P38 Kinase, SGK1 and NFκB Dependent Up-Regulation of Na^+/Ca^{2+} Exchanger Expression and Activity Following TGFß1 Treatment of Megakaryocytes

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

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Tübingen

2019

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	08.09.2020
Stellvertretender Dekan:	Prof. Dr. József Fortágh
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ABBREVIATIONS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
CAEND	Camurati-Engelmann Disease
Da.	Dalton
ER	Endoplasmic Reticulum
fw	Forward
GAPDH	Glyceraldehyd-3-phosphate-Dehydrogenase
HSCs	Hematopoietic Stem Cells
IKK	IκB kinase complex
IκB-α	a member of a family of proteins that inhibit NF-κB
MK	Megakaryocyte
NCKX	K^+-dependent Na^+/Ca^{2+} exchanger
NCX	K^+-independent Na^+/Ca^{2+} exchanger
NFκB	Nuclear Factor κB
PCR	Polymerase Chain Reaction
PDK1	Pyruvate Dehydrogenase Kinase 1
PMCA	Plasma Membrane Ca^{+2} Transport ATPase
rev	Reverse
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
SGK1	Serum/Glucocorticoid Inducible kinase 1
SR	Sarcoplasmic Reticulum
STIM1	Stromal interaction molecule 1
TGFß1	Transforming Growth Factor ß1
TβRI	TGF-β type I receptor
TβRII	TGF-β type II receptor
UV	Ultraviolet

ABSTRACT

Transforming Growth Factor β1 (TGFβ1) plays an important role in the maturation of megakaryocyte and formation of platelets. TGFβ1 can up-regulate Ca^{2+} entry through store operated Ca^{2+} entry (SOCE) and on the contrary, it can up-regulate Ca^{2+} exclusion by upregulating the activity of Na^{+}/Ca^{2+} exchangers. TGFβ1 first enhances the increase of intracellular Ca^{2+} triggered by the release of Ca^{2+} from intracellular stores, then it enhances the subsequent decline of $[Ca^{2+}]i$.

The mechanism of action, by which TGFβ1 up-regulates SOCE, is based on a signalling pathway requires the activation of p38 MAP Kinase, Serum & Glucocorticoid inducible Kinase (SGK1), and Nuclear Factor κB (NFκB). On the other hand, the mechanism of action, by which TGFβ1 upregulates Na^{+}/Ca^{2+} exchangers remained unidentified, as well as the specific Na^{+}/Ca^{2+} exchanger isoforms involved in the process of up-regulation. The present study aimed to identify, whether TGFβ1 influences the expression and activity of K^{+}-independent (NCX) and K^{+}-dependent (NCKX) Na^{+}/Ca^{2+} exchangers, and aimed also to explore the signalling involved.

Methods: In human megakaryocytic cells (MEG01), Fura-2 fluorescence was utilized to observe cytosolic Ca^{2+} activity $[Ca^{2+}]i$. The activity of Na^{+}/Ca^{2+} exchanger was studied by observing the rise in $[Ca^{2+}]i$ resulting from changing the extracellular solution from a solution with 0 mM Ca^{2+} and 130 mM Na^{+} to a solution with 2 mM Ca^{2+} and 0 Na^{+}. For analysis of NCX, the concentration of K^{+} was 0 mM. For analysis of NCKX, the concentration of K^{+} was 40 mM. In order to quantify transcription levels of NCX/NCKX isoform, RT-PCR was applied.

Results: TGFβ1 (60 ng/ml, 24 h) was found to increase significantly the transcription levels of certain isoforms of NCX/NCKX including: NCX1, NCKX1, NCKX2 and NCKX5. Additionally, the activity of NCX and NCKX was shown to be increased significantly in the presence of TGFβ1 (60 ng/ml, 24 h). Skepinone-L (1 μM), a p38 MAP Kinase inhibitor, caused a significant downregulation of the effect of TGFβ1 on both transcription levels and activity of NCX and NCKX. GSK-650394 (10 μM), an inhibitor of SGK1, and Wogonin (100 μM), and inhibitor of NFκB, caused a significant downregulation of the effect of TGFβ1 on the activity of NCX and NCKX.

Conclusions: P38 MAP Kinase, SGK1 and NFκB are involved in the signaling pathway by which TGFβ1 increases the activity of Na^{+}/Ca^{2+} exchanger and the transcription levels of NCX1, NCKX1, NCKX2, and NCKX5.

Zusammenfassung

Der Transforming Growth Factor β1 (TGFβ1) spielt eine wichtige Rolle in der Regulation von Megakaryocyten-Reifung und Thrombocyten-Bildung. TGFβ1 steigert sowohl die Speicher-abhängige Ca^{2+}-Freisetzung [store operated Ca^{2+} entry (SOCE)] als auch den Ca^{2+} Export über Na^+/Ca^{2+} Austauscher. Somit steigert TGFβ1 sowohl die Zunahme der cytosolische Ca^{2+} Aktivität ($[Ca^{2+}]i$) durch Freisetzung von Ca^{2+} aus intracellulären Speichern, als auch die folgende Abnahme von $[Ca^{2+}]i$.

Die Wirkung von TGFβ1 auf SOCE wird durch einen Signalweg mit p38 MAP Kinase, Serum & Glucocorticoid inducible Kinase (SGK1) und Nuclear Factor κB (NFκB) vermittelt. Der Signalweg, über den TFβ1 den Na^+/Ca^{2+} Austausch beeinflußt und welcher Na^+/Ca^{2+} Austauscher beteiligt ist, blieb unbekannt. Die Doktorarbeit befasst sich mit dem Einfluss von TGFβ1 auf Expression und Akitivität von K^+-unabhängigen (NCX) und K^+-abhängigen (NCKX) Na^+/Ca^{2+} Austauschern, sowie mit den beteiligten Signalwegen.

Methoden: In humanen Megakaryocyten (MEG01) wurde mit Hilfe von Fura-2 Fluoreszenz $[Ca^{2+}]i$ gemessen und die Na^+/Ca^{2+} Austauscher Aktivität vom $[Ca^{2+}]i$ Anstieg nach Wechsel von einer extrazellulären Lösung mit 130 mM Na^+ und 0 mM Ca^{2+} zu einer extrazellulären Lösung mit 0 Na^+ und 2 mM Ca^{2+}. Die K^+ Konzentration war 0 mM zur Analyse von NCX und 40 mM zur Analyse von NCKX. RT-PCR wurde zur Quantifizierung der NCX/NCKX Isoform-Transcripte eingesetzt.

Ergebnisse: TGFβ1 (60 ng/ml, 24 h) steigerte significant die Transcription von NCX1, NCKX1, NCKX2 und NCKX5. TGFβ1 (60 ng/ml, 24 h) steigerte ferner significant die Aktivität sowohl von NCX als auch von NCKX. Die Wirkung von TGFβ1 auf die NCX und NCKX Transcription und Aktivität wurde significant durch den p38 Kinasehemmer Skepinone-L (1 µM) gehemmt. Die Wirkung von TGFβ1 auf die NCX und NCKX Aktivität wurde durch den SGK1-Hemmer GSK-650394 (10 µM) und den NFκB-Hemmer Wogonin (100 µM) reduziert.

Schlussfolgerung: TGFβ1 steigert significant die Transcription von NCX1, NCKX1, NCKX2 sowie NCKX5 und damit die Na^+/Ca^{2+} exchanger Aktivität, eine Wirkung, die p38 kinase, SGK1 und NFκB erfordert.

1. Introduction

1.1. Calcium in Human Body

Calcium is an important intracellular messenger in the human body and also in all living organisms. Calcium is counted as one of the most profuse minerals in the body. Many cellular progressions and physiological functions such as the polymerisation of fibrin, the transmission of impulses in the nervous system and the function of Skeletal muscles are regulated by calcium which plays as well a central structural role in the body. One of the essential physiological roles of calcium is its role as an intracellular messenger (Bagur and Hajnoczky 2017).

Hormonal and exocrine secretion and muscular motility as well as motility in nonmuscle cells in addition to many other cellular functions are regulated by Calcium signalling which plays a significant role in a variety of metabolic pathways (Carafoli 1988).

Most of the intracellular Ca^{2+} is stored in the ER. The release of Ca^{2+} from endoplasmic reticulum (ER) activates specific Ca^{2+} channels on the plasma membrane of the cell, which contributes additionally to the increase of intracellular Ca^{2+} levels. Eventually, the intracellular Ca^{2+} concentration might pass by (10-100 times) its levels in rest mode (Groenendyk, Lynch et al. 2004).

1.2. Calcium Homeostasis and Signalling

Free Ca^{2+} concentrations can be extensively varied inside different intracellular organelles. Some organelles can store Ca^{2+} and accumulate higher $[Ca^{2+}]$ than the cytoplasm. Those organelles are called Ca^{2+} stores, and among them Calcium can be basically stored in endoplasmic reticulum (ER) and, in muscle cells, sarcoplasmic reticulum (SR). However, the concentrations of free Ca^{2+} in other organelles such as the mitochondrial matrix and nuclear matrix are similar to the cytoplasmic $[Ca^{2+}]$. On the other hand, the extracellular milieu has higher levels of $[Ca^{2+}]$

compared to the cytoplasm in resting mode. The cytoplasmic [Ca^{2+}] can be kept lower in resting cells by the activity of Na^+/Ca^{2+} exchanger (NCX) and the plasma membrane Ca^{2+} transport ATPase (PMCA) (Bagur and Hajnoczky 2017).

Cytoplasmic [Ca^{2+}] can be tuned through active and passive mechanisms. The first includes Na^+/Ca^{2+} exchangers (NCX), plasma membrane and ER/SR- Ca^{2+} - ATPases, while Ca^{2+} channels represent the passive mechanism. Both active and passive mechanisms are vital for the cell to keep its intracellular Ca^{2+} concentration levels always in a favorable range for intracellular signalling and other cellular activities (Martinez-Zaguilan and Wesson 1996).

The ER particularly stores the majority of intracellular Ca^{2+}. Ca^{2+} channels in the plasma membrane are activated when ER releases Ca^{2+}, resulting in an intracellular [Ca^{2+}] increase between 10-100 fold. (Groenendyk, Lynch et al. 2004)

Ca^{2+} homeostasis is vital in the process of aging, heart disease, cancer, and neurodegeneration (Squier and Bigelow 2000).

For instance, a dysregulation of intracellular Ca^{2+} homeostasis in neurons causes misfunction in Ca^{2+}-dependent signalling pathways, can be associated with Brain aging. (Hartmann, Eckert et al. 1994)

1.3. Na^+/Ca^{2+} Exchangers

Na^+/Ca^{2+} exchanger molecules in mammalian cells are categorized in two sequenced and cloned families (Blaustein and Lederer 1999, Philipson and Nicoll 2000, Shigekawa and Iwamoto 2001).

The first family is K^+-independent (NCX) exchangers, which has an exchanging ratio of $1Ca^{2+}:3Na^+$ (Philipson and Nicoll 2000). NCX exchangers family has 3 isoforms that have been identified: NCX1, NCX2, NCX3 (Aneiros, Philipp et al. 2005).

The second family of Na^+/Ca^{2+} exchangers is K^+-dependent (NCKX) exchangers, which has an exchanging ratio of $(1\ Ca^{2+} + 1\ K^+):4\ Na^+$ (Schnetkamp, Basu et al. 1989). NCKX exchangers family has six isoforms (Cai and Lytton 2004)

NCX1 is generally expressed in every mammalian cell (Kofuji, Lederer et al. 1994, Quednau, Nicoll et al. 1997). NCX2 is mainly found in the brain and spinal cord, and can be expressed kidney tissues and the gastrointestinal tract. NCX3 is largely expressed in the skeletal muscles and the brain, but it can also be expressed in osseous tissue and the immune system (Quednau, Nicoll et al. 1997, Michel, Verkaart et al. 2014, Michel, Hoenderop et al. 2015)

NCKX1 is predominantly expressed in retinal photoreceptors (Kang and Schnetkamp 2003). The expression of NCKX2 is mainly in the brain. NCKX3 is generally expressed mostly in the brain, aorta, lung, and intestine. NCKX4 is expressed mainly in the brain (Visser, Valsecchi et al. 2007). NCKX5 is expressed in melanocytes and seems to play an important role in skin pigmentation (Altimimi and Schnetkamp 2007). NCKX6 has a ubiquitous expression in all tissues (Cai and Lytton 2004).

Na^+/Ca^{2+} exchangers can function in both directions: forward mode or reverse mode (Philipson, Nicoll et al. 2002). In the forward mode, the activity of the Na^+/Ca^{2+} exchanger results in the exclusion of Ca^{2+} in exchange for Na^+ entry, the opposite way is the reverse mode which leads to the influx of Ca^{2+} and the extrusion of Na^+. (Philipson, Nicoll et al. 2002, Annunziato, Pignataro et al. 2004)

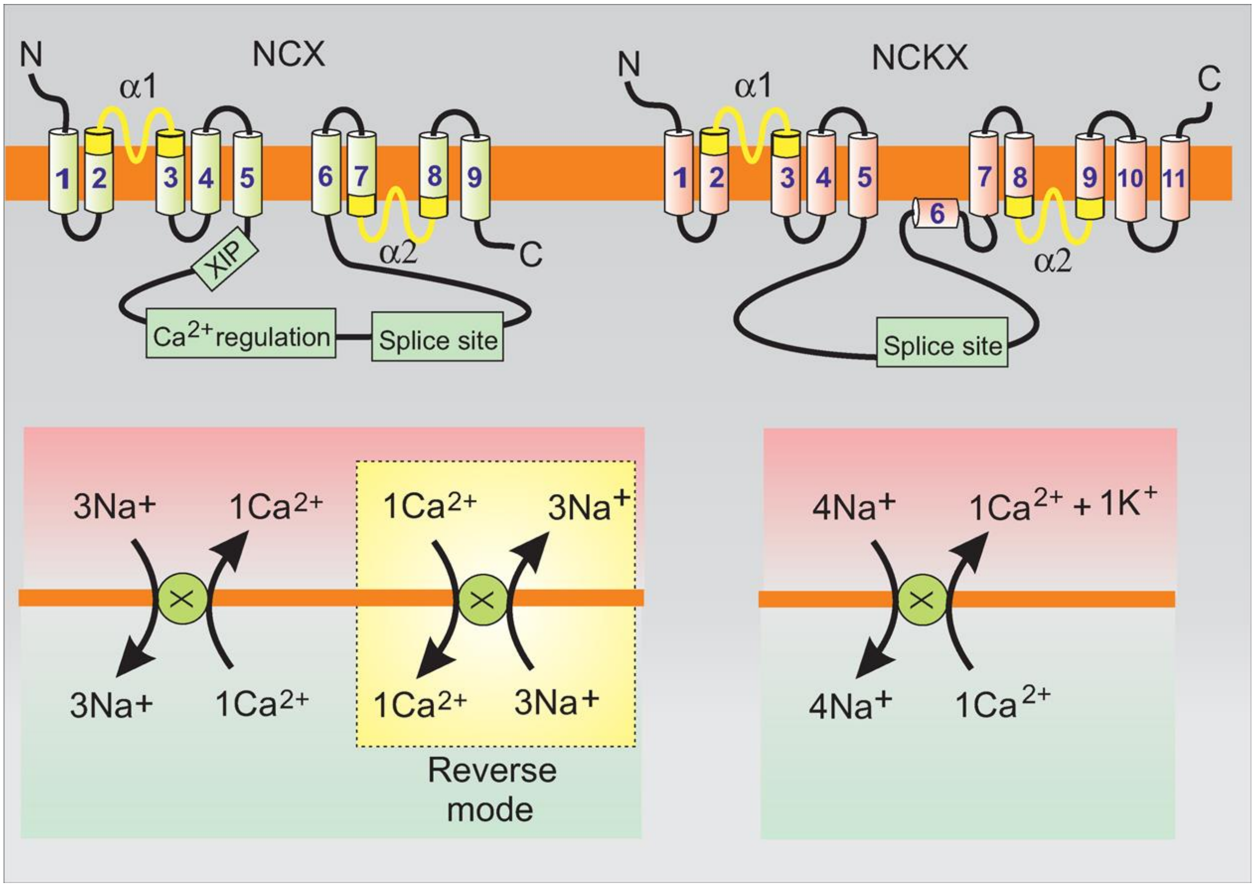

Figure.1. Structure and function of sodium/calcium exchangers (Berridge 2014).

