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Effect of TGFβ on calcium signaling in megakaryocytes

Jing Yan [a], Evi Schmid [a, b], Ahmad Almilaji [a], Ekaterina Shumilina [a], Oliver Borst [a, c], Stefan Laufer [d], Meinrad Gawaz [c], Florian Lang [a, *]

[a] Department of Physiology I, University of Tübingen, Tübingen, Germany
[b] Department of Pediatric Surgery and Pediatric Urology, University Children's Hospital Tübingen, Tübingen, Germany
[c] Department of Cardiology & Cardiovascular Medicine, University of Tübingen, Tübingen, Germany
[d] Department of Pharmacy, University of Tübingen, Tübingen, Germany

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ABSTRACT

TGFβ is a powerful regulator of megakaryocyte maturation and platelet formation. As previously shown for other cell types, TGFβ may up-regulate the expression of the serum & glucocorticoid inducible kinase SGK1, an effect requiring p38 kinase. SGK1 has in turn recently been shown to participate in the regulation of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) in megakaryocytes and platelets. SGK1 phosphorylates the IκB kinase (IKKα/β), which in turn phosphorylates the inhibitor protein IκBα resulting in nuclear translocation of nuclear factor NFκB. Genes up-regulated by NFκB include Orai1, the pore forming ion channel subunit accomplishing store operated Ca^{2+} entry (SOCE). The present study explored whether TGFβ influences Ca^{2+} signaling in megakaryocytes. $[Ca^{2+}]_i$ was determined by Fura-2 fluorescence and SOCE from the increase of $[Ca^{2+}]_i$ following re-addition of extracellular Ca^{2+} after store depletion by removal of extracellular Ca^{2+} and inhibition of the sarcoendoplasmatic Ca^{2+} ATPase (SERCA) with thapsigargin (1 μM). As a result, TGFβ (60 ng, 24 h) increased SOCE, an effect significantly blunted by p38 kinase inhibitor Skepinone-L (1 μM), SGK1 inhibitor EMD638683 (50 μM) and NFκB inhibitor wogonin (100 μM). In conclusion, TGFβ is a powerful regulator of store operated Ca^{2+} entry into megakaryocytes, an effect mediated by a signaling cascade involving p38 kinase, SGK1 and NFκB.

1. Introduction

Platelets are pivotal for primary hemostasis at sites of vascular injury and by the same token decisive for the development of acute thrombosis and thrombotic vascular occlusion [1]. Following activation platelets degranulate, participate in the triggering of the coagulation cascade, expose phosphatidylserine, aggregate and form thrombi [2]. Those functions depend on an increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [3,4], which results from stimulation of Ca^{2+} release from intracellular stores with subsequent stimulation of store operated calcium entry (SOCE) [5]. SOCE is accomplished by the Ca^{2+} permeable pore forming calcium release-activated channel (CRAC) moiety Orai1 (CRACM1) and its regulator stromal interaction molecule 1 (STIM1), which senses the Ca^{2+} content of the intracellular Ca^{2+} stores [6–8].

Orai1 abundance in platelets is up-regulated by phosphoinositide 3-kinase (PI3K) signaling [9,10]. Disruption of PI3K signaling thus compromises Ca^{2+} influx into platelets [11,12]. PI3K dependent signaling includes the serum- and glucocorticoid-inducible kinase 1 (SGK1) [13,14], which has recently been shown to be a powerful stimulator of Orai1 expression [15]. SGK1 is effective by phosphorylating and thus activating the IκB kinase (IKKα/β), which in turn phosphorylates the inhibitor protein IκBα resulting in nuclear translocation of nuclear factor NFκB [15,16].

SGK1 is strongly up-regulated by transforming growth factor TGFβ [17], which is effective by activating p38 kinase [18]. TGFβ is, on the other hand, a powerful inhibitor of megakaryocyte maturation [19].

The present study explored, whether TGFβ1 influences Ca^{2+} signaling in megakaryocytes.

* Corresponding author. Department of Physiology, University of Tübingen, Gmelinstr. 5, 72076 Tübingen, Germany. Fax: +49 7071 29 5618.
E-mail address: florian.lang@uni-tuebingen.de (F. Lang).

