AGG TCT GC - 3′ (reverse) for human *PGAM1*; 5′ - CCA AAG AGG ATC GCC AA - 3′ (forward) and 5′ - CCC CGA ACG ATG AGA CA - 3′ (reverse) for human *ENO1*; and 5′ - GAT CAT TGC TCC TCC TGA GC - 3′ (forward) and 5′ - ACT CCT GCT TGC TGA TCC AC - 3′ (reverse) for human *ACTB (β-actin)*.

2.5. Gene silencing

For *CCN2* silencing via RNAi we used a small interfering RNA (siRNA) cocktail composed of 3 distinct siRNAs directed against human *CCN2* (Santa Cruz Biotechnology, Santa Cruz, CA, USA), together with a non-targeting control siRNA (Santa Cruz Biotechnology). MDA-MB-231 cells were seeded into 12-well plates and transfected at sub-confluence with 10 nM of each siRNA using the Lipofectamine® RNAiMAX

Reagent (Thermo Fisher Scientific, Carlsbad, CA), according to the manufacturer's indications. Forty-eight hours post-transfection, the total cellular RNA was extracted and conditioned media were collected from the cell cultures for further analyses.

2.6. Gene Expression Omnibus (GEO) data

A dataset with the accession number GSE111653 was downloaded from the Gene Expression Omnibus (GEO) website (https://www.ncbi.nlm.nih.gov/geo/) at the National Center for Biotechnology Information (NCBI). This dataset, which was provided by Fertig and Gilkes [17], contains exhaustive RNA sequencing data obtained using the Illumina Hiseq 3000/4000 (Illumina, San Diego, CA). The dataset also quantified the RNA from both breast cancer and normal mammary epithelial cells. The quality of the data was checked by Illumina SAV (Illumina).

2.7. Statistical Analysis

The statistical comparisons between 2 experimental groups were performed by using the Student's *t*-test, whereas Tukey's test was employed for multiple comparisons of more than 2 groups.

3. Results

3.1. The effects of CCN2 silencing on glycolysis and CCN3 expression in MDA-MB-231 breast cancer cells

According to our previous studies, CCN2 and glycolysis regulate each other by

forming a positive feedback loop in chondrocytes [6,10], which strongly suggests that CCN2 could be a key molecule in the development of the Warburg effect (Fig. 1A). Therefore, as we suspected that CCN2 played a critical role in the Warburg effect, we evaluated the effect of *CCN2* silencing on glycolysis in human breast cancer MDA-MB-231 cells, which are characterized by elevated glycolysis. Thus, by using a siRNA cocktail against *CCN2*, we successfully performed knockdown of *CCN2* expression in those cells (Fig. 1B). We also confirmed that the non-targeting control siRNA itself did not affect the expression of CCN2 (Suppl. Fig. 1). However, we found that lactate production, which represents glycolytic activity, was not significantly affected in *CCN2*-silenced cells (Fig. 1C). It is known that CCN2 deficiency induces *CCN3* expression, as well as impaired glycolysis in chondrocytes [20]. This *CCN3* induction is thought to be mediated by the impairment of glycolytic activity, as glycolysis negatively regulates *CCN3* in chondrocytes (Fig. 1A) [10]. However, CCN2 deficiency in MDA-MB-231 cells was shown to have no effect on *CCN3* expression (Fig. 1D), suggesting that CCN2 may not regulate glycolysis and/or glycolysis may not regulate *CCN3* expression in these cells. To further clarify this, MDA-MB-231 cells were treated with MIA, and then *CCN3* expression levels were evaluated. As shown in Fig. 1E, *CCN3* expression was increased by MIA treatment in a dose-dependent manner, confirming that an active regulatory system of CCN3 by glycolysis is also present in breast cancer cells. Collectively, our results show that, unlike in chondrocytic cells, glycolysis is not controlled by CCN2 in MDA-MB-231 cells.

3.2. The effects of CCN2 silencing on glycolytic enzyme gene expression

In *Ccn2*-deficient chondrocytes, the expression of three genes that encode enzymes

which catalyze three steps of the glycolysis process was found to be attenuated (Fig. 2A) [6,21]. These genes encode phosphoglycerate kinase 1 (*PGK1*), phosphoglycerate mutase 1 (*PGAM1*), and enolase 1 (*ENO1*). In contrast to the results in chondrocytes, *CCN2* silencing did not affect the expression of these genes and even led to an increase in the expression of two out of the three genes (Fig. 2). These results are consistent with those shown in Fig. 1, therefore solidly confirming that glycolysis is independent of CCN2 in MDA-MB231 cells.