What regulates the activity of Na^+/Ca^{2+} exchangers in favor of forward mode (Ca^{2+}-efflux) or reverse mode (Ca^{2+}-influx) are the prevailing electrochemical driving forces for Ca^{2+} and Na^+ (Armoundas, Hobai et al. 2003).

Thus, the membrane potential (Baczko, Giles et al. 2003) and the transmembrane gradients of Na^+ can control how the Na^+/Ca^{2+} exchanger functions (Philipson and Nicoll 2000). The Na^+/Ca^{2+} exchanger is driven by transmembrane sodium motive force which can let the exchanger force Ca^{2+} transport even against its concentration gradient (Marinelli, Almagor et al. 2014). In K^+-dependent Na^+/Ca^{2+} exchangers (NCKX), exchanging Ca^{2+} requires both the K^+ and Na^+ electrochemical gradients in order to power the extrusion or influx of Ca^{2+} (Blaustein and Lederer 1999)

For instance, when the intracellular Na^+ concentration increases, it sets the exchanger in the reverse mode. Na^+/Ca^{2+} exchanger can be shifted into reverse mode and start contributing to the influx of Ca^{2+} even when the alterations in intracellular Na^+ concentration and/or membrane potential are relatively small (Armoundas, Hobai et al. 2003).

This increase in intracellular $[Ca^{2+}]_i$ which is mediated by Na^+/Ca^{2+} exchangers can be functionally relevant and can be the trigger of some physiological operations in the cell (Paluzzi, Alloisio et al. 2007, Reyes, Verkhratsky et al. 2012).

1.4. Calcium in Megakaryocytes

Megakaryocytes descend from hematopoietic stem cells (HSCs), which represent a lifetime source of all blood cells in circulation (Ogawa 1993, Pang, Weiss et al. 2005).

This process of producing different distinct types of blood cells starting from HSCs requires a chain of sequential differentiations in which the proliferative and developmental capacities of descendant cells become gradually more limited (Pang, Weiss et al. 2005).

In normal cases, approximately 1 in each 10,000 nucleated cells in human marrow is Megakaryocyte (Branehog, Ridell et al. 1975).

megakaryocyte is a large cell (~50–100 μm diameter) with a single, large, polyploid nucleus(Pang, Weiss et al. 2005). each megakaryocyte produces about 10^4 platelets (Long 1998).

The production of platelets by megakaryocytes is controlled by a group of environmental and autocrine elements (Di Buduo, Moccia et al. 2014). The autocrine factors can be represented basically in the release of TGFß1 (Sakamaki, Hirayama et al. 1999, Ponce, de Lourdes F. Chauffaille et al. 2012, Badalucco, Di Buduo et al. 2013) and adenosine diphosphate (Di Buduo, Moccia et al. 2014) by human megakaryocytes which lead to an increase in cytosolic calcium concentration in megakaryocyte itself (Di Buduo, Moccia et al. 2014, Yan, Schmid et al. 2015).

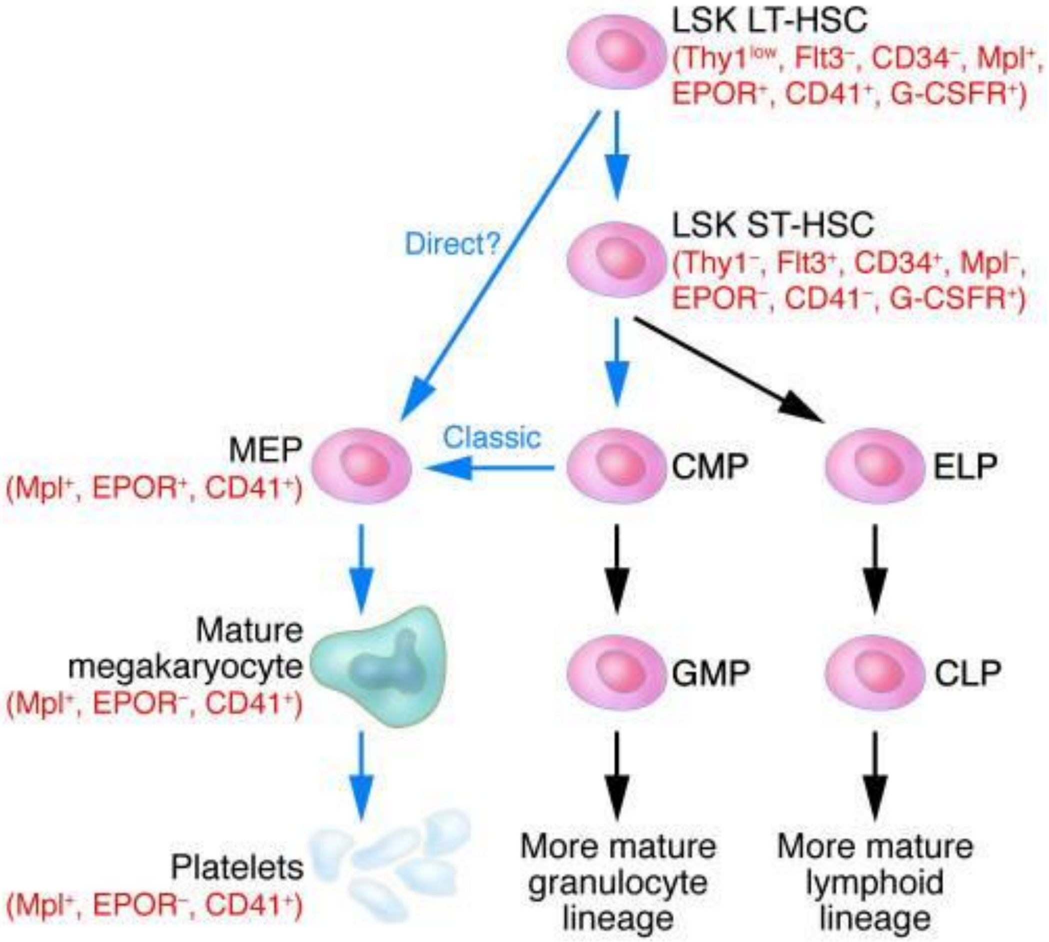

Figure.2. Megakaryopoiesis pathways. (Pang, Weiss et al. 2005)
Megakaryocytes descend from hematopoietic stem cells (HSCs), which represent a lifetime source of all blood cells in circulation

Calcium release from intracellular stores doesn’t only trigger signaling pathways that activates megakaryocyte differentiation and proplatelet formations, but it also induces extracellular calcium entry which mainly plays important role in the regulation of the contractile force controlling megakaryocyte motility (Di Buduo, Moccia et al. 2014).

Motility is crucial to the formation of platelets as Mks are supposed to migrate during differentiation, from the osteoblastic to the vascular niche (Avecilla, Hattori et al. 2004).

This shows that not only calcium release from intracellular stores (mainly ER) but also calcium entry from extracellular milieu into the cell are essential to the regulation of the functions of human megakaryocytes (Di Buduo, Moccia et al. 2014).

A variety of Ca^{+2} channels have been shown to play functional roles in platelet activation (Sun, Li et al. 1998, Mahaut-Smith 2012). In addition to Ca^{2+} channels, many other ion channels have been proved to participate in the regulation of the function of platelets including Kv1.3 voltage gated channels, P2X1 ATP-gated channels, connexin gap junction channels and kainate glutamate receptors (Mahaut-Smith 2012).

1.5. Transforming Growth Factor ß1 (TGFß1)

TGFß1 is a member of the transforming growth factor TGF-β superfamily. This multipotent cytokine superfamily has important roles in regulating a wide range of cellular pathways and functions.(Hwangbo, Tae et al. 2016)

TGFß1 is expressed in a broad variety of the body tissues including largely expression in spleen, bone marrow and 23 other tissues. (Fagerberg, Hallstrom et al. 2014)

1.5.1. The Role of TGFß1

TGF-β1 regulates cellular functions by binding to heteromeric complexes of TGF-β receptors which includes two types: TGF-β type I receptor (TβRI) and TGF-β type II receptor (TβRII). (Attisano and Wrana 2002). By binding to its TβR receptors TGF-β activates them. Activated TβR in turn initiate the phosphorylation of SMAD2 and SMAD3, which are both coupled to the receptor. The phosphorylation of SMAD2 and SMAD3 leads to the formation of strong combinations with SMAD4. Those complexes go then through a translocation to the nucleus, where the targeted genes are located. Once the SMAD complexes are translocated to the nucleus they start regulating the transcription of those genes (Zavadil and Bottinger 2005, Bierie and Moses 2006). Beside SMAD pathway, TGF-β1 can also activate a variety of other signaling pathways including mitogen-activated protein kinase, c-Jun-N-terminal kinase, the phosphoinositide 3-kinase/Akt pathways and p38 kinase pathway. (Zavadil and Bottinger 2005, Zhang 2009).

Transforming growth factor ß1 (TGFß1) is described as a multifunctional protein controlling a variety of functions including differentiation and proliferation in different cell types. TGFß1 is produced and released by many cells and those cells have specific receptors for TGFß1. (Chen, Lee et al. 2014)

Megakaryocytes are one of those cells. TGFβ1 is produced and released by megakaryocytes (Bock, Loch et al. 2005, Ponce, de Lourdes et al. 2012, Badalucco, Di Buduo et al. 2013) . TGFβ1 then binds to its receptors on the membrane of Mks and thus triggers signalling downstreams, and so TGFβ1 can be considered an autocrine regulator of megakaryocytes. The resulting effect of TGFβ1 on Mks includes the activation of differentiation and eventually the formation of proplatelets. (Sakamaki, Hirayama et al. 1999, Badalucco, Di Buduo et al. 2013). Besides its role in Mk maturation and platelet formation, TGFβ1 is found to play the major role in stimulating the expression of bone marrow stromal thrombopoietin. Thrombopoietin itself plays a role in the activation of the expression of TGF-beta receptors in Megakaryocytes, which means that TGF-beta1 plays also a role as a feedback regulator of megakaryopoiesis. (Sakamaki, Hirayama et al. 1999).

TGFβ1 can also control the cytosolic Ca^{2+} activity $[Ca^{2+}]_i$ in megakaryocytes and platelets, by its ability to up-regulate the expression of serum/glucocorticoid inducible kinase (SGK1), which is a novel regulator of $[Ca^{2+}]_i$ in megakaryocytes and platelets. The up-regulation of (SGK1) through TGFβ1 pathway occurs through the activation of P38 MAPK. (Yan, Schmid et al. 2015).

The highest concentration of TGFß in the body is found in platelets, and the largest amount of TGFß is produced in bones with a concentration of 200 micrograms of TGFß in each 1kg of bone tissue. (Bonewald and Mundy 1990).

The mechanism of action of TGFß1 activity includes the regulation (positively and negatively) of a variety of other growth factors. For instance, TGFß1 has a significant role in bone remodeling as it is an effective motivator of osteoblastic bone formation, leading to proliferation or differentiation in osteoblasts. (Chen, Lee et al. 2014). TGF-beta can play different roles in bone cells based on their phenotype and phase of differentiation.(Bonewald and Mundy 1990). TGFß1 acts also as a potent stimulator of the sustained synthesize and secretion of collagen in fibroblasts. (Sakamaki, Hirayama et al. 1999, Chen, Lee et al. 2014). By activating the differentiation of fibroblasts to myofibroblasts which is more effective than the former in producing collagen. (Sakamaki, Hirayama et al. 1999).

1.5.2. Deficiency of TGFß1

The occurrence of mutations affecting the gene encoding TGFß1 is responsible for a case known as "Camurati-Engelmann disease (CAEND)"

This disease is an autosomal dominant disorder with symptoms including sclerosis and hyperostosis in the diaphysis of long bones. The symptoms can be noticed starting from early childhood including muscular weakness, pain, and myopathic gait, and in some cases the symptoms can also include difficulties in hearing, paralysis of facial muscles, or eye disorders including exophthalmos or vision loss. (Kinoshita, Saito et al. 2000, Janssens, ten Dijke et al. 2003, McGowan, MacPherson et al. 2003)

1.6. Serum & Glucocorticoid Inducible Kinase 1 (SGK1)

Serum- and glucocorticoid-inducible kinases (SGKs) are members of the AGC family (protein kinase A, G, C families: PKA-, PKG-, PKC-related) of serine/threonine kinases, which can be found in most of the cells (Arencibia, Pastor-Flores et al. 2013).

SGK is remarkably expressed in the liver and contributes to the regulation of cell survival in response to environmental changes and stress stimulators (Leong, Maiyar et al. 2003).

Other members of the AGC family are AKT (protein kinase B) and PKC (protein kinase C) and others. SGK kinases have highest proportion of homology with the AKT family (Firestone, Giampaolo et al. 2003, Pearce, Komander et al. 2010).

The SGK family includes three isoforms (SGK1, SGK2, and SGK3) that are encoded by three different genes found on different chromosomes, but those isoform still have high structural similarity (Lang and Cohen 2001).

1.6.1. The Role of SGK1

SGK1 is an important regulator of cytosolic Ca^{2+} and plays an important role in the regulation of megakaryocytes maturation and platelets functions. (Borst, Schmidt et al. 2012)

SGK was initially considered to be under intense transcriptional regulation by glucocorticoids and serum (Webster, Goya et al. 1993), but later, SGK has been reported to be controlled by a variety of regulators including hormones, growth factors and oxidative and osmotic stress (Buse, Tran et al. 1999, Bell, Leong et al. 2000, Mizuno and Nishida 2001, Leong, Maiyar et al. 2003).

The function of SGK can be regulated at different levels in the cell including transcriptional level, subcellular translocation and regulation of enzymatic activity. (Meng, Yamagiwa et al. 2005)

Several hormones and other factors involve in the regulation of SGK1 including thrombin, ischemia, oxidative stress, transforming growth factor TGF-β and other growth factors such as IGF-1. (Borst, Schmidt et al. 2012)

The mechanism of transcription of SGK1 is regulated by a wide range of elements including nuclear factor KappaB (NFκB), and SMAD3 and SMAD4 complexes which are transcription factors regulated by TGFβ. The regulation of transcription of SGK1 includes also receptors for progesterone, mineralocorticoid, glucocorticoid and other factors. (Lang, Artunc et al. 2009)

SGK1 regulates a variety of transporters, enzymes, ion channels such as ENaC (Faletti, Perrotti et al. 2002), and transcription factors such as nuclear factor kappa B NFκB. (Lang, Bohmer et al. 2006). SGK1 plays a role also in different cellular functions including the regulation of neuroexcitability, hormone release, cell proliferation and apoptosis. (Lang, Bohmer et al. 2006)

The effects of SGK1 on platelets include the stimulation of coagulation, through tissue factor expression (Lang, Artunc et al. 2009) and SGK1 can, by up-regulating NFκB, contribute to the development of strokes (Dahlberg, Smith et al. 2011) and thrombosis (Borst, Schmidt et al. 2012), through the NFκB-induced up-regulation of expression of Ca^{+2} channels such as Orai1/STIM1 in the platelets. (Dahlberg, Smith et al. 2011, Borst, Schmidt et al. 2012)

1.6.2. GSK-650394 (SGK1 Inhibitor)

GSK-650394 has higher selectivity for SGK1 compared to that for Akt, which has the highest homology to SGK1 among AGC kinase family. GSK-650394 is rather non-toxic. For example, the LC_{50} values are 68 times higher than its IC_{50} in HeLa cells. The selectivity of GSK-650394 for SGK1 was shown to be more than 30 times higher when compared to that for Akt, while compared to PDK1 (another AGC kinase family member) the selectivity of GSK-650394 for SGK1 was 60 times higher (Sherk, Frigo et al. 2008).

