Effect of transforming growth factor-β1 on function and expression of monocarboxylate transporter 1 in alveolar epithelial cells

肺胞上皮細胞におけるモノカルボン酸トランスポーター**MCT1**の発現・機能に及ぼす**transforming growth factor-β1**の影響解析

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Abbreviations

AR	AR-C155858
ATII cells	Alveolar type II epithelial cells
CHC	α-cyano-4-hydroxycinnamate
CK19	Cytokeratin 19
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial Mesenchymal Transition
FBS	Fetal bovine serum
GHB	Gamma-hydroxybutyrate
GLUT1	Glucose transporter 1
IPF	Idiopathic pulmonary fibrosis
LDH	Lactate dehydrogenase
MCT1	Monocarboxylate transporter 1
MTX	Methotrexate
OXPHOS	Oxidative phosphorylation
PBS	Phosphate-buffered saline
SB	SB431542
TGF-β1	Transforming growth factor beta 1
α-SMA	Alpha-smooth muscle actin

General Introduction

During inspiration, healthy adults inhale oxygen containing air. Through trachea, bronchi and bronchioles, oxygen rich air reaches to the air sacs. These air sacs or alveoli are surrounded by fine blood vessels called capillaries which carry the blood flowing from the heart. The inhaled oxygen is then transported to the blood capillary from alveoli which is then pumped into our whole body. Oxygen plays very important role in many processes of our body.

Pulmonary fibrosis (PF) is a set of interstitial lung diseases that cause progressive scarring on the wall of tiny air sacs of the lungs. When the air sacs in lungs gradually become replaced with scar, the wall becomes thicker and stiff. As a result, transport of oxygen from alveoli to blood capillary is severely hindered.

The lungs cannot breathe deeply. So, the amount of oxygen that gets into the lung is also reduced. Pulmonary fibrosis can also cause weakening of heart. Risk factors for pulmonary fibrosis include chronic inflammation, cigarette smoke, infection, radiation therapy, genetic reasons, environmental factors, certain medications etc.

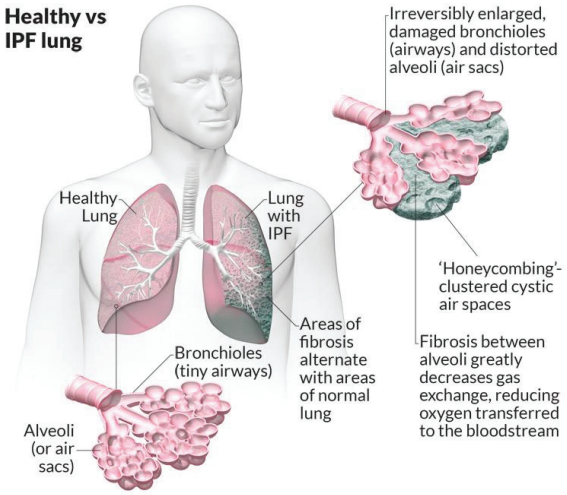


Figure 1: Idiopathic pulmonary fibrosis

https://www.lungsandyou.com/facts/effects-of-ipf

When the cause of PF was unknown, the condition is termed idiopathic pulmonary fibrosis (IPF). IPF is a chronic, progressive, irreversible, and lethal lung disease (Fig. 1). For many years, it is thought that inflammation is the driving force of IPF, but it's not the case. IPF is the result of an irreversible proliferation of fibroblast and abnormal wound-healing cascade (Martinez et al. 2005; Raghu et al. 2006). It initially manifests with symptoms including exercise-induced breathlessness, fatigue, dry coughing, unexpected weight loss, etc. As the disease progresses, they become hypoxemic and need supplemental oxygen. Physicians can hear inspiratory crackles during auscultation. Patients might have a phenomenon called clubbing of fingers. A patchy or pied appearance and honeycombing where fibrotic changes have occurred are evident on CT scan and are highly predictive of IPF.

Various factors predisposing pulmonary fibrosis comprise genetic factors, environmental exposure of certain agents, chemotherapy, aging, etc. (Abid et al. 2001; Wolters et al. 2014). Regarding the initiation of PF, numerous triggers initiate the fibrogenic process, the most important of which is the central regulator Transforming growth factor beta (TGF-β) followed by its activation, recruitment of fibroblast and their transformation to myofibroblasts and release of inflammatory mediators (Kottmann et al. 2009; Willis and Borok, 2007). Finally, the disease propagates by persistent pathologic fibroblast differentiation,

fibro proliferative processes, matrix deposition, lung remodeling and formation of scar and stiff tissue (Wynn, 2008).

Although etiology and mechanism underlying PF and IPF are complex, it is postulated that **Epithelial Mesenchymal Transition** (**EMT)** plays a significant role in the process of pulmonary fibrosis. (Kalluri and Neilson 2003; Kalluri and Weinberg 2009; Willis and Borok 2007).

EMT is a dynamic process in which epithelial cells acquire mesenchymal fibroblast like properties. It has been involved in both normal physiological event like embryonic development as well as in disease development such as fibrosis and metastasis. In this process, epithelial cells loose cell-cell interaction and acquire migratory and invasive properties (Figure 2). Epithelial cells have apico basal polarity, tight and adherent junctions and desmosomes and express E-cadherin. On the other hand, mesenchymal cells have front-back polarity. Instead of expressing E-Cadherin, mesenchymal cells express N-Cadherin, vimentin, fibronectin, integrins, MMP (matrix metalloproteinase enzyme), etc. (Nieto et al. 2016; Onder et al. 2008). The morphological change is accompanied by molecular change initiated by several pathways and signaling factors which regulate expression of transcription factors including Snail, SLUG, ZEB1, ZEB2, Twist1, Twist2 and others (Peinado et al. 2007). Once activated by external signals, these transcription factors inhibit the expression of genes related to epithelial state of cell and activate the expression of genes related to mesenchymal state of cell.

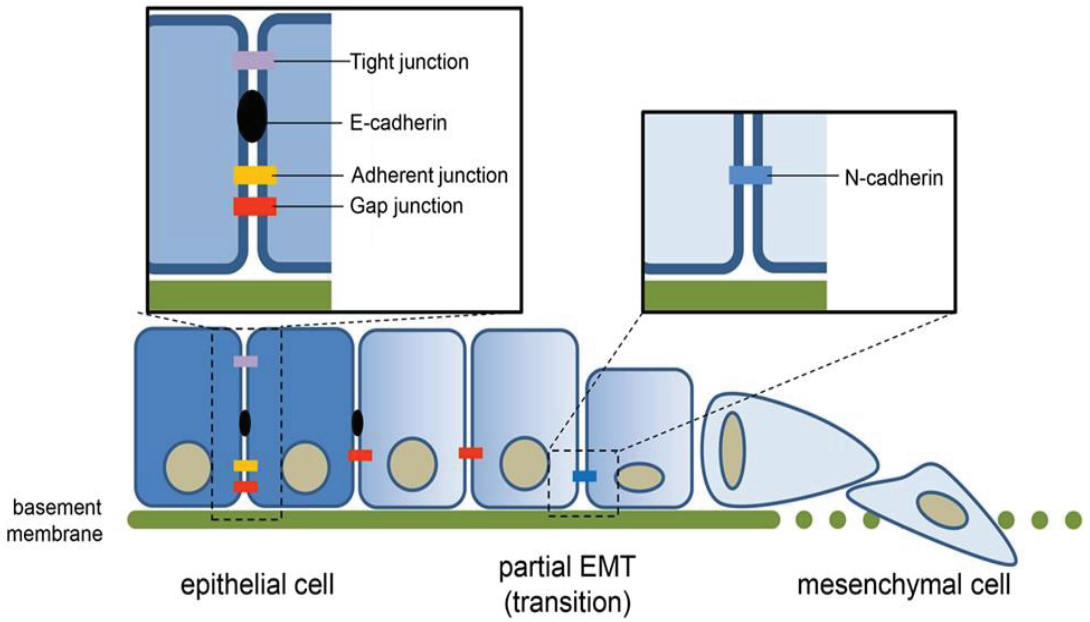


Figure 2: Epithelial Mesenchymal Transition (EMT) (Morandi et al. 2017)

The most important signalling pathway that induce EMT is the TGF-β signalling pathway. Other pathways include Wnt/ β-catenin pathway, EGF, HGF etc (Battle et al. 2000; Cano et al. 2000; Peinado et al. 2007).

In recent times, it has been reported that the EMT and metabolic changes are intertwined (Kang et al. 2019). Monocarboxylate transporters (MCTs) play significant physiological role in these metabolic changes via regulation of lactate transport (Fig. 3) (Fishbein et al. 2002; Halestrap and Wilson 2012). Among all MCTs, **monocarboxylate transporter 1** (**MCT1)** is the best characterized isoform and the major function of MCT1 is to facilitate the transport of monocarboxylates such as lactate across the plasma membrane. So MCT1 could play a crucial role in metabolic reprograming via regulation of the lactate shuttle.

However, little information is available concerning MCT1 function in alveolar epithelial cells, which is mostly injured during pulmonary fibrosis and the role of MCT1 to EMT has not been studied. If the relationship between MCT1 and EMT can be determined, MCT1 inhibition may be used to prevent EMT-related diseases, like pulmonary fibrosis. Currently, A549 pulmonary adenocarcinoma cells have been regarded as a suitable model for EMT studies (Kawami et al. 2016, 2018a), and it has been clarified that TGF-β1 induces EMT via upregulation of its signaling pathway. There is a report that indicates the expression of MCT1 in A549 cells (Eilertsen et al. 2014).

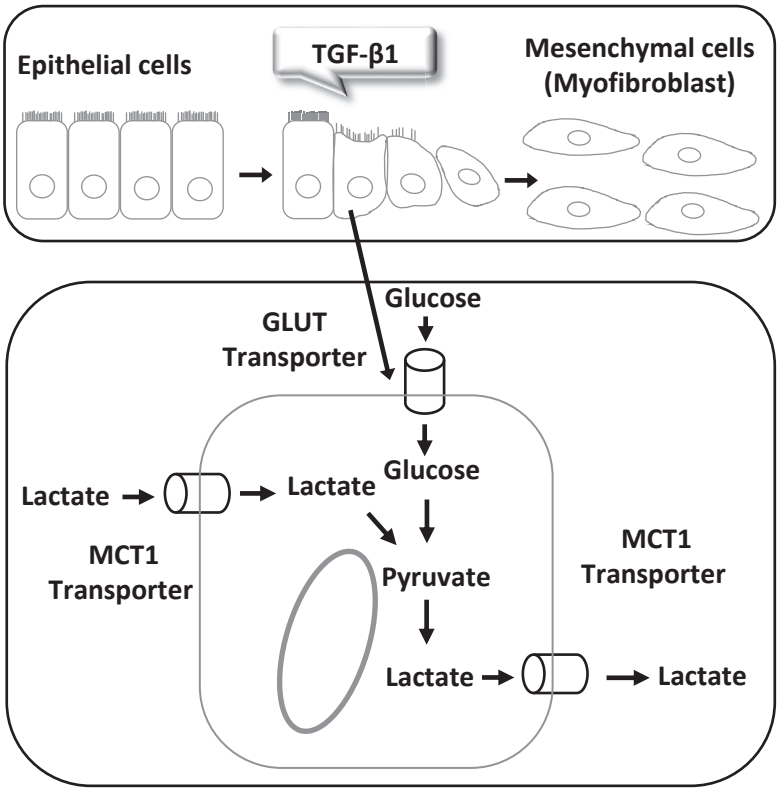


Figure 3: Epithelial Mesenchymal Transition & MCT1

Taking everything into account, A549 cells should be a good model for clarifying the role of TGF-β1 on the function and expression of MCT1.

So, the aims of the present study:

- To characterize MCT1 function.
- To examine the changes in expression and function of MCT1 during TGF-β1-induced EMT using A549 cells, an in vitro model of human alveolar type II epithelial cells derived from human lung carcinoma.

Monocarboxylate transporters

There are 14 members in MCT family. Among them, MCTs 1–4 play significant metabolic roles depending on the metabolic needs essential for the tissue.

Table I. Classification of Monocarboxylate Transporter Family (Halestrap 2013)

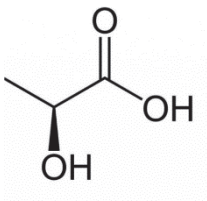
Human gene name	Protein name	Transport type/ coupling ions	Predominant substrate	Tissue distribution and cellular/ subcellular expression
SLC16A1	MCT1	C/H^+ or E/monocarboxylate	Lactate, pyruvate, ketone bodies	Ubiquitous except β cell of endocrine pancreas
SLC16A2	MCT8	F	T2, T3, T4	Most tissues
SLC16A3	MCT4	C/H+	Lactate, Ketone bodies	Skeletal muscle, chondrocytes, leucocytes, testis, lung, ovary, placenta, heart
SLC16A4	MCT5	O		Brain, muscle, liver, kidney, lung, ovary, placenta, heart
SLC16A5	MCT6	O		Kidney, muscle, brain, heart, pancreas, prostate, lung, placenta
SLC16A6	MCT7	O		Brain, pancreas, muscle, prostate
SLC16A7	MCT2	C/H+	Pyruvate, lactate, ketone bodies	High expression in testis, moderate to low in spleen, heart, kidney, pancreas, skeletal, muscle, brain and leucocyte
SLC16A8	MCT3	C/H+	Lactate	Retinal pigment epithelium, choroid plexus
SLC16A9	MCT9	O		Endometrium, testis, ovary, breast, brain, kidney, spleen adrenal, retina
SLC16A10	TAT1, MCT10	F	Aromatic amino acids, T3, T4	Kidney (basolateral), intestine, muscle, placenta, heart
SLC16A11	MCT11	O		Skin, lung, ovary, breast, lung, pancreas, retinal pigment, epithelium, choroid plexus
SLC16A12	MCT12	O		Kidney, retina, lung, testis
SLC16A13	MCT13	O		Breast, bone marrow, stem cells
SLC16A14	MCT14	O		Brain, heart, muscle, ovary, prostate, breast, lung, pancreas liver, spleen, thymus

C: Cotransporter E: Exchanger F: Facilitated transporter O: Orphan transporter.

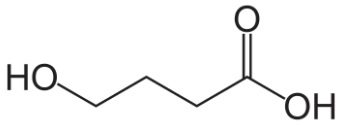
MCT1

MCT1 is expressed in almost all tissues (table I). It plays significant role in transport of L-lactate across the plasma membrane. Depending of the existing intracellular and extracellular substrate concentrations and the pH gradient, MCT1 may influx or efflux lactate across the plasma membrane. Transport of lactate has shown stereoselectivity. MCT1 has a greater affinity for L-lactate than D-lactate. However, MCT1 can also act as an exchanger, with the exchange of one monocarboxylate for another without the net movement of protons.

MCT1 is responsible for transport of some naturally occurring monocarboxylates such as L-lactate, pyruvate, β-hydroxybutyrate, and acetoacetate across the plasma membrane. Apart from endogenous monocarboxylates, MCTs are known to mediate the transport of some drugs like salicylate, gamma-hydroxybutyrate (GHB) and nicotinic acid (Halestrap and Meredith 2004; Halestrap and Wilson 2012).



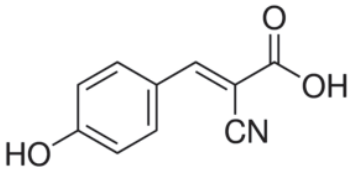
Lactate



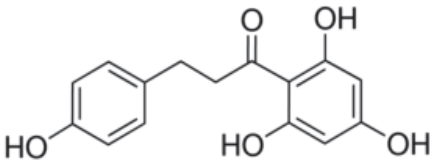
GHB

GHB acts as a neurotransmitter. There are two binding sites of GHB, GABA B and GHB receptor. Therapeutically, GHB is used for the treatment of alcohol withdrawal.

A range of MCT inhibitors have been confirmed, including α-cyano-4-hydroxycinnamate (CHC) and its analogues, stilbene disulphonates, phloretin, bioflavanoids such as quercetin, etc. Among them CHC is used by several groups and has shown promise in inhibiting MCTs. But it is at least two orders of magnitude more potent as an inhibitor of the mitochondrial pyruvate transporter. Besides AR-C155858 which is a pyrrole pyrimidine derivative, developed by AstraZeneca is a selective and potent inhibitor of MCT1.

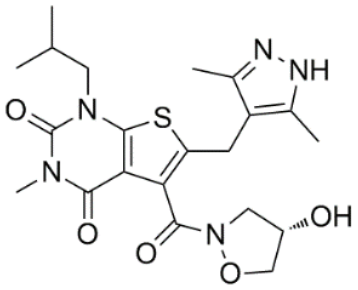


CHC



Phloretin

In rat erythrocytes, AR-C155858 inhibited MCT1-mediated L-lactate transport dose-dependently with a Ki value of 2 nM (table III). When expressed in *Xenopus laevis* oocytes, MCT1 and MCT2 were potently inhibited by AR-C155858 whilst MCT4 was not (Ovens et al. 2010).



AR-C155858

Other Monocarboxylates:

The expression of MCT2 is relatively little in human tissues. It is expressed in mouse, rat and hamster (table I). MCT2 has shown higher affinity for most monocarboxylates than MCT1. Km values for pyruvate and L-lactate were determined to be about 0.025 and 0.74 mM respectively, in Xenopus oocytes (table II). MCT4 is highly expressed in tissues that rely on glycolysis. The key role of MCT4 is to facilitate export of lactate derived from glycolysis. MCT8 is important for transport of thyroid hormone. MCT10 also known as TAT1 (T-type amino acid transporter 1) is essential for transport of aromatic amino acid. Eight orphan members of MCT family are ambiguous.

Table II. Comparison of substrates for various MCT isoforms in Human and Rat (Morris 2008)

Isoform	Species	Expression system	Substrate	Km (mM)
MCT1	Human	Xenopus oocytes	L-lactate	3.5-6
			Pyruvate	1.8-2.5
			Acetoacetate	5.5
	Rat	Xenopus oocytes	Lactate	3.5
			GHB	4.6
MCT2	Human	Xenopus oocytes	Pyruvate	0.025
	Rat	Xenopus oocytes	Lactate	0.74
MCT4	Human	Xenopus oocytes	L-lactate	28
			D-lactate	519
			Pyruvate	153
			Acetoacetate	216
	Rat	Xenopus oocytes	L-lactate	34
			Pyruvate	36.3
			Acetoacetate	31

Table III. Comparison of inhibitors for various MCT isoforms in Human and Rat (Morris 2008)

Isoform	Species	Expression system	Inhibitor	Ki
MCT1	Human	Xenopus oocytes	Phloretin CHC	28 µM 425 µM
MCT1	Rat	Rat erythrocytes	AR-C155858	2 nM
MCT4	Human	Xenopus oocytes	CHC Phloretin	991 µM 41 µM

Metabolic roles of SLC16 family members

MCTs 1-4 have been involved in the proton-dependent transport of L-lactate, pyruvate and ketone bodies in a wide variety of tissues. Among them, L-lactate is by far the most abundant and physiologically significant substrate.

Lactate efflux from cells

Lactate is the by-product of anaerobic glycolysis. When glycolysis is stimulated in anaerobic condition, lactate production is enhanced. MCT1 is responsible for efflux of this lactate (Fig.4). This plays an important part in pH homeostasis within the cell. Increased rate of glycolysis occurs not only in presence of oxygen but also under aerobic conditions, a phenomenon called "Warburg effect". However, most cells with high glycolytic activity express MCT4 than MCT1 (Halestrap and Wilson 2012).

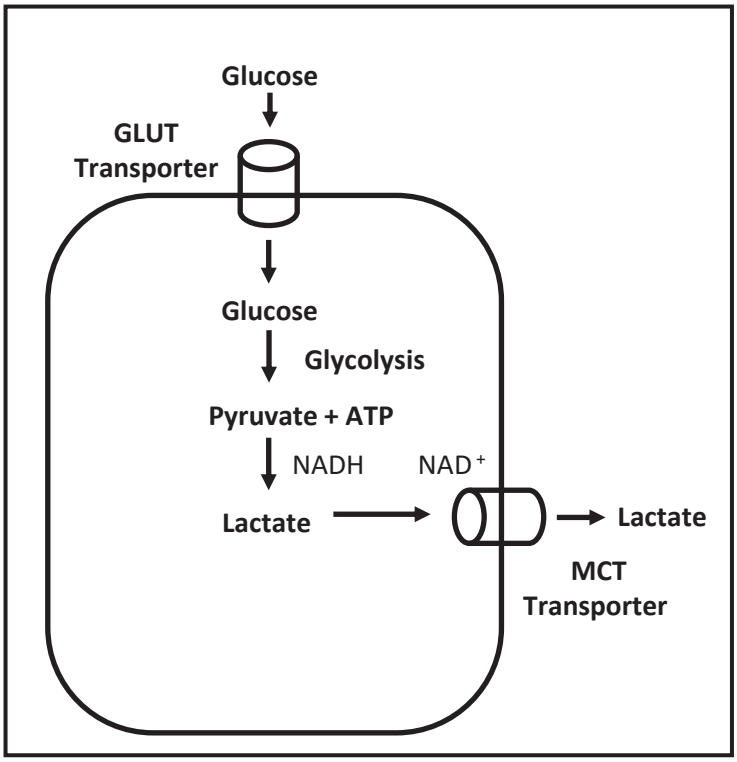


Figure 4: Scheme for cellular lactate efflux

Lactate uptake into cells

Sometimes lactate is used as a substrate for lipogenesis and gluconeogenesis by particular tissues (liver, kidney, adipose tissue). These cells express either MCT1 or MCT2 depending on species (Halestrap and Meredith 2004; Halestrap and Price 1999; Halestrap and Wilson 2012). Lactate and ketone bodies are also used as respiratory substrates for production of energy in some tissues (Fig. 5). (Bergersen 2007; Halestrap and Meredith 2004).