3.3. The effects of glycolysis inhibition on CCN2 expression in breast cancer cells with distinct phenotypes

Next, in order to determine whether *CCN2* expression is also positively regulated by ATP and/or glycolysis in highly metastatic breast cancer cells, *CCN2* expression was analyzed in the presence of increasing concentrations of MIA. However, MIA did not significantly alter *CCN2* expression in MDA-MB-231 cells (Fig. 3A), which indicates that *CCN2* expression is not positively controlled by glycolysis. As *CCN2* expression in MDA-MB-231 cells showed a tendency of increase after MIA treatment (Fig. 3A), we performed a similar analysis using another breast cancer cell line, MCF7, as we suspected that it may have a different *CCN2* regulatory system. Surprisingly, *CCN2* expression was strongly induced upon MIA treatment in a dose-dependent manner, which is opposite to the effect found in chondrocytes (Fig. 3B). In order to further confirm that impaired glycolysis causes *CCN2* induction in MCF7 cells, another glycolysis inhibitor, namely NaF, which targets enolase (Fig. 4A), was employed. As expected, NaF also induced *CCN2* expression in a dose-dependent manner (Fig. 4B). The difference in the *CCN2* regulation by glycolysis could be related to the phenotypic differences between the 2

breast cancer cell lines (Fig. 4C). It is well known that hypoxia enhances glycolysis. Therefore, we analyzed a dataset of RNA expression profiles from a variety of breast cancer and normal mammary epithelial cells exposed to hypoxia, which was available on the GEO website. We compared the CCN2 mRNA levels under normoxic and hypoxic conditions and found that CCN2 expression was repressed after exposure to 1% O_2 for 24 h in both MCF7 and normal mammary epithelial cells (Fig. 5A). In contrast, hypoxia increased *CCN2* expression in MDA-MB-231 cells, which is consistent with previous findings [22]. These results suggest a mammary epithelial cell-specific negative regulation of *CCN2*, which may be related to the malignant characteristics of breast cancer cells and is completely different from that observed in chondrocytes.

4. Discussion

Our previous research on energy metabolism in *Ccn2*-null mice and human chondrocytic cells clearly indicated that CCN2 plays a central role in supporting ATP production through glycolysis in chondrocytes [6,20]. Since the cartilage is an avascular tissue with a low oxygen supply, these findings were consistent with the notion that glycolysis is the dominant energy production system in chondrocytes. Moreover, the positive feedback system created via the mutual enhancement of CCN2 and energy production should be a critical system used by chondrosarcoma cells (Fig. 5B), including the HCS-2/8 cells used in this study, to enhance glycolysis. Therefore, CCN2 can be regarded as a Warburg effector, at least in this particular type of tumor. Since the MDA-MB-231 cell line, which exhibits a prominent Warburg effect, produces higher levels of

CCN2 than MCF7, which has a lower glycolytic activity [23], we suspected the involvement of the same metabolic system in the Warburg effect in MDA-MB-231 cells. However, the results of our study suggest that CCN2 is regulated via a novel system in breast cancer cells, which is distinct from the one observed in chondrocytes.

Previous studies have already revealed that CCN2 is a key molecule involved in promoting the bone metastasis of breast cancer cells [12,13]. Kang *et al*. reported that the combination of CCN2 and interleukin 11 (IL-11) drive breast cancer cells to osteolytic metastasis [12]. Our study also showed that CCN2 is required for the bone metastasis of MDA-MB-231 cells in an animal model *in vivo* [13]. It should be noted that CCN2 is an angiogenesis inducer [24,25] and also enhances matrix metalloproteinase (MMP) production [22], which are generally required for the invasion and metastasis of tumors. Furthermore, CCN2 was also shown to promote osteoclastogenesis through multiple mechanisms [26,27]. This suggests that breast cancer cells are exploiting these functional properties of CCN2, rather than its metabolic function, as observed in chondrocytes, in order to invade and expand in the bone tissue.