1.7. Nuclear Factor kappa B (NFκB)

Nuclear factor KappaB (NFκB) is a cytokine-regulated transcription factor that has an important role in the regulation of transcription of genes responsible basically for cell survival and inflammation (Ghosh, May et al. 1998).

NFκB is a protein complex that can regulate gene expression by binding to κB sites in the promoters/enhancers of the targeted genes. NFκB can either activate or suppress the expression of genes (Hoffmann and Baltimore 2006).

In addition to the fact that NFκB is expressed in nearly all cells, it has been found also that κB sites, which are the NFκB binding sites on the DNA sequences, exist in the promoters/enhancers of a broad variety of genes (Oeckinghaus and Ghosh 2009).

It is estimated that there are approximately 10^6 κB sites for NFκB to bind in the human genome, and about 500 genes are regulated by NFκB (Natoli, Saccani et al. 2005, Antonaki, Demetriades et al. 2011).

1.7.1. The Role of NFκB

NFκB was initially considered a transcriptional activator (Seto 2003), but a variety of studies emerged later suggesting and reporting the transcriptional repression activity of NFκB also (Ashburner, Westerheide et al. 2001, Baetz, Regula et al. 2005).

In mammalians, five NFκB family members are discovered: RelA (p65), RelB, c-Rel, p50/p105 (NFκB1) and p52/p100 (NFκB2) (Tieri, Termanini et al. 2012)

However, 12 distinct dimers can be formed out of those five members through their ability to homo- or hetero-dimerise (Christian, Smith et al. 2016).

The available data demonstrate that nuclear factor-κB (NFκB) consists of a group of transcription factors that have major roles in cellular responses in a variety of aspects including inflammation, immunity, cell survival, differentiation, and proliferation (Oeckinghaus and Ghosh 2009).

NFκB is found in its inactive mode in the cytoplasm of the cell (Sheppard, Rose et al. 1999). Being in its inactive mode is caused by binding to inhibitory proteins, such as IκB-α. The activation of NFκB requires phosphorylation of the inhibitory protein. This phosphorylation is accomplished by an IκB kinase complex (IKK) leading to the degradation of the inhibitory protein. The activation of NFκB, by the phosphorylation of as IκB, acts as a trigger for NFκB dimer to be translocated to the nucleus, where it starts the regulation of expression of specific genes (Maniatis 1997, Sheppard, Rose et al. 1999).

As NFκB has the ability to regulate the expression of a wide range of genes, thus the activity of NFκB is firmly regulated and fine-tuned at different levels in the cell. The first step in the process of regulation of NFκB activity is represented by the inhibitory proteins (IκBs) and the (IKK) kinase complex phosphorylating the (IκB) (Oeckinghaus and Ghosh 2009).

SGK1 can regulate the activation of NFκB, as SGK1 has the ability to phosphorylates the IκB kinase complex (IKK), which is responsible for phosphorylation of the inhibitory protein IκB-α what leads eventually to translocation of nuclear factor NFκB to the nuclear (Yan, Schmid et al. 2015).

The transcriptional activity of NFκB is regulated also by p38 MAP kinase. The regulation of NFκB, which takes place at the nuclear level, is unrelated to the translocation of transcription factor to its site in the nucleus and independent from the potency of the transcription factor to attach to the targeted genes. P38 MAP kinase was found to regulate the phosphorylation of NFκB ReIA in RAW264.7 cells (Olson, Hedrick et al. 2007).

In general, the activity of NFκB is regulated through a variety of mechanisms including post-translational modifications such as acetylation or phosphorylation of NFκB (Hayden and Ghosh 2004, Xiao 2004).

As NFκB regulates a variety of essential cellular physiological activities including proliferation, immunity and cell survival, so in cases of malfunction of the regulation of NFκB activity, the outcomes are represented in severe disorders and diseases including immunodeficiency, autoimmunity, arthritis and cancer (Courtois and Gilmore 2006).

In Megakaryocytes, NFκB is crucial to the regulation of megakaryocytopoiesis. Up-regulation of the activity of p38 MAPK in megakaryocytes leads to up-regulation of the activity of NFκB which enhances maturation of megakaryocytes and thus platelet production (Wu, Xie et al. 2015).

1.7.2. Wogonin (NFκB inhibitor)

Wogonin (5,7-dihydroxy-8-methoxyflavone) is an effective NFκB pathway inhibitor (Wu, Xie et al. 2015). It is extracted from *Scutellaria baicalensis Georgi* (known also as *Huang-Qin*) (Xu, Yang et al. 2016). Wogonin plays a role in several medical cases including inflammatory diseases, neurological diseases (Lin 2011), allergy (Lucas, Dorward et al. 2015) and tumors (Chirumbolo 2013).

The mechanism by which Wogonin inhibits NFκB is based on a characteristic of NFκB that it can be regulated by the redox potential in the cell (Pantano, Reynaert et al. 2006). In cases of oxidative stress, elevated intracellular levels of reactive oxygen species (ROS) causes the activation of NFκB (Fas, Baumann et al. 2006).

Wogonin has an effect on the cellular redox homeostasis. It causes an alteration in the redox state of the cell towards less oxidative state (H202), by scavenging oxidative radicals (O2-). Through this mechanism Wogonin can cause the ROS-mediated NFκB activation to be down-regulated.(Fas, Baumann et al. 2006, Li-Weber 2009)

1.8. P38 Mitogen-Activated Protein Kinase (p38 MAPK) Pathway

1.8.1. The Role of p38 Kinase

p38 MAP kinase is a stimulator of transcription factors through phosphorylation. Its activity involves the activation of nuclear kinases among other kinases also (Deak, Clifton et al. 1998).

Similar to SGK1, the p38 MAP kinase functions in response to environmental stimulators. P38 MAPK has an important role in the regulation of those intracellular responses. The activation of p38 MAPK pathway can be triggered by external elements and environmental stress, such as: UV radiation, heat shock, proinflammatory cytokines, bacterial lipopolysaccharide (LPS) or hormones (Meng, Yamagiwa et al. 2005). A variety of downstream signaling intracellular responses result from the activation of p38 MAPK. Those cellular responses include apoptosis, differentiation, cell cycle regulation, inflammatory responses (Nebreda and Porras 2000).

In general, the regulatory effect of P38 for certain stimuli depends on two factors including cell type and the condition of the cell (Conde, Pabon et al. 2010).

As a result of variation of those two factors, different responses for p38 activity can be observed. p38 MAPK can induce differentiation (Yosimichi, Nakanishi et al. 2001), apoptosis (Wang, Li et al. 2005) or cell survival through inhibition of apoptosis (Zhang, Shan et al. 2003).

The p38 MAPK family includes four major isoforms: p38α (MAPK14), p38β (MAPK11), p38γ (MAPK12), and p38δ (MAPK13). These isoforms are encoded by different genes and expressed in different tissues. However, under situations of extracellular changes the p38 MAPKs act in correspondence in order to produce intracellular signaling complexes resulting in specific reactions in correspondence to each external stimulation (Bachstetter and Van Eldik 2010). In cases of p38 activation in response to inflammatory stimulation, the process of activation includes phosphorylation of two residues in p38, which are Thr and Tyr. This activation of p38 is accomplished by upstream kinases, and the activated p38 in turn activates downstream pathways by phosphorylating specific substrates which ends up eventually in the upregulation of proinflammatory cytokines production (Schieven 2009).

P38 MAPK can regulate Serum and Glucocorticoid-inducible Kinase (SGK). The regulation can take place through more than one possible mechanism. it can occur at the transcriptional level or by phosphorylating the Ser^{78} residue of SGK. Even p38 MAPK activity in its constitutive level can regulate SGK (Meng, Yamagiwa et al. 2005).

Inhibitors of p38 MAPK block the phosphorylation of SGK at Ser^{78} and thus inhibits the activation of SGK. On the contrary, when the expression of p38 MAPK is up-regulated it results in the augmentation of the constitutive phosphorylation and thus the constitutive activity of SGK (Meng, Yamagiwa et al. 2005).

In Megakaryocytes it is suggested that the downregulation of P38 MAPK has a major effect on the differentiation of Megakaryocytes (Jacquel, Herrant et al. 2006).

P38 plays a key role in regulating the progress of the differentiation and the final phenotype in Megakaryocytes (Conde, Pabon et al. 2010).

P38 is suggested also to have an important effect on the progress of cell cycle through negative regulation (Bulavin and Fornace 2004, Hui, Bakiri et al. 2007).

In general, p38 MAPKs pathway is involved in Megakaryocyte differentiation along with ERK1/2 and PI3K pathways. The available data show that p38 MAPK plays a key role in the regulation of megakaryocytopoiesis. (Conde, Pabon et al. 2010).

1.8.2. Skepinone-L, a selective P38 Kinase Inhibitor

Skepinone-L, which is a dibenzosuberone compound, is a p38 MAPK inhibitor with a high *in vivo* potency and a superior selectivity compared to the majority of other p38 inhibitors (Koeberle, Romir et al. 2011).

Most of other p38 MAPK inhibitors have a molecular weight >500 Da., which makes them considered large molecules, a characteristic that counts unfavourable for *in vivo* efficacy. In comparable whole-blood assays, the majority of p38 MAPK inhibitors are found to have moderate potency (Goldstein and Gabriel 2005) which demands higher *in vivo* plasma concentration in order to reach the required inhibitory effect. Many p38 MAPK inhibitors are ATP-competitive inhibitors, which is considered relatively a weakness point in the domain of selectivity as the ATP-site retains unchanged a wide range of proteins, and thus most of the p38 MAPK inhibitors, including (SB203580) and (BIRB796), have poor selectivity. Skepinone-L has two features in its structure that grant the outstanding selectivity for p38 MAPK. Those two features are represented in the L-glycine–flip at Gly110 and the linear binding ability. Beside its selectivity, Skepinone-L has high in vivo potency which ensures the ability of having a clear-cut examining of the p38 MAPK signalling pathway (Koeberle, Romir et al. 2011).

1.9. AIM OF THE STUDY

The aim of the present study was to investigate whether p38 MAPK, NFκB and SGK1 can up-regulate Na^+/Ca^{2+} exchanger in Megakaryocytes following treatment with TGFß1.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Culture of Megakaryocytes

Equipments

Name	Manufacturer and country of origin
Heraeus Incubator	Thermo Electron Corporation, Dreieich, Deutschland
Cell culture flask	Darstedt AG, Nümbrecht, Deutschland
Centrifuge RotoFix 32	Hettich Zentrifugen, Tuttlingen, Deutschland
Eppendorf pipettes 10, 100, 1000 µL	Eppendorf AG, Hamburg, Deutschland
6-well plates	BD Biosciences, Franklin Lakes, NJ, USA
Eppendorf cups 1.5 mL	Eppendorf AG, Hamburg, Deutschland
Neubauer counting chamber	Brand, Wertheim, Deutschland
Vortex Genie	Scientific Industries, Bohemia NY, USA

Chemicals

Name	Manufacturer and country of origin
RPMI-1640	Gibco, Carlsbad, Deutschland
FBS (Fetal bovine serum)	Gibco, Carlsbad, Deutschland
Penicillin/streptomycin	Gibco, Carlsbad, Deutschland
PBS (Phosphate buffered saline)	Gibco, Carlsbad, Deutschland
TGFß1	Sigma, Taufkirchen, Deutschland
Skepinone-L	Merck, Darmstadt, Deutschland
GSK-650394	Tocris, Wiesbaden-Nordenstadt, Deutschland
Wogonin	Sigma, Darmstadt, Deutschland

2.1.2. Intracellular Calcium Imaging

- **Equipments for calcium measurment**

Name	Company
Camera Proxitronic	Proxitronic, Bensheim, Deutschland
Centrifuge RotoFix 32	Hettich Zentifugen, Tuttlingen, Deutschland
Cover glasses round, 30mm, Thickness No. 1	VWR, Darmstadt, Deutschland
Discofix® Stopcock for Infusion Therapy	B.Braun, Melsungen AG (global), Deutschland
Eppendorf pipettes 1000 µL, 100 µL, 10 µL	Eppendorf AG, Hamburg, Deutschland

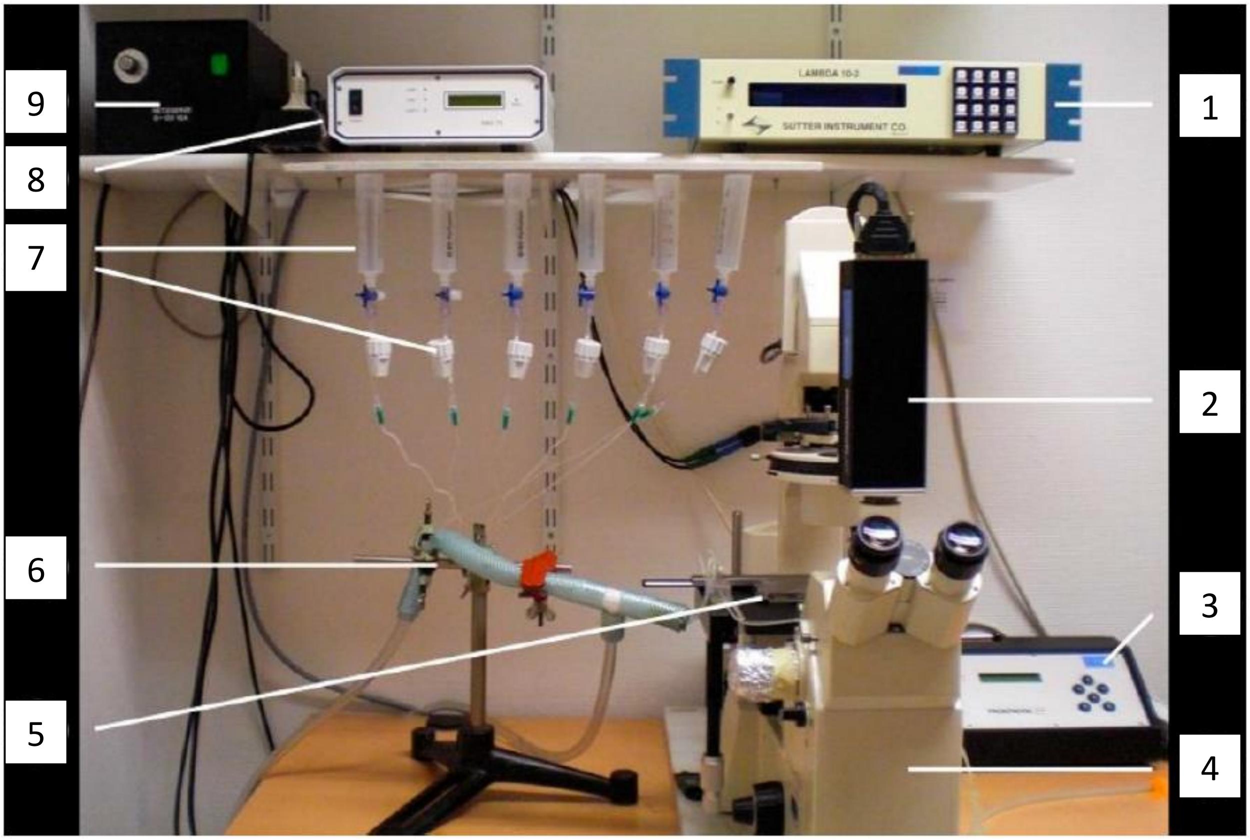

Figure. 3. Calcium measurement equipments

1. Shutter instrument, 2. Camera, 3. control panel of the Camera, 4. Microscope, 5. Cell chamber, 6. Warming system of Extracellular solutions, 7. Transmission system of extracellular solutions, 8. Control panel of xenon lamp, 9. Control panel of Light.