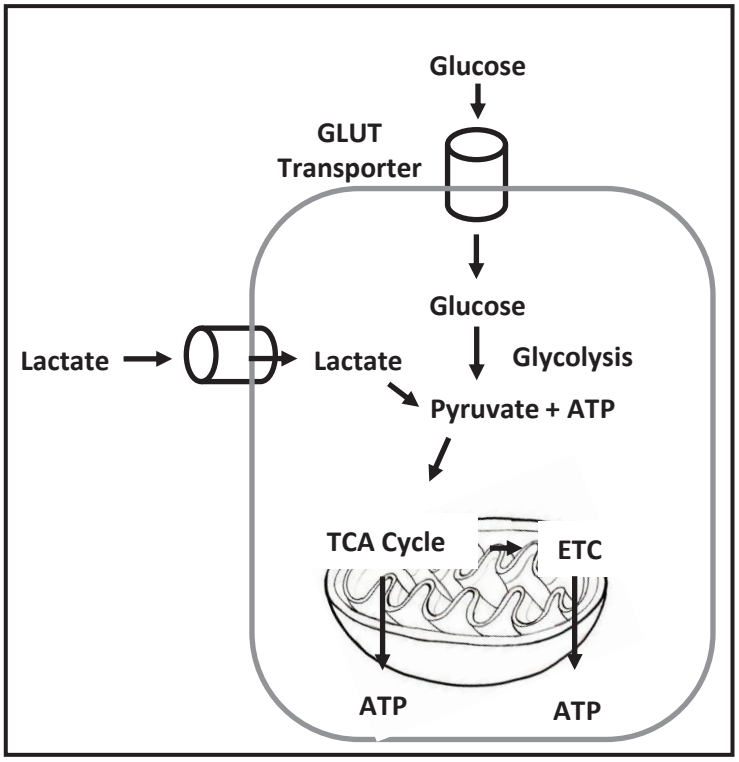


Figure 5: Scheme for cellular lactate uptake

MCTs may be involved in shuttling lactate between different cell types within a tissue

MCTs plays a fundamental role in shuttling lactate between cells that produce it and those that use it within the same tissue (skeletal muscle, brain) (Bergersen 2007; Brooks 2009).

Chapter 1

Basic characterization of Monocarboxylate Transporter 1 (MCT1) in A549 cells

1.1. Introduction

Halestrap and his research group first determined the presence of a proton coupled MCT1 in human red blood cells. They showed that transport of L-lactate and pyruvate into human red blood cells was clearly inhibited by CHC (Halestrap and Denton 1974). Later both Halestrap's and Deuticke's laboratories characterized the detailed kinetics of this transporter (Deuticke 1982; Poole and Halestrap 1993). Finally, the molecular identity of the protein responsible was confirmed by Christine Kim Garcia in the laboratory of Goldstein and Brown. The transporter was named MCT1 by these authors (Kim-Garcia et al. 1994).

However, very few information is available regarding function and expression of MCT1 in alveolar epithelial cells. That's why we attempted to characterize MCT1 function in A549 cells, an in vitro model of human alveolar type II epithelial cells. There are several substrates and inhibitors of MCT1. For our study we used lactic acid and GHB as substrates and CHC, phloretin, lactic acid and AR-C155858 as inhibitors of MCT1. In this study we examined time-, temperature- and pH-dependence of GHB and lactic acid uptake in A549 cell. Then effect of the inhibitors on the function of MCT1 was observed.

1.2. Results

1.2.1. Time-, temperature- and pH-dependence of GHB uptake in A549 cells

MCT1 has been shown to facilitate transport of some drugs like GHB across the plasma membrane. In our study, we confirmed mRNA expression of MCT1 in A549 cells by gel electrophoresis (Fig. 6). Then, we examined time-, temperature- and pH-dependence of GHB uptake in A549 cells. Time dependent study clearly illustrates a linear increase in [^{3}H] GHB uptake for up to 1 min (Fig. 7a). The rate of GHB uptake was remarkably enhanced at 37^{o}C indicating that the uptake process is temperature dependent (Fig. 7a). The effect of extracellular pH on GHB uptake was examined. [^{3}H] GHB uptake at pH 6.0 was much higher than that at pH 7.4 (Fig. 7b) which demonstrates that MCT1 is a proton coupled symporter.

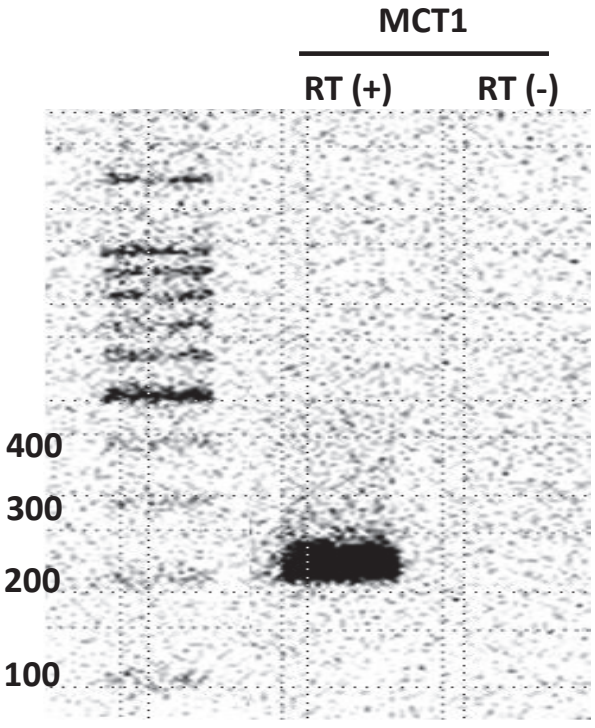


Figure 6: mRNA expression of MCT1 in A549 cells by gel electrophoresis. Predicted size of MCT1 is 187 bp.

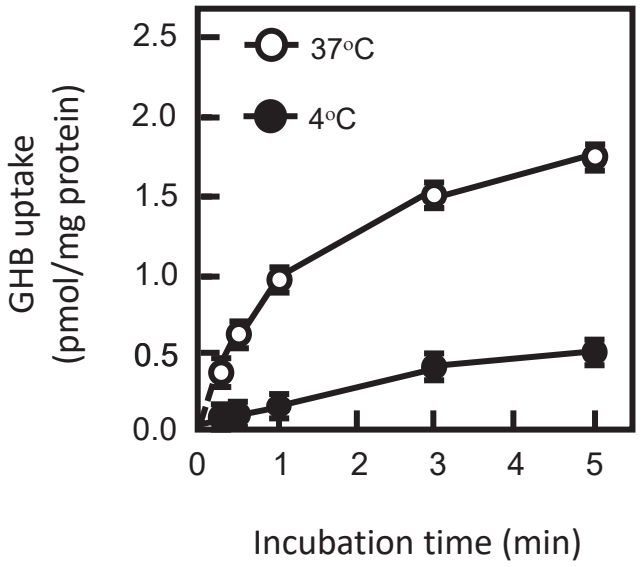


Figure 7 (a). Time- and temperature-dependence of [^{3}H]GHB in A549 cells. The cells were incubated with [^{3}H]GHB (100 nM) for 0.25, 0.5, 1, 3, and 5 min at 37°C (open circles) or 4°C (solid circles) at pH 7.4. Each value represents the mean ± S.E.M. of three monolayers (Uddin et al. 2020).

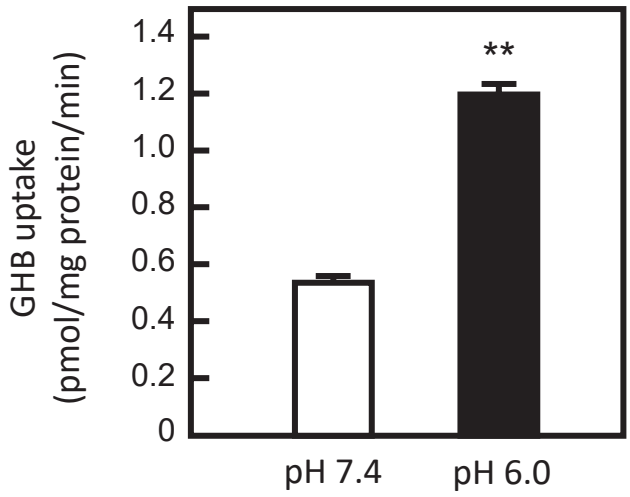


Figure 7 (b): Effect of pH on the uptakes of [^{3}H]GHB in A549 cells. The cells were incubated with [^{3}H]GHB (100 nM) for 1 minute at pH 7.4 (open column) or pH 6.0 (solid column). Each value represents the mean ± S.E.M. of three monolayers. **p<0.01, significantly different from the control (Uddin et al. 2020).

1.2.2. Time-, temperature- and pH-dependence of lactate uptake in A549 cells: MCT1 function was characterized using another substrate, lactate in A549 cells. The findings were like GHB. With increase of time and temperature, lactate uptake was enhanced (Fig. 8a). And the uptake of lactate at pH 6.0 was much higher than that at pH 7.4 (Fig. 8b).

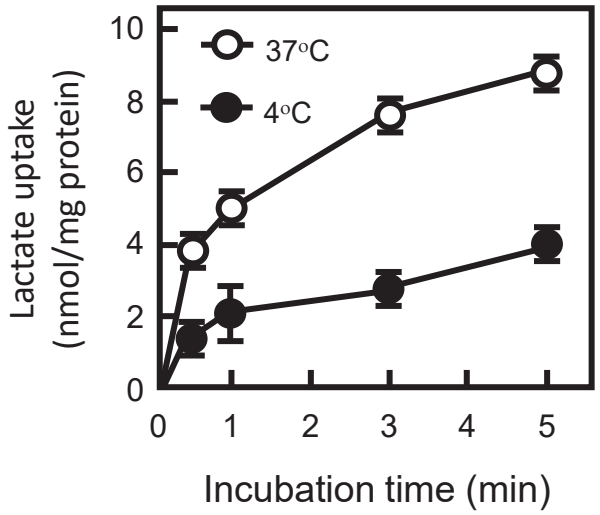


Figure 8 (a). Time- and temperature-dependence of [^{3}H]lactate uptake in A549 cells. The cells were incubated with [^{3}H]lactate (1 mM) for 0.25, 0.5, 1, 3, and 5 min at 37°C (open circles) or 4°C (solid circles) at pH 7.4. Each value represents the mean ± S.E.M. of three monolayers (Uddin et al. 2020).

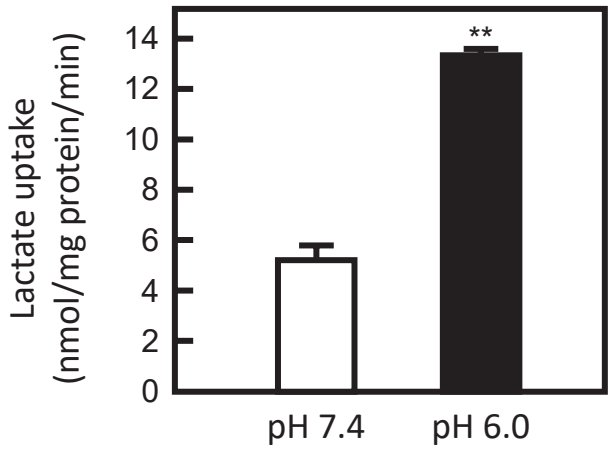


Figure 8 (b): Effect of pH on the uptake of [^{3}H]lactate in A549 cells. The cells were incubated with [^{3}H]lactate (1 mM) for 1 minute at pH 7.4 (open column) or pH 6.0 (solid column). Each value represents the mean ± S.E.M. of three monolayers. **p<0.01, significantly different from the control (Uddin et al. 2020).

1.2.3. Effect of inhibitors on the function of MCT1

There are some inhibitors of MCT1. Among them, CHC, phloretin, lactic acid, and quercetin are non-specific inhibitors, while AR-C155858 and AZD3965 are specific inhibitors. In this case, we examined the effect of two non-specific inhibitors, phloretin and lactic acid on the function of MCT1. Both inhibitors significantly suppressed GHB uptake in a concentration-dependent manner (Fig. 9).

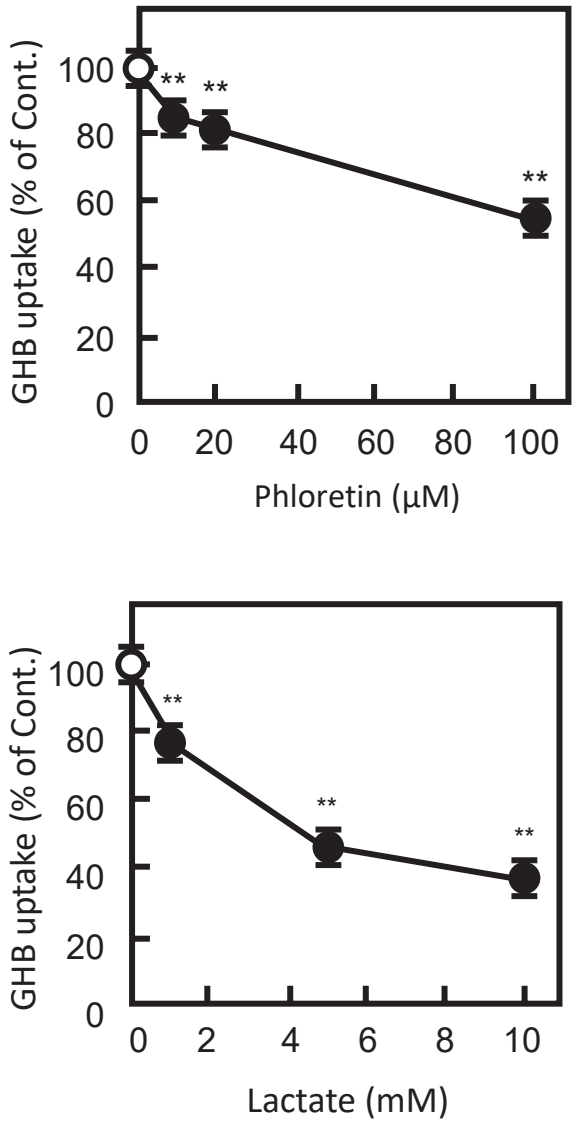


Figure 9: Effect of phloretin and Lactate on the uptake of GHB in A549 cells. The cells were incubated with [^{3}H]GHB (50 nM) for 1 min in the absence or presence of various concentrations of phloretin (10, 20, 100 μM) or lactate (1, 5, 10 mM) at pH 6.0 (Uddin et al. 2020).

Later, we examined the effect of CHC, a non-specific inhibitor on the function of MCT1. From Dixon plot, it was confirmed that concentration-dependent inhibition of [^{3}H]GHB and [^{3}H]lactate uptake by CHC was competitive inhibition with Ki values of 0.84 and 1.75 mM, respectively (Fig. 10). These values were not quite different from the previously reported Ki value for CHC to MCT1 (Bröer et al. 1999).

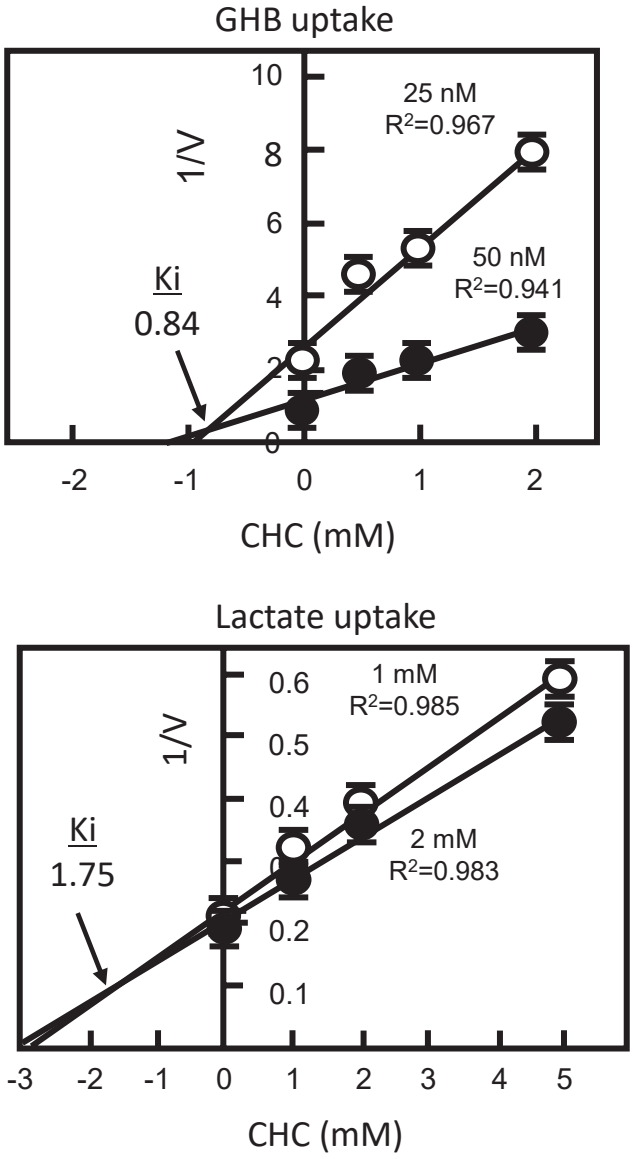


Figure 10: Dixon plot analysis of the inhibitory effects of CHC on MCT1-mediated transport. A549 cells were incubated with [^{3}H] GHB (25 nM or 50 nM) and [^{3}H]lactate (1 mM or 2 mM) for 1 min in the absence or presence of varying concentrations of CHC (0.5, 1, 2 mM) at pH 6.0 (Uddin et al. 2020).

Effect of AR-C155858 on the function of MCT1

AR is a specific inhibitor against MCT1. We examined the effect of AR on GHB and lactate uptake. AR also significantly suppressed uptakes of GHB and lactate in a concentration-dependent manner (Fig. 11).

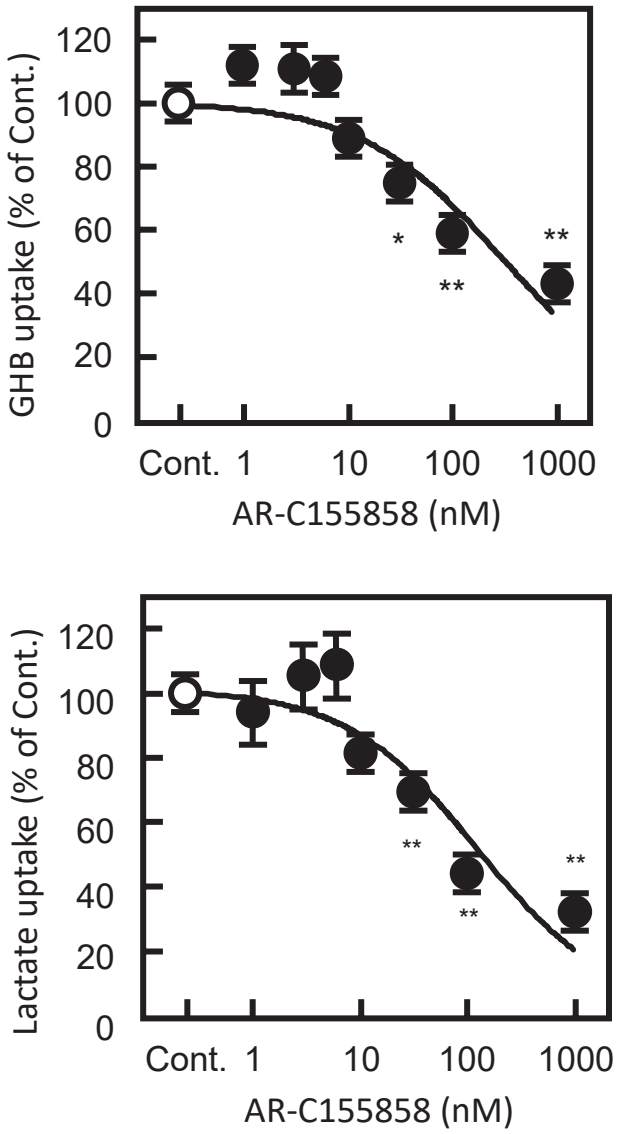


Figure 11: Effect of AR on the uptakes of GHB and lactate in A549 cells. The cells were incubated with [^{3}H]GHB (50 nM) or [^{3}H]lactate (1 mM) for 1 min in the absence or presence of various concentrations of AR (1, 3, 6, 10, 30, 100, 1000 nM) at pH 6.0. Each value represents the mean ± S.E.M. of three monolayers. *p<0.05, **p<0.01, significantly different from the control (Uddin et al. 2020).

1.3. Discussion

It was observed that GHB and lactate uptake showed time-, temperature-, and pH-dependence (Fig. 7, 8). All inhibitors against MCT1 significantly suppressed uptakes of GHB and lactate in a concentration-dependent manner (Fig. 9, 10, 11). This is the first time MCT1 function was characterized in A549 cells.