Another question arising here is how CCN2 production can be constitutively elevated in MDA-MB-231 cells in the absence of the positive feedback system found in chondrocytes. In this context, the fact that CCN2 gene expression is negatively regulated by glycolytic activity in the less invasive MCF7 breast cancer cells (Fig. 3B), while this regulation is not observed in MDA-MB-231 cells (Fig. 3A), is of significant importance. Based on these findings, we hypothesized that, in less aggressive breast cancer cells and possibly, in normal mammary epithelial cells (Fig. 5A), CCN2 is negatively regulated by glycolysis (Fig. 5B), while this regulatory system is inactivated in highly metastatic breast cancer cells (Fig. 5C). Indeed, this hypothesis is supported by the analysis of the GEO

data. Interestingly, MMP-3, which is occasionally produced by breast cancer cells, is taken up by the cell and enhances *CCN2* expression by acting as a transcription factor [28]. This may be another mechanism of *CCN2* induction in highly malignant breast cancers.

Breast cancer patients are often treated for bone metastasis using high doses of bisphosphonates or antibodies targeting RANKL, which repress bone resorption. This strategy is shown to be effective. However, it also exposes the patients to the risk of developing MRONJ, a serious oral complication. Although CCN2 may not be associated with enhanced energy metabolism in breast cancer cells, this molecule enhances tumor angiogenesis and invasion, as well as bone resorption. Therefore, CCN2-targeted molecular therapies may lead to better clinical outcomes without adverse oral complications.

5. Conclusions

In chondrocytes, CCN2 and glycolysis support each other by forming a positive feedback loop. However, this study revealed that glycolysis is not regulated by CCN2 and CCN2 is repressed by glycolysis in breast cancer cells. CCN2 overproduction caused by the impairment of this negative regulatory system may drive breast cancer cells to become highly invasive.

Ethical approval

No ethical approval was necessary for this work since neither human subjects nor experimental animals were used.

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Conflict of interest

The authors have no conflict of interests to declare.

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Figure captions

Fig. 1. The effects of *CCN2* silencing on glycolysis and its downstream target, *CCN3*, in breast cancer MDA-MB-231 cells. **A.** Summary of the regulatory network among *CCN2*, *CCN3*, and glycolysis found in chondrocytes. *CCN2* is highly expressed and supports all cellular activities by enhancing glycolysis and forming a positive feedback loop with glycolysis. **B.** Efficient *CCN2* silencing using a siRNA cocktail in MDA-MB-231 cells. Data are represented as the mean and standard deviation (SD) of 3 independent samples. **C.** *CCN2* silencing has no effect on lactate production, which represents glycolytic activity. Medium lactate concentrations from 6 independent cultures of MDA-MB-231 cells were measured and compared with those in regular cell cultures. **D.** *CCN2* silencing has no effect on *CCN3* expression, which is regulated by glycolysis. Mean and SD values from 3 independent samples are shown. **E.** *CCN3* is regulated by glycolysis in MDA-MB-231 cells. The cells from 4 independent cultures were treated with the indicated concentrations of monoiodoacetic acid (MIA) for 12 h, following which *CCN3* expression levels were evaluated. Mean values are shown with error bars for SDs. $*p < 0.05$, $**p < 0.01$; significantly different from the control (NC or 0). The RNA data was standardized against the respective *ACTB* expression level. NC, non-targeting control siRNA.

Fig. 2. The effect of CCN2 deficiency on the gene expression of glycolytic enzymes in MDA-MB-231 cells. **A.** Three genes encoding glycolytic enzymes that are downregulated by CCN2 deficiency in murine chondrocytes. The glycolytic steps, which are catalyzed by these enzymes, are shown at the top with the relevant metabolites. Genes are represented by murine gene symbols. **B.** CCN2 gene silencing has no effect on the

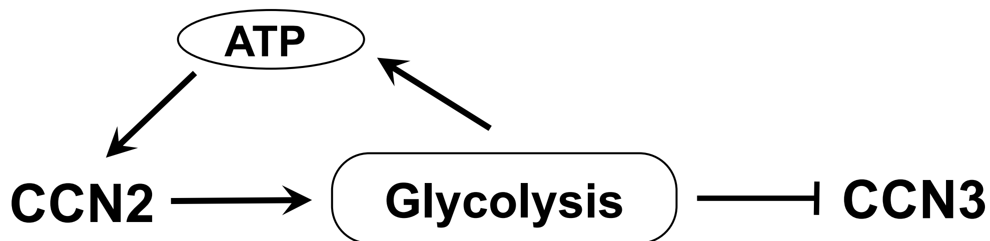
expression of the enzymatic genes *PGK1* (left), *PGAM1* (center), and *ENO1* (right) in MDA-MB-231 cells. RNAi-mediated *CCN2* silencing was performed under the same conditions used in Fig. 1. The relative expression levels standardized using the β-actin (*ACTB*) expression levels are shown. Data represent averages from 3 independent cultures with SDs.