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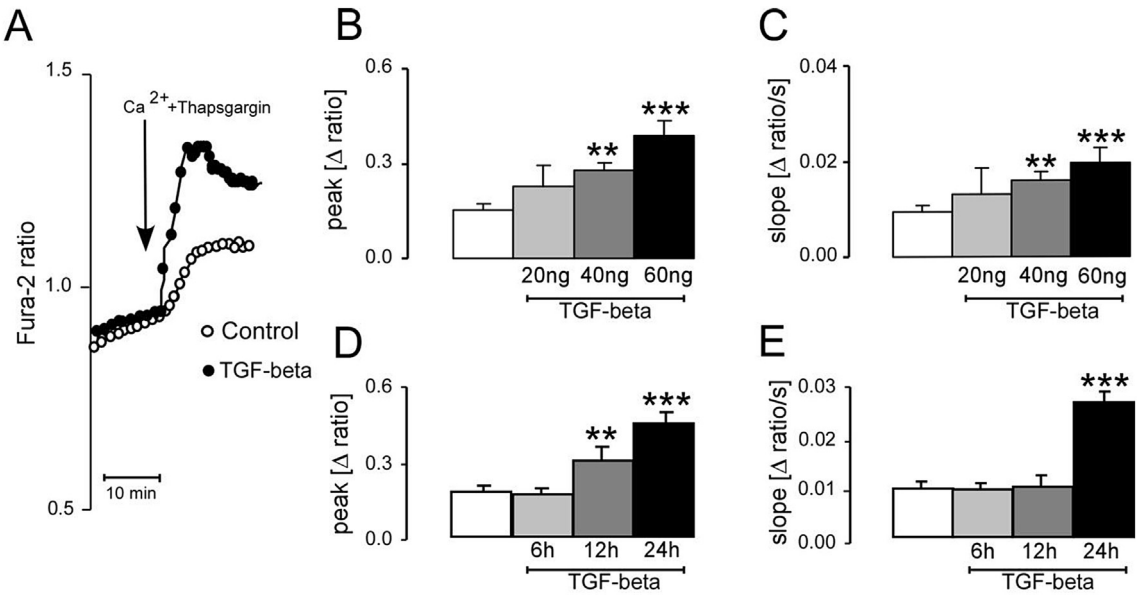

Fig. 1. TGFß1 sensitive store operated Ca^{2+} entry (SOCE) in megakaryocytes. **A**. Representative tracings of fura-2 fluorescence-ratio in megakaryocytes prior to, during and after extracellular Ca^{2+} removal and addition of sarcoendoplasmatic Ca^{2+} ATPase (SERCA) inhibitor thapsigargin (1 μM) without (white circles) or with (black circles) a prior 24 h treatment with 60 ng/ml TGFβ1. **B,C**. Arithmetic means (±SEM, n = 6, each experiment 15-12-10-10 cells) of peak (**B**) and slope (**C**) increase of fura-2-fluorescence-ratio in megakaryocytes without (white bars) and with (grey and black bars) a prior 24 h treatment with TGFβ1 (20–60 ng/ml). **D, E** Arithmetic means (±SEM, n = 5, each experiment 12-8-11-12 cells) of peak (**D**) and slope (**E**) increase of fura-2-fluorescence-ratio in megakaryocytes without (white bars) and with (grey and black bars) a prior 2–24 h treatment with TGFβ1 (60 ng/ml). **(p < 0.01), ***(p < 0.001) indicate statistically significant difference to respective value in absence of TGFβ1.