Eppendorf cups 1.5, 2 mL	Eppendorf AG, Hamburg, Deutschland
Filter tips 10, 100, 1000 µL	Biozym Scie., Hess.Oldendorf, Deutschland
Filter wheel	Sutter Inst. Co., Novato, USA
Incubator	Thermo Electro. Cor., Dreieich, Deutschland
Lamp XBO 75	Leistungselektronik GmbH, Jena, Deutschland
Metafluor Software	Universal Imaging, Downingtown, USA
Microscope Axiovert 100	Zeiss, Oberkochen, Deutschland
Multiwell™ 6 well	Becton Dichinson Labware, Franklin Lakes, USA
Needles BD Microlance™3, 1.2X40mm	Becton Dichinson Labware, Franklin Lakes, USA
Objective neo fluar 40X/1.3 oil	Carl Zeiss, Oberkochen, Deutschland
Syringe BD 10ml. Luer-Lok™ Tip	Becton Dichinson labware, Franklin Lakes, USA
Syringe BD, Perfusion™ 50 mL	Becton Dichinson labware, Franklin Lakes, USA

- **Calcium Measurement Chemicals**

Name	Company
Fura-2 AM	Invitrogen, Göttingen, Deutschland
Thapsigargin	Invitrogen, Karlsruhe, Deutschland
Poly-L-lysine	Sigma-Aldrich Chemie GmbH, Munich, Deutschland
Immersol 518F	Carl Zeiss, Göttingen, Deutschland
Silicone Gel	Carl Roth, Karlsruhe, Deutschland
NaCl	Sigma, Taufkrchen, Deutschland
KCl	Carl Roth, Karlsruhe, Deutschland
TAE^+ (Tetraethylammonium-chloride-Monohydrat)	Sigma, Taufkirchen, Deutschland
HEPES	Sigma, Taufkirchen, Deutschland
Glucose	Carl Roth, Karlsruhe, Deutschland
NMDG (N-Methyl-D-glucamine)	Sigma, Taufkirchen, Deutschland
CaCl2.2H2O	Carl Roth, Karlsruhe, Deutschland
$\text{MgCl2.6H}_2\text{O}$	Sigma, Taufkirchen, Deutschland

- **Calcium measurement Buffer Composition**

Table.1. Standard Extracellular Solution with 0mM KCl (for NCX)

Substance	concentration	
NaCl	130	mM
KCl	0	mM
$MgCl_2.6H_2O$	2	mM
HEPES	10	mM
Glucose	5	mM

H_2O (37^0C), pH 7.4 (NaOH)

Table.2. ONa^+-Extracellular Solution with 0mM KCl (for NCX)

Substance	Concentration	
NMDG	90	mM
KCl	0	mM
$CaCl_2.2H_2O$	2	mM
$MgCl_2.6H_2O$	2	mM
HEPES	10	mM
Glucose	5	mM

H_2O (37^0C), pH 7.4 (HCl)

Table.3. Standard Extracellular Solution with 40mM KCl (for NCKX)

Substance	Concentration	
NaCl	130	mM
KCl	40	mM
TAE^+	20	mM
$MgCl_2.6H_2O$	2	mM
HEPES	10	mM
Glucose	5	mM

H_2O (37^0C), pH 7.4 (NaOH)

Table.4. ONa$^+$-Extracellular Solution with 40mM KCl (for NCKX)

Substance	Concentration	
NMDG	90	mM
KCl	40	mM
TAE$^+$	20	mM
$CaCl_2.2H_2O$	2	mM
$MgCl_2.6H_2O$	2	mM
HEPES	10	mM
Glucose	5	mM

H_2O (37^{0}C), pH 7.4 (HCl)

2.1.3. Real Time PCR

Technical Equipment

Equipment	Company
Densitometer	BioRad, München, Deutschland
Low Profile 96 Well PCR Plates	PEQLAB Biotechnologie GMBH; Erlangen, Deutschland
CFX96 Real Time System	BioRad, München, Deutschland
PCR Plate Sealing films / Foils	Biozym Biotch Trading GMBH

Chemicals

Product	Company
Chloroform	Carl Roth, Karlsruhe, Deutschland
DEPC Water	Promega, Mannheim, Deutschland
dNTP mix	Promega, Mannheim, Deutschland
Ethanol 99.7%	VWR, Darmstadt, Deutschland
2 X GoTaq®qPCR Master Mix	Promega, Hilden, Deutschland

PeqGOLD TriFast	Peqlab Biotechnologi GMBH, Erlangen, Deutschland
Primers	Invitrogen, Darmstadt, Deutschland
Transcriptor High Fidelity cDNA Synthsis Kit	Roche Diagnostics, Penzberg, Deutschland
2-Propanol (Isopropanol)	Sigma, Taufkirchen, Deutschland

Primers

Name	Company
NCX1, NCX3, NCKX1, NCKX2, NCKX5, NCKX6, GAPDH	Invitrogen, Darmstadt, Deutschland

2.2. Methods

2.2.1. Culture of Megakaryocytes

The source of Megakaryocytes (MEG-01) was the American Type Culture collection (ATCC). Human Megakaryocytes MEG-01 (ATCC® CRL-2021™) were cultured in cell culture flask. The medium was changed every third day. An RPMI-1640 (Roswell Park Memorial Institute) medium (Gibco, Carlsbad, Deutschland) containing 1% Penicillin/Streptomycin and 10% Fetal Bovin Serum (FBS) (Gibco, Carlsbad, Deutschland) was used. The cells were cultured in humidified atmosphere at 37°C with a consistent 5% CO2.

For experiments, Meg-01 cells were moved into 6-well plates (BD Biosciences, Franklin Lakes, NJ, USA) at a density of 2 × 105 cells/well.

In the first stage of the experiment, Concentration dependence and time dependence of TGFβ-stimulated Na^+/Ca^{2+} exchanger activity in megakaryocytes were detected. In order to detect the concentration dependence, the Meg-01 cells were categorized into a control group and other three groups. Untreated cells were used as controls. The other three groups were treated with 20, 40 or 60 ng/ml of TGFß1 (Sigma, Taufkirchen, Deutschland) for 24 hours. Then, the TGFß1 concentration of 60 ng/ml was adopted for next step of the experiment, as it showed significant upregulation of the activity of Na^+/Ca^{2+} exchangers after 24 hours.

In order to detect the time dependence of TGFβ-stimulated Na^+/Ca^{2+} exchanger activity in megakaryocytes for 60 ng/ml of TGFβ1, the Meg-01 cells were categorized into a control group and other three groups. Untreated cells were used as controls. The other three groups were treated with 60 ng/ml of TGFß1 for 6, 12 or 24 hours.

Treatment of Meg-01 cells with 60 ng/ml of TGFβ1 for 24 hours was adopted for the next stage of the experiment.

In the second stage of the experiment, the role of p38 kinase, SGK1 and NFκB in the regulation of the TGFß-induced activity and expression of Na^+/Ca^{2+} exchangers were detected.

In order to detect the role of p38 kinase, Meg-01 cells were distributed into three groups. The first group was treated with 60 ng/ml of TGFß1 for 24 hours. The second group was treated with 60 ng/ml of TGFß1 and p38 kinase inhibitor, Skepinone-L (1 μM, Merck) for 24 hours. Untreated Meg-01 cells were used as a control group.

The role of SGK1 was detected through making three groups of Meg-01 cells. The first group was treated with 60 ng/ml of TGFß1 for 24 hours. The second group was treated with 60 ng/ml of TGFß1 and SGK1 inhibitor, GSK-650394 (10 μM, Tocris) for 24 hours. Untreated Meg-01 cells were used as controls.

In order to detect the role of NFκB, Meg-01 cells were categorized into three groups. The first group was treated with 60 ng/ml of TGFß1 for 24 hours. The second group was treated with 60 ng/ml of TGFß1 and NFκB inhibitor, Wogonin (100 μM, Sigma) for 24 hours. Untreated Meg-01 cells were used as a control group.

2.2.2. Measurement of Intracellular Calcium

The utilization of Fura-2 fluorescent dye was required in order to determine the intracellular Ca^{+2} activity. The Meg01 cells were loaded with Fura-2/AM (2 μM, Invitrogen, Goettingen, Deutschland) and Thapsigargin (1 μM, Invitrogen) for 20-60 minutes at 37 °C.

At wavelengths of 340 nm and 380 nm alternatively the cells were excited through a fluorescence microscope (Axiovert 100, Zeiss, Oberkochen, Deutschland). A dichroic mirror deflected the light either to an objective (Fluor 40×/1.30 oil) or to a camera (Proxitronic, Bensheim, Deutschland). At a wavelength of 505 nm the emitted fluorescence intensity was recorded. A specialized computer software (Metafluor, Universal Imaging, Downingtown, USA) was used to acquire the data.

The removal of extracellular sodium would cause the cytosolic Ca^{+2} of the cell to be changed through the activity of Na^+/Ca^{+2} exchanger which gives the ability to estimate this activity using the calcium imaging technique. In order to achieve the removal of extracellular Sodium, two solutions are required. The first solution is a Standard Ringer containing Na^+ and the second solution is a ONa^+-Ringer Solution. In case of measuring the activity of potassium-dependent NCKX, both solutions should be also containing potassium K^+; but in case of measuring the activity of NCX both solutions should be 0 K^+.

In order to perform the experiment, the extracellular standard Na^+-containing solution was replaced by 0 Na^+-solution.

The standard ringer solution required to determine the activity of NCX is composed of (in mM): 130 NaCl, 0 KCl, 2 $MgCl_2$, 10 HEPES, 5 Glucose, pH 7.4. The Sodium-free ringer solution for NCX is composed of (in mM): 90 NMDG, 0 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 5 Glucose, pH 7.4.

In order to determine the activity of Potassium dependent Sodium/Calcium Exchanger NCKX, Na^+-containing standard ringer solution is required and it is composed of (in mM): 130 NaCl, 40 KCl, 20 TAE^+, 2 $MgSO_4$, 10 HEPES, 5 Glucose, pH 7.4 whereas the Na^+-free ringer solution is composed of (in mM): 90 NMDG, 40 KCl, 20 TAE^+, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 5 Glucose, pH 7.4.

For quantification of Ca^{+2} entry, the peak (delta ratio) and slope (delta ratio/s) were calculated subsequent to the removal of Na^+.

2.2.3. Real-Time PCR

The extraction of total RNA from MEG01 cells was carried out by TriFast (Peqlab, Erlangen, Deutschland) in correspondence to the procedures mentioned in the manufacturer’s protocol. DNAse digestion was performed and then it was followed by a reverse transcription of total RNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Penzberg, Deutschland). RT-PCR of the targeted sequences were constructed in a total volume of 20 µl using 40 ng of cDNA, 500 nM forward and reverse primer and 2x GoTaq® qPCR Master Mix (Promega, Hilden, Deutschland). The procedures were performed based on the manufacturer's instructions. For efficient amplification Cycling parameters were set as following: initial denaturation step was carried out at 95°C for 5 min, then 40 cycles of 95°C for 15 sec, 58°C for 15 sec and 68°C for 20 sec. The primers used for amplification were as following (5`->3`orientation):

for NCX1:
fw: ACAAGAGGTATCGAGCTGGC
rev: ATGCCATTTCTCGCCTAGC

for NCX3:
fw: GCATTGCCAGGGTCATTGTCT
rev: CCATAAGGGTCAGGTTGGAGA

for NCKX1:
fw: TCCACGCAGAAGATGGTG
rev: GTGATGGAGGGGATAGCG

for NCKX2:
fw: GAGACAGATACACAGAGCACAGG
rev: GAGAATAGTACAGATCACGCCC

for NCKX5:
fw: CTCCATCATCGGAGTTTCC
rev: CTTCCTACCCTCCCTGGAA

for NCKX6:
fw: CGTGCTGGTTACCACAGTGG
rev: CTTCCGTGGCAGGGTCAG

for GAPDH:
fw: TGAGTACGTCGTGGAGTCCAC
rev: GTGCTAAGCAGTTGGTGGTG

Melt curve analysis was performed to assess the specificity of PCR products.

CFX96 Real-Time System (Bio-Rad) was utilized to perform the Real-time PCR amplifications. All the experiments were performed in duplicate. GAPDH (Glyceraldehyd-3-phosphate-Dehydrogenase) was used as a reference gene; the amplification of this house-keeping gene served thus in the standardization of the amount of sample RNA. The previously described ΔCT method was used to carry out relative quantification of gene expression.

2.3. Statistical Analysis

By using paired or unpaired Student t-test and one-way ANOVA (Analysis of variance between groups), relative differences between results were tested for significance.

Data were provided as means ± SEM (standard error of the mean), n represents the number of independent experiments.

$P<0.05$ was considered statistically significant.

§/*($p<0.05$), §§/**($p<0.01$) or §§§/***($p<0.001$)

3. RESULTS

The aim of the present study was to explore the process by which the upregulation of Na^{+}/Ca^{+2} exchangers by TGFß1 is carried out.

3.1. Determining Concentration and Time Course for TGFβ1-Stimulated Na^{+}/Ca^{2+} Exchanger Activity in Megakaryocytes

In the first step of the experiment, the concentration dependence of TGFβ-stimulated Na^{+}/Ca^{+2} exchanger activity in megakaryocytes was detected, which required comparing the cytosolic Ca^{+2} activity ([Ca^{+2}]i) of untreated control MEG01 cells with other groups of MEG01 cells treated with different concentration of TGFß1, including: 20 ,40 or 60 ng/ml TGFß1.

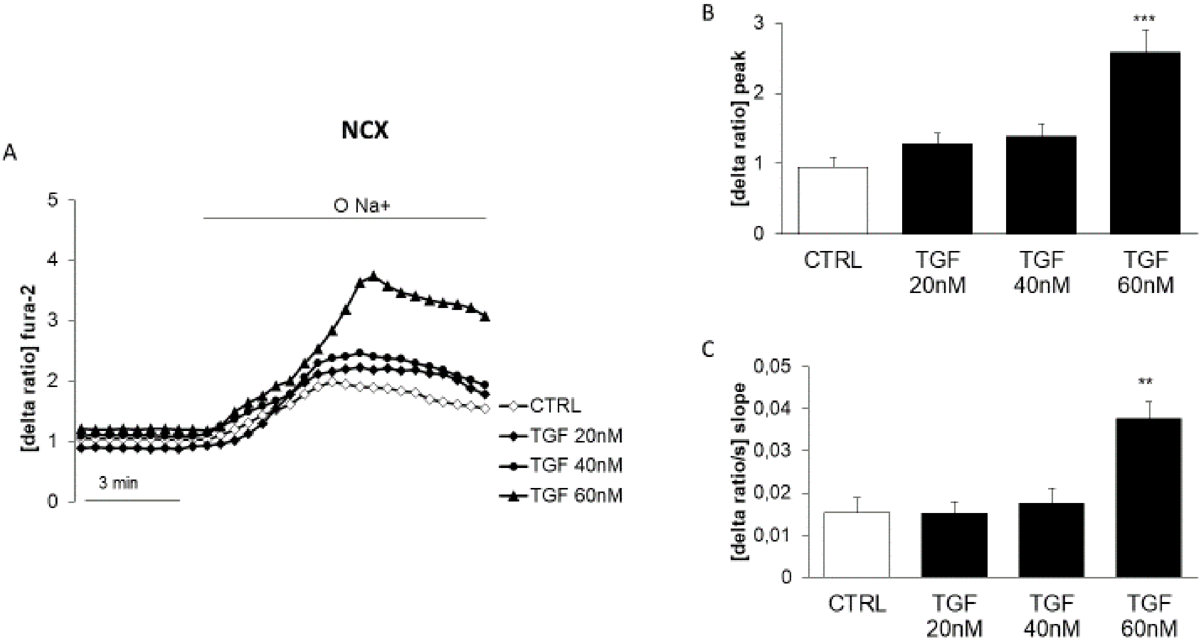

Figure. 3. TGFß1-induced Ca^{+2} entry mediated by NCX in megakaryocytes. (Al-Maghout, Pelzl et al. 2017)

A. Representative original tracings illustrating intracellular Ca^{+2} concentrations in megakaryocytes loaded in Fura-2/ AM for 20-60 minutes with and without a pretreatment of 20, 40 or 60 ng/ml TGFß1 for 24 hours before and after the removal of external Na^{+} (0 Na^{+}) and adding 2 mM Ca^{+2} at 0 mM K^{+}.