The human epithelial lung adenocarcinoma cell line A549 is a useful in vitro model for studying drug transport and metabolic processes in alveolar type II epithelial (ATII) cells (Lieber et al. 1976). ATII cells have a very high metabolic rate, and consequently they mostly depend on mitochondria for energy production (Lottes et al. 2014). It is reported that these cells import lactate through MCT1 and use it as a substrate for mitochondrial energy production (Lottes et al. 2015). Our findings concerning activity of MCT1 in A549 cells are similar with the concept of mitochondrial metabolism as alternative source of energy in ATII cells. Kottmann et al. (2012) demonstrated that the concentration of lactate was remarkably increased in lung tissue of IPF patients. Adjacent cells which express MCT1 can import this lactate and use it to produce ATP via oxidative phosphorylation.

Chapter 2

Basic characterization of Glucose Transporter 1 (GLUT1) in A549 cells

2.1. Introduction

Glucose is the most abundant and essential source of energy for almost all organisms. It does not diffuse through the plasma membrane easily due to its polar nature. That's why role of glucose transporter (GLUT) transporter is very significant. Glucose is imported by a process under facilitated diffusion. These facilitative glucose transporters mediate the transport of glucose from blood into the cell within the body to assure a continuous supply of glucose to cells for metabolism. GLUT family has fourteen members in total. Among them, GLUT1 was the first isoform to be studied (Thorens and Mueckler 2010).

The function and expression of the GLUT isoforms depend on their physiological and pathological conditions, metabolic needs, substrate specificities, etc. (Thorens 1996). GLUT1 is mostly expressed in the endothelial cells of blood-tissue barriers and GLUT3 maintains glucose homeostasis in neurons. Both transporters play a key role in maintaining a basal rate of glucose uptake. Basal blood glucose level is approximately 5 mM. The Km value for GLUT1 and GLUT3 is 1 mM.

GLUT1 and GLUT3 are high affinity glucose transporter. On the other hand, GLUT2 is a low affinity glucose transporter with a Km value of 15-20 mM. GLUT2 is expressed in kidney, liver, intestine and pancreatic beta cells. GLUT4 is insulin sensitive and is expressed in muscle and adipose tissue.

Several studies have been performed to determine the kinetic properties of these GLUT isoforms. As glucose is readily metabolized, it is difficult to determine the true kinetics of glucose transport. For this reason, various nonmetabolizable glucose analogues such as 2-deoxyglucose, 3-o-methylglucose have been used as a substrate for kinetic analysis.

However, there is less information concerning GLUT1 activity in alveolar epithelium, which is mainly injured during pulmonary fibrosis. Therefore, we attempted to characterize GLUT1 function in A549 cells, an in vitro model of human alveolar type II epithelial cells. For our study we used [^{3}H]D-glucose and [^{3}H]3-o-methyl-D-glucose as substrates and phloretin as inhibitor of GLUT1. In this study we examined time- and temperature-dependence of [^{3}H]D-glucose uptake in A549 cells. Then effect of inhibitors on the function of GLUT1 was observed.

2.2. Results

2.2.1. Time- and temperature-dependence of Glucose uptake in A549 cells: Firstly, we confirmed mRNA expression of GLUT1 in A549 cells by gel electrophoresis (Fig. 12). Then, we investigated the general characteristics of [^{3}H] D-glucose uptake in A549 cells. Here, the uptake of [^{3}H]D-glucose at different time periods and different temperatures (37 & 4^{o}C) were examined. The uptake of [^{3}H]D-glucose was enhanced with increase of time and temperature (Fig. 13).

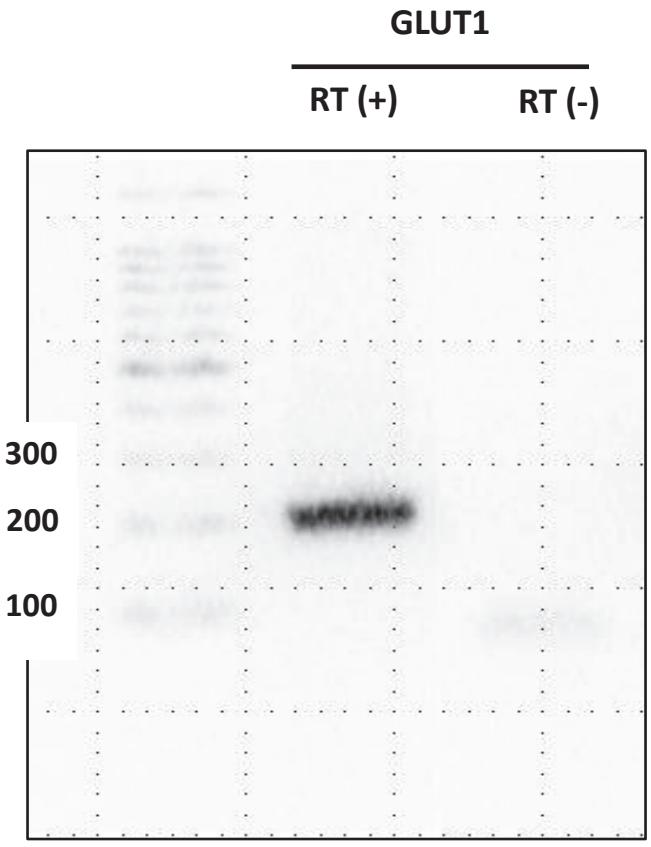


Figure 12: mRNA expression of GLUT1 in A549 cells by gel electrophoresis. Predicted size of GLUT1 is 185 bp.

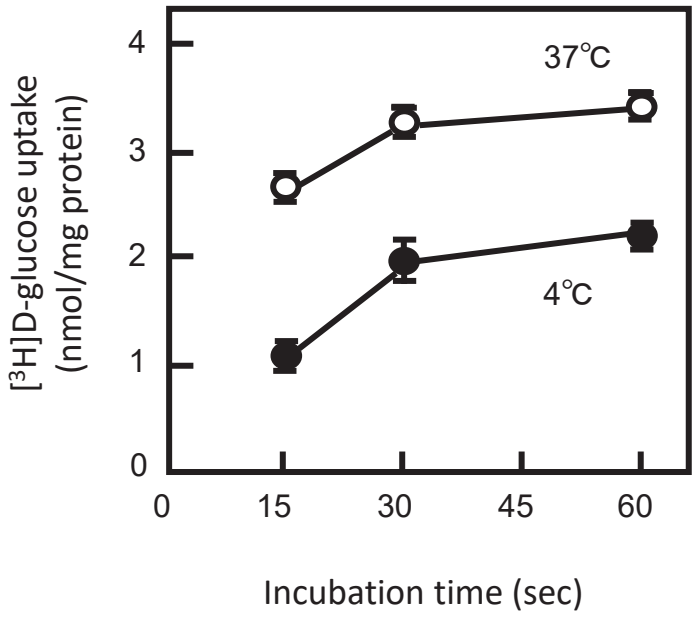


Figure 13. Time- and temperature-dependence of [^{3}H]D-glucose uptake in A549 cells. The cells were incubated with [^{3}H]D-glucose (1 mM) for 15, 30 & 60 second at 37° C (open circles) or 4° C (solid circles). Each value represents the mean ± SE of three monolayers. **p <0.01 is significantly different from the control.

2.2.2. Effect of inhibitors on GLUT1 function in A549 cells

Phloretin is an inhibitor of GLUTs and phloridzin is an inhibitor of SGLT (sodium/glucose cotransporter).

Sodium/glucose cotransport: In this process glucose moves from lower concentration to higher concentration. The energy that drives glucose across a membrane against the concentration gradient does not come directly from ATP. Rather it comes from a sodium ion gradient which was created using ATP. Although SGLT2 is a major cotransporter involved in glucose reabsorption in kidney, we investigated the effects of both inhibitors on the uptake of glucose in A549 cells.

The inhibitory effect of Phloretin on D-glucose was very significant compared with the case of phlorizin (Fig:14). Phloretin also suppressed 3-o-methyl-D-glucose uptake in concentration-dependent manner (Fig:15). So, it may be the GLUT transporter which was mainly involved in glucose uptake in A549 cells.

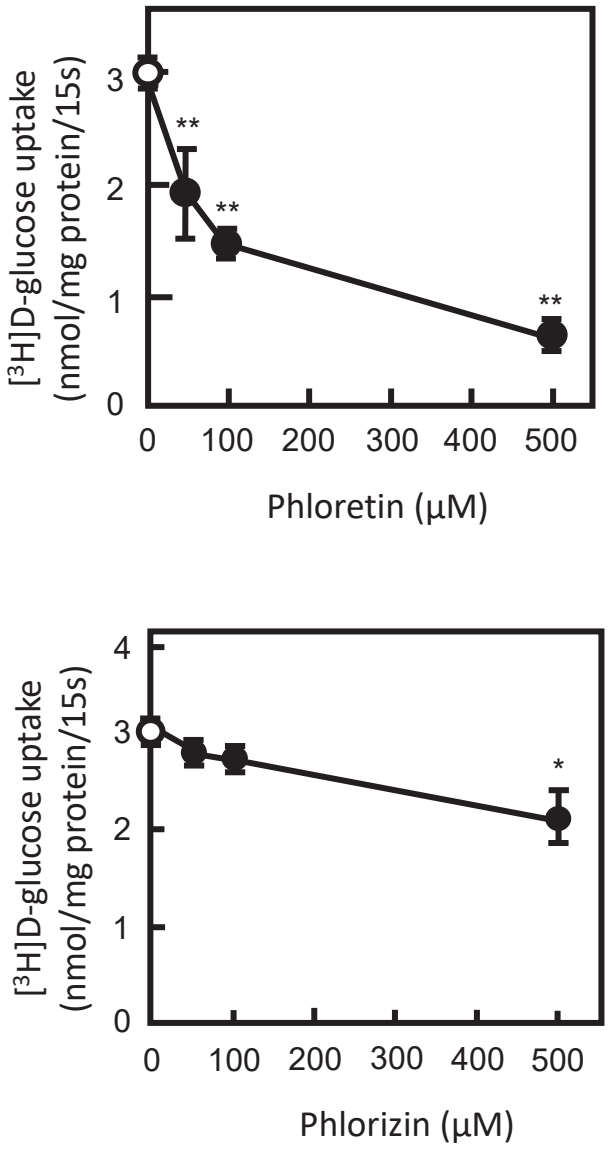


Figure 14. Effect of phloretin and phlorizin on the uptakes of [^{3}H]D-glucose in A549 cells. The cells were incubated with [^{3}H]D-glucose (1 mM) for 15s in the absence or presence of various concentration of phloretin (inhibitor of GLUTs) or phlorizin (inhibitor of SGLT). Each value represents the mean ± SE of three monolayers. *p<0.05, **p<0.01 are significantly different from the control.

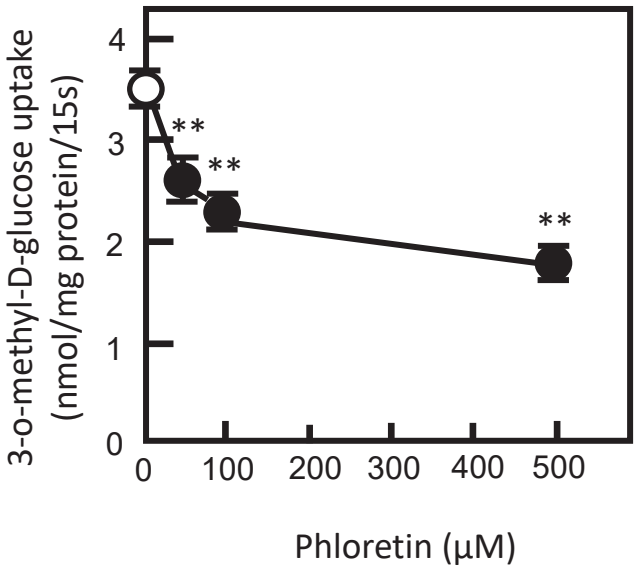


Figure 15. Effect of phloretin on the uptakes of [^{3}H]3-o-methyl-D-glucose in A549 cells. The cells were incubated with [^{3}H]3-o-methyl-D-glucose (1 mM) for 15s in the absence or presence of various concentration of Phloretin (inhibitor of GLUTs). Each value represents the mean ± SE of three monolayers. **p<0.01 is significantly different from the control.

2.3. Discussion

It was observed that the uptake of D-glucose showed time- and temperature-dependence. Phloretin significantly suppressed uptakes of [^{3}H]D-glucose and [^{3}H]3-o-methyl-D-glucose in a concentration-dependent manner (Fig. 14, 15). So, GLUT1 is a functioning glucose transporter in A549 cells. GLUT1 might play an important role in energy production in alveolar type II epithelial cells through the intake of glucose.

Chapter 3

Effect of TGF-β1 on the functional expression of Monocarboxylate Transporter 1 (MCT1) in A549 cells

3.1. Introduction

We know that TGF-β1 is a master regulator of EMT, in which epithelial cells lose their apico-basal polarity and cell-cell interaction, changing into mesenchymal cells and acquire migratory and invasive abilities. There are report that demonstrate that EMT is correlated with metabolic changes where monocarboxylate transporters play a key role（Kang et al. 2019). As MCT1 is the best characterized isoform found in almost all tissues, it may play a significant role in these metabolic changes during EMT.

We already characterized MCT1 function in A549 cells. However, the role of TGF-β1 in metabolic reprogramming in A549 cells is poorly understood. To date, the effect of TGF-β1 on the expression of MCT1 in A549 cells is unknown. That's why in this study we aimed to explore the effect of TGF-β1 on the function and expression of MCT1 and tried to elucidate its underlying mechanism. Finally, we investigated the contribution of MCT1 to TGF-β1 induced EMT.

3.2. Results

3.2.1. Effect of the TGF-β1 in mRNA expression of α-SMA in A549 cells

It was reported that 10 ng/mL of TGF-β1 treatment for 72 hr markedly induced EMT-like phenotypical changes in A549 cells (Kawami et al. 2016). On the other hand, α-SMA is used as an EMT indicator. That's why we examined the effect of TGF-β1 on the expression of α-SMA mRNA. TGF-β (10 ng/mL) treatment for 72 hr significantly enhanced mRNA expression of α-SMA (Fig. 16).

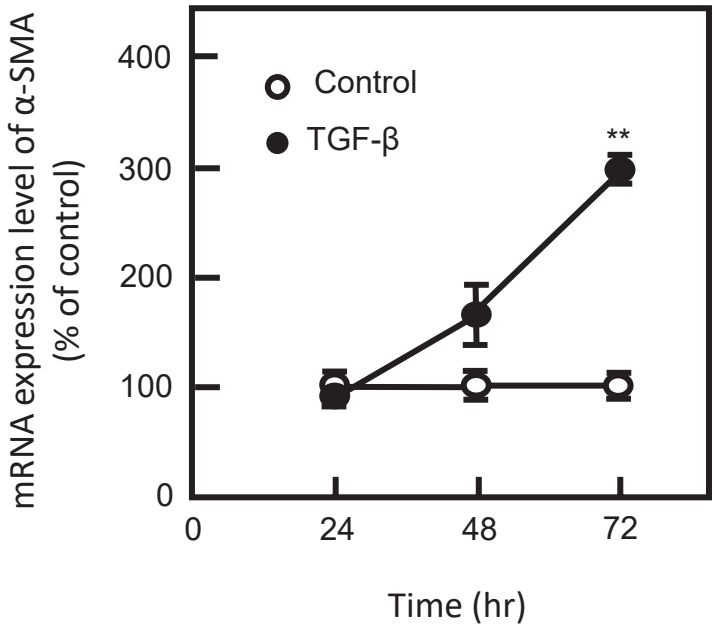


Figure 16. Effect of TGF-β1 treatment on α-SMA mRNA expression in A549 cells. Total RNA was extracted from the cells and real time PCR analysis was performed to evaluate the expression of each mRNA in A549 cells. A549 cells were not treated (control, open circle) or were treated with TGF (solid circle) for (a) 24 hr and (b) 48 hr and (c) 72 hr, respectively. The percentage of control of each mRNA expression estimated by real-time PCR was calculated after normalization by GAPDH, a housekeeping gene. Each value represents the mean ± SE of three RNA samples. **$p<0.01$ is significantly different from control.

3.2.2. Effect of the TGF-β1 in mRNA expression of MCT1 in A549 cells

Then, we examined the effect of TGF-β treatment on mRNA expression of MCT1. It was observed that TGF-β (10 ng/mL) treatment (48 hr, 72 hr) significantly enhanced mRNA expression of MCT1 at late phase (Fig. 17).

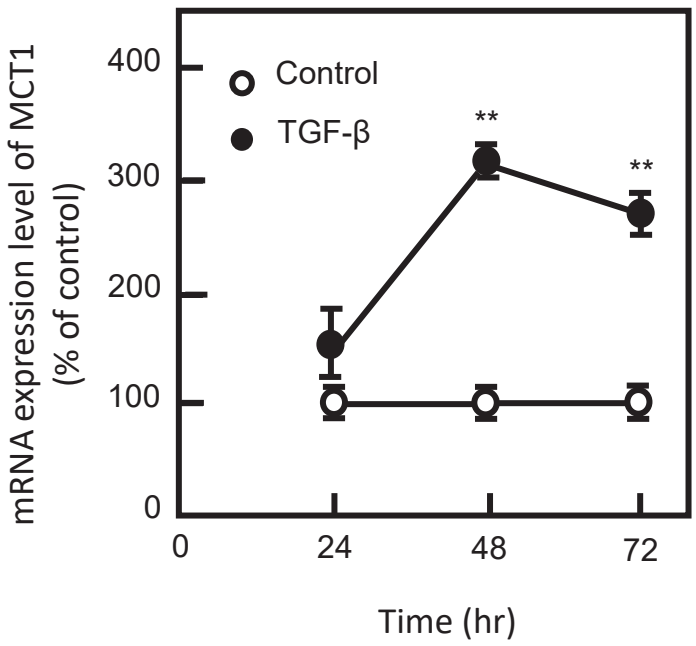


Figure 17. Effect of TGF-β1 treatment on MCT1 mRNA expression in A549 cells. Total RNA was extracted from the cells and real time PCR analysis was performed to evaluate the expression of each mRNA in A549 cells. A549 cells were not pretreated (control, open circle) or were pretreated with TGF (solid circle) for (a) 24 hr and (b) 48 hr and (c) 72 hr respectively. The percentage of control of each mRNA expression estimated by real-time PCR was calculated after normalization by GAPDH, a housekeeping gene. Each value represents the mean ± SE of three RNA samples. **p<0.01 is significantly different from control.

3.2.3. Effect of the TGF-β1 on the function of MCT1 in A549 cells

After that, we investigated the effect of TGF-β treatment on GHB uptake as MCT1 function in A549 cells. It was found that at 24 and 48 hr, TGF-β1 had no effect on MCT1 function, whereas the uptake of GHB was significantly enhanced by the treatment for 72 hr at pH 6 in A549 cells (Fig. 18).

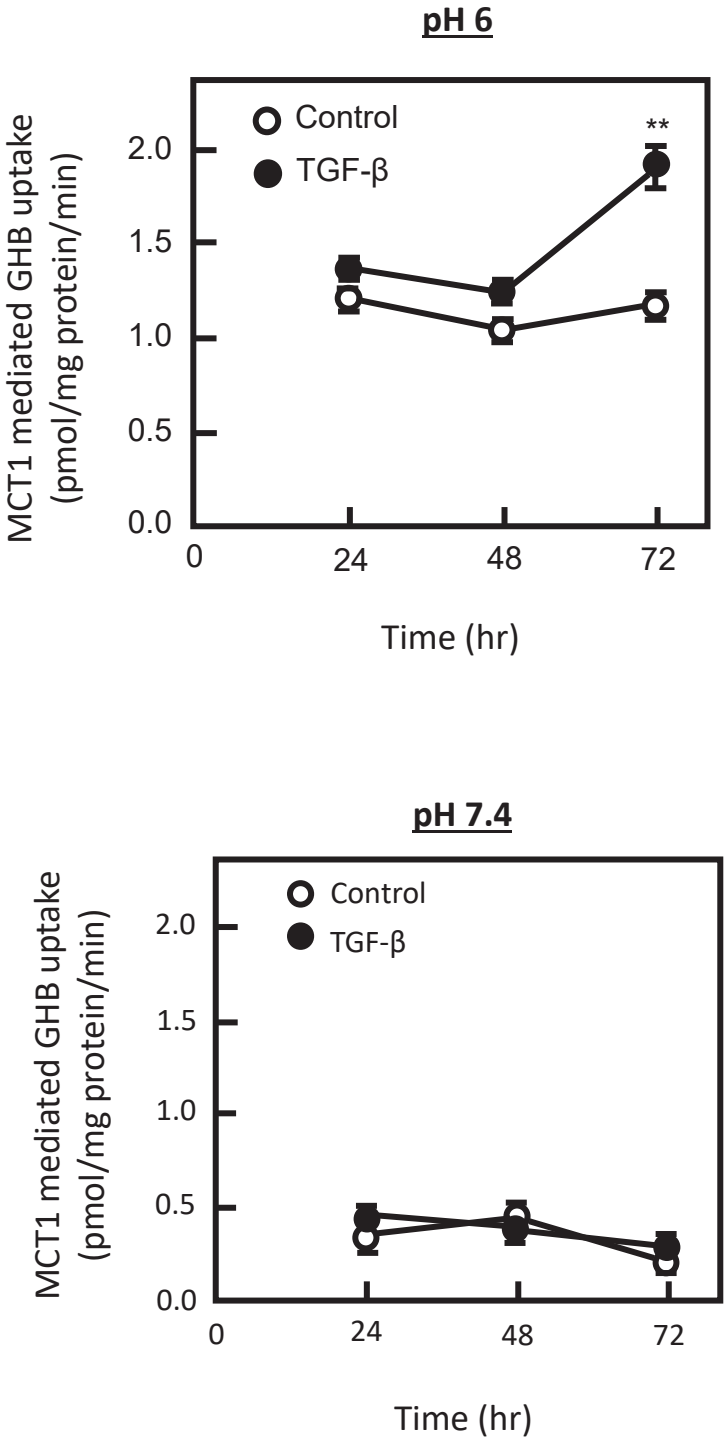


Figure 18. Effect of TGF-β1 on MCT1 function (MCT1 dependent uptake of GHB) in A549 cells at pH 6 & 7.4. The cells were treated with TGF-β1 (10ng/mL) for 72 hr. After that, the treated cells were incubated with [^{3}H]GHB (100 nM) in the absence or presence of CHC (2 mM). GHB uptake value was calculated by subtracting [^{3}H]GHB uptake with CHC from that without CHC. Each value represents the mean ± S.E.M. of three monolayers. **p< 0.01, significantly different from control cells.