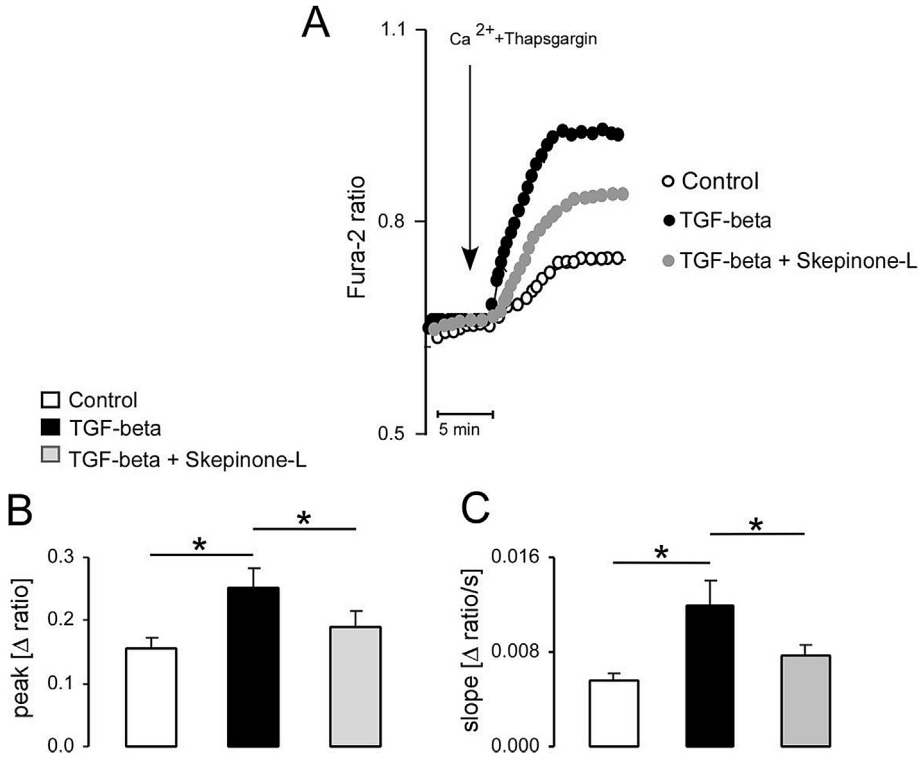

Fig. 2. Effect of TGFß1 on SOCE in megakaryocytes in absence and presence of p38 kinase inhibitor Skepinone-L. **A**. Representative tracings of fura-2 fluorescence-ratio in megakaryocytes prior to, during and after extracellular Ca^{2+} removal and addition of sarcoendoplasmatic Ca^{2+} ATPase (SERCA) inhibitor thapsigargin (1 μM) without (white circles) or with a prior 24 h treatment with 60 ng/ml TGFβ1 in the absence (black circles) and presence (grey circles) of p38 kinase inhibitor Skepinone-L (1 μM). **B,C**. Arithmetic means (±SEM, n = 4, each experiment 16-21-22 cells) of peak (**B**) and slope (**C**) increase of fura-2-fluorescence-ratio in megakaryocytes without (white bars) and with a 24 h treatment with TGFβ1 (60 ng/ml) in the absence (black bars) and presence (grey bars) of p38 kinase inhibitor Skepinone-L (1 μM). *(p < 0.05) indicate statistically significant difference.