B. C. Arithmetic means (± SEM, n = 46 - 59 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{+2} concentrations following removal of external Na^{+} (0 Na^{+}) and adding 2 mM Ca^{+2} at 0 mM K^{+} in megakaryocytes with and without a pretreatment of 20, 40 or 60 ng/ml TGFß1 for 24 hours. ** ($p<0.01$), *** ($p<0.001$) refer to a statistically significant difference from untreated control group of megakaryocytes (ANOVA). (Al-Maghout, Pelzl et al. 2017)

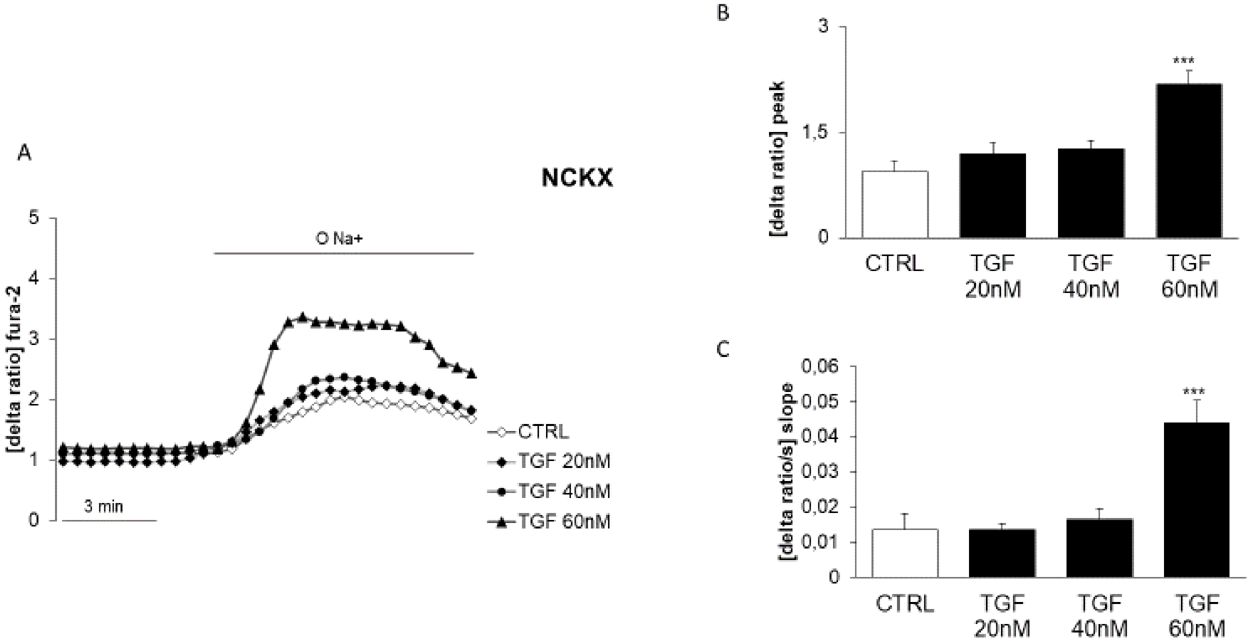

Figure. 4. TGFß1-induced Ca^{2+} entry mediated by NCKX in megakaryocytes. (Al-Maghout, Pelzl et al. 2017)
A. Representative original tracings illustrating intracellular Ca^{2+} concentrations in megakaryocytes loaded in Fura-2/ AM for 20-60 minutes with and without a pretreatment of 20, 40 or 60 ng/ml TGFß1 for 24 hours before and after the removal of external Na^{+} (0 Na^{+}) and adding 2 mM Ca^{2+} at 0 mM K^{+}. **B. C.** Arithmetic means (± SEM, n = 46 - 59 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations in megakaryocytes after removing extracellular Na^{+} (0 Na^{+}) and adding 2 mM Ca^{2+} at 40 mM K^{+} with and without a pretreatment of 20, 40 or 60 ng/ml TGFß1 for 24 hours. *** ($p<0.001$) refers to a statistically significant difference from untreated megakaryocytes (ANOVA). (Al-Maghout, Pelzl et al. 2017)

Fura-2 fluorescent dye was used to determine the cytosolic Ca^{+2} activity ([Ca^{+2}]i) in order to study the activity of Na^{+}/ Ca^{+2} exchanger in Megakaryocytes. The activity of this exchanger was able to be evaluated in each MEG01 treatment group and compared to other groups. The cytosolic Ca^{+2} activity was increased differently throughout the different treatment groups as a result of the activation of the reverse mode of Na^{+}/ Ca^{+2} exchanger which leads to an influx of the extracellular calcium into the cell. The activation of the reverse mode of the Na^{+}/ Ca^{+2} exchanger is carried out by replacing the Na^{+}-containing extracellular standard ringer solution with a 0Na^{+}-solution which in turn contains Ca^{+2}. This change in extracellular solution triggers the reverse mode of the Na^{+}/ Ca^{+2} exchanger. In order to selectively study NCX activity, the experiments were performed in the absence of extracellular K^{+}. As shown in Figure 3, only the Meg01 cells which were treated with 60 ng/ml TGFß1 demonstrated statistically significant increase of NCX activity compared to the control group, while treatment with 20 or 40 ng/ml TGFß1 didn't show significant increase in the activity of NCX.

In order to study the concentration dependence of TGFß1-stimulated activity of K^+-dependent Na^+/Ca^{2+} exchanger (NCKX), the change in cytosolic Ca^{2+} activity was studied after adding 40nM K^+ to both extracellular solutions used to trigger the reverse mode of Na^+/Ca^{2+} exchanger. Adding 40nM K^+ to both standard and $0Na^+$-solution served in providing the ability to detect the activity of NCKX and compare the cytosolic Ca^{2+} activity ($[Ca^{2+}]$i resulting from NCKX in different treatment groups of MEG01 cells. As shown in Figure 4, the Meg01 cells which were treated with 60 ng/ml TGFß1 were the only group that demonstrated statistically significant increase of K^+-dependent Na^+/Ca^{2+} exchanger NCKX activity compared to the control group. Treatment of MEG01 cells with 20 or 40 ng/ml TGFß1 didn't show significant increase in the activity of NCKX.

The second step of the study was to explore the time course of the TGFß1-stimulated Na^+/Ca^{2+} exchangers activity.

After studying the concentration dependence of TGFß1-stimulated Na^+/Ca^{2+} exchangers activity, the TGFß1 concentration of 60ng/mL was adopted for further investigations for both NCX and NCKX in Meg01 cells.

In order to study the time course of the effect of TGFß1, the cytosolic Ca^{2+} activity ($[Ca^{2+}]$i) of untreated control MEG01 cells was compared to $[Ca^{2+}]$i of other groups of MEG01 cells treated with 60ng/mL TGFß1 but for different periods of time, including: 6, 12 or 24 hours.

The activity of K^+-independent Na^+/Ca^{2+} exchangers (NCX) was explored by using 0 K^+ extracellular solutions when performing cytosolic calcium activity measurements. As shown in Figure 5, only the Meg01 cells which were treated with 60 ng/ml TGFß1 for 24 hours demonstrated statistically significant increase of NCX activity compared to the control group, while treatment with 60 ng/ml TGFß1 for 6 or 12 hours didn't show significant increase in the activity of K^+ independent Na^+/ Ca^{2+} exchanger NCX.

By using K^+-containing extracellular solution during the measurement of cytosolic Ca^{2+} activity ($[Ca^{2+}]$i , the time course of TGFß1-stimulated K^+-dependent Na^+/Ca^{2+} exchangers activity was elucidated.

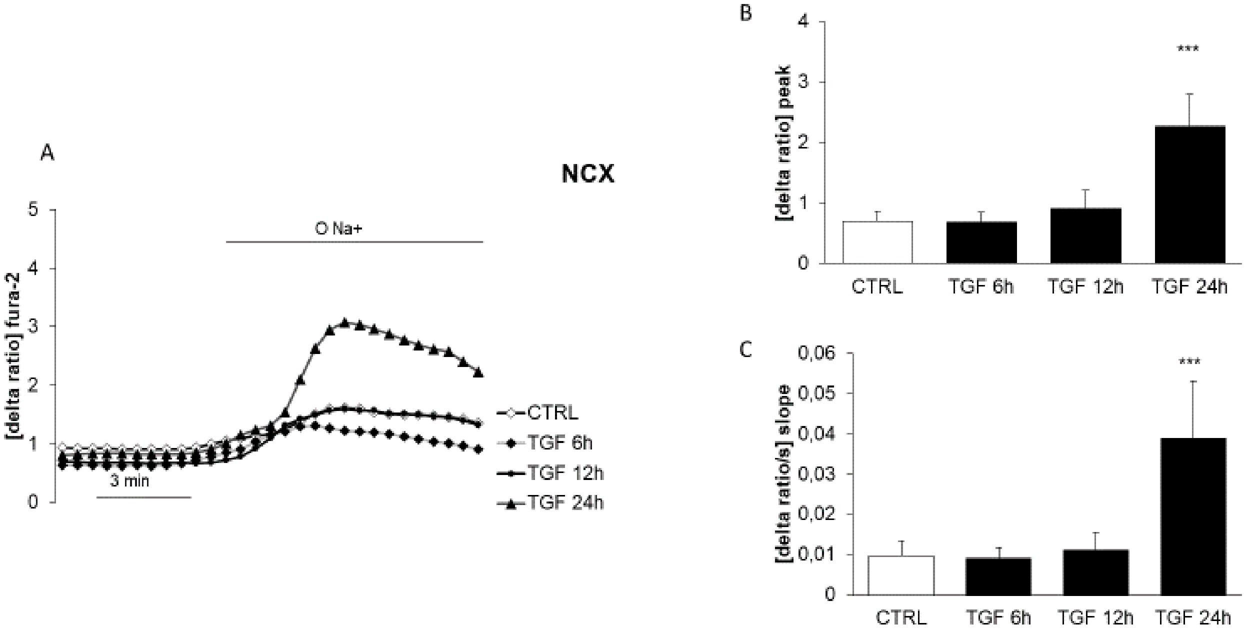

Figure. 5. Time dependence of TGFß1-stimulated increase of Ca^{2+} entry mediated by NCX in megakaryocytes. (Al-Maghout, Pelzl et al. 2017) **A.** Representative original tracings illustrating intracellular Ca^{2+} concentrations in megakaryocytes loaded in Fura-2/ AM for 20-60 minutes with and without a pretreatment of 60 ng/ml TGFß1 for 6, 12 or 24 hours before and after the removal of external Na^{+} (0 Na^{+}) and adding 2 mM Ca^{2+} at 0 mM K^{+}.
B, C. Arithmetic means (± SEM, n = 40 - 52 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations in megakaryocytes after removing extracellular Na^{+} (0 Na^{+}) and adding 2 mM Ca^{2+} at 0 mM K^{+} with and without a pretreatment of 60 ng/ml TGFß1 for 6, 12 or 24 hours. *** ($p<0.001$) refers to a statistically significant difference from untreated control group of megakaryocytes (ANOVA). (Al-Maghout, Pelzl et al. 2017)

As shown in Figure 6, the Meg01 cells which were treated with 60 ng/ml TGFß1 for 24 hours were the only group that demonstrated statistically significant increase of K^{+}-dependent Na^{+}/Ca^{2+} exchanger (NCKX) activity compared to the control group. Treatment of MEG01 cells with 60 ng/ml TGFß1 for 6 or 12 hours didn't show significant increase in the activity of NCKX.

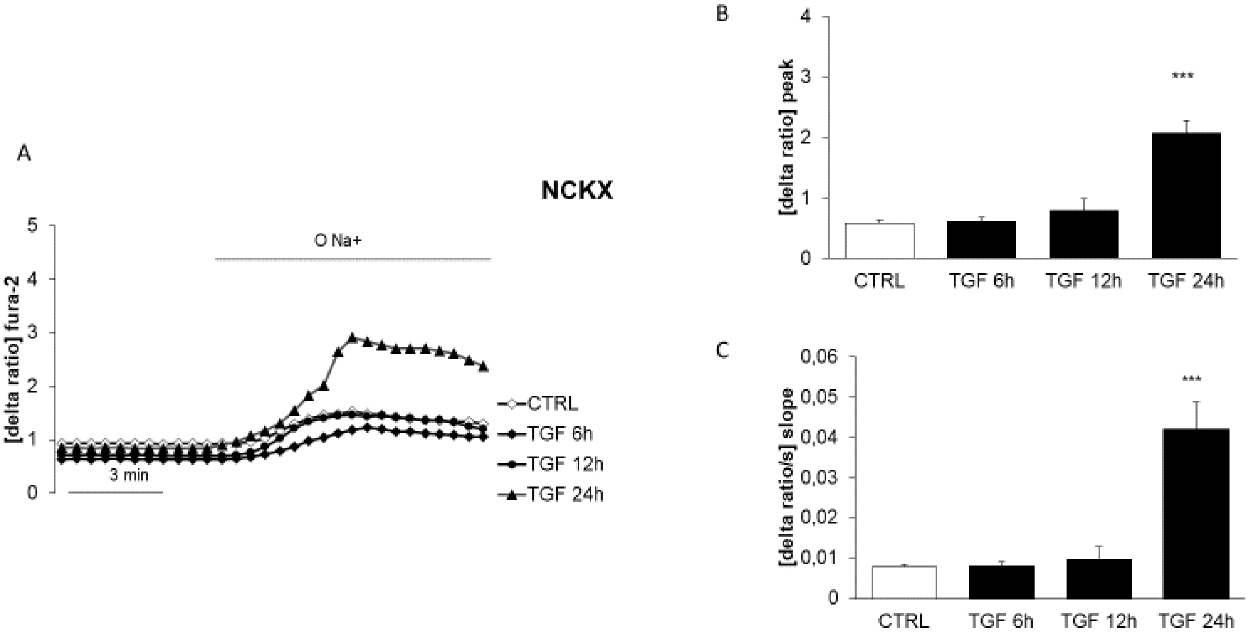

Figure. 6. Time dependence of TGFß1-stimulated increase of Ca^{2+} entry mediated by NCKX in megakaryocytes. (Al-Maghout, Pelzl et al. 2017)
A. Representative original tracings illustrating intracellular Ca^{2+} concentrations in megakaryocytes loaded in Fura-2/AM for 20-60 minutes with and without a pretreatment of 60 ng/ml TGFß1 for 6, 12 or 24 hours before and after the removal of external Na^{+} (0 Na^{+}) and adding 2 mM Ca^{2+} at 0 mM K^{+}.
B, C. Arithmetic means (± SEM, n = 40 - 52 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations in megakaryocytes after removal of external Na^{+} (0 Na^{+}) and adding 2 mM Ca^{2+} at 40 mM K^{+} with and without a pretreatment of 60 ng/ml TGFß1 for 6, 12 or 24 hours. *** ($p<0.001$) refers to a statistically significant difference from untreated control group of megakaryocytes (ANOVA) (Al-Maghout, Pelzl et al. 2017)

3.2. TGFß1 Increases the Expression of Certain NCX and NCKX Isoforms

After elucidating the concentration and time dependence of the TGFß1-induced activity of Na^{+}/Ca^{2+} exchangers, Treatment of Meg-01 cells with 60 ng/ml of TGFβ1 for 24 hours was adopted for further investigation of the isoforms and mechanisms involved TGFß1 activity.