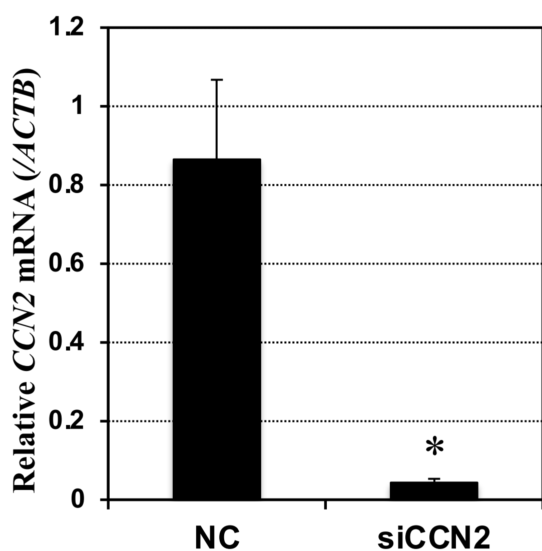
Fig. 3. The effects of monoiodoacetic acid (MIA) on *CCN2* expression in breast cancer MDA-MB-231 (**A**) and MCF7 (**B**) cells. The cells were treated with MIA at the indicated concentrations for 12 h, after which *CCN2* mRNA levels were quantified. The relative gene expression levels *versus* the β-actin (*ACTB*) expression levels are shown. Mean values from 4 independent cell cultures are shown, with error bars representing SDs. ** $p < 0.01$; significantly different from the control (0 μg/mL).

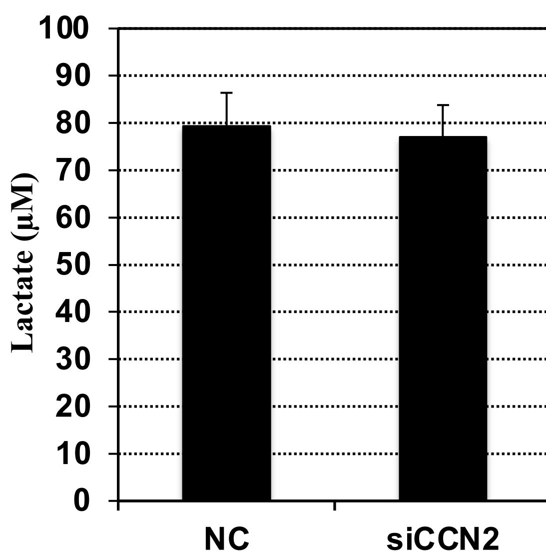
Fig. 4. Negative regulation of *CCN2* by glycolysis in the less malignant breast cancer cell line, MCF7. **A.** The distinct glycolytic steps inhibited by monoiodoacetic acid (MIA) and sodium fluoride (NaF). **B.** Dose-dependent induction of CCN2 gene expression by NaF in MCF7 cells. The cells were treated with the indicated concentrations of NaF for 12 h, following which *CCN2* mRNA levels were measured. Values were standardized against *ACTB* mRNA levels. Mean values from 3 independent cell cultures are shown with error bars representing SDs. ** $p < 0.01$; significantly different from the control (0 mM). **C.** Summary of the characteristics of the 2 breast cancer cell lines used in this study.

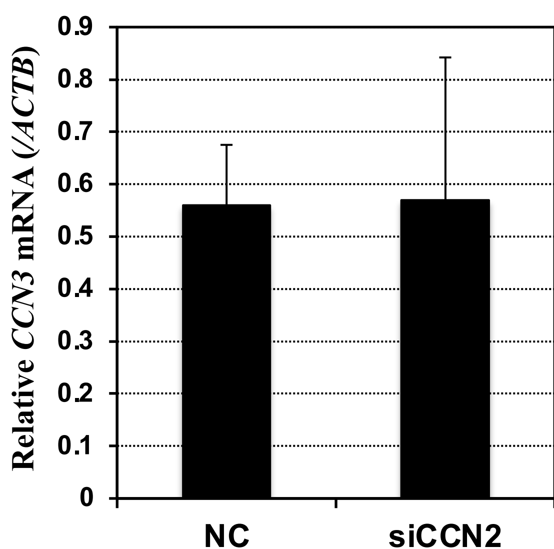
Fig. 5. A. Hypoxia enhances the effect of glycolysis on *CCN2* expression in MCF7, human mammary epithelial (HME), and MDA-MB-231 cells. Data are represented as

gene counts and were extracted from a quantitative RNA sequencing dataset downloaded from the GEO website. H and N indicate the data from the cells cultured in a 1% and 20% O_2 atmosphere for 24 h, respectively. **B.** CCN2 is negatively regulated by glycolysis in MCF7 cells. *CCN2* expression was suppressed by glycolytic activity, which leads to a less malignant phenotype. **C.** Impaired negative regulation of *CCN2* by glycolysis in MDA-MB-231 cells, which allows *CCN2* overproduction for metastasis. The mechanism by which glycolysis is overactivated remains unclear. Arrows and T-bar represent positive and negative regulation, respectively.