3.2.4. Role of the TGF-β signaling pathway in functional expression of MCT1 in A549 cells

3.2.4.1. Effect of SB on mRNA and protein expression changes induced by TGF-β1 in A549 cells: We confirmed that 10 ng/mL of TGF-β1 treatment for 72 hr significantly enhanced function and expression of MCT1. Later we examined the pathway by which TGF-β1 enhancing function and expression of MCT1. SB is a TGF-β type I receptor kinase inhibitor. So, the effect of SB on the function and expression of MCT1 was observed. TGF-β1 treatment significantly increased mRNA and protein expression levels of MCT1, and cotreatment with SB markedly suppressed the upregulation of MCT1 induced by TGF-β1 (Fig. 19, 20).

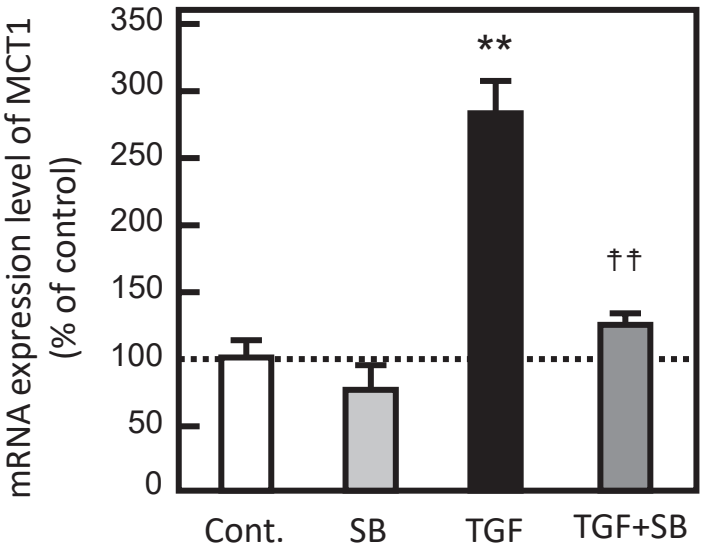


Figure 19: Role of TGF-β1 signaling cascade pathway on the mRNA expression levels of MCT1 in A549 cells. The cells were treated with TGF-β1 (10ng/mL) for 72 hr in the absence or presence of SB (10 μM). mRNA expression levels were evaluated by real-time PCR using total RNA. Each value represents the mean ± S.E.M. of three monolayers. **p< 0.01, significantly different from control cells. ††p< 0.01, significantly different from TGF-β1 treated cells (Uddin et al. 2020).

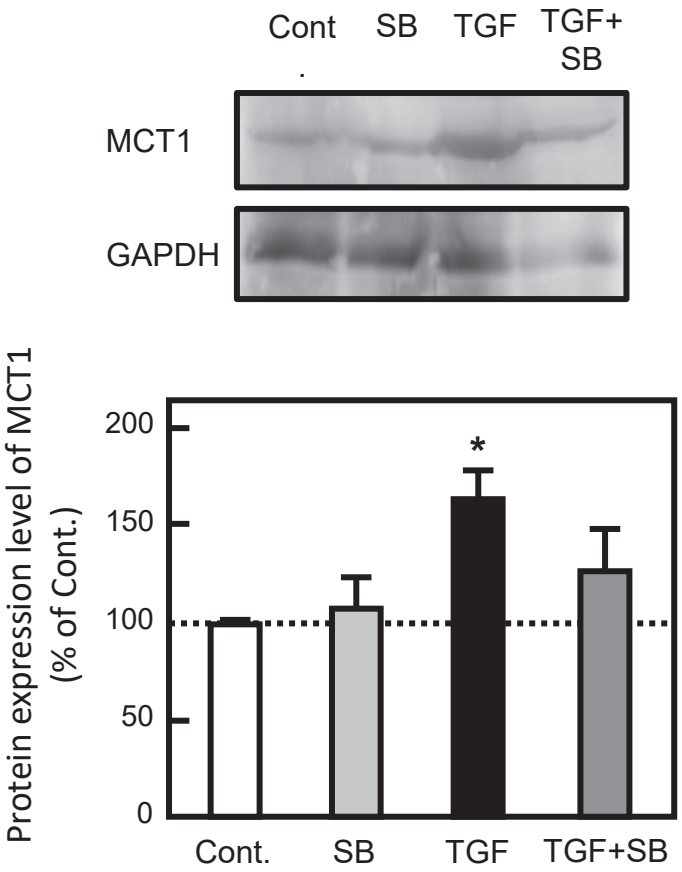


Figure 20: Role of TGF-β1 signaling cascade pathway on the protein expression levels of MCT1 in A549 cells. The cells were treated with TGF-β1 (10ng/mL) for 72 hr in the absence or presence of SB (10 μM). Protein expression levels were evaluated by western blot using total RNA and crude membrane fraction extracted from the treated cells, respectively. Each value represents the mean ± S.E.M. of three monolayers. *p< 0.05, significantly different from control cells (Uddin et al. 2020).

3.2.4.2. Effect of SB on changes of MCT1 function induced by TGF-β1 in A549 cells:

TGF-β1 treatment significantly enhanced [^{3}H]GHB and [^{3}H]lactic acid uptake in A549 cells, and these changes were suppressed by co-treatment with SB. (Fig. 21). These findings indicate that the TGF-β signaling pathway may be closely related with the functional expression of MCT1 in A549 cells.

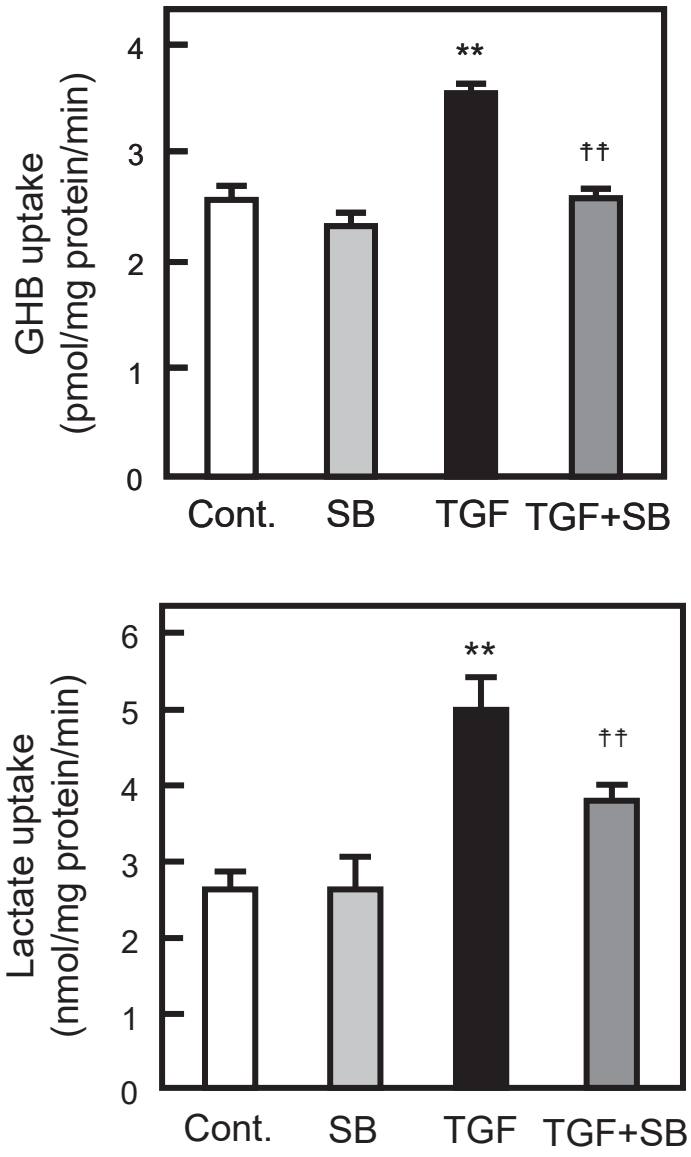
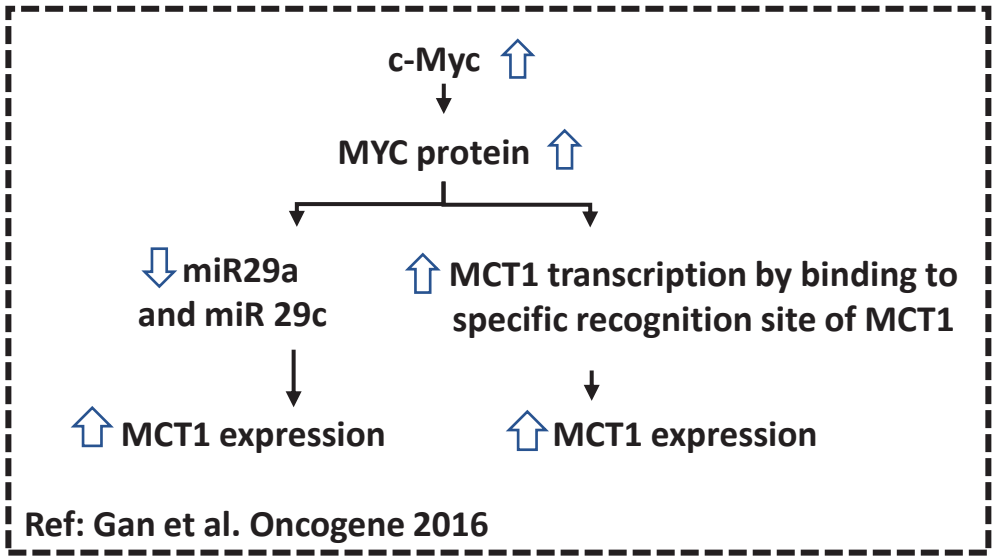


Figure 21. Role of TGF-β1 signaling cascade pathway on the function of MCT1 in A549 cells. The cells were treated with TGF-β1 (10 ng/mL) for 72 hr in the absence or presence of SB (10 μM). After that, the treated cells were incubated with [^{3}H]GHB (100 nM) or [^{3}H] lactate (1 mM). Each value represents the mean ± S.E.M. of three monolayers. **p< 0.01, significantly different from control cells. ††p< 0.01, significantly different from TGF-β1 treated cells (Uddin et al. 2020).

We found that TGF-β signaling pathway may be involved in the regulation of the functional expression of MCT1. But the detailed mechanisms of regulation of MCT1 by TGF-β1 is still unclear at this stage.

However, c-myc is reported to directly control the transcription of MCT1 as described in below shceme.



So, we examined the effect of TGF-β1 on expression of c-myc. TGF-β1 treatment increased the mRNA expression of c-myc, which was suppressed by SB. So, TGF-β1 may regulate MCT1 expression via c-myc in A549 cells (Fig. 22)

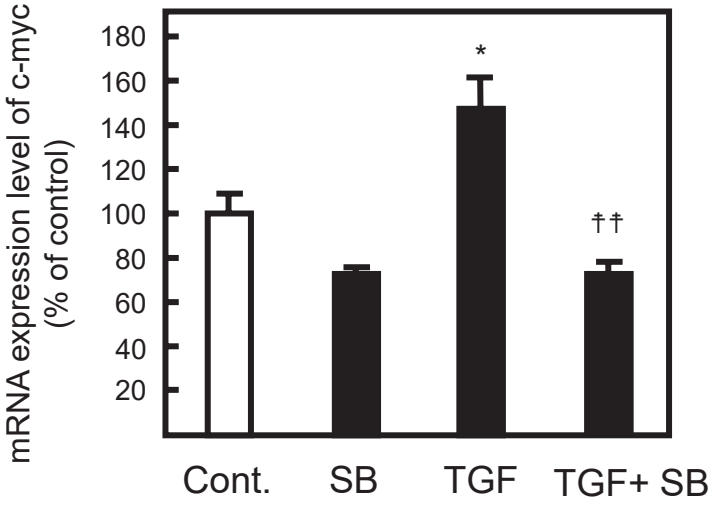


Figure 22: Effect of TGF-β1 on mRNA expression of c-Myc in A549 cells. The cells were treated with TGF-β1 (10ng/mL) for 72 hr in the absence or presence of SB (10 µM). mRNA expression levels were evaluated by real-time PCR using total RNA. Each value represents the mean ± S.E.M. of three monolayers. *p< 0.05, significantly different from control cells. ††p< 0.01, significantly different from TGF-β1 treated cells (Uddin et al. 2020).

MCT family has 14 members. Among them, four isoforms (MCT1-MCT4) are known to be involved in transport of monocarboxylates, like lactate, pyruvate, ketone bodies etc. But the expression of MCT2 is relatively less in human tissue and is expressed in restricted tissues, such as liver parenchyma and proximal convoluted tubule of kidney. MCT3 is expressed in retinal pigment epithelium and choroid plexus epithelium. In addition, we observed that mRNA expression of MCT4 was decreased by TGF-β1 in A549 cells (Fig. 23). Therefore, MCT1 may be a predominant isoform among MCTs that play a key role in transport of lactate during TGF-β1 induced EMT in A549 cells.

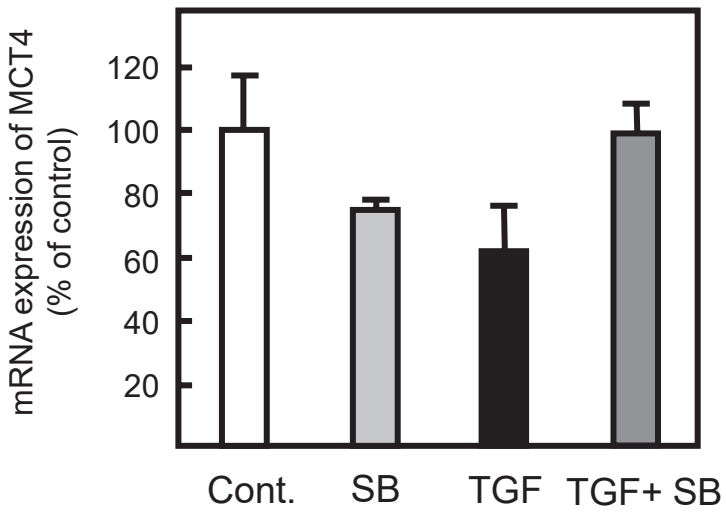


Figure 23: Effect of TGF-β1 on mRNA expression of MCT4 in A549 cells. The cells were treated with TGF-β1 (10 ng/mL) for 72 hr in the absence or presence of SB (10 μM). mRNA expression levels were evaluated by real-time PCR using total RNA. Each value represents the mean ± S.E.M. of three monolayers (Uddin et al. 2020).

3.2.4.3. Role of TGF-β signaling pathway in functional expression of MCT1 in other cell line: A549 is an in vitro model of human alveolar type II epithelial cells derived from human lung carcinoma. We observed that TGF-β1 induced EMT-like morphological change and enhanced the function and expression of MCT1 in A549 cells. Therefore, we further tried to clarify the effect of TGF-β1 on functional expression of MCT1 in other cell lines than A549 cells. We have experienced that primary cultured cells from rat cannot be used for EMT studies due to its trans differentiation. Actually, the cells cultured for 2 days exhibited cuboidal type II epithelial morphology with lamellar bodies inside the cells, while the cells cultured for 6 days exhibited squamous type I epithelial morphology (Ikehata et al. 2008). Therefore, we further tried to use RLE/Abca3 cells derived from rat normal alveolar epithelial cells to clarify the effect of TGF-β1 on functional expression of MCT1. We observed that TGF-β1 induced EMT-like morphological change and increase in mRNA expression of α-SMA in RLE/Abca3 cells (Takano et al. 2015). Therefore, we examined the role of TGF-β signaling pathway in functional expression of MCT1 using RLE/Abca3 cells. In RLE/Abca3 cells, mRNA expression level of α-SMA was clearly upregulated by TGF-β1, which was completely canceled by the co-treatment with SB (Fig. 24). However, mRNA expression level of MCT1 was decreased by TGF-β1, and SB suppressed the TGF-β1-induced alteration (Fig. 25). In addition, TGF-β1 treatment had no effect on GHB uptake in RLE/Abca3 cells (Fig. 26).

Also, the uptake amounts of GHB was quite lower than the case of A549 cells. It seems that MCT1 activity was quite low in RLE/Abca3 cells. Considering above-mentioned observations, it may be difficult to clarify the relationship between TGF-β1-induced EMT and functional expression of MCT1 using RLE/Abca3 cells. Further investigations using other human cell lines than A549 cells may be required for complete understanding of relationship between EMT and functional expression of MCT1.

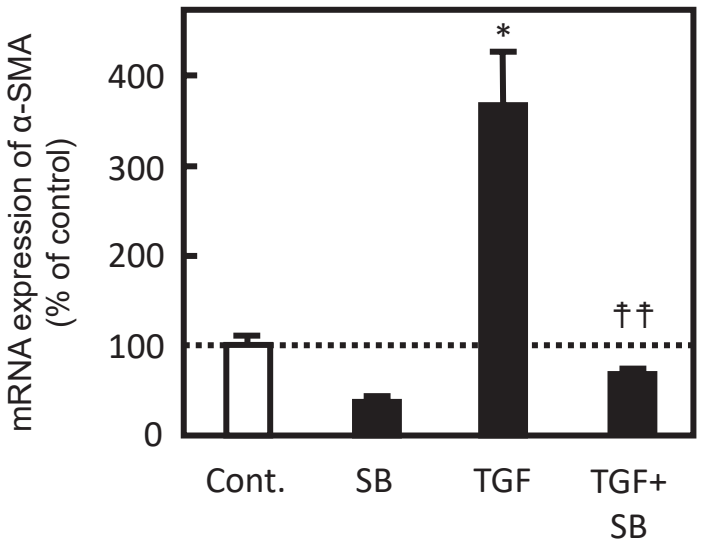


Figure 24: Effect of TGF-β1 on the mRNA expression levels of α-SMA in RLE/Abca3 cells derived from rat normal alveolar epithelial cells. The cells were treated with TGF-β1 (10 ng/mL) for 72 hr in the absence or presence of SB (10 μM). mRNA expression levels were evaluated by real-time PCR using total RNA. Each value represents the mean ± S.E.M. of three monolayers. *p< 0.05, significantly different from control cells. ††p< 0.01, significantly different from TGF-β1 treated cells.

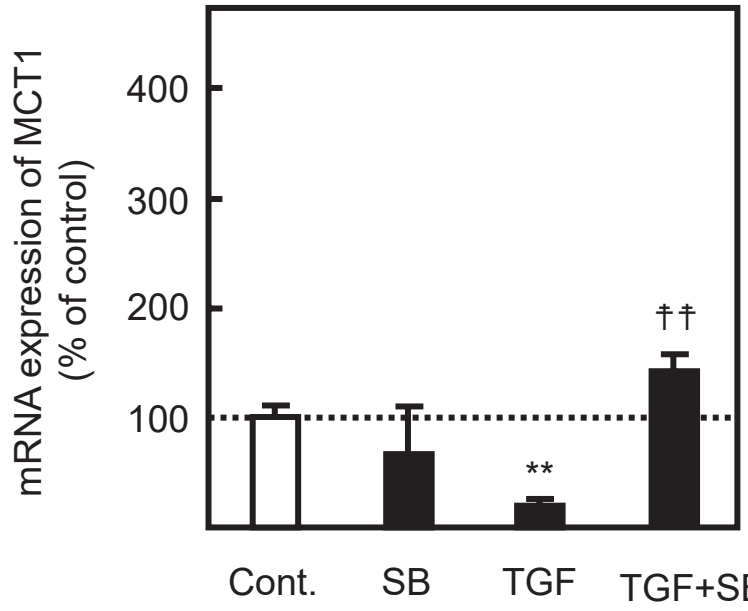


Figure 25: Effect of TGF-β1 on the mRNA expression level of MCT1 in RLE/Abca3 cells derived from rat normal alveolar epithelial cells. The cells were treated with TGF-β1 (10ng/mL) for 72 hr in the absence or presence of SB (10 μM). mRNA expression levels were evaluated by real-time PCR using total RNA. Each value represents the mean ± S.E.M. of three monolayers. **p< 0.01, significantly different from control cells. ††p< 0.01, significantly different from TGF-β1 treated cells.