RT-PCR was utilized in order to define the NCX and NCKX isoforms which undergoes upregulation of expression in response to pretreatment with TGFß1. As illustrated in Figure 7, the treatment of Meg01 cells by 60 ng/ml TGFß1 for 24 hours resulted in a significant stimulation of the expression of several NCX and NCKX isoforms, including: NCX1, NCKX1, NCKX2 and NCKX5.

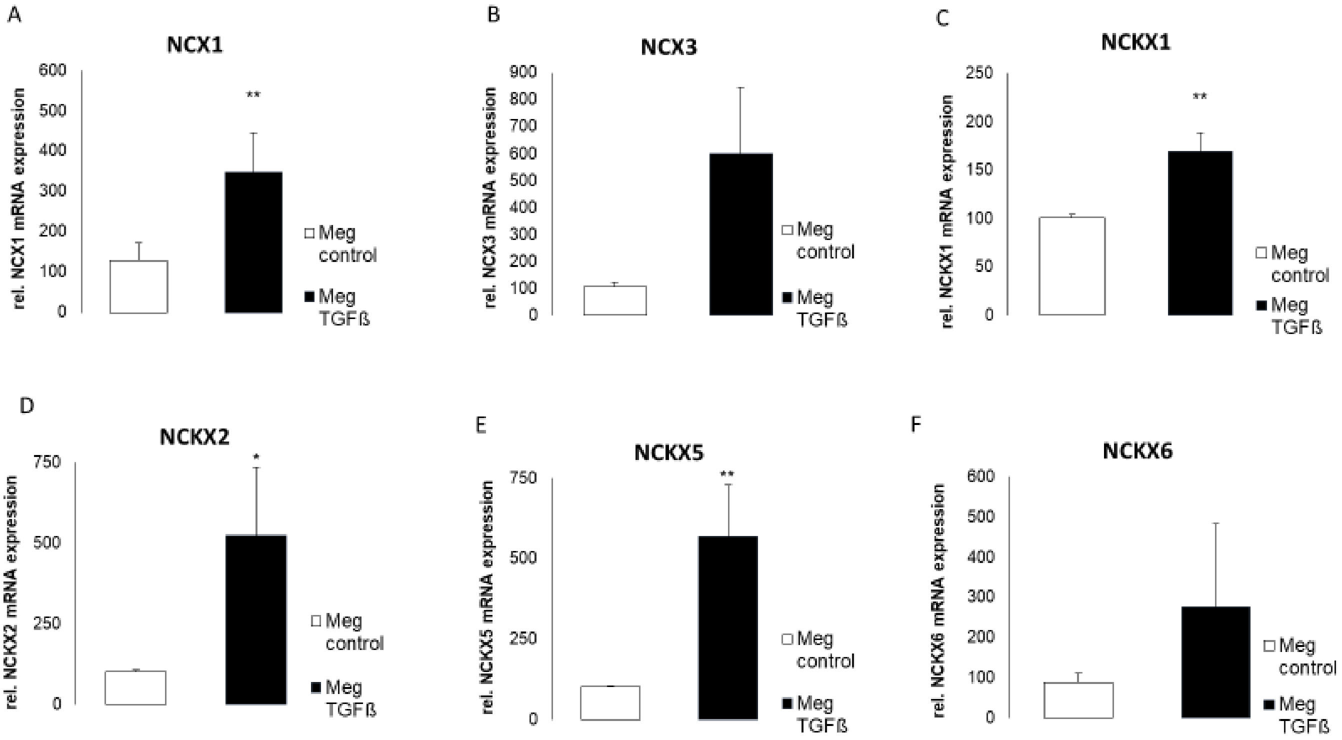

Figure. 7. TGFß1-induced expression of NCX and NCKX isoforms in megakaryocytes (Al-Maghout, Pelzl et al. 2017). A-F: Arithmetic means (± SEM, n = 5-11 preparations) of (A) NCX1, (B) NCX3, (C) NCKX1, (D) NCKX2, and (E) NCKX5, (F) NCKX6 over GAPDH transcript levels in megakaryocytes with and without a treatment of 60 ng/ml TGFß1 for 24 hours. *($p<0.05$), ** ($p<0.01$) refer to a statistically significant difference from untreated control group of megakaryocytes (student´s t-test) (Al-Maghout, Pelzl et al. 2017).

3.3. P38 Kinase Mediates the Role of TGFß1 in Upregulating Activity and Expression of Na^+/Ca^{2+} Exchangers

In order to detect the role of p38 kinase in the regulation of the TGFß1-induced activity and expression of Na^+/Ca^{2+} exchangers, Skepinone-L (1 µM), which is a p38 kinase inhibitor was utilized. TGFß1 activity in up-regulating Na$^+$/Ca^{2+} exchangers activity and transcript was observed with the presence or absence of p38 kinase inhibitor, Skepinone-L.

As shown in Figure 8, treatment with 60 ng/ml TGFß1 for 24 hours increased the expression of NCX1, NCKX1, NCKX2, and NCKX5 significantly in the absence of Skepinone-L; whereas in the presence of Skepinone-L, the expression of those isoforms was not upregulated, as Skepinone-L can, by inhibiting p38 kinase, preclude the role of TGFß1 in upregulating transcription levels of those isoforms.

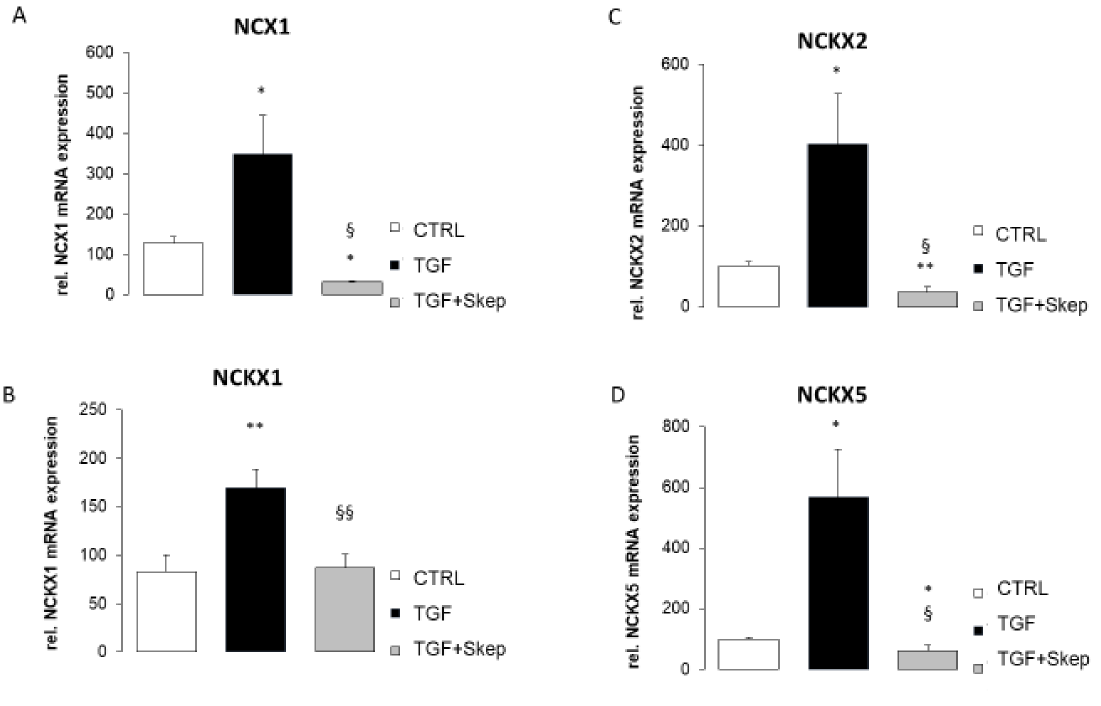

Figure. 8. Inhibition of p38 kinase by Skepinone-L abrogates the TGFß1-induced expression of NCX1, NCKX1, NCKX2, and NCKX5 isoforms in megakaryocytes (Al-Maghout, Pelzl et al. 2017). A-D: Arithmetic means (± SEM, n = 4-11 preparations) of (A) NCX1, (B) NCKX1, (C) NCKX2, and (D) NCKX5 over GAPDH transcript levels in megakaryocytes with and without a pretreatment with 60 ng/ml TGFß1 for 24 hours in the absence or presence of p38 kinase inhibitor Skepinone-L (1 µM). *(p<0.05), ** (p<0.01) refer to a statistically significant difference from untreated control group of megakaryocytes, § (p<0.05), §§ (p<0.01) refer to a statistically significant difference from megakaryocytes treated with TGFß1 alone (student´s t-test) (Al-Maghout, Pelzl et al. 2017).

The activity of K^+ independent Na^+/Ca^{2+} exchanger NCX was significantly increased as a result of the treatment with 60ng/ml TGFß1 for 24 hours, but in the presence of p38 kinase inhibitor Skepinone-L the effect of TGFß1 is abrogated. (Figure. 9).

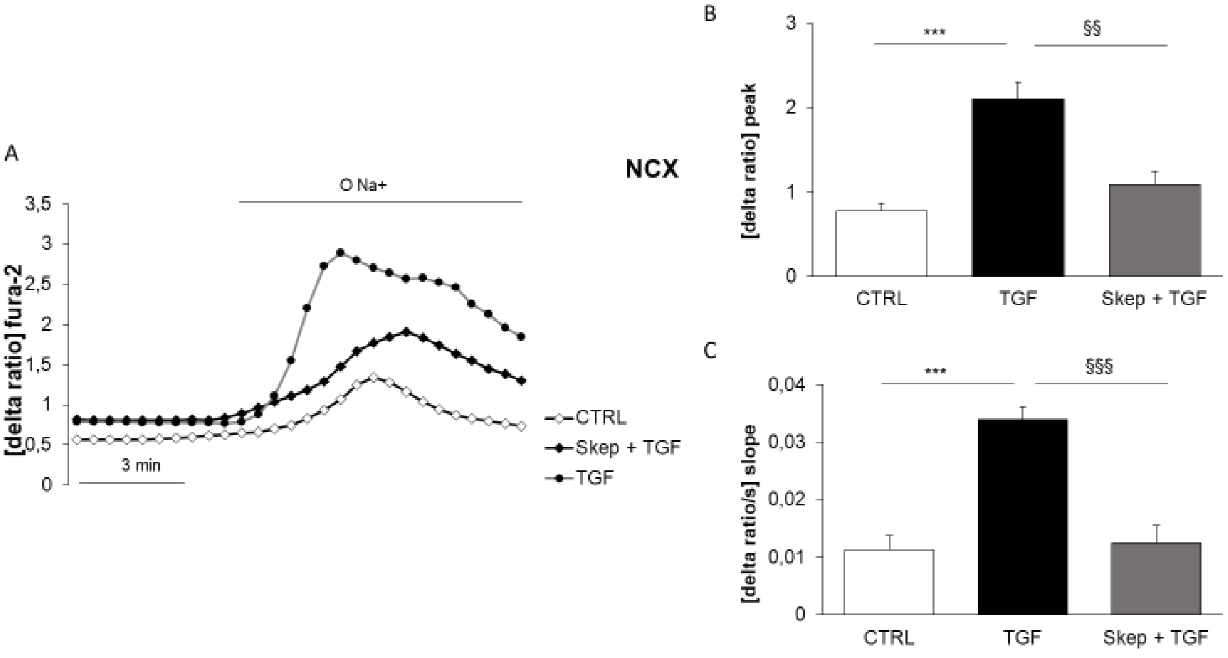

Figure. 9. Inhibition of p38 kinase by Skepinone-L abrogates the TGFß1-induced Ca^{2+} entry mediated by NCX in megakaryocytes (Al-Maghout, Pelzl et al. 2017).

A. Representative original tracings illustrating intracellular Ca^{2+} concentrations in megakaryocytes loaded in Fura-2/AM for 20-60 minutes with and without a pretreatment for 24 hours with 60 ng/ml TGFß1 alone or TGFß1 with p38 kinase inhibitor Skepinone-L (1 µM) before and after the removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+.

B, C. Arithmetic means (± SEM, n = 32 - 36 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations in megakaryocytes after removing external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+ with and without a pretreatment for 24 hours with 60 ng/ml TGFß1 alone or TGFß1 with p38 kinase inhibitor Skepinone-L (1 µM). *** ($p<0.001$) refers to a statistically significant difference from untreated control group of megakaryocytes (ANOVA), §§ ($p<0.01$), §§§ ($p<0.001$) refer to statistically significant difference from megakaryocytes treated with TGFß1 alone (ANOVA) (Al-Maghout, Pelzl et al. 2017).

The activity of K^+-dependent Na^+/Ca^{2+} exchanger NCKX was also significantly upregulated after 24 hours of treatment with 60ng/ml TGFß1, but in the presence of p38 kinase inhibitor Skepinone-L the effect of TGFß1 is abrogated on NCKX also. (Figure. 10).

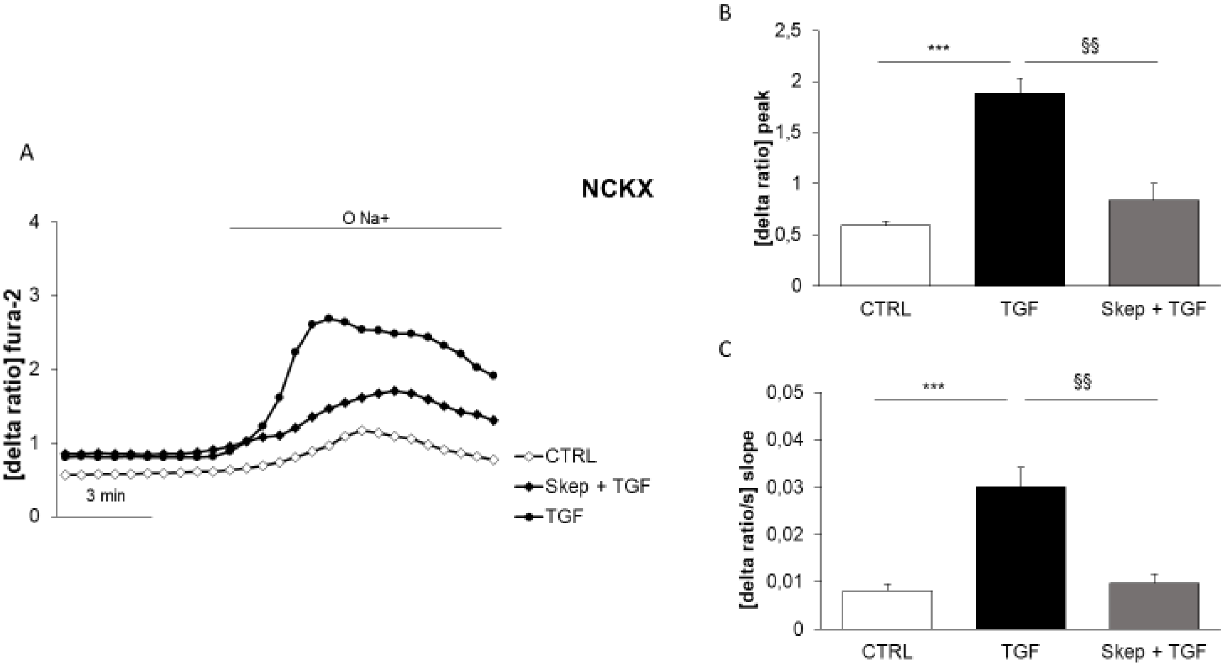

Figure. 10. Inhibition of p38 kinase by Skepinone-L abrogates the TGFß1-induced Ca^{2+} entry mediated by NCKX in megakaryocytes (Al-Maghout, Pelzl et al. 2017).