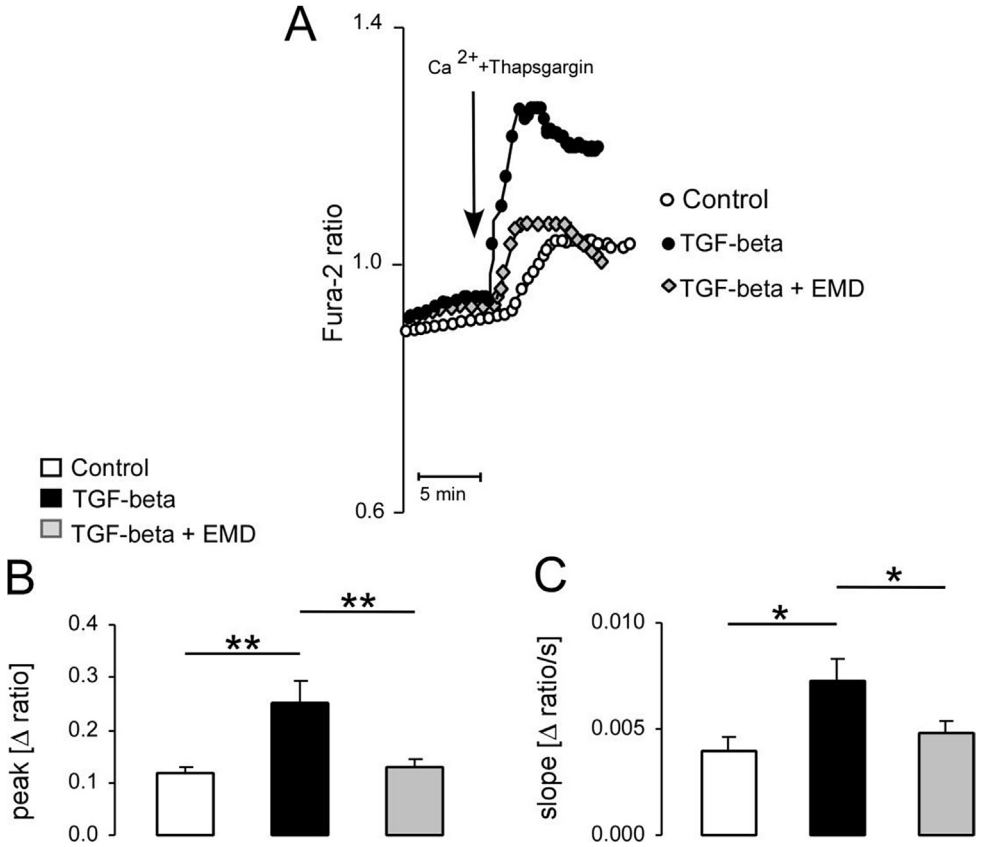

Fig. 3. Effect of TGFß1 on SOCE in megakaryocytes in absence and presence of SGK1 inhibitor EMD 638683. **A**. Representative tracings of fura-2 fluorescence-ratio in megakaryocytes prior to, during and after extracellular Ca^{2+} removal and addition of sarcoendoplasmatic Ca^{2+} ATPase (SERCA) inhibitor thapsigargin (1 μM) without (white circles) or with a prior 24 h treatment with 60 ng/ml TGFβ1 in the absence (black circles) and presence (grey circles) of SGK1 inhibitor EMD638683 (50 μM). **B,C**. Arithmetic means (±SEM, n = 4, each experiment 16-21-22 cells) of peak (**B**) and slope (**C**) increase of fura-2-fluorescence-ratio in megakaryocytes without (white bars) and with a 24 h treatment with TGFβ1 (60 ng/ml) in the absence (black bars) and presence (grey bars) of SGK1 inhibitor EMD638683 (50 μM). *($p < 0.05$), **($p < 0.01$) indicate statistically significant difference.

2. Materials and methods

2.1. Ethics

The work described in this manuscript has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and EU Directive 2010/63/EU for animal experiments.

2.2. Isolation and culture of murine megakaryocytes

For the isolation of murine megakaryocytes, bone marrow cells were harvested by flushing the femurs and tibiae with phosphate-buffered saline as described previously [20,21]. The obtained cells were separated over Percoll (GE Healthcare) and cultured in specific growth medium (MethoCult®, Stemcell) containing 50 ng/ml thrombopoietin (Invitrogen) as described previously [22]. After 5–7 days, differentiation into megakaryocytes was tested by microscopy and glyocoprotein Ib (GPIb) staining.

2.3. Calcium measurements

Fura-2/AM fluorescence was utilized to determine intracellular Ca^{2+} measurements [23]. Cells were loaded with Fura-2/AM (2 μM, Invitrogen, Goettingen, Germany) for 15 min at 37 °C. Cells were excited alternatively at 340 nm and 380 nm through an objective (Fluor 40 × /1.30 oil) built in an inverted phase-contrast microscope (Axiovert 100, Zeiss, Oberkochen, Germany). Emitted fluorescence intensity was recorded at 505 nm. Data were acquired using specialized computer software (Metafluor, Universal Imaging, Downingtown, USA). Cytosolic Ca^{2+} activity was estimated from the 340 nm/380 nm ratio. SOCE was determined by extracellular Ca^{2+} removal and subsequent Ca^{2+} re-addition in the presence of thapsigargin (1 μM, Invitrogen) [24]. For quantification of Ca^{2+} entry, the slope (delta ratio/s) and peak (delta ratio) were calculated following re-addition of Ca^{2+}. Experiments were performed with Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 $CaCl_2$, 2 Na_2HPO_4, 32 HEPES, 5 glucose, pH 7.4. To reach nominally Ca^{2+}-free conditions, experiments were performed using Ca^{2+}-free Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 Na_2HPO_4, 32 HEPES, 0.5 EGTA, 5 glucose, pH 7.4. The changes in $[Ca^{2+}]_i$ upon removal of extracellular Na^+ were taken as measure of Na^+/Ca^{2+} exchange. N-methyl-D-glucamine (NMDG) was used to replace Na^+. The Na^+-standard and Na^+-free solution contained either 5 mM or 40 mM KCl. Experiments were performed with Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 $CaCl_2$, 2 Na_2HPO_4, 32 HEPES, 5 glucose (pH 7.4). To measure Na^+/Ca^{2+} exchange the standard solution contained (in mM): 130 or 90 NaCl, 5 or 40 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 5 glucose, pH 7.4, and the Na^+- free solution contained (in mM): 130 or 90 NMDG, 5 or 40 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 5 glucose, pH 7.4.