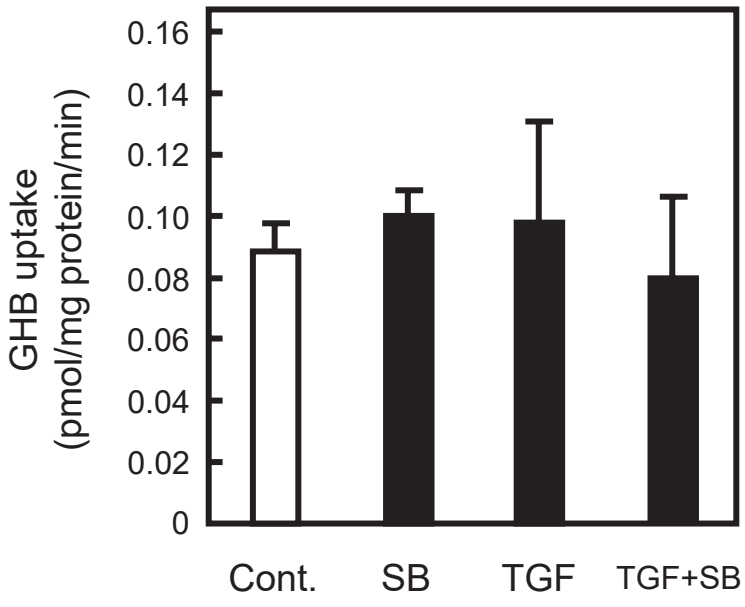


Figure 26. Effect of TGF-β1 on the function of MCT1 in RLE/Abca3 cells. The cells were treated with TGF-β1 (10ng/mL) for 72 hr in the absence or presence of SB (10 μM). After that, the treated cells were incubated with [^{3}H]GHB (100 nM) in the absence or presence of CHC (2 mM). GHB uptake value was calculated by subtracting [^{3}H]GHB uptake with CHC from that without CHC. Each value represents the mean ± S.E.M. of three monolayers.

3.2.5. Contribution of MCT1 to TGF-β1-induced EMT in A549 cells

3.2.5.1. Effect of CHC on TGF-β1-induced changes in A549 cells

CHC is a non-specific MCT1 inhibitor. In the prior section, We found that CHC significantly suppressed uptakes of GHB and lactic acid in a concentration dependent manner for shorter period of time (1 minute). So, we investigated the effect of CHC on TGF-β1-induced EMT in A549 cells. However, CHC had no effect on morphological changes induced by TGF-β1 in A549 cells (Fig. 27). In addition, known TGF-β1-induced alterations of mRNA expression such as decrease in CK19 and increase in α-SMA were not changed by cotreatment with CHC (Fig.28).

These findings suggest that TGF-β1-induced EMT may be independent of MCT1 function.

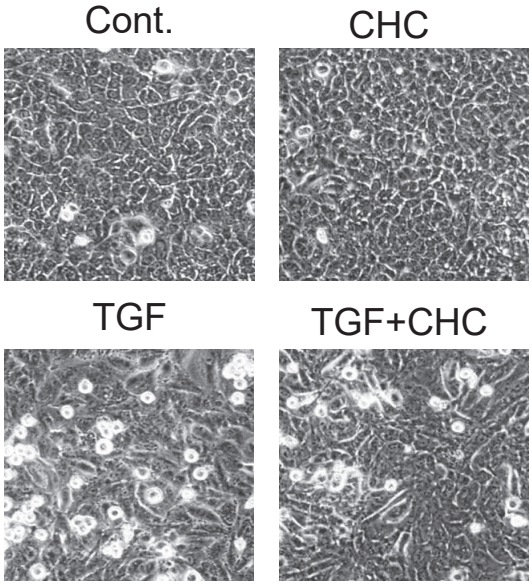


Figure 27. Effect of CHC on TGF-β1-induced changes in morphology in A549 cells. The cells were treated with TGF-β1 (10 ng/mL) in the absence or presence of CHC (2 mM) for 72 hr. After that, the morphology was observed by phase-contrast microscopy (Uddin et al. 2020).

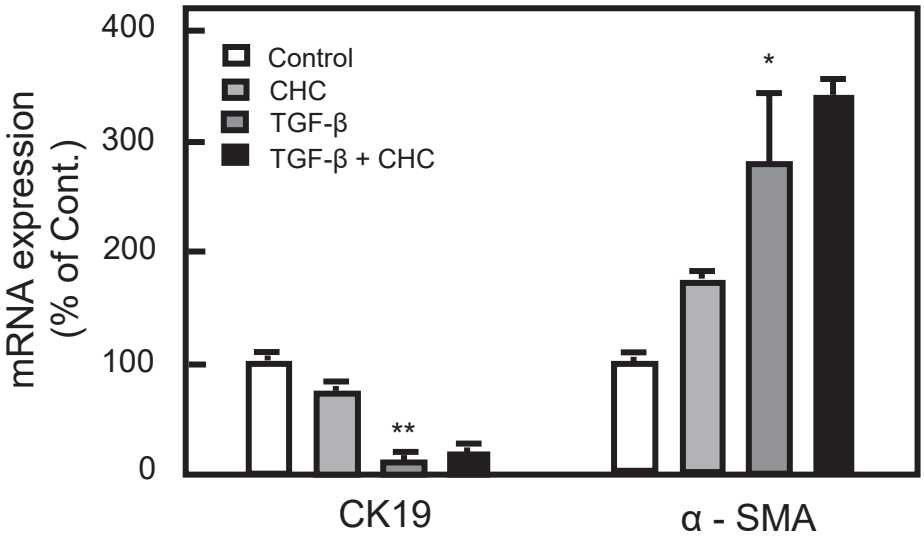


Figure 28. Effect of CHC on mRNA expression level of CK19 and α-SMA in A549 cells. The cells were treated with TGF-β1 (10 ng/mL) in the absence or presence of CHC (2 mM) for 72 hr. After that, mRNA expression level of CK19 and α-SMA in the case of co-treatment with CHC was analyzed by real-time PCR using total RNA extracted from the treated cells. Each value represents the mean ± S.E.M. of three monolayers. *p< 0.05, **p< 0.01, significantly different from control cells (Uddin et al. 2020).

3.2.5.2. Effect of AR-C155858 on TGF-β1-induced changes in A549 cells

AR-C155858 is a potent MCT1 inhibitor. AR also significantly suppressed uptake of GHB and lactic acid in a concentration dependent manner for shorter period of time (1 minute). So, we investigated the effect of AR on TGF-β1-induced EMT in A549 cells. AR had no effect on morphological changes induced by TGF-β1 in A549 cells (Fig. 29). In addition, known TGF-β1 induced alterations of mRNA expression such as decrease in CK19 and increase in α-SMA were not changed by cotreatment with AR (Fig.30).

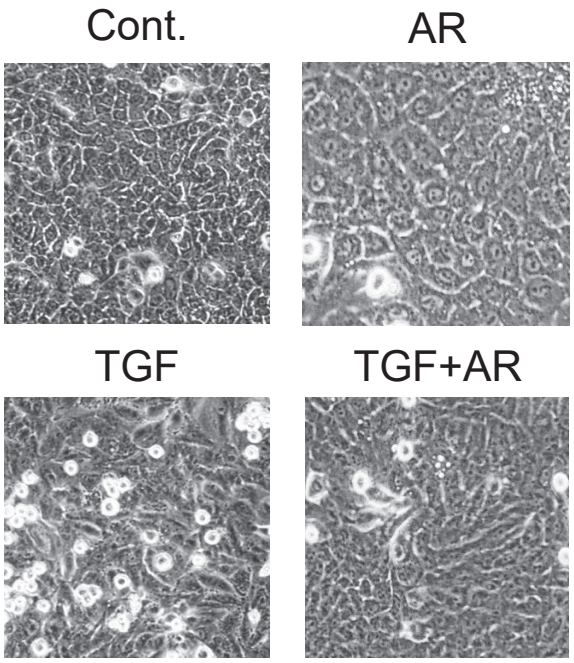


Figure 29. Effect of AR on TGF-β1-induced changes in morphology in A549 cells. The cells were treated with TGF-β1 (10 ng/mL) in the absence or presence of AR (1 μM) for 72 hr. After that, the morphology was observed by phase-contrast microscopy (Uddin et al. 2020).

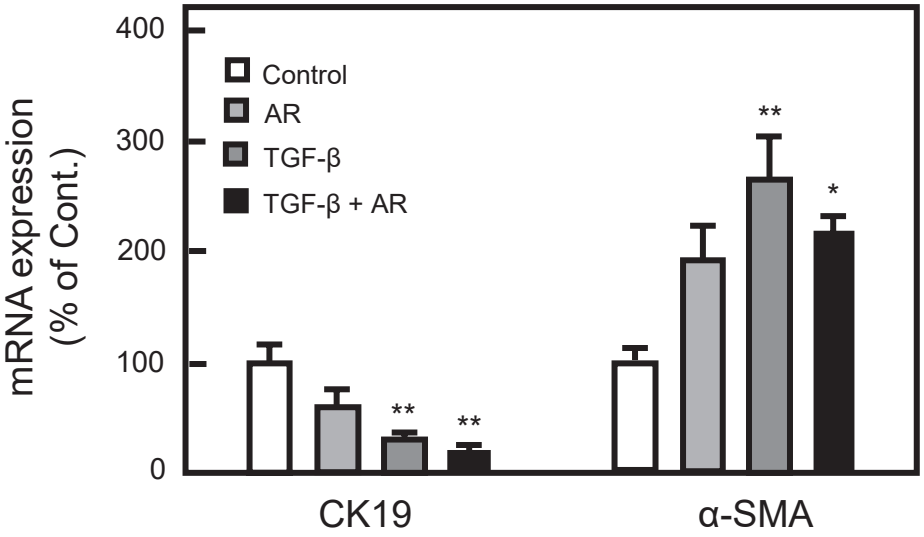


Figure 30. Effect of AR on mRNA expression level of CK19 and α-SMA in A549 cells. The cells were treated with TGF-β1 (10 ng/mL) in the absence or presence of AR (1 μM) for 72 hr. After that, mRNA expression level of CK19 and α-SMA in the case of co-treatment with AR was analyzed by real-time PCR using total RNA extracted from the treated cells. Each value represents the mean ± S.E.M. of three monolayers. *$p < 0.05$, **$p < 0.01$, significantly different from control cells (Uddin et al. 2020).

3.3. Discussion:

So far, the underlying mechanism involved in regulation of MCT1 is unclear. In the current study, we observed that TGF-β1 treatment enhanced GHB and lactic acid uptakes as well as upregulated mRNA and protein expression levels of MCT1 in A549 cells, and that these effects were canceled by cotreatment with SB. Thus, TGF-β signaling pathway may be involved in the regulation of the functional expression of MCT1.

On the other hand, c-myc is reported to regulate the transcription of MCT1 (Doherty et al. 2014). We observed that TGF-β1 significantly upregulated mRNA expression levels of c-myc. Furthermore, SB suppressed TGF-β1-induced enhancement of c-myc mRNA expression. So, there is a possibility that c-myc may be involved with the regulation of MCT1 by TGF-β1 in A549 cells. Besides it is reported that Myc targets the promoter region of snail, suppresses expression of E-Cadherin and promotes EMT (Smith et al. 2009).

Both MCT1 inhibitors of CHC and AR had no significant effect on the morphological changes induced by TGF-β1. TGF-β1-induced alterations of mRNA expression such as decrease in epithelial marker, CK19 and increase in mesenchymal marker, α-SMA were unaffected by co-treatment with CHC and AR. So, there may be less contribution of MCT1 to TGF-β1-induced EMT in A549 cells.

On the other hand, Gray et al. (2016) demonstrated that knockdown of MCT1 (but not AZD3965, a promising MCT1 inhibitor) suppressed hepatocyte growth factor- and epidermal growth factor-induced tumor cell scattering and wound healing. Thus, our results suggest that MCT1 transport activity may not be crucial to the EMT process. Therefore, factors other than the transporter activity of MCT1 may be responsible for TGF-β1-induced EMT in A549 cells.

On the other hand, when the inhibitory effect of CHC and AR was observed, it was examined for short time (1-5 minute). But when we examined the effect of these inhibitors on TGF-β1 induced EMT, we checked it for 72 hr. We are not sure whether these inhibitors are effective for long time.

Besides there are some reports that TGF-β1 is produced as a latent complex, and activation of TGF-β1 generally requires the binding of αv integrin to an RGD motif in the latent form of TGF-β1 (Shi et al. 2011). Alterations in pH resulting from endogenous production and release of lactate are reported to be capable of activating latent TGF-β1 (Kottmann et al. 2012). In this context, lactate concentration, which is regulated by MCT1, may be a key component during TGF-β1- induced EMT under in vivo condition. Therefore, the relationship between MCT1 expression level, transporter activity of MCT1, and induction of EMT by TGF-β1 needs to be studied further.

Chapter 4

Effect of TGF-β1 on the functional expression of Glucose Transporter 1 (GLUT1) in A549 cells

4.1. Introduction

GLUT1 is the most common isoform of glucose transporter which is expressed in almost all living organism. It plays a key role in assuring constant supply of glucose to cells for metabolism. Among all GLUT isoform expressed, GLUT1 is a high-affinity, low-capacity glucose transporter with a Ki value of 1 mM. For this reason, it reaches saturation below the basal glucose concentration. Therefore, changes in GLUT-1 expression and function are essential for cells to significantly increase their basal glucose uptake.

On the other hand, It has been reported that Warburg-like metabolic reprogramming in alveolar epithelial cells derived from IPF patients would result in increased glucose uptake mediated by GLUT1 (Zank et al. 2018). Another report showed that aerobic glycolysis was enhanced during myofibroblast differentiation and lung fibrosis (Xie et al. 2015). Therefore, we examined the effect of TGF-β1 treatment on [^{3}H]D-glucose and [^{3}H]3-o-methyl-D-glucose uptake in A549 cells.

4.2. Results

4.2.1. Effect of the TGF-β1 in mRNA expression of GLUT1 in A549 cells

We investigated the effect of TGF-β1 treatment on mRNA expression of GLUT1 at shorter (6 & 9 hr) and longer (48 & 72 hr) time period. TGF-β1 treatment significantly enhanced mRNA expression of GLUT1 at different time period (Figure 31).

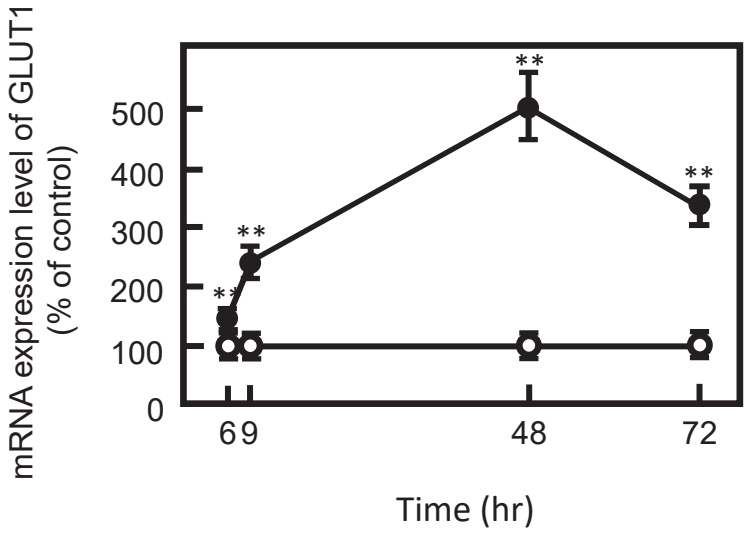


Figure 31. Effect of TGF-β1 treatment on GLUT1 mRNA expression in A549 cells. Total RNA was extracted from the cells and real time PCR analysis was performed to evaluate the expression of each mRNA in A549 cells. A549 cells were not pretreated (control, open circle) or were pretreated with TGF (solid circle) for shorter period (6 & 9 hr) and longer period (48 & 72 hr) respectively. The percentage of control of each mRNA expression estimated by real-time PCR was calculated after normalization by GAPDH, a housekeeping gene. Each value represents the mean ± SE of three RNA samples. **p<0.01 is significantly different from control.

4.2.2. Effect of the TGF-β1 on the function of GLUT1 in A549 cells

We investigated the effect of TGF-β treatment on the uptake of [^{3}H] D-glucose and [^{3}H]3-o-methyl-D-glucose in A549 cells. Surprisingly, it was observed that TGF-β1 treatment significantly enhanced glucose uptake for up to 12 hr, but not at 24 and 72 hr (Fig. 32, 33).

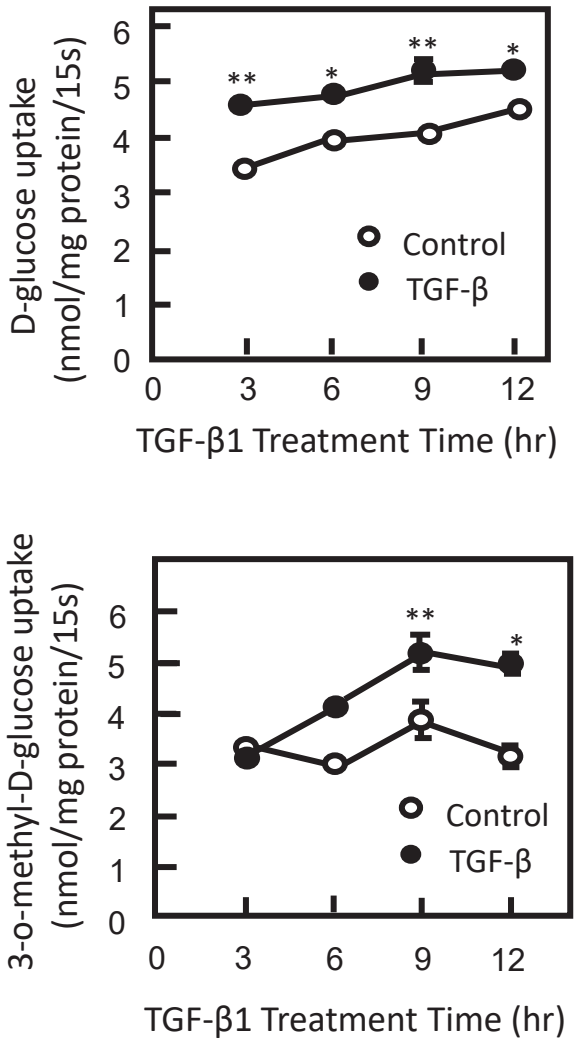


Figure 32. Effect of short term TGF-β1 treatment on GLUT1 function in A549 cells. The cells were not pretreated (control, open circle) or were pretreated with TGF-β1 (solid circle) for (a) 3, 6, 9 & 12 hr, respectively, and then uptake operation was performed using [^{3}H] D-glucose or [^{3}H] 3-o-methyl-D-glucose(100 nM) as a substrate. Each value represents the mean ± SE of three monolayers. **p<0.01 is significantly different from control (Uddin et al. 2020).

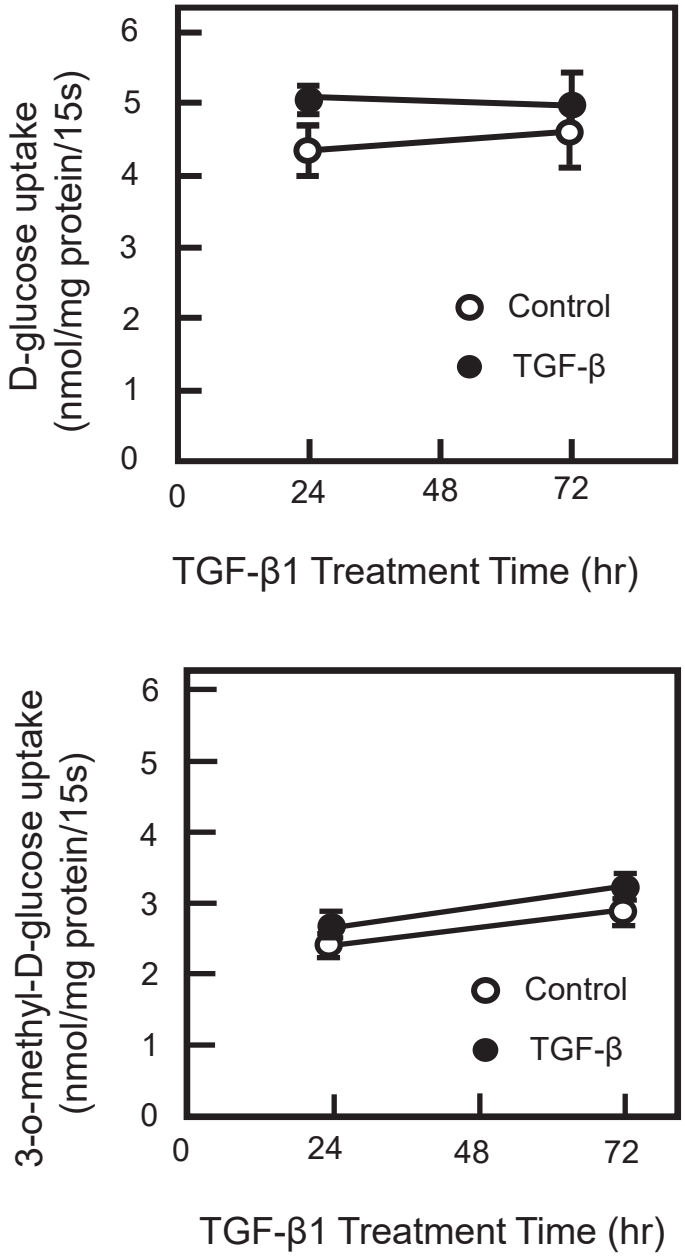


Figure 33. Effect of long term (24 hr, 72 hr) TGF-β1 treatment on GLUT1 function in A549 cells. The cells were not pretreated (control, open circle) or were pretreated with TGF-β1 (solid circle) for (b) 24 hr and (c) 72 hr, and then uptake operation was performed using [^{3}H]D-glucose or [^{3}H]3-o-methyl-D-glucose (100 nM) as a substrate. Each value represents the mean ± SE of three monolayers. **p<0.01 is significantly different from control (Uddin et al. 2020).