A. Representative original tracings illustrating intracellular Ca^{2+} concentrations in megakaryocytes loaded in Fura-2/AM for 20-60 minutes with and without a pretreatment for 24 hours with 60 ng/ml TGFß1 alone or TGFß1 with p38 kinase inhibitor Skepinone-L (1 µM) before and after the removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+.

B, C. Arithmetic means (± SEM, n = 32 - 36 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations in megakaryocytes after removing external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+ with and without a pretreatment for 24 hours with 60 ng/ml TGFß1 alone or TGFß1 with p38 kinase inhibitor Skepinone-L (1 µM). *** ($p<0.001$) refers to a statistically significant difference from untreated control group of megakaryocytes (ANOVA), §§ ($p<0.01$) refers to a statistically significant difference from megakaryocytes treated with TGFß1 alone (ANOVA) (Al-Maghout, Pelzl et al. 2017).

3.4. SGK1 Mediates the Role of TGFß1 in Upregulating the Activity of Na^+/Ca^{2+} Exchangers

In order to detect the role of SGK1 in the regulation of TGFß1-induced activity and expression of Na^+/Ca^{2+} exchangers, GSK-650394 (10 µM), which is an SGK1 inhibitor was utilized. TGFß1 activity in up-regulating Na^+/Ca^{2+} exchangers activity and transcript were observed with the absence or presence of SGK1 inhibitor GSK-650394.

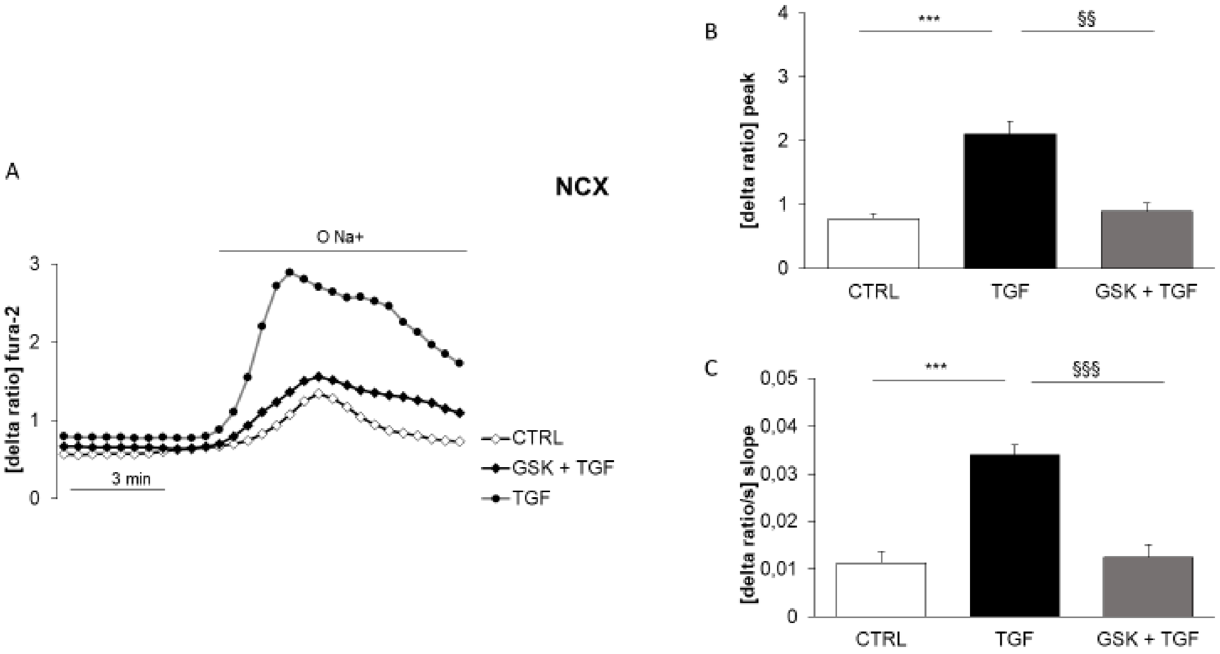

Figure. 11. Inhibition of SGK1 by GSK-650394 abrogates the TGFß1-induced Ca^{2+} entry mediated by NCX in megakaryocytes (Al-Maghout, Pelzl et al. 2017).

A. Representative original tracings illustrating intracellular Ca^{2+} concentrations in megakaryocytes loaded in Fura-2/ AM for 20-60 minutes with and without a pretreatment for 24 hours with 60 ng/ml TGFß1 alone or TGFß1 with SGK1 inhibitor GSK-650394 (10 µM) before and after the removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+.

B, C. Arithmetic means (± SEM, n = 34 - 36 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations in megakaryocytes after removing external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+ with and without a pretreatment for 24 hours with 60 ng/ml TGFß1 alone or TGFß1 with SGK1 inhibitor GSK-650394 (10 µM). *** ($p<0.001$) refers to a statistically significant difference from untreated control group of megakaryocytes (ANOVA), §§§ ($p<0.001$), §§ ($p<0.01$) refer to statistically significant difference from megakaryocytes treated with TGFß1 alone (Al-Maghout, Pelzl et al. 2017).

As illustrated in Fig. 11, Treatment of Meg01 cells with 60 ng/ml TGFß1 for 24 hours upregulated the activity of NCX significantly in the absence of SGK1 inhibitor GSK-650394; whereas TGFß1 in the presence of GSK-650394 (10 µM) couldn't upregulate the activity of NCX, as GSK-650394 can, by inhibiting SGK1, attenuate the role of TGFß1 in upregulating the activity of NCX. (Figure 11)

As shown in Fig. 12, the activity of K^+ dependent Na^+/Ca^{2+} exchanger NCKX was also significantly upregulated after 24 hours of treatment with 60 ng/ml TGFß1, but in the presence of SGK1 inhibitor GSK-650394 (10 µM), the effect of TGFß1 was abrogated on NCKX also.

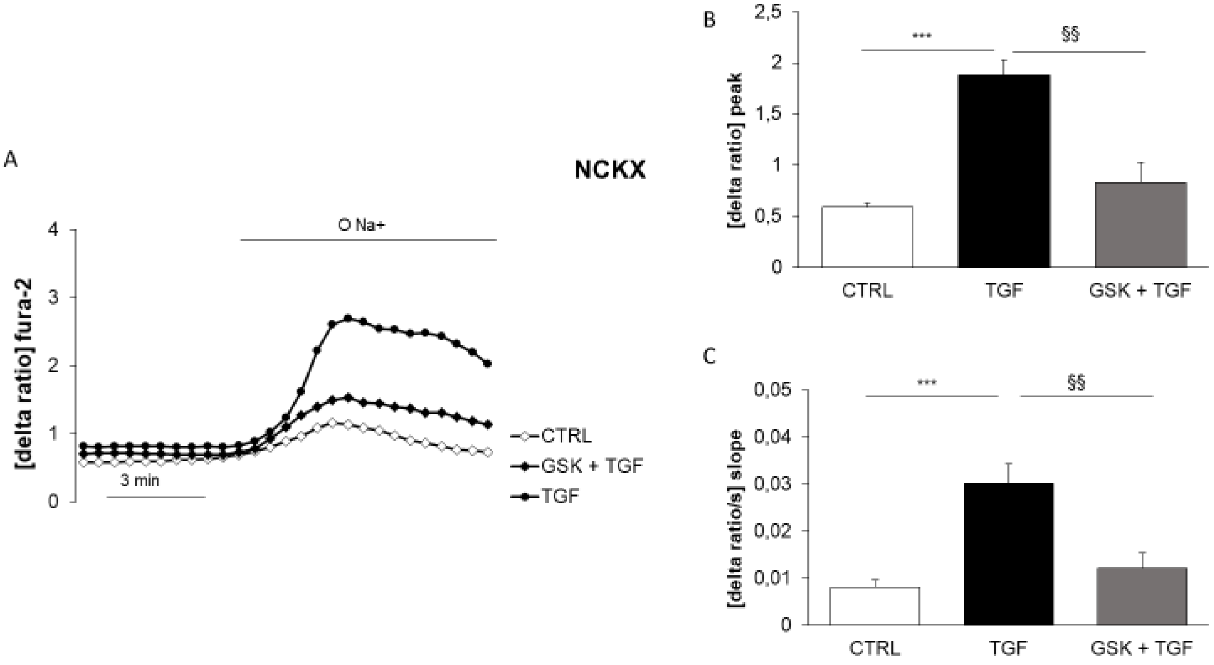

Figure. 12. Inhibition of SGK1 by GSK-650394 abrogates the TGFß1-induced Ca^{2+} entry mediated by NCKX in megakaryocytes (Al-Maghout, Pelzl et al. 2017). A. Representative original tracings illustrating intracellular Ca^{2+} concentrations in megakaryocytes loaded in Fura-2/ AM for 20-60 minutes with and without a pretreatment for 24 hours with 60 ng/ml TGFß1 alone or TGFß1 with SGK1 inhibitor GSK-650394 (10 µM) before and after the removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+. B, C. Arithmetic means (± SEM, n = 34 - 36 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations in megakaryocytes after removing external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+ with and without a pretreatment for 24 hours with 60 ng/ml TGFß1 alone or TGFß1 with SGK1 inhibitor GSK-650394 (10 µM). *** ($p<0.001$) refers to a statistically significant difference from untreated control group of megakaryocytes (ANOVA), §§ ($p<0.01$) refers to a statistically significant difference from megakaryocytes treated with TGFß1 alone (Al-Maghout, Pelzl et al. 2017).

Treatment with TGFß1 increased the transcript levels encoding NCX1, NCKX1, NCKX2, and NCKX5 significantly in the absence of SGK1 inhibitor, GSK-650394, as well as in the presence of GSK-650394 (10 μM). The role of TGFß1 in increasing the expression of specific isoforms of NCX and NCKX was not affected significantly by GSK-650394. The alteration of Na^+/Ca^{2+} exchanger activity caused by GSK-650394 was not aligned with a significant change in the transcript levels of genes encoding specific isoform of NCX and NCKX.

3.5. NFκB Mediates the Role of TGFß1 in Upregulating the Activity of Na^+/Ca^{2+} Exchangers

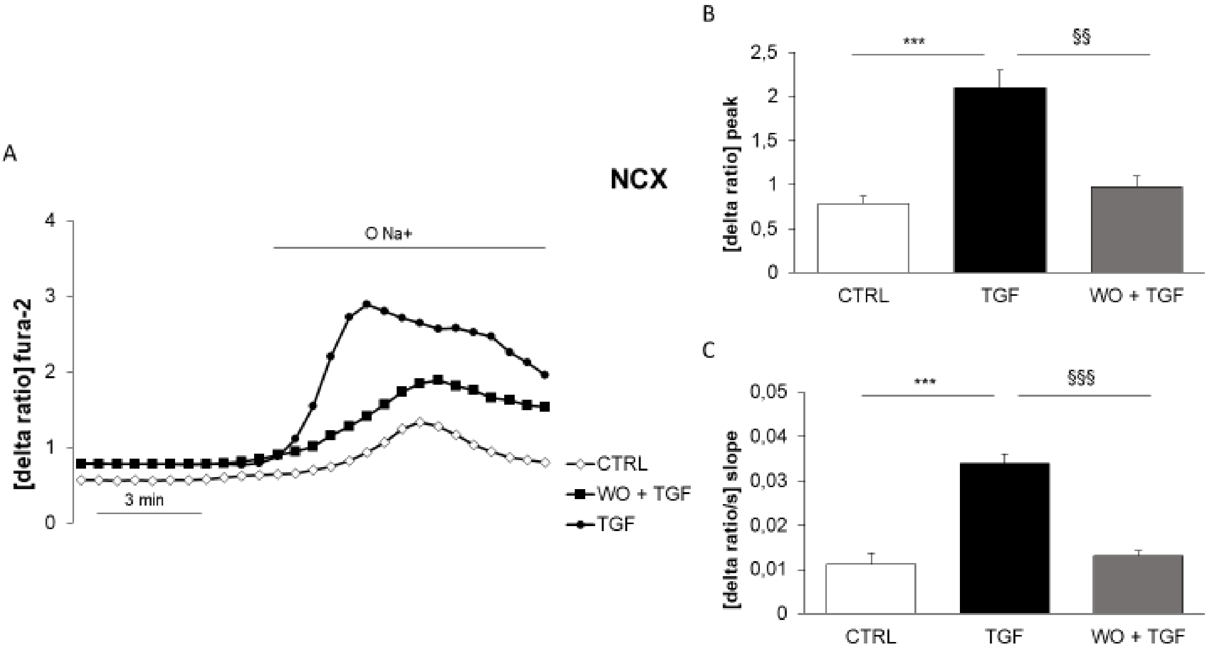

Figure. 13. Inhibition of NFκB by Wogonin abrogates the TGFß1-induced Ca^{2+} entry mediated by NCX in megakaryocytes (Al-Maghout, Pelzl et al. 2017).
A. Representative original tracings illustrating intracellular Ca^{2+} concentrations in megakaryocytes loaded in Fura-2/AM for 20-60 minutes with and without a pretreatment for 24 hours with 60 ng/ml TGFß1 alone or TGFß1 with NFκB inhibitor Wogonin (100 μM) before and after the removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+.
B,C. Arithmetic means (± SEM, n = 31 - 39 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations in megakaryocytes after removing external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 0 mM K^+ with and without a pretreatment for 24 hours with 60 ng/ml TGFß1 alone or TGFß1 with NFκB inhibitor Wogonin (100 μM). *** ($p<0.001$) refers to a statistically significant difference from untreated control group of megakaryocytes (ANOVA), §§ ($p<0.01$) §§§ ($p<0.001$) refer to statistically significant difference from megakaryocytes treated with TGFß1 alone (Al-Maghout, Pelzl et al. 2017).