2.4. Statistical analysis

Data are provided as means ± SD or SEM, *n* represents the number of experiments. All data were tested for significance using paired or unpaired Student t-test and one-way ANOVA with

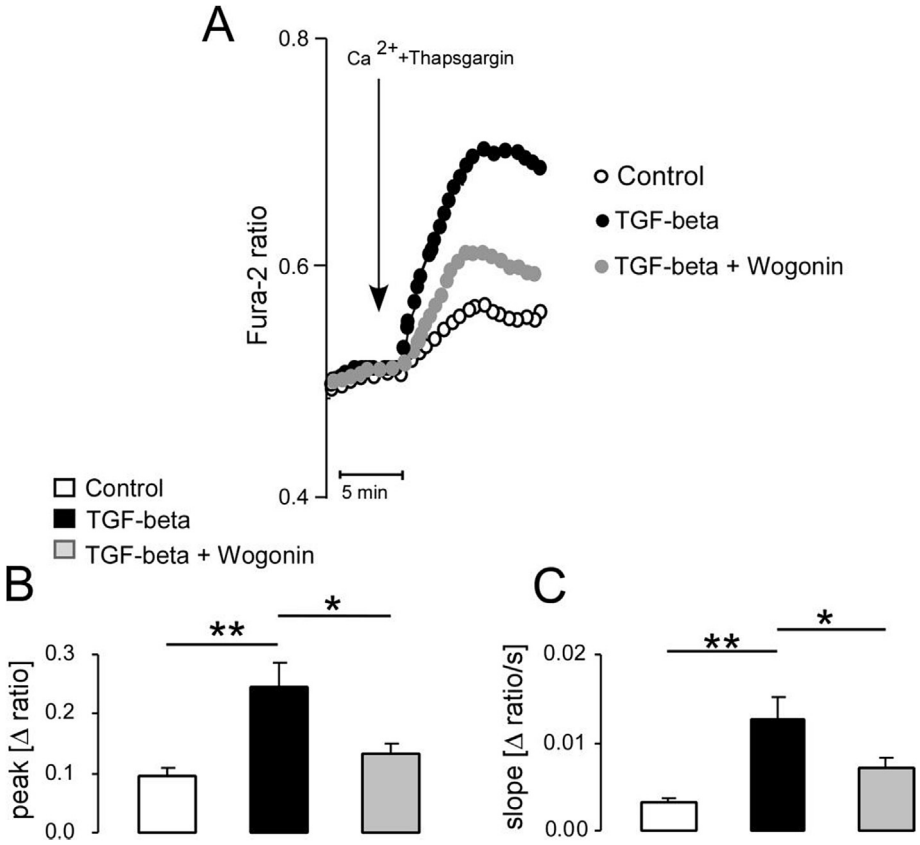

Fig. 4. Effect of TGFß1 on SOCE in megakaryocytes in absence and presence of NFκB inhibitor wogonin. **A**. Representative tracings of fura-2 fluorescence-ratio in megakaryocytes prior to, during and after extracellular Ca^{2+} removal and addition of sarcoendoplasmatic Ca^{2+} ATPase (SERCA) inhibitor thapsigargin (1 μM) without (white circles) or with a prior 24 h treatment with 60 ng/ml TGFβ1 in the absence (black circles) and presence (grey circles) of NFκB inhibitor wogonin (100 μM). **B,C**. Arithmetic means (±SEM, n = 4, each experiment 16-21-22 cells) of peak (**B**) and slope (**C**) increase of fura-2-fluorescence-ratio in megakaryocytes without (white bars) and with a 24 h treatment with TGFβ1 (60 ng/ml) in the absence (black bars) and presence (grey bars) of NFκB inhibitor wogonin (100 μM). *($p < 0.05$), **($p < 0.01$) indicate statistically significant difference.

Dunnets post-hoc test. Results with *($p < 0.05$), **($p < 0.01$) or ***($p < 0.001$) were considered statistically significant.

3. Results

The present study addressed the impact of TGFβ1 on Ca^{2+} signaling in megakaryocytes. To this end, cytosolic Ca^{2+} activity was determined utilizing Fura-2 fluorescence. Intracellular Ca^{2+} stores were emptied by removal of extracellular Ca^{2+} and addition of sarcoendoplasmatic Ca^{2+} ATPase (SERCA) inhibitor thapsigargin (1 μM). Store operated Ca^{2+} entry (SOCE) was quantified by subsequent re-addition of extracellular Ca^{2+}. As illustrated in Fig. 1, both slope and peak of SOCE were increased by a 24 h pretreatment of megakaryocytes with TGFβ1, an effect reaching statistical significance at 40 ng/ml TGFβ1 (Fig. 1B and C). Pretreatment of megakaryocytes with 60 ng/ml TGFβ1 resulted in a statistically significant increase of peak within 12 h (Fig. 1D) and of slope within 24 h (Fig. 1E).