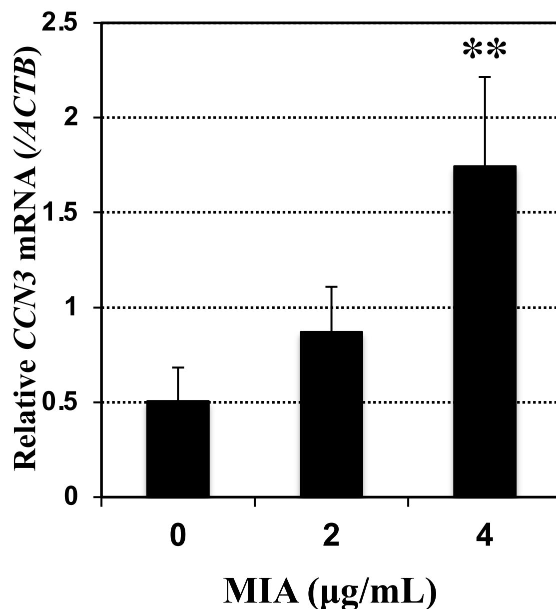
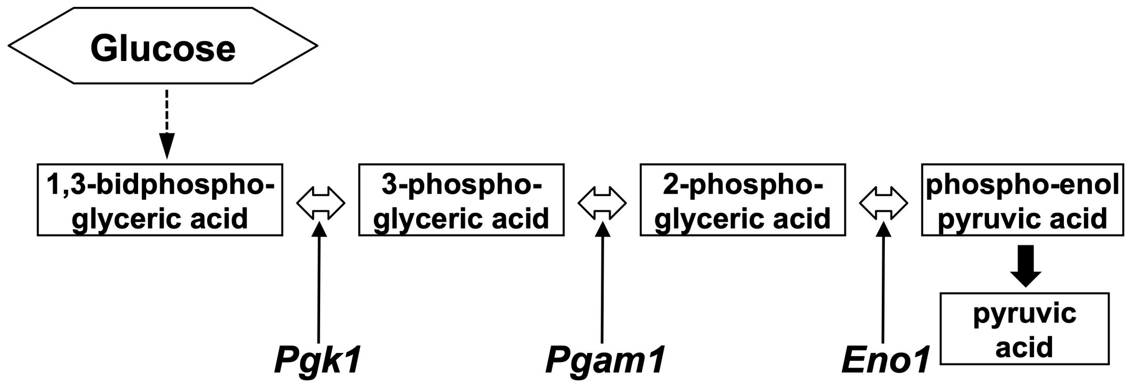


Fig. 1. Akashi et al.