Treatment of Meg01 cells with 60 ng/ml TGFß1 for 24 hours upregulated the activity of NCX significantly in the absence of NFκB inhibitor Wogonin; whereas TGFß1 in the presence of Wogonin couldn't upregulate the activity of NCX, as Wogonin can, by inhibiting NFκB, abrogate the role of TGFß1 in upregulating the activity of NCX. (Figure 13)

The activity of K^+-dependent Na^+/Ca^{2+} exchanger NCKX was also significantly upregulated after 24 hours of treatment with 60ng/ml TGFß1, but in the presence of NFκB inhibitor Wogonin, the effect of TGFß1 was abrogated on NCKX also. (Figure 14)

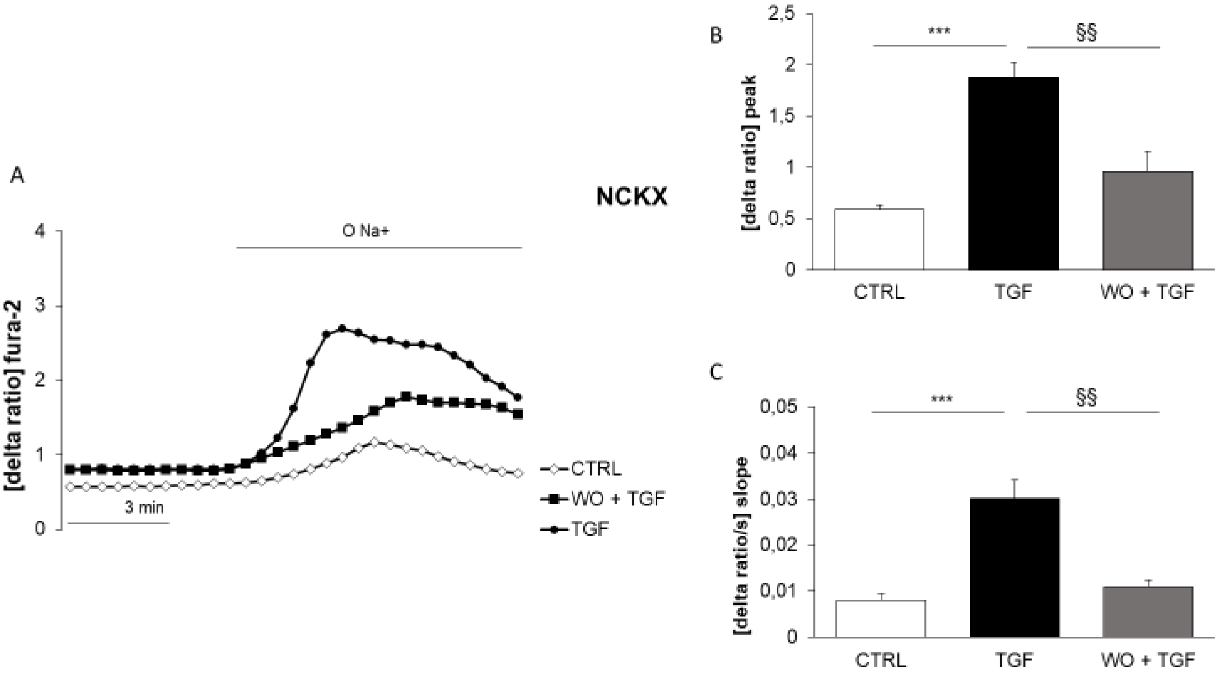

Figure. 14. Inhibition of NFκB by Wogonin abrogates the TGFß1-induced Ca^{2+} entry mediated by NCKX in megakaryocytes (Al-Maghout, Pelzl et al. 2017). **A.** Representative original tracings illustrating intracellular Ca^{2+} concentrations in megakaryocytes loaded in Fura-2/ AM for 20-60 minutes with and without a pretreatment for 24 hours with 60 ng/ml TGFß1 alone or TGFß1 with NFκB inhibitor Wogonin (100 µM) before and after the removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+. **B, C.** Arithmetic means (± SEM, n = 31 - 39 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations in megakaryocytes after removing external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} at 40 mM K^+ with and without a pretreatment for 24 hours with 60 ng/ml TGFß1 alone or TGFß1 with NFκB inhibitor Wogonin (100 µM). *** ($p<0.001$) refers to a statistically significant difference from untreated control group of megakaryocytes (ANOVA), §§ ($p<0.01$) refers to a statistically significant difference from megakaryocytes treated with TGFß1 alone (Al-Maghout, Pelzl et al. 2017).

Treatment with 60 ng/ml TGFß1 for 24 hours increased the transcript levels encoding NCX1, NCKX1, NCKX2, and NCKX5 significantly in the absence of NFκB inhibitor, Wogonin. In the presence of Wogonin (100 μM), the effect of TGFß1 in up-regulating the expression of specific isoforms of NCX and NCKX didn't show significant change. The change of Na^+/Ca^{2+} exchanger activity, in the presence of GSK-650394, was not aligned with a significant change in the transcript levels encoding NCX1, NCKX1, NCKX2, and NCKX5.

4. DISCUSSION

In the present study, TGFß1 is shown to be an effective activator of Na^+/Ca^{2+} exchangers in megakaryocytes. TGFß1 can upregulate Ca^{2+} entry or Ca^{2+} extrusion by Na^+/Ca^{2+} exchange, as the exchanger plays a role in the mobility of Ca2+ in both directions. Depending on the gradient of Ca^{2+} and Na^+, the Na^+/Ca^{2+} exchanger can carry out Ca^{2+} extrusion at high levels of intracellular Ca^{2+} and/or hyperpolarized cell membrane potential; and on the contrary, in cases of high levels of intracellular Na^+ and/or depolarized cell membrane the Na^+/Ca^{2+} exchanger can accomplish Ca^{2+} entry (Roberts, Matsuda et al. 2012, Pulcinelli, Trifiro et al. 2013, Shumilina, Nurbaeva et al. 2013). Na^+/Ca^{2+} exchanger activity in both directions can be upregulated by TGFß1 (Almilaji, Yan et al. 2016), TGFß1 has also a major role in regulating Ca^{2+} entry via Orai1 (Yan, Schmid et al. 2015).

TGFß1 can simultaneously activate Orai1 to carry out Ca^{2+} entry and stimulate Na^+/Ca^{2+} exchangers to extrude Ca^{2+}. Through this concurrent dual activation of Ca^{2+} entry and Ca^{2+} extrusion, TGFß1 may trigger Ca^{2+} oscillations [39] which in turn play a role in regulating a variety of cellular functions (Parekh and Penner 1997, Lang, Busch et al. 1998, Berridge, Bootman et al. 2003).

Ca^{2+} oscillations are also important for cell survival (Heise, Palme et al. 2010, Parkash and Asotra 2010) and during cell cycle (Steinhardt and Alderton 1988, Taylor, Zeng et al. 2008), as Ca^{2+} oscillations are important for the cell to develop into the synthesis *phase (S phase)* and the Metosis (M phase) of the cell cycle (Steinhardt and Alderton 1988, Taylor, Zeng et al. 2008). Both Ca^{2+} extrusion and Ca^{2+} entry are important for cell survival and development. The lack of Ca^{2+} extrusion leads to sustained increase of cytosolic Ca^{2+} activity as Ca^{2+} entry continues to increase intracellular Ca^{2+} levels which eventually stimulates apoptosis (Parekh and Penner 1997, Berridge, Lipp et al. 2000, Towhid, Schmidt et al. 2013).

In the present study, TGFß1 is found to up-regulate Na^+/Ca^{2+} exchangers through certain isoforms of NCX and NCKX. Not only the involved isoforms have been defined, but also the signaling pathway required for the accomplishment of the effect of TGFß1 has been explored.

The effect of TGFß1 is found to be abrogated by a variety of elements, including: Skepinone-L (p38 kinase inhibitor), GSK-650394 (SGK1 inhibitor) and Wogonin (NFκB inhibitor). The inhibition of p38 kinase, SGK1 or NFκB is found to lead to the inhibition of TGFß1-stimulated up-regulation of Na^+/Ca^{2+} exchangers. Thus, the effect of TGFß1 in this case is suggested to be carried out through the upregulation of p38 kinase, which leads to the up-regulation of SGK1 (Borst, Schmidt et al. 2012), what in turn is followed by an activation of nuclear factor NFκB (Borst, Schmidt et al. 2012, Eylenstein, Schmidt et al. 2012).

Through this mechanism TGFß1 can play a role in the direct regulation of intracellular Ca^{2+} activity in Megakaryocytes. In addition to this direct role, TGFß1 has also an indirect effect through its effect on Na^+/K^+ ATPase in megakaryocytes. TGFß1 can through upregulating Na^+/K^+ ATPase affect the activity of Na^+/Ca^{2+} exchangers indirectly and thus affect intracellular Ca^{2+} activity in Megakaryocytes indirectly, as up-regulating the Na^+/K^+ ATPase increases the Na^+ gradient and potential difference, what activates Na^+/Ca^{2+} exchangers and eventually leads to Ca^{2+} extrusion [57]. p38 kinase, SGK1 and NFκB are also involved in this effect of TGFß1 on Na^+/K^+ ATPase (Hosseinzadeh, Schmid et al. 2014).

TGFß1 is important for the maturation of megakaryocytes and the production of platelets (Sakamaki, Hirayama et al. 1999). The Megakaryocytic cell itself produce and release TGFß1 (Bock, Loch et al. 2005, Ponce, de Lourdes et al. 2012). Thrombopoietin, which plays a role in the regulation of platelet production, stimulates the expression of megakaryocytic TGFß receptors. The expression of thrombopoietin itself is stimulated by TGFß (Sakamaki, Hirayama et al. 1999). TGFß1 is thus a powerful regulator of megakaryopoiesis (Sakamaki, Hirayama et al. 1999) and excessive TGFß1 expression leads to myelofibrosis (Ponce, de Lourdes et al. 2012).

In addition to the role of TGFß1 in several cellular functions in megakaryocytes including proliferation, maturation and survival, the effect of TGFß1 involves also the enhancement of functions in platelets. This includes the role of TGFß1 in enhancing Ca^{2+} entry via Orai1 in platelets in addition to enhancing Ca^{2+} entry and extrusion via

Na^+/Ca^{2+} exchanger, which in turn boost the response of platelets for activators such as thrombin or collagen related peptide (Borst, Schmidt et al. 2012).

In conclusion, TGFß1 has a role in up-regulating certain isoforms of Na^+/Ca^{2+} exchanger in Megakaryocytes including NCX1, NCKX1, NCKX2 and NCKX5. TGFß1 plays a role in regulating the cytosolic calcium activity $[Ca^{2+}]$ in Megakaryocytes through a variety of mechanisms including upregulating Ca^{2+} extrusion by specific Na^+/Ca^{2+} exchanger isoforms besides upregulating store operated Ca^{2+} entry. p38 kinase, SGK1 and NFκB are involved in the mechanism of which TGFß1 regulates the function of and Na^+/Ca^{2+} exchange.

5. Summary

Transforming Growth Factor ß1 (TGFß1) is produced and released by many cell types as it is expressed in a wide range of tissues. The cells that produce TGFß1 tend to have specific receptors for TGFß1.

Megakaryocytes are one of those cells. TGFβ1 is produced and released by megakaryocytes, then it binds to its receptors on the membrane of Megakaryocytes and thus triggers signalling downstreams, and so TGFβ1 can be considered an autocrine regulator of megakaryocytes. The resulting effect of TGFβ1 on Megakaryocytes includes the activation of differentiation and eventually the formation of proplatelets.

The production of platelets by megakaryocytes is controlled by a group of environmental and autocrine elements. The autocrine factors can be represented basically in the release of TGFß1 and adenosine diphosphate by human megakaryocytes which lead to an increase in cytosolic calcium concentration in megakaryocyte itself.

Calcium release from intracellular stores and calcium entry from extracellular milieu into the cell are essential to the regulation of the functions of human megakaryocytes.

TGFβ1, which is a key regulator of megakaryocyte maturation and platelet formation, up-regulates both, store operated Ca^{2+} entry (SOCE) and Ca^{2+} extrusion by Na^{+}/Ca^{2+} exchangers. By upregulating SOCE, TGFβ1 thus enhances the increase of cytosolic Ca^{2+} activity ([Ca^{2+}]i) caused by the release of Ca^{2+} from intracellular stores and later TGFβ1 enhances the following decline of [Ca^{2+}]i by upregulating Na^{+}/Ca^{2+} exchangers.

P38 Mitogen-Activated Protein Kinase, Serum/Glucocorticoid Inducible kinase (SGK1) and Nuclear Factor κB (NFκB) are important components of the TGFβ1 signaling pathway, which controls a variety of cellular functions. This pathway plays an important role in differentiation and proliferation of different cell types.

The aim of the present study is to investigate whether P38 Kinase, SGK1 and NFκB play a role in the upregulation of Na^{+}/Ca^{2+} exchanger activity and expression in Megakaryocytes following treatment with TGFß1.

To conclude, TGFβ1 significantly upregulates transcription of certain isoforms of Na^{+}/Ca^{2+} exchanger, namely: NCX1, NCKX1, NCKX2, and NCKX5 and thus TGFβ1 upregulates Na^{+}/Ca^{2+} exchanger activity, an effect requiring p38 kinase, SGK1 and NFκB.

6. Publications

Inhibition of Lithium Sensitive Orai1/ STIM1 Expression and Store Operated Ca2+ Entry in Chorea-Acanthocytosis Neurons by NF-κB Inhibitor Wogonin. Sukkar B, Hauser S, Pelzl L, Hosseinzadeh Z, Sahu I, **Al-Maghout T**, Bhuyan AAM, Zacharopoulou N, Stournaras C, Schöls L, Lang F. Cell Physiol Biochem. 2018;51(1):278-289. doi: 10.1159/000495229. Epub 2018 Nov 19. PMID:30453283

Epigallocatechin-3-gallate (EGCG) up-regulates miR-15b expression thus attenuating store operated calcium entry (SOCE) into murine CD4$_+$ T cells and human leukaemic T cell lymphoblasts. Zhang S, **Al-Maghout T**, Bissinger R, Zeng N, Pelzl L, Salker MS, Cheng A, Singh Y, Lang F. Oncotarget. 2017 Aug 8;8(52):89500-89514. doi: 10.18632/oncotarget.20032. eCollection 2017 Oct 27.

P38 Kinase, SGK1 and NF-κB Dependent Up-Regulation of Na+/Ca2+ Exchanger Expression and Activity Following TGFß1 Treatment of Megakaryocytes. **Al-Maghout T**, Pelzl L, Sahu I, Sukkar B, Hosseinzadeh Z, Gutti R, Laufer S, Voelkl J, Pieske B, Gawaz M, Lang F. Cell Physiol Biochem. 2017;42(6):2169-2181. doi: 10.1159/000479992. Epub 2017 Aug 15.

Istaroxime Inhibits Motility and Down-Regulates Orai1 Expression, SOCE and FAK Phosphorylation in Prostate Cancer Cells. Stagno MJ, Zacharopoulou N, Bochem J, Tsapara A, Pelzl L, **Al-Maghout T**, Kallergi G, Alkahtani S, Alevizopoulos K, Dimas K, Calogeropoulou T, Warmann SW, Lang F, Schmid E, Stournaras C. Cell Physiol Biochem. 2017;42(4):1366-1376. doi: 10.1159/000479200. Epub 2017 Jul 14.

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7. Contributions

The present study describes the role of TGFβ1 in the regulation of Ca^{2+} signaling in Megakarycytes. The data included in this dissertation is mostly produced and collected from experiments I accomplished personally. Howerver, I obtained support from Basma Sukkar, Itishri Sahu and Lisann Pelzl in performing PCR and maintaining cultured megakaryocytic cells.

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ACKNOWLEDGMENTS

I would like to thank my supervisors **Prof. Dr. Florian Lang** and **Prof. Dr. Peter Ruth** for granting me the opportunity to carry out my PhD work in **Eberhard Karls Universität Tübingen**. I have been so lucky to have such supervisors who never hesitate to provide the patient guidance, heartful encouragement and invaluable advice whenever I need it throughout my time as a doctoral candidate in the **Institute of Physiology I**. I am thankful also to all **my colleagues** in the mentioned institute.

I am extremely thankful to the **German Academic Exchange Service - Deutscher Akademischer Austauschdienst (DAAD)** - for funding me during this work by granting a scholarship in their program: “Research Grants for Doctoral Candidates and Young Academics and Scientists (2014/15)”.

The deepest and warmest thanks are to my beloved wife **Hadil** and our little angel **Ellen** for being my strength throughout the time and for giving me motivation. You are the reason I am smiling today. Thank you for fulfilling my life.

My parents, without the guidance and affection of you, I couldn’t have achieved my goals. I have no words to explain how much I look up to you.