TGFβ1 is known to activate p38 [18]. The involvement of the p38 kinase in the up-regulation of SOCE following TGFβ1 treatment of megakaryocytes was tested by application of the p38 kinase inhibitor Skepinone-L [25]. As illustrated in Fig. 2A–C, the up-regulation of both, slope and peak, by TGFβ1 pretreatment was significantly blunted in the presence of p38 kinase inhibitor Skepinone-L.

TGFβ1 strongly up-regulates the serum and glucocorticoid inducible kinase SGK1 [17], a powerful stimulator of SOCE [15]. The involvement of SGK1 in the up-regulation of SOCE following TGFβ1 treatment of megakaryocytes was tested by application of the SGK1 inhibitor EMD638683 (50 μM). As illustrated in Fig. 3A–C, the up-regulation of both, slope and peak, by TGFβ1 pretreatment was significantly blunted in the presence of SGK1 inhibitor EMD638683 (50 μM).

The effect of SGK1 on Orai1 and SOCE has previously been shown to be mediated at least in part by phosphorylation of the inhibitor protein IκBα leading to activation of nuclear factor NFκB [15,16]. The involvement of NFκB in the up-regulation of SOCE following TGFβ1 treatment of megakaryocytes was tested by application of the NFκB inhibitor wogonin (100 μM). As illustrated in Fig. 4A–C, the up-regulation of both, slope and peak, by TGFβ1 pretreatment was significantly blunted in the presence of NFκB inhibitor wogonin (100 μM).

4. Discussion

The present study discloses a completely novel function of TGFβ1, i.e. the up-regulation of store operated Ca^{2+} entry into megakaryocytes. The effect of TGFβ1 is virtually abrogated in the presence of p38 kinase inhibitor Skepinone-L, of SGK1 inhibitor EMD638683 and of NFκB inhibitor wogonin. TGFβ1 is thus presumably effective by upregulating p38 kinase [18] with subsequent up-regulation of SGK1 [15], which in turn activates nuclear factor NFκB [15,16]. Nuclear factor NFκB has previously been shown to stimulate the expression of pore forming calcium release-activated

channel (CRAC) moiety Orai1 (CRACM1) [15,16], which accomplishes store operated Ca^{2+} entry in a wide variety of cells [5] including platelets [6–8] and megakaryocytes [15].

An effect of TGFβ1 on SOCE has been shown in two other cell types [26,27], but to the best of our knowledge, an effect of TGFβ1 on Ca^{2+} entry into megakaryocytes or platelets has never been shown.

The NFκB dependent up-regulation of SOCE is at least in part due to stimulation of Orai1 expression [15,16]. The enhanced expression of Orai1 in megakaryocytes leads to increased Orai1 abundance in platelets [15]. Thus, activation of megakaryocytes with TGFβ1 yields platelets with enhanced Orai1 abundance, Ca^{2+} entry and thus exaggerated response to activators such as thrombin or collagen related peptide [15].

Besides its effect on SOCE, TGFβ1 up-regulates the Na^+/K^+ ATPase in megakaryocytes [28], an effect similarly involving p38 kinase, SGK1 and NF-κB [28]. Up-regulation of Na^+/K^+ ATPase is expected to decrease cytosolic Na^+ activity, which, at least in theory, could lower cytosolic Ca^{2+} concentration under resting conditions.

TGFβ1 is produced by megakaryocytes [29,30] and is thus an autokrine regulator of megakaryocytes. TGFβ1 is required for regulation of megakaryocyte maturation and platelet formation [19] and is the most powerful stimulator of bone marrow stromal thrombopoietin expression [19]. Thrombopoietin in turn stimulates the megakaryocytic expression of TGF-beta receptors [19]. TGF-beta1 is thus a feedback regulator of megakaryopoiesis [19]. Excessive expression of TGFβ1 leads to myelofibrosis [30]. To which extent the up-regulation of SOCE contributes to the TGFβ1 sensitive regulation of megakaryocyte survival, maturation or function remains to be clarified.

In conclusion, TGFβ1 is a powerful stimulator of store operated Ca^{2+} entry (SOCE) into megakaryocytes. Accordingly, activation of SOCE is an integral element in the regulation of megakaryocyte function.

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Authorship contributions

JY, ES, and AA performed experiments and analyzed data; OB and ES analysed data, FL and MG designed research, FL drafted the manuscript. All authors corrected and approved the manuscript.

Conflict of interest

The authors state that they have no conflict of interest.

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