A



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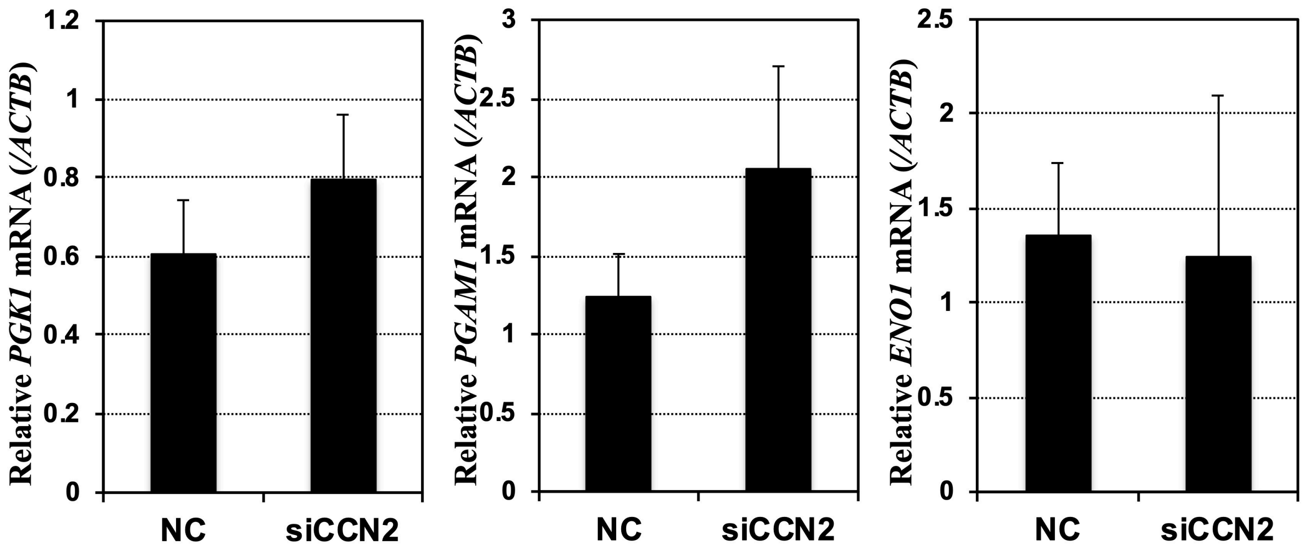
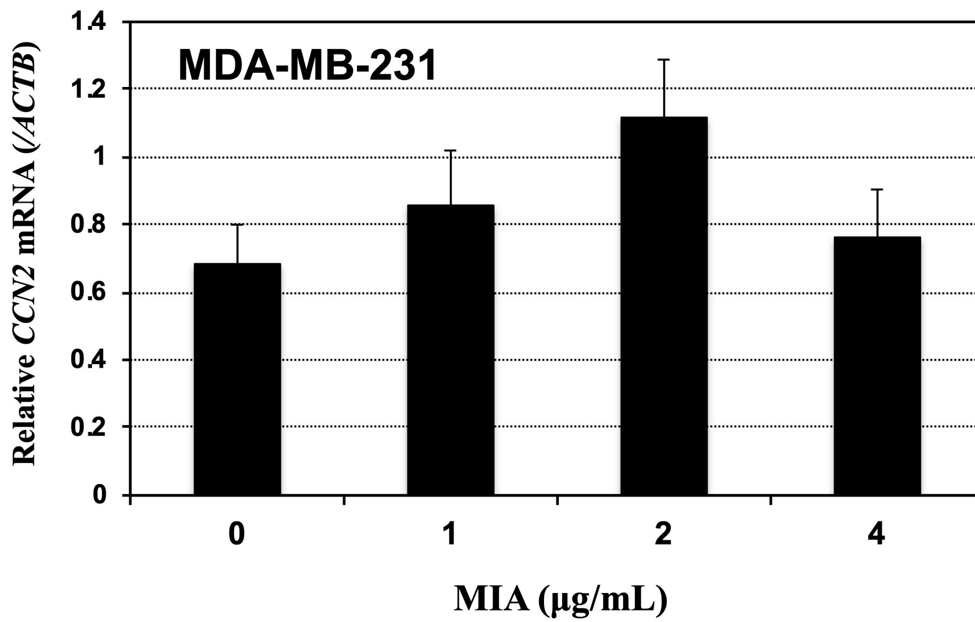


Fig. 2. Akashi et al.