4.3. Discussion:

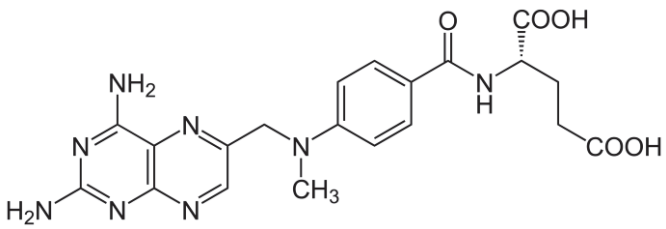
We examined the effect of TGF-β1 treatment on mRNA expression and function of GLUT1 in A549 cells. TGF-β1 treatment significantly enhanced mRNA expression of GLUT1 at different time periods (Fig. 31). which supports the report of Warburg-like metabolic reprogramming in alveolar epithelial cells derived from IPF patients (Zank et al. 2018). On the other hand, D-glucose uptake was enhanced at 6, 9, and 12 hr, but not at 24 and 72 hr after treatment (Fig. 32, 33). In our study, we observed that GHB uptake was not affected by TGF-β1 at 24 hr (data not shown). These findings suggest that, in an early phase, TGF-β1 may stimulate glucose uptake, while in a later phase, MCT1 function is upregulated for oxidative metabolism in A549 cells. However, further studies are needed to clarify the mechanisms underlying such a metabolic shift during TGF-β1-induced EMT.

Chapter 5

Effect of MTX treatment on MCT1 function in A549 cells

5.1. Introduction

Methotrexate (MTX) is a chemotherapy agent and immune system suppressant. It is used to treat several cancers including breast cancer, leukemia, lung cancer, lymphoma and autoimmune diseases like psoriasis, rheumatoid arthritis, and Crohn's disease etc. (Benedek et al. 2010; Bleyer WA et al. 1978; Rajitha P et al. 2017). But MTX cause serious lung diseases such as pulmonary fibrosis. Considering the wide use of MTX in clinical practice, it is important to establish a preventive approach against MTX-induced lung injury.



Methotrexate (MTX)

However, the underlying mechanisms of lung injury induced by MTX are not well understood. Recently, some reports indicated that epithelial-mesenchymal transition (EMT) induces the development of fibrosis in several organs including the lungs (Kim et al. 2006; Yu et al. 2018).

Several studies have elucidated that MTX induced EMT-like phenotypical changes in the alveolar epithelium in vitro and in vivo (Kawami et al. 2016; Ohbayash et al. 2014; Takano et al. 2015,). These findings strongly indicated that severe MTX induced lung injury might be associated with the EMT in alveolar epithelial cells.

It is reported that EMT and metabolic reprogramming are intertwined (Kang et al. 2019). We observed that MCT1 function and expression were enhanced during TGF-β1 induced EMT. But, the effect of MTX on the expression and function of MCT1 in A549 cells is unknown. That's why, in this study, we aimed to explore the effect of MTX on the function and expression of MCT1 in A549 cells.

5.2. Result

5.2.1. Effect of MTX on morphological changes in A549 cells

In a previous study of our lab (Kawami et al., 2016), MTX treatment induced EMT-like phenotypic changes with decrease in epithelial markers (CK19 and E-cadherin) and increase in mesenchymal markers (α-SMA, vimentin) in A549 cells. In our study, we examined the effect of MTX at different concentrations (0.1μM, 0.2μM, 0.3μM) on the morphological changes in A549 cells. MTX treatment induced EMT like morphological changes in A549 cells (Fig. 34).

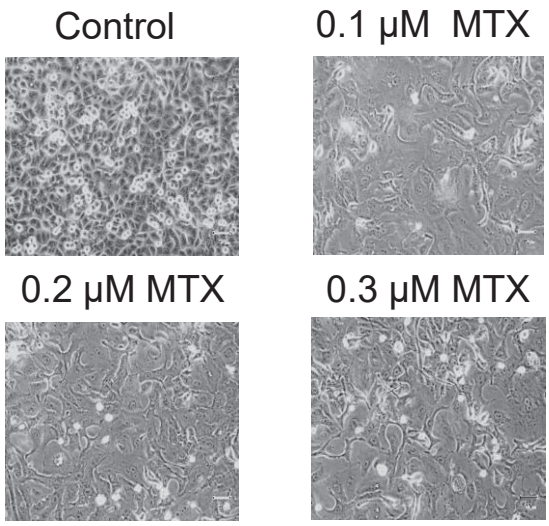


Figure 34. Effect of MTX on the morphology of A549 cells. The cells were treated with MTX at a concentration of 0.1 μM, 0.2 μM and 0.3 μM respectively for 72 hr. After that, the morphology was observed by phase-contrast microscopy.

5.2.2. Comparison of TGF-β1 and MTX on mRNA expression of MCT1 and MCT4 in A549 cells

Here, we compared the effect of TGF-β1 and MTX on mRNA expression of MCT1 and MCT4 in A549 cells. TGF-β1 treatment significantly enhanced the expression of MCT1, but there was substantial decline in the expression of MCT4 by TGF-β1 treatment. On the other hand the effect of MTX on mRNA expression of MCT1 and MCT4 was marginal (Fig. 35).

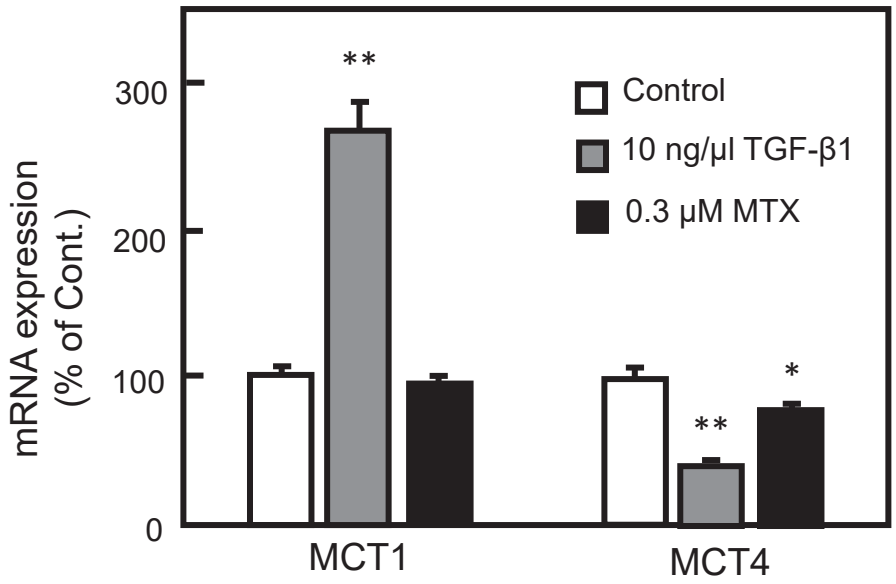


Figure 35: Effect of TGF-β1 and MTX on mRNA expression of MCT1 and MCT4 in A549 cells. The cells were treated with TGF-β1 (10 ng/mL) and MTX (0.3 μM) for 72 hr. mRNA expression levels were evaluated by real-time PCR using total RNA. Each value represents the mean ± S.E.M. of three monolayers. *p< 0.05, **p< 0.01, significantly different from control cells.

5.2.3. Effect of MTX on MCT1 function in A549 cells

We observed that MTX treatment has no clear effect on the mRNA expression of MCT1. Then, we examined the effect of different concentration (0.1 µM, 0.2 µM, 0.3 µM) of MTX on the uptake of GHB at pH 6 and 7.4. MTX treatment at a concentration of 0.2 µM and 0.3 µM significantly enhanced uptake of GHB at pH 6 (Fig. 36).

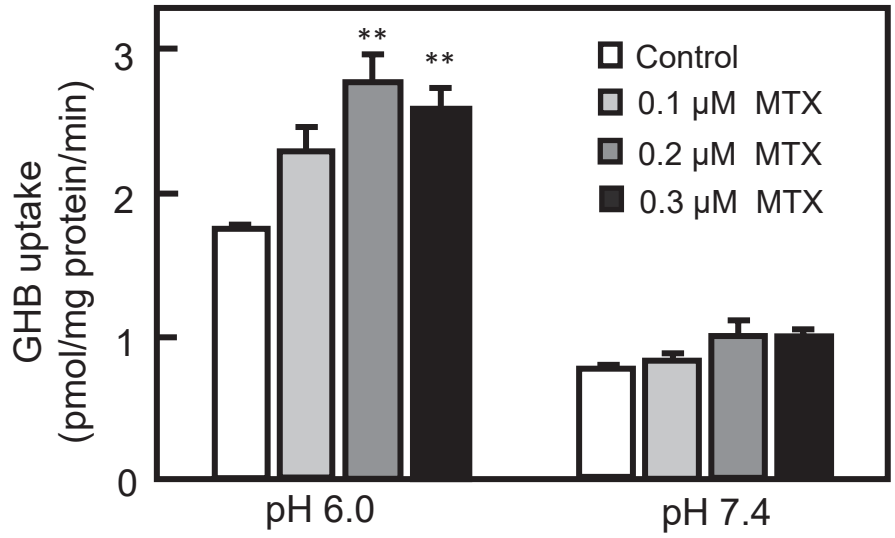


Figure 36. Effect of MTX on the function of MCT1 in A549 cells. The cells were treated with MTX at a concentration of 0.1 µM, 0.2 µM and 0.3 µM respectively for 72 hr. After that, the treated cells were incubated with [^{3}H]GHB (100 nM). Each value represents the mean ± S.E.M. of three monolayers. **p< 0.01, significantly different from control cells.

5.2.4. Effect of CHC on MTX induced EMT in A549 cells

CHC is a non-specific MCT1 inhibitor. We investigated the effect of CHC on MTX-induced EMT in A549 cells. It was observed that CHC had no effect on morphological changes induced by MTX in A549 cells (Fig. 37). In addition, known MTX-induced alterations of mRNA expression such as increase in α-SMA were not changed by cotreatment with CHC (Fig. 38). These findings suggest that the effect of MCT1 was less significant in MTX induced EMT.

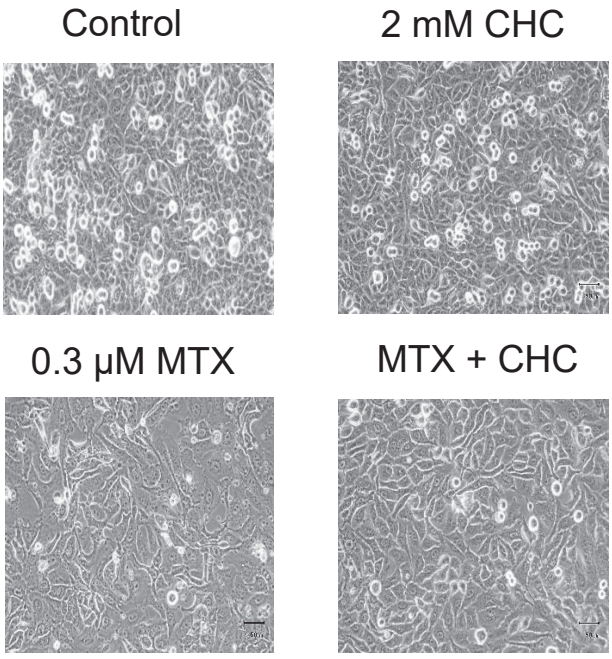


Figure 37. Effect of CHC on MTX-induced changes in morphology in A549 cells. The cells were treated with MTX (0.3 μM) in the absence or presence of CHC (2 mM) for 72 hr. After that, the morphology was observed by phase-contrast microscopy.

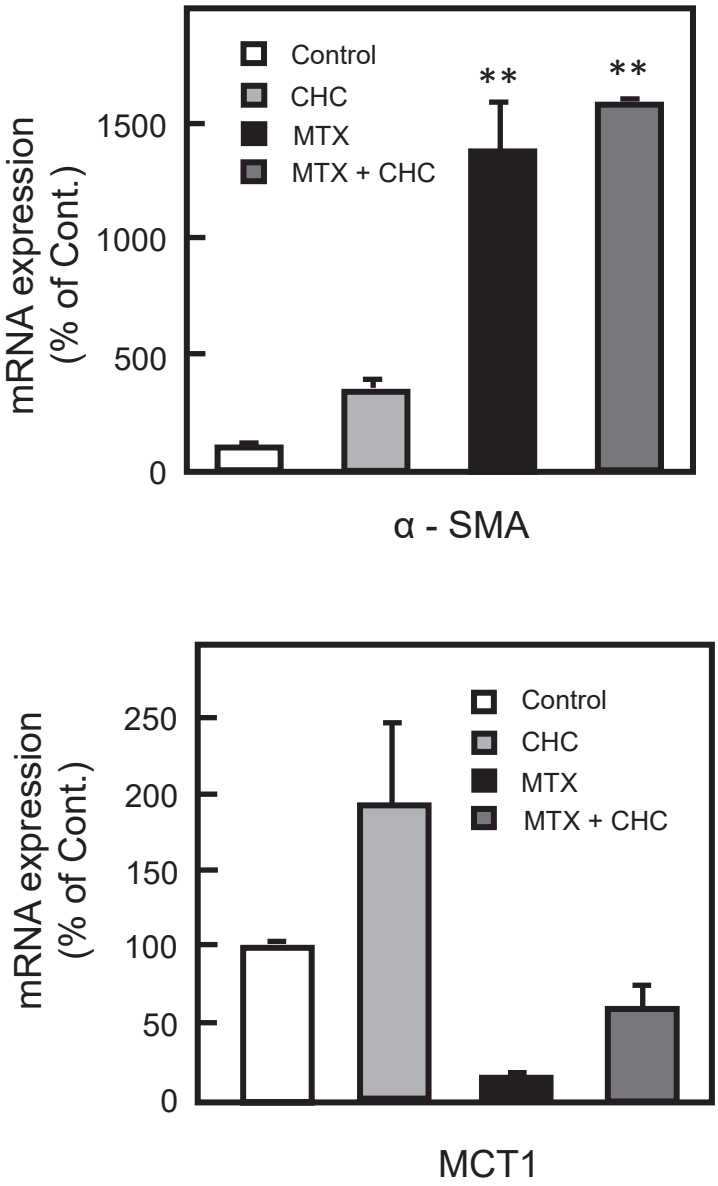


Figure 38. Effect of CHC on mRNA expression level of α-SMA and MCT1 in A549 cells. The cells were treated with MTX (0.3 μM) in the absence or presence of CHC (2 mM) for 72 hr. After that, mRNA expression level of α-SMA and MCT1 was analyzed by real-time PCR using total RNA extracted from the treated cells. Each value represents the mean ± S.E.M. of three monolayers. **p< 0.01, significantly different from control cells.

5.2.5. Effect of AR-C155858 on MTX induced EMT in A549 cells

AR-C155858 is a potent MCT1 inhibitor. We investigated the effect of AR on MTX-induced EMT in A549 cells. It was observed that AR had no effect on morphological changes induced by MTX in A549 cells (Fig. 39). In addition, known MTX-induced alterations of mRNA expression such as increase in α-SMA were not changed by cotreatment with AR (Fig. 40). These findings suggest that the effect of MCT1 was not clear in MTX induced EMT.

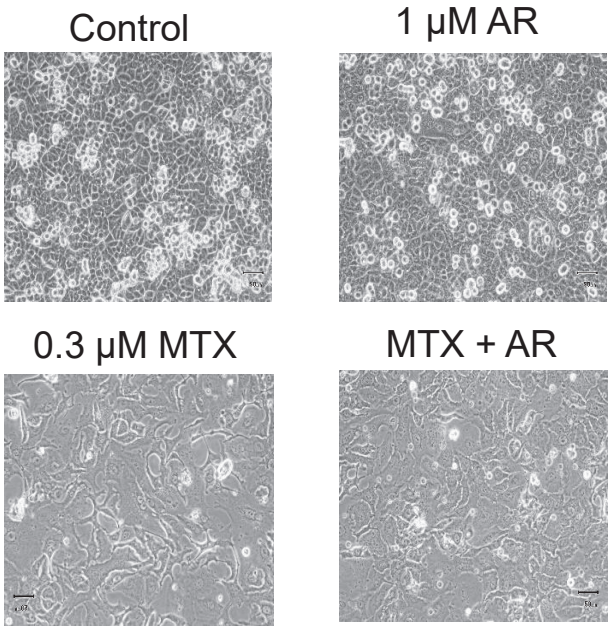


Figure 39. Effect of AR on MTX-induced changes in morphology in A549 cells. The cells were treated with MTX (0.3 μM) in the absence or presence of AR (1 μM) for 72 hr. After that, the morphology was observed by phase-contrast microscopy.

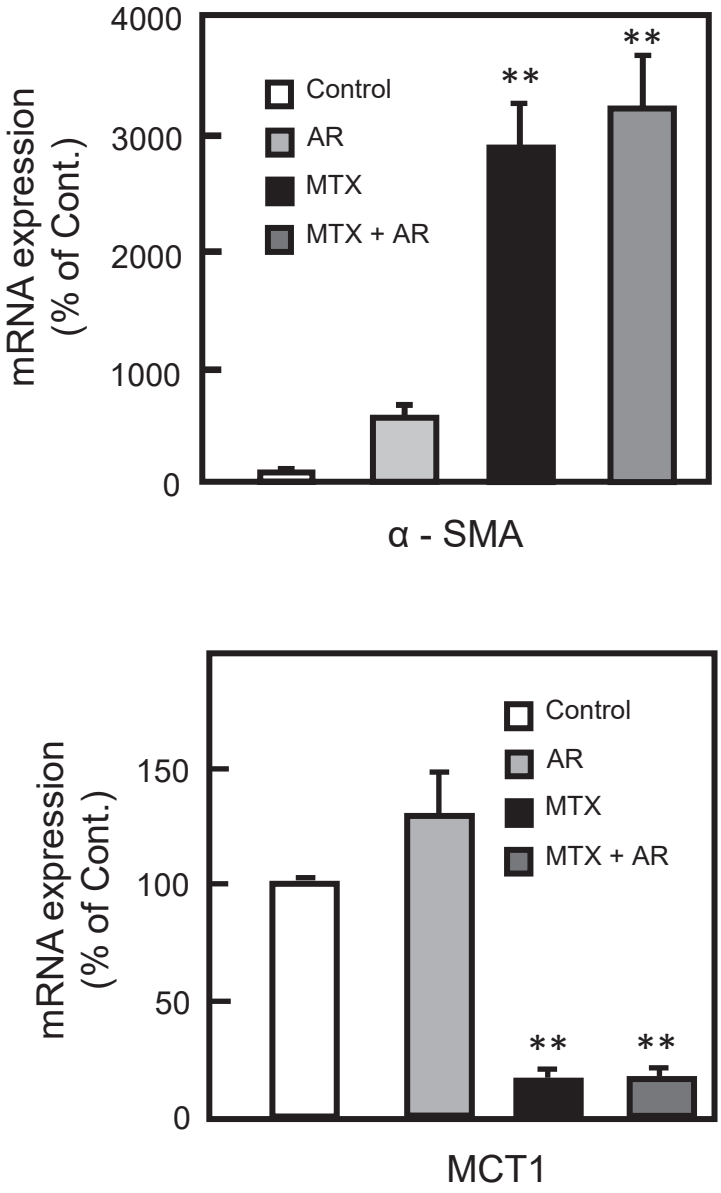


Figure 40. Effect of AR on mRNA expression level of α-SMA and MCT1 in A549 cells. The cells were treated with MTX (0.3 μM) in the absence or presence of AR (1 μM) for 72 hr. After that, mRNA expression level of α-SMA and MCT1 was analyzed by real-time PCR using total RNA extracted from the treated cells. Each value represents the mean ± S.E.M. of three monolayers. **p< 0.01, significantly different from control cells.

5.3. Discussion

Comparing the effect of TGF-β1 and MTX on mRNA expression of MCT1 in A549 cells, it was observed that TGF-β1 treatment significantly enhanced the expression of MCT1, while the expression of MCT1 was not affected by treatment with MTX (Fig. 35). Therefore, underlying mechanism of MTX-induced EMT may be different from TGF-β1-induced EMT. On the other hand, MTX treatment enhanced GHB uptake (Fig. 36). So, we need further studies on metabolic changes during MTX-induced EMT.

During TGF-β1-induced EMT, we observed that lactate dehydrogenase (LDH) mRNA expression was enhanced (Chapter 6). So, we may predict that lactate production was enhanced during TGF-β1-induced EMT, followed by enhancement of MCT1 function. To elucidate the metabolic changes during MTX induced EMT, LDH mRNA expression and lactate production assay should be also investigated.

Chapter 6
Metabolic changes during TGF-β1-induced EMT in A549 cells

6.1. Introduction

In general, cells produce energy in the form of ATP through cellular respiration. Initially, glucose enters the cytoplasm via GLUT transporter. Then glucose is converted to pyruvate by glycolysis. In aerobic condition, pyruvate is carried to mitochondria and converted to acetyl coenzyme A, Then ATP is generated through TCA cycle and Oxidative phosphorylation (OXPHOS) (Fig. 41). Under anaerobic condition, pyruvate is broken down to lactate in the cytosol (Fig. 42). Augmented glycolysis takes place not only in the absence of oxygen, but also under aerobic conditions, a phenomenon called “ Warburg effect” (DeBerardinis et al. 2008; Koppenol et al. 2011)

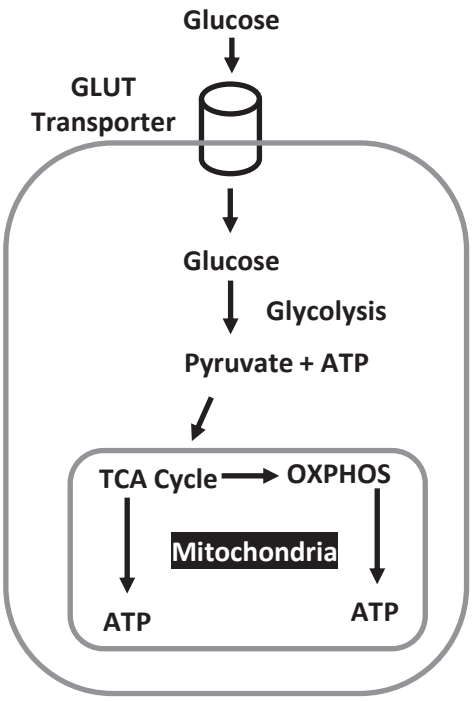


Figure 41. Cellular respiration
In presence of O_2

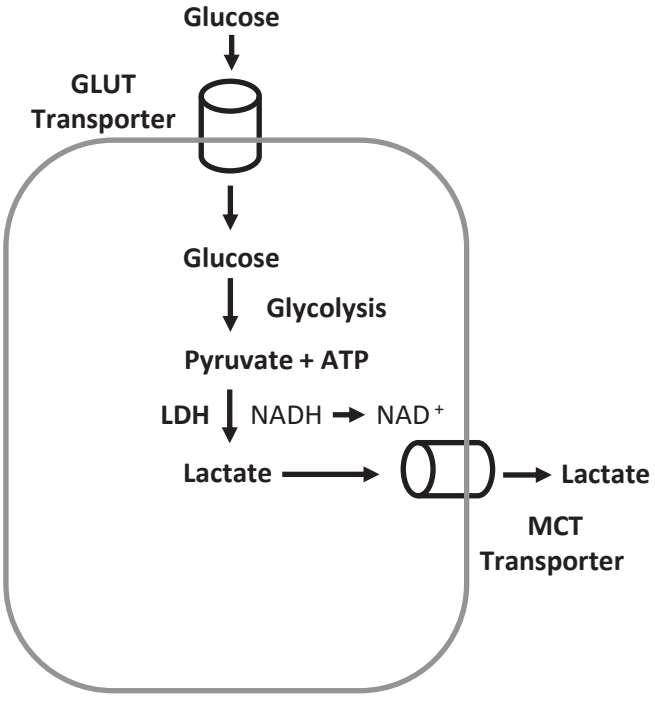


Figure 42. Cellular respiration
In absence of O_2

Lactate produced at sites with high rates of glycolysis can be shuttled to adjacent sites and may be taken up by adjacent cells having oxidative phenotype (Fig. 43). Shuttled lactate can be oxidized to pyruvate providing additional energy through the TCA cycle and driving OXPHOS.

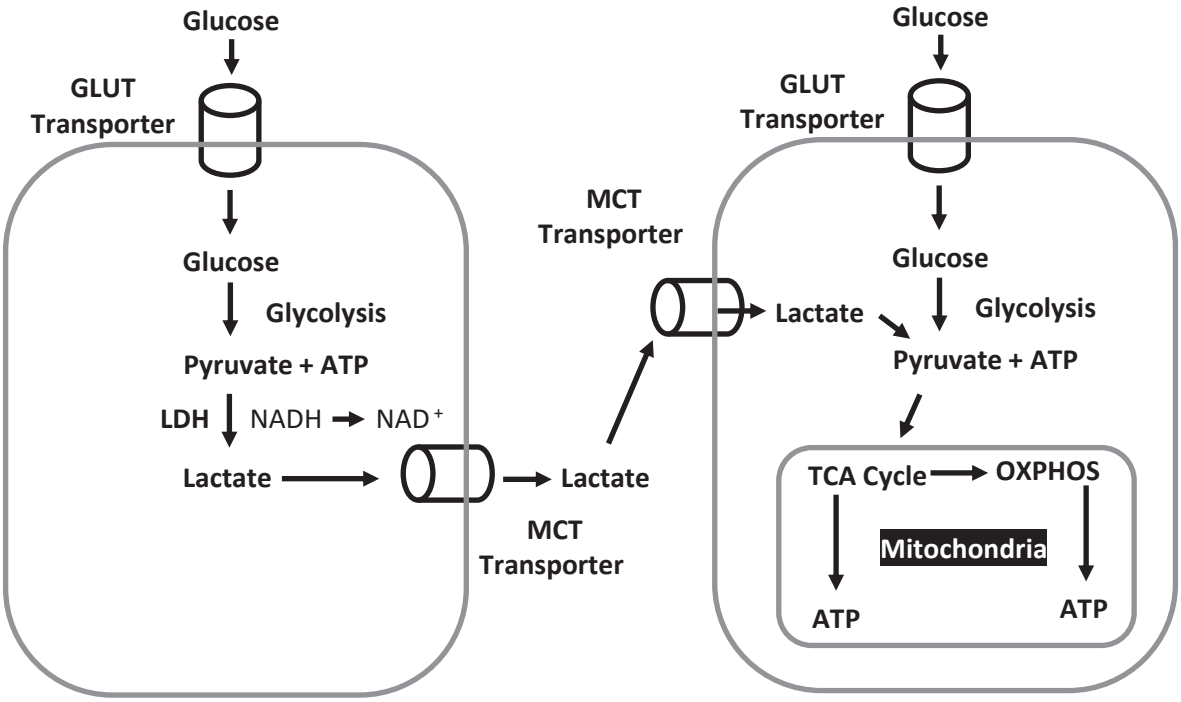


Figure 43. Lactate shuttle

It is assumed that TGF-β1 can mediate metabolic reprogramming. However, the mechanism is not fully understood. It is also unknown how this modulation of metabolic pathway affects the progression of EMT. So, to understand the relationship, we focused on two more factors in addition to MCT1, which are related to metabolism.

- Expression of lactate dehydrogenase (LDH)
- Production of ATP

6.2. Result

6.2.1. Effect of TGF-β1 on mRNA expression of LDH in A549 cells

LDH converts lactate to pyruvate and back. We investigated effect of TGF-β1 on the expression of LDHA and LDHB. TGF-β1 treatment significantly enhanced the expression of LDHA, which was suppressed by SB (Fig. 44).

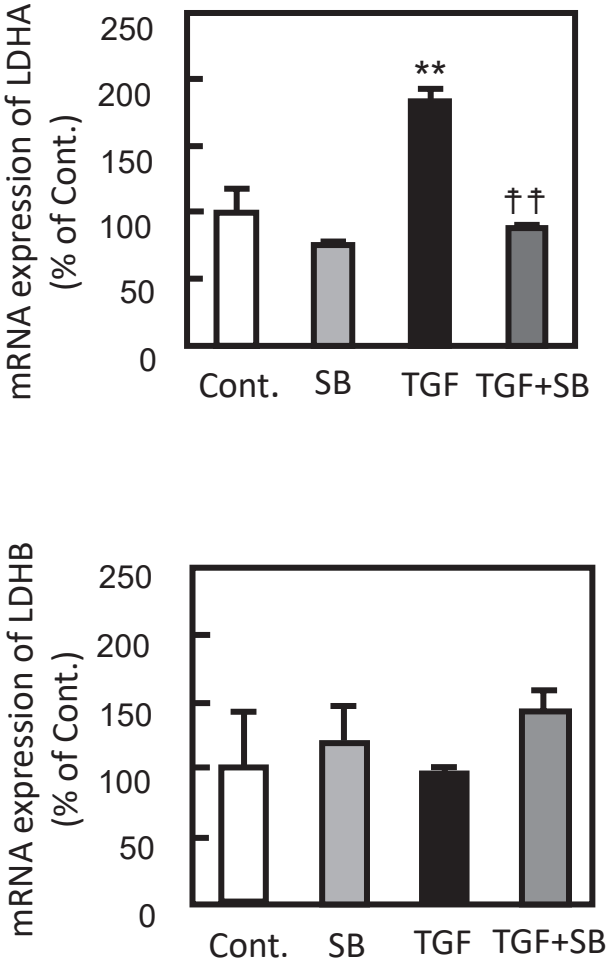


Figure 44: Role of TGF-β1 on the mRNA expression levels of LDHA and LDHB in A549 cells. The cells were treated with TGF-β1 (10 ng/mL) for 72 hr in the absence or presence of SB (10 μM). mRNA expression levels were evaluated by real-time PCR using total RNA. Each value represents the mean ± S.E.M. of three monolayers. **p< 0.01, significantly different from control cells. ††p< 0.01, significantly different from TGF-β1 treated cells.

6.2.2. Effect of TGF-β1 on production of ATP in A549 cells

We investigated effect of TGF-β1 on total production of ATP. TGF-β1 treatment significantly enhanced the expression of LDHA, which was suppressed by SB (Fig. 45).

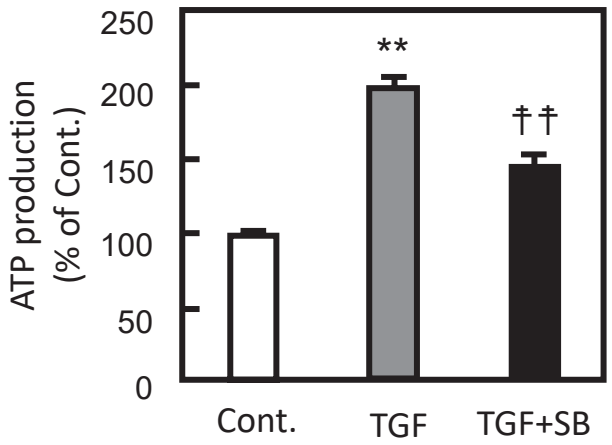


Figure 45: Effect of TGF-β1 on ATP production rate in A549 cells. The cells were treated with TGF-β1 (10 ng/mL) for 72 hr in the absence or presence of SB (10 μM). ATP production rate was examined by using ATP detection kit. Each value represents the mean ± S.E.M. of three monolayers. **p< 0.01, significantly different from control cells. ††p< 0.01, significantly different from TGF-β1 treated cells.

6.3. Discussion

We observed that during TGF-β1-induced EMT, the expression of LDHA mRNA was significantly enhanced. It is reported that LDHA has higher affinity for pyruvate and convert it to lactate and NADH to NAD^+, whereas LDHB has higher affinity for lactate and convert it to pyruvate. NAD^+ is usually regenerated through OXPHOS, but when cell depends mostly on glycolysis as a source of energy, NAD^+ is regenerated from NADH by LDHA in order to maintain glycolysis, generating lactate as a by-product (Valvona et al. 2016). So, we can assume that during TGF-β1 induced EMT in A549 cells, lactate production is enhanced. To facilitate transport of lactate, MCT1 function was enhanced.

Some reports state that Warburg-like metabolic reprogramming is observed in IPF cells, resulting in increased glucose uptake and an accumulation of TCA cycle metabolites and byproducts that act as signaling mechanisms. Kottmann and colleagues (2012) demonstrated that glycolytic flux increases lactate production and lowers the local tissue pH resulting in increased activation of TGF-β and increased transcription of LDH, which synergizes with TGF-β to induce differentiation of fibroblasts to myofibroblasts. LDH produces lactate, which can be exported by MCT-4 and may be taken up by adjacent cells expressing MCT-1. Shuttled lactate can be oxidized to pyruvate providing additional energy through the TCA cycle and driving OXPHOS. This phenomenon is termed the reverse Warburg effect and promotes fibroblast proliferation (Zank et al. 2018)

Fibroblasts also have been shown to undergo a metabolic shift away from the highly efficient method of ATP production, OXPHOS to the less efficient method of glycolysis despite adequate oxygen to continue OXPHOS. Primary myofibroblasts derived from IPF lungs and lung fibroblasts treated with TGF-β also demonstrate increased lactate contents (Xie et al. 2015).

On the other hand, we observed that TGF-β1 treatment enhanced ATP production. So, one of the reasons of this metabolic reprogramming is to generate more energy. This energy may be used for migration and invasion of cell during EMT. However, further studies are needed to confirm the metabolic changes during TGF-β1-induced EMT and to determine how these alterations might modify disease progression.

7. Discussion

At present, IPF represents one of the most lethal lung diseases of unknown origin. A profound understanding of IPF pathogenesis might offer novel therapeutic strategies for this disease. Currently, EMT is considered to play a significant role in the development of pulmonary fibrosis. Besides, some reports demonstrated a correlation between MCT1 and EMT (Liu et al. 2016; Morandi et al. 2017). In this study, therefore, we have focused on the relationship between MCT1 function and TGF-β1-induced EMT in A549 cells.

The human epithelial lung adenocarcinoma cell line A549 is a useful in vitro model for studying drug transport and metabolic processes in alveolar type II epithelial (ATII) cells (Lieber et al. 1976). ATII cells have a very high metabolic rate, and consequently they mostly depend on mitochondria for energy production (Lottes et al. 2014). It is reported that these cells import lactate through MCT1 and use it as a substrate for mitochondrial energy production (Lottes et al. 2015). Our findings concerning activity of MCT1 in A549 cells are similar with the concept of mitochondrial metabolism as alternative source of energy in ATII cells. Kottmann et al. (2012) demonstrated that the concentration of lactate was remarkably increased in lung tissue of IPF patients. Adjacent cells which express MCT1 can import this lactate and use it to produce ATP via oxidative phosphorylation.

To date, the regulation of MCT1 expression remains poorly understood. In our study, we observed that TGF-β1 induced GHB and LA uptake as well as upregulate mRNA and protein expression levels of MCT1 in A549 cells and these effects were canceled by cotreatment with SB. Therefore, TGF-β signaling pathway may be involved in the regulation of the functional expression of MCT1. But A549 is adenocarcinoma cell. So, we further tried to clarify the regulation of MCT1 by TGF-β1 in other normal cell lines. Actually, primary cultured cells from rat cannot be used for EMT studies due to its trans differentiation (Ikehata et al. 2008). Therefore, we selected RLE/Abca3 cells which is derived from rat normal alveolar epithelium. Our previous reports demonstrated that the RLE/Abca3 cell line is a useful model to evaluate EMT (Takano et al. 2015; Kawami et al. 2017). But, unfortunately, MCT1 activity was much lower in RLE/Abca3 cells than in A549 cells (data not shown), and it was difficult to evaluate the effect of TGF-β1 on the functional expression of MCT1 using this cell line. Further investigations using other alveolar epithelial cell lines with significant MCT1 activity may provide a better understanding of the relationship between EMT and functional expression of MCT1.

It has been reported that Warburg-like metabolic reprogramming in alveolar epithelial cells derived from IPF patients would result in increased glucose uptake mediated by GLUT1 (Zank et al. 2018). We examined the effect of TGF-β1 treatment on mRNA expression and function of GLUT1 in A549 cells.

TGF-β1 treatment significantly enhanced mRNA expression of GLUT1 at different time periods which supports the report of Warburg-like metabolic reprogramming in alveolar epithelial cells derived from IPF patients. On the other hand, D-glucose uptake was enhanced at 6, 9, and 12 hr, but not at 24 and 72 hr after treatment. In our study, we observed that GHB uptake was not affected by TGF-β1 at 24 hr. These findings suggest that, in an early phase, TGF-β1 may stimulate glucose uptake, while in a later phase, MCT1 function is upregulated for oxidative metabolism in A549 cells. However, further studies are needed to clarify the mechanisms underlying such a metabolic shift during TGF-β1-induced EMT.

In addition to organ fibrosis, EMT also contributes to cancer progression, and metabolic reprogramming in cancer cells is well characterized. Tumors frequently display a glycolytic phenotype with increased uptake of glucose for glycolysis and concomitant synthesis of lactate (Morais-Santos et al. 2015). The lactate produced by hypoxic tumor cells may diffuse and be taken up by oxygenated tumor cells (Feron 2009). MCT1 regulates the entry of lactate into oxidative tumor cells (Fiaschi et al. 2012). Therefore, higher MCT1 expression is assumed to be a prognostic indicator in human neuroblastoma and melanoma cell lines (Fang et al. 2006; Zhang et al. 2018). Moreover, Choi et al. (2014) reported that high MCT1 expression levels are correlated with high tumor grade, advanced tumor stage, and lymphatic tumor invasion.

Based on these studies, we examined the effect of CHC and AR on TGF-β1- induced EMT in A549 cells. Although CHC and AR clearly inhibited GHB and LA uptakes in A549 cells, these inhibitors did not affect TGF-β1-induced EMT-like morphological changes. Moreover, TGF-β1- induced changes in mRNA expression related to EMT are not affected by these inhibitors indicating that MCT1 transport function may not directly contribute to TGF-β1-induced EMT in A549 cells. Gray et al. (2016) demonstrated that knockdown of MCT1 (but not AZD3965, a promising MCT1 inhibitor) suppressed hepatocyte growth factor- and epidermal growth factor-induced tumor cell scattering and wound healing. Therefore, MCT1 and GLUT1 may have different roles independent of transporter activity in TGF-β1-induced EMT in A549 cells.

On the other hand, When the inhibitory effect of CHC and AR on the function of MCT1 was observed, it was examined for short time (1-5 minute). But when we examined the effect of these inhibitors on TGF-β1 induced EMT, we checked it for 72 hr. We are not sure whether these inhibitors are effective for long time. Besides some reports demonstrate that TGF-β1 is produced as a latent complex, and activation of TGF-β1 generally requires the binding of αv integrin to an RGD motif in the latent form of TGF-β1 (Shi et al. 2011). Alterations in pH resulting from endogenous production and release of Lactate are reportedly capable of activating latent TGF-β1 (Kottmann et al. 2012; Tuder et al. 2012).

In this context, Lactate concentration, which is regulated by MCT1, may be a key component during TGF-β1- induced EMT under in vivo conditions. Therefore, the relationship between MCT1 expression level, transporter activity of MCT1, and induction of EMT by TGF-β1 needs to be studied further.

8. Conclusion

We characterized MCT1 function in A549 cells and found that functional expression of MCT1 was upregulated by TGF-β1 treatment via the TGF-β1 signaling pathway, as evidenced by an inhibitory effect of SB. These findings may help in understanding the association of metabolic reprogramming with TGF-β1-induced EMT in alveolar epithelial cells.

9. Materials and Methods

9.1. Materials

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and Nutrient Mixture F-12 (Ham) were purchased from MP Biomedicals (Solon, OH, USA), Trypsin-ethylenediaminetetraacetic acid (EDTA) and penicillin-streptomycin were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA), TGF-β1 was purchased from BioLegend, Inc. (San Diego, CA, USA), SB431542 (SB) and Methotrexate (MTX) were purchased from Wako Pure Chemicals Ltd. (Osaka, Japan), [^{3}H] GHB and [^{3}H] lactate were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA), [^{3}H]D-Glucose and [^{3}H]3-o-methyl-D-Glucose were purchased from Moravek Biochemicals and radiochemicals (California, USA). All the other chemicals used for the experiments were of the highest purity that was commercially available.

9.2. Cell culture

A549 cells: A549 cell line was obtained from RIKEN BioResource Research Center (Tokyo, Japan) were cultured in DMEM containing 100-IU/mL penicillin, 100-µg/mL streptomycin, and 10% FBS in 5% CO_2 at 37^{o}C and were sub-cultured every 7 days (after cell treatment with 1 mM EDTA and 0.25% trypsin) as described previously (Kawami et al. 2016). The medium was replaced every 2 or 3 days.

RLE/Abca3: RLE/Abca3 cell line was established by transfecting RLE-6TN cells with rat Abca3 gene using a retroviral vector. Cells were cultured in DMEM/ F12 (1:1) as previously reported (Kawami et al. 2017).