A



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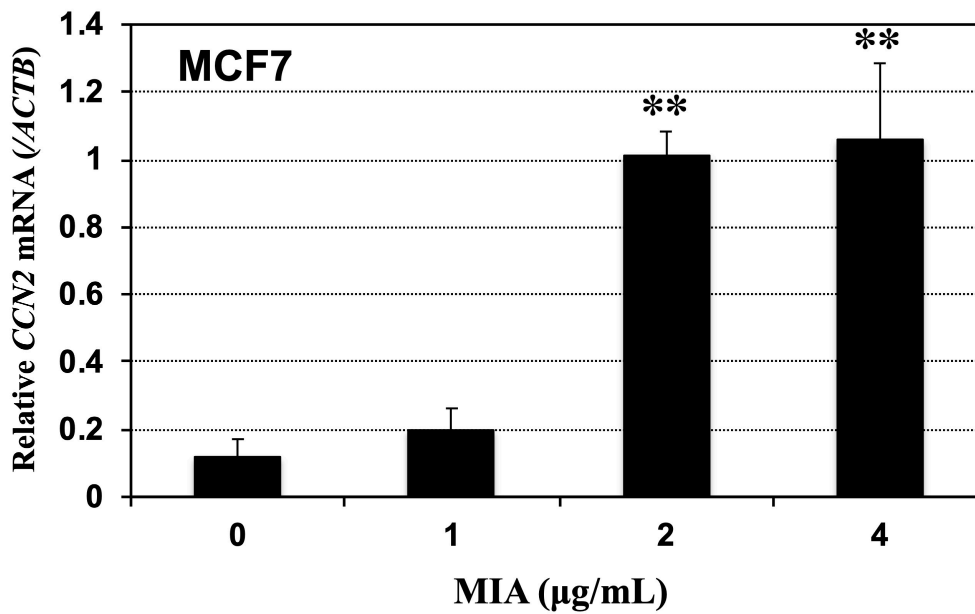
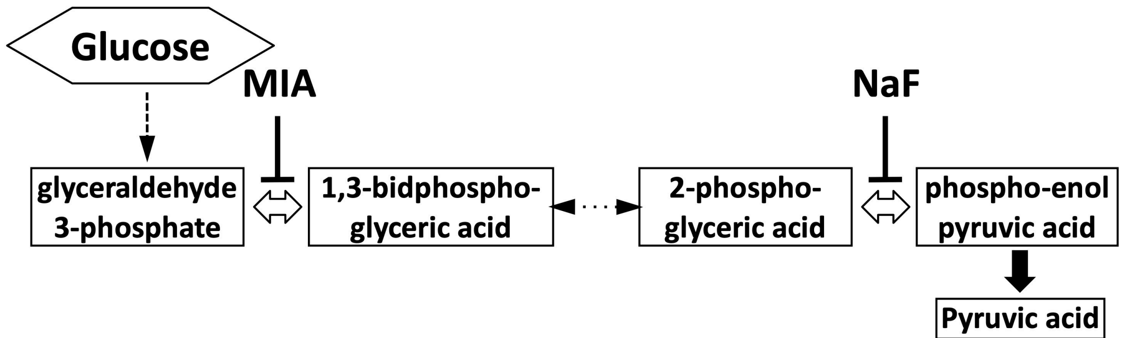


Fig. 3. Akashi et al.

A



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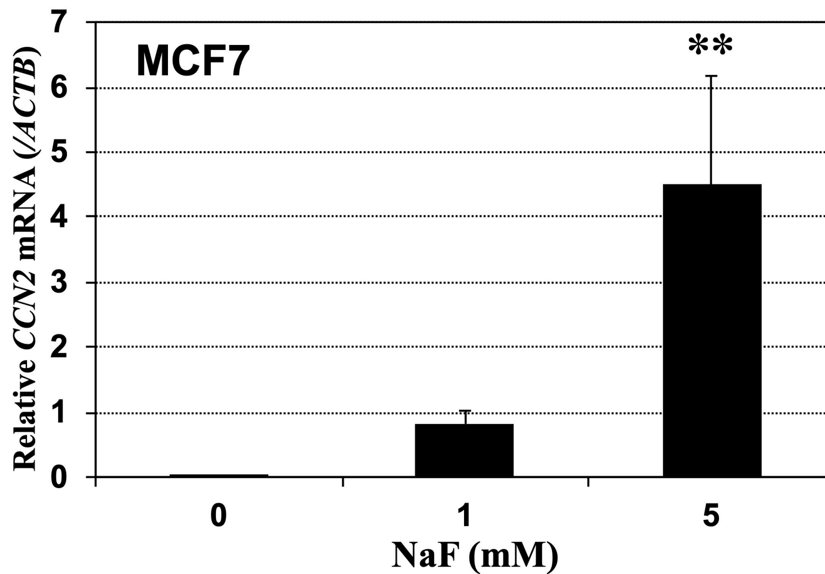
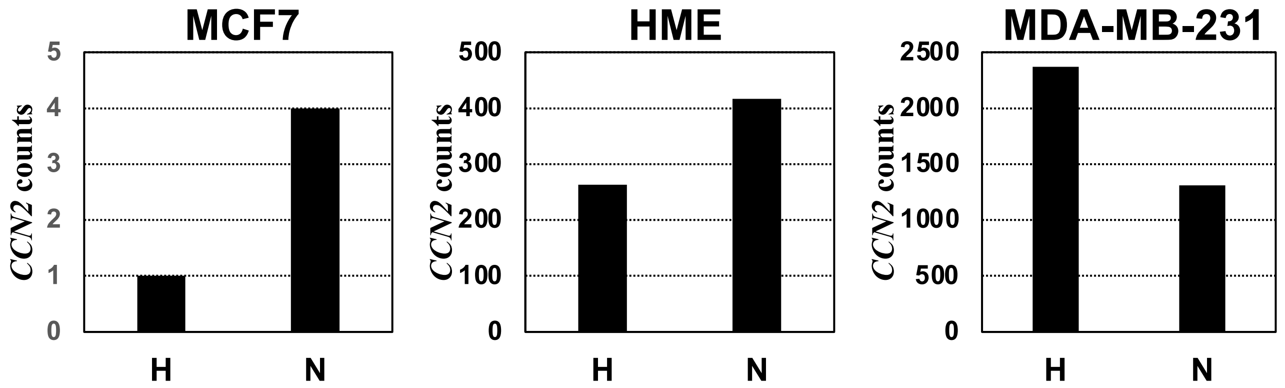