9.3. Uptake studies

9.3.1. Uptake studies of GHB or Lactic acid

A549 cells grown on a 24-well plate for 6 days were used for uptake studies. After removing the culture medium, the cells were washed twice with HEPES-buffered saline (HBS; 5 mM HEPES, 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 mM glucose, pH 7.4) and preincubated with HBS at 37°C for 10 min. Then, the cells were incubated with HBS or MES-buffered saline (MBS; 5 mM MES, 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 mM glucose, pH 6) containing 100 nM GHB at 37°C or 4°C for 0.25–5 min. For inhibition studies, the cells were incubated with [^{3}H] GHB or [^{3}H] Lactate at 37°C for 1 min in the absence or presence of various concentrations of CHC (0.5, 1, 2 mM), phloretin (10, 20, 100 μM), non-labeled Lactate (1, 5, 10 mM), and AR-C155858 (AR) (1, 3, 10, 30, 60, 100, 1000 nM) in HBS or MBS. After the incubation, the uptake buffer was aspirated, and the cells were rinsed with ice-cold phosphate buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4). The cells were scraped and solubilized with NaOH for 30 min at approximately 22°C and then centrifuged at 9838 × g for 5 min.

The supernatant was then used for either radioactivity counting or protein assay. For the measurement of [^{3}H] GHB or [^{3}H] Lactate uptake, 3 mL of Ultima GOLD (PerkinElmer, MA, USA) was added, and the radioactivity was measured by liquid scintillation counting on a LSC5100 (Hitachi Aloka Medical Ltd., Tokyo, Japan). Protein concentration was measured by the Bradford method using bovine serum albumin as a standard. Calculation of the Ki value of CHC to MCT1 using a Dixon plot was achieved by linear regression analysis of reciprocal saturable uptake (1/v) for different GHB or Lactate concentrations (25 or 50 nM) as a function of CHC inhibition concentrations. The MCT mediated uptake of GHB was calculated by subtracting [^{3}H] GHB uptake with CHC (2 mM) from that without CHC.

9.3.2. Uptake studies of D-Glucose or 3-o-methyl-D-Glucose

A549 cells grown on a 24-well plate for 6 days and uptake studies were performed on the confluent cells attached to the plate. After removing the culture medium, the cells were washed twice with HBS and preincubated with HBS at 37^{o}C for 10 min. Then, the cells were incubated with HBS containing 1-mM [^{3}H]D-Glucose at 37 or 4^{o}C for 0.25–1 min. For inhibition studies, the cells were incubated with [^{3}H]D-Glucose or [^{3}H]3-o-methyl-D-Glucose at 37^{o}C for 15 s in the absence or presence of various concentrations of phloretin (50, 100, 500 µM), phloridzin (50, 100, 500 µM) in HBS. After incubation, the uptake of [^{3}H]D-glucose and [^{3}H] 3-o-methyl-D-glucose was measured as described in 9.3.1.

Real-time PCR

The total RNA was extracted from the cells and reverse transcribed into cDNA using ReverTra Ace (Toyobo, Osaka, Japan). Real-time PCR was performed on a Bio-Rad CFX96 (Bio Rad, Hercules, CA, USA) using KAPA SYBR® FAST qPCR Kit, as described previously (Kawami et al. 2018).

The PCR conditions were as follows:

initial denaturation for 1 cycle of 30 s at 95°C,

followed by specified number of cycles of 5 s at 95°C (denaturation),

20 s at 60°C (annealing)

15 s at 72°C (extension).

The primer sequences were as follows:

MCT1

5′-TGGCTGTCATGTATGGTGGA-3′ (sense) and

5′-AAGTTGAAGGCAAGCCCAAG-3′ (antisense).

GLUT1

5′-TGGCTACAACACTGGAGTCATC-3′ (sense) and

5′-TAACGAAAAGGCCCACAGAG-3′ (antisense).

CK19

5′-TACAGCCACTACTACACGACCATCC-3′ (sense) and

5′-GGACAATCCTGGAGTTCTCAATG-3′ (antisense).

α-SMA

5′-TCAGAGAGAGGAAGCCGAAA-3′ (sense) and

5′-TTTGCTCTGTGCTTCGTCAC-3′ (antisense).

LDHA

5′-TGAAGGACTTGGCAGATGAA-3′ (sense) and

5′-AATGACCAGCTTGGAGTTTG-3′ (antisense).

LDHB

5′-AAGTTGGTATGGCGTGTGCT-3′ (sense) and

5′-ATGTTCCCCCAAAATCCATC-3′ (antisense).

GAPDH

5′-ACGGGAAGCTTGTCATCAAT-3′ (sense) and

5′-TGGACTCCACGACGTACTCA-3′ (antisense).

The mRNA expression level of MCT1 was normalized to that of GAPDH, a housekeeping gene.

Western blotting

MCT1 protein in A549 cells was analyzed using the crude membrane fraction of the treated cells as described previously (Kawami et al. 2018b). The expression of MCT1 was examined using rabbit polyclonal anti-MCT1 antibodies (PA5– 12335; 1:250 dilution) and HRP-linked donkey anti-rabbit IgG secondary antibodies (NA934; 1:5000 dilution).

In these studies, GAPDH was used as a loading control. GAPDH was detected with rabbit polyclonal anti-GAPDH antibodies (G9545; 1:5000 dilution) using a luminescent image analyzer, LAS 4000 plus (GE Healthcare Japan Corporation, Tokyo, Japan).

ATP measurement:

A549 cells were seeded on 96-well plate, and treated with TGF-β1 in the absence or presence of SB for 72 h. After that, the supernatants were removed, and washed by PBS 2 times. Then, ATP measuring reagent included in ATP measurement Kit (FUJIFILM Wako Chmicals, Tokyo) was added on each well, and left it for 20 min. The chemiluminescence intensity during the reaction between ATP and the reagent was detected by EnSpireTM (PerkinElmer Japan Co., Ltd., Kanagawa, Japan). Using the standard for ATP, the ATP amounts were calculated, and compared between the experimental conditions.

Statistical analysis:

Data were expressed as means ± S.E.M. Statistical analysis was performed by Student's *t* test or one-way ANOVA followed by Tukey's test for multiple comparisons. The level of significance was set at $*p < 0.05$ or $**p < 0.01$.

10. Publication list

- Uddin M, Kawami M, Yumoto R, Takano M (2020) Effect of transforming growth factor-β1 on functional expression of monocarboxylate transporter 1 in alveolar epithelial A549 cells. Naunyn Schmiedebergs Arch Pharmacol 393:889-896. https://doi:10.1007/s00210-019-01802-3

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12. References

- Abid SH, Malhotra V, Perry MC (2001) Radiation-induced and chemotherapy-induced pulmonary injury. Curr Opin Oncol 13:242-8. https://doi.org/10.1097/00001622-200107000-00006

- Batlle E, Sancho E, Francí C, et al. (2000) The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol 2:84-89. https://doi:10.1038/35000034

- Benedek TG (2010) Methotrexate: from its introduction to non-oncologic therapeutics to anti-TNF-α. Clin Exp Rheumatol 28 (5 Suppl 61):S3-S8.

- Bergersen LH (2007) Is lactate food for neurons? Comparison of monocarboxylate transporter subtypes in brain and muscle. Neuroscience 145:11-19. https://doi:10.1016/j.neuroscience.2006.11.062

- Bleyer WA (1978) The clinical pharmacology of methotrexate: new applications of an old drug. Cancer 41:36-51. https://doi:10.1002/1097-0142(197801)41:1<36::aid-cncr2820410108>3.0.co;2-i

- Bröer S, Bröer A, Schneider HP, Stegen C, Halestrap AP, Deitmer JW (1999) Characterization of the high-affinity monocarboxylate transporter MCT2 in Xenopus laevis oocytes. Biochem J 341:529–535. https://doi.org/10.1042/0264-6021:3410529

- Brooks GA. Cell-cell and intracellular lactate shuttles (2009) J Physiol 587:5591-5600. https://doi:10.1113/jphysiol.2009.178350

- Cano A, Pérez-Moreno MA, Rodrigo I, et al. (2000) The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol 2(2):76-83. https://doi:10.1038/35000025

- Choi JW, Kim Y, Lee JH, Kim YS (2014) Prognostic significance of lactate/proton symporters MCT1, MCT4, and their chaperone CD147 expressions in urothelial carcinoma of the bladder. Urology 84:245.e9-15. https://doi.org/10.1016/j.urology.2014.03.031

- DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB (2008) The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab 7:11–20. https://doi.org/10.1016/j.cmet.2007.10.002

- Deuticke B (1982) Monocarboxylate transport in erythrocytes. J Membr Biol 70:89-103. https://doi:10.1007/BF01870219

- Doherty JR, Yang C, Scott KE, et al. (2014) Blocking lactate export by inhibiting the Myc target MCT1 Disables glycolysis and glutathione synthesis. Cancer Res 74:908–920. https://doi.org/10.1158/00085472.CAN-13-2034

- Eilertsen M, Andersen S, Al-Saad S et al. (2014) Monocarboxylate transporters 1-4 in NSCLC: MCT1 is an independent prognostic marker for survival. PLoS One 9:e105038. https://doi.org/10.1371/journal.pone.0105038

- Fang J, Quinones QJ, Holman TL, et al. (2006) The H+-linked monocarboxylate transporter (MCT1/SLC16A1): a potential therapeutic target for high-risk neuroblastoma. Mol Pharmacol 70:2108–2115. https://doi.org/10.1124/mol.106.026245

- Feron O (2009) Pyruvate into lactate and back: from the Warburg effect to symbiotic energy fuel exchange in cancer cells. Radiother Oncol 92:329–333. https://doi.org/10.1016/j.radonc.2009.06.025

- Fiaschi T, Marini A, Giannoni E, et al. (2012) Reciprocal metabolic reprogramming through lactate shuttle coordinately influences tumor-stroma interplay. Cancer Res 72:5130–5140. https://doi.org/10.1158/0008-5472.CAN-12-1949

- Fishbein WN, Merezhinskaya N, Foellmer JW (2002) Relative distribution of three major lactate transporters in frozen human tissues and their localization in unfixed skeletal muscle. Muscle Nerve 26:101–112. https://doi.org/10.1002/mus.10168

- Gan L, Xiu R, Ren P, et al. (2016) Metabolic targeting of oncogene MYC by selective activation of the proton-coupled monocarboxylate family of transporters. Oncogene 35(23):3037-3048. https://doi:10.1038/onc.2015.360

- Garcia CK, Goldstein JL, Pathak RK, Anderson RG, Brown MS (1994) Molecular characterization of a membrane transporter for lactate, pyruvate, and other monocarboxylates: implications for the Cori cycle. Cell 76:865–873. https://doi.org/10.1016/0092-8674(94)90361-1

- Gray AL, Coleman DT, Shi R, Cardelli JA (2016) Monocarboxylate transporter 1 contributes to growth factor-induced tumor cell migration independent of transporter activity. Oncotarget 7:32695–32706. https://doi.org/10.18632/oncotarget.9016

- Halestrap AP, Denton RM (1974) Specific inhibition of pyruvate transport in rat liver mitochondria and human erythrocytes by acyano-4-hydroxycinnamate. Biochem J 138:313–316. https://doi.org/10.1042/bj1380313

- Halestrap AP, Meredith D (2004) The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. Pflugers Arch 447:619–628. https://doi.org/10.1007/s00424-003-1067-2

- Halestrap AP, Price NT (1999) The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. Biochem J 343:281-299.

- Halestrap AP, Wilson MC (2012) The monocarboxylate transporter family–role and regulation. IUBMB Life 64:109–119. https://doi.org/10.1002/iub.572

- Ikehata M, Yumoto R, Nakamura K, Nagai J, Takano M (2008) Comparison of albumin uptake in rat alveolar type II and type I-like epithelial cells in primary culture. Pharm Res 25:913-922. https://doi:10.1007/s11095-007-9426-x

- Kalluri R, Neilson EG (2003) Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest 112:1776–1784. https://doi.org/10.1172/JCI20530

- Kalluri R, Weinberg RA (2009) The basics of epithelial-mesenchymal transition. J Clin Invest 119:1420–1428. https://doi.org/10.1172/JCI39104

- Kang H, Kim H, Lee S et al. (2019) Role of metabolic reprogramming in epithelial–mesenchymal transition (EMT). Int J Mol Sci 20:2042. https://doi.org/10.3390/ijms20082042

- Kawami M, Deguchi J, Yumoto R, Sakakibara N, Tsukamoto I, Konishi R, Takano M (2017) Effect of COA-Cl on transforming growth factor-β1-induced epithelial-mesenchymal transition in RLE/Abca3 cells. Drug Metab Pharmacokinet 32:224–227. https://doi.org/10.1016/j.dmpk.2017.05.001

- Kawami M, Harabayashi R, Harada R, Yamagami Y, Yumoto R, Takano M(2018) Folic acid prevents methotrexate-induced epithelial mesenchymal transition via suppression of secreted factors from the human alveolar epithelial cell line A549. Biochem Biophys Res Commun 497:457–463. https://doi.org/10.1016/j.bbrc.2018.02.111

- Kawami M, Harabayashi R, MiyamotoM, Harada R, Yumoto R, Takano M (2016) Methotrexate-induced epithelial–mesenchymal transition in the alveolar epithelial cell line A549. Lung 194:923–930. https://doi.org/10.1007/s00408-016-9935-7

- Kawami M, Yamada Y, Issarachot O, Junyaprasert VB, Yumoto R, Takano M (2018) P-gp modulating effect of Azadirachta indica extract in multidrug-resistant cancer cell lines. Pharmazie 73:104–109. https://doi.org/10.1691/ph.2018.7116

- Kim KK, Kugler MC, Wolters PJ et al. (2006) Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. Proc Natl Acad Sci USA 103:13180–13185. https://doi.org/10.1073/pnas.0605669103

- Koppenol WH, Bounds PL, Dang CV (2011) Otto Warburg's contributions to current concepts of cancer metabolism. Nat Rev Cancer 11:325–337. https://doi.org/10.1038/nrc3038

- Kottmann RM, Hogan CM, Phipps RP, Sime PJ (2009) Determinants of initiation and progression of idiopathic pulmonary fibrosis. Respirology 14:917-933. https://doi:10.1111/j.1440-1843.2009.01624.x

- Kottmann RM, Kulkarni AA, Smolnycki KA, et al. (2012) Lactic acid is elevated in idiopathic pulmonary fibrosis and induces myofibroblast differentiation via pH-dependent activation of transforming growth factor-β. Am J Respir Crit Care Med 186:740–751. https://doi.org/10.1164/rccm.201201-0084OC

- Lieber M, Smith B, Szakal A et al (1976) A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int J Cancer 17:62–70. https://doi.org/10.1002/ijc.2910170110

- Liu M, Quek LE, Sultani G, Turner N (2016) Epithelial-mesenchymal transition induction is associated with augmented glucose uptake and lactate production in pancreatic ductal adenocarcinoma. Cancer Metab 4:19. https://doi.org/10.1186/s40170-016-0160-x

- Lottes RG, Newton DA, Spyropoulos DD, Baatz JE (2014) Alveolar type II cells maintain bioenergetic homeostasis in hypoxia through metabolic and molecular adaptation. Am J Physiol Lung Cell Mol Physiol 306:L947–L955. https://doi.org/10.1152/ajplung.00298.2013

- Lottes RG, Newton DA, Spyropoulos DD, Baatz JE (2015) Lactate as substrate for mitochondrial respiration in alveolar epithelial type II cells. Am J Physiol Lung CellMol Physiol 308:L953–L961. https://doi.org/10.1152/ajplung.00335.2014

- Martinez FJ, Safrin S, Weycker D, et al. (2005) The clinical course of patients with idiopathic pulmonary fibrosis. Ann Intern Med 142:963-967. https://doi.org/10.7326/0003-4819-142-12_part_1-200506210-00005

- Morais-Santos F, Granja S, Miranda-Gonçalves V, et al. (2015) Targeting lactate transport suppresses in vivo breast tumour growth. Oncotarget 6:19177–19189. https://doi.org/10.18632/oncotarget.3910

- Morandi A, Taddei ML, Chiarugi P, Giannoni E (2017) Targeting the metabolic reprogramming that controls epithelial-to-mesenchymal transition in aggressive tumors. Front Oncol 7:40. https://doi.org/10.3389/fonc.2017.00040

- Morris ME, Felmlee MA (2008) Overview of the proton-coupled MCT (SLC16A) family of transporters: characterization, function and role in the transport of the drug of abuse gamma-hydroxybutyric acid. AAPS J 10:311-321. https://doi:10.1208/s12248-008-9035-6

- Nieto MA, Huang RY, Jackson RA, Thiery JP (2016) EMT:2016. Cell 166:21-45. https://doi:10.1016/j.cell.2016.06.028

- Ohbayashi M, Kubota S, Kawase A, Kohyama N, Kobayashi Y, Yamamoto T (2014) Involvement of epithelial-mesenchymal transition in methotrexate-induced pulmonary fibrosis. J Toxicol Sci 39:319-330. https://doi.org/10.2131/jts.39.319

- Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA (2008) Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer Res. 68:3645-3654. https://doi:10.1158/0008-5472.CAN-07-2938

- Ovens MJ, Davies AJ, Wilson MC, Murray CM, Halestrap AP (2010) AR-C155858 is a potent inhibitor of monocarboxylate transporters MCT1 and MCT2 that binds to an intracellular site involving transmembrane helices 7-10. Biochem J 425:523-530. https://doi:10.1042/BJ20091515

- Peinado H, Olmeda D, Cano A (2007) Snail Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer 7:415-428. https://doi:10.1038/nrc2131

- Poole RC, Halestrap AP (1993) Transport of lactate and other monocarboxylates across mammalian plasma membranes. Am J Physiol 264:C761– C782. https://doi.org/10.1152/ajpcell.1993.264.4.C761

- Poole RC, Halestrap AP (1994) N-Terminal protein sequence analysis of the rabbit erythrocyte lactate transporter suggests identity with the cloned monocarboxylate transport protein MCT1. Biochem J 303: 755–759. https://doi.org/10.1042/bj3030755

- Rajitha P, Biswas R, Sabitha M, Jayakumar R (2017) Methotrexate in the treatment of psoriasis and rheumatoid arthritis: mechanistic insights, current issues and novel delivery approaches. Curr Pharm Des 23:3550-3566. https://doi.org/10.2174/1381612823666170601105439

- Raghu G, Weycker D, Edelsberg J, Bradford WZ, Oster G (2006) Incidence and prevalence of idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 174:810-816. https://doi.org/10.1164/rccm.200602-163OC

- Shi M, Zhu J, Wang R, et al. (2011) Latent TGF-β structure and activation. Nature 474:343–349. https://doi.org/10.1038/nature10152

- Smith AP, Verrecchia A, Fagà G, et al (2009) A positive role for Myc in TGFbeta-induced Snail transcription and epithelial-to-mesenchymal transition. Oncogene 28(3):422-430. https://doi:10.1038/onc.2008.395

- Thorens B, Mueckler M (2010) Glucose transporters in the 21st Century. Am J Physiol Endocrinol Metab 298:E141-E145. https://doi.org/10.1152/ajpendo.00712.2009

- Takano M, Yamamoto C, Yamaguchi K et al. (2015) Analysis of TGF-β1-and drug-induced epithelial-mesenchymal transition in cultured alveolar epithelial cell line RLE/Abca3. Drug Metab Pharmacokint 30:111–118. https://doi.org/10.1016/j.dmpk.2014.10.007

- Thorens B (1996) Glucose transporters in the regulation of intestinal, renal, and liver glucose fluxes. Am J Physiol. 270:G541-G553. https://doi.org/10.1152/ajpgi.1996.270.4.G541

- Thorens B, Mueckler M (2010) Glucose transporters in the 21st Century. Am J Physiol Endocrinol Metab 298: E141-5. https://doi.org/10.1152/ajpendo.00712.2009

- Tuder RM, Lara AR, Thannickal VJ (2012) Lactate, a novel trigger of transforming growth factor-β activation in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 186:701–70. https://doi.org/10.1164/rccm.201208-1491ED

- Valvona CJ, Fillmore HL, Nunn PB, Pilkington GJ (2016) The Regulation and Function of Lactate Dehydrogenase A: Therapeutic Potential in Brain Tumor. Brain Pathol 26:3-17. https://doi:10.1111/bpa.12299

- Willis BC, Borok Z (2007) TGF-β-induced EMT: mechanisms and implications for fibrotic lung disease. Am J Physiol Lung Cell Mol Physiol 293:L535–L534. https://doi.org/10.1152/ajplung.00163

- Wolters PJ, Collard HR, Jones KD (2014) Pathogenesis of idiopathic pulmonary fibrosis. Annu Rev Pathol 9:157–179. https://doi.org/10.1146/annurev-pathol-012513-104706

- Wynn TA (2008) Cellular and molecular mechanisms of fibrosis. J Pathol 214:199-210. https://doi.org/10.1002/path.2277

- Xie N, Tan Z, Banerjee S, et al. (2015) Glycolytic Reprogramming in Myofibroblast Differentiation and Lung Fibrosis. Am J Respir Crit Care Med 192:1462–1474. https://doi.org/10.1164/rccm.201504-0780OC

- Yu K, Li Q, Shi G, Li N (2018) Involvement of epithelial-mesenchymal transition in liver fibrosis. Saudi J Gastroenterol 24:5-11. https://doi.org/10.4103/sjg.SJG_297_17

- Zank DC, Bueno M, Mora AL, Rojas M (2018) Idiopathic pulmonary fibrosis: aging, mitochondrial dysfunction, and cellular bioenergetics. Front Med 5:10. https://doi.org/10.3389/fmed.2018.00010

- Zhang G, Zhang Y, Dong D, et al. (2018) MCT1 regulates aggressive and metabolic phenotypes in bladder cancer. J Cancer 9:2492–2501. https://doi.org/10.7150/jca.25257