C

	MDA-MB-231	MCF7
Invasive phenotype	++	-
Mammary epithelial phenotype	±	+
Estrogen receptor	-	+
Warburg effect	++	±
CCN2 expression	++	±

Fig. 4. Akashi et al.

A



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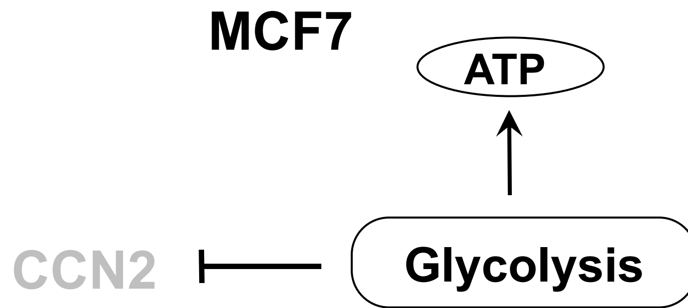

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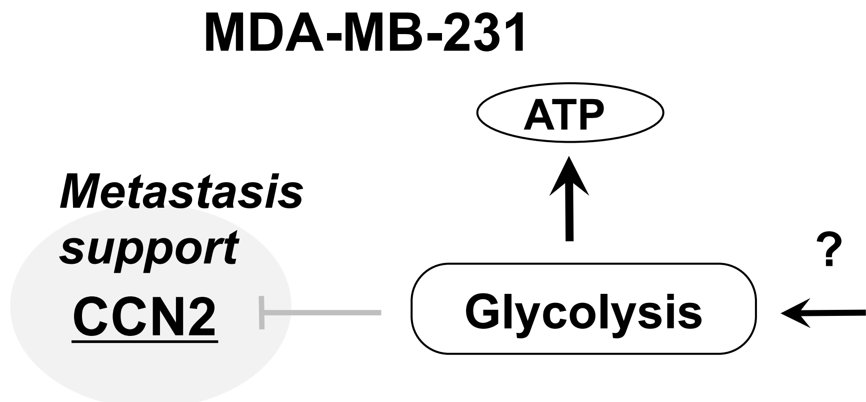
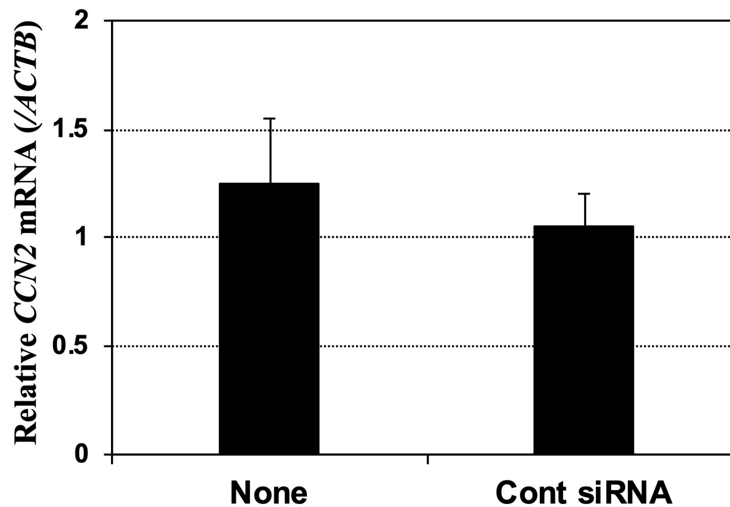


Fig. 5. Akashi et al.



Supplementary Figure 1. ***CCN2*** **expression was not affected by a non-targeting control siRNA.** MDA-231 cells were transfected with the non-targeting control siRNA used in this study, and the *CCN2* expression was compared with that in untreated cells as described in Materials and Methods. Results are from 4 independent cell cultures, and relative expression levels against those of *ACTB* are shown with SDs. No statistically significant difference was found between them.