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# Calcium Signaling in Platelet Migration

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# Contents

# Abbreviation

| | |
|---|---|
| ACD | Acid citrate dextrose |
| ADP | Adenosine diphosphate |
| AM | Acetoxymethyl |
| ANOVA | Analysis of variance |
| ATP | Adenosine triphosphate |
| BK | Big conductance calcium activated potassium channels |
| BTP2 | N-[4-[3,5-Bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl]-4-methyl-1,2,3-thiadiazole-5-carboxamide |
| CCD | Charged couple device |
| CRP | C-reactive protein |
| CXCR4 | C-X-C chemokine receptor type 4 |
| DAG | 1,2-idacyl-glycerol |
| $ddH_2O$ | Distilled water |
| DIC | Differential interference contrast |
| DMSO | Dimethyl sulfoxide |
| EACA | Epsilon-aminocaproic acid |
| EGTA | Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid |
| eNaC/DEG | Epithelial sodium channel/degenerin |
| ER | Endoplasmic reticulum |
| ESCs | Embryonic stem cells |
| FACS | Fluorescence activated cell sorting |
| Fbg | Fibrinogen |
| FcR | Fc receptor |
| FITC | Fluorescein isothiocyanate |
| fMLP | N-formyl-methionyl-leucyl-phenylalanine |
| GP | Glycoprotein |
| GPCR | G protein-coupled receptors |
| GsMTx4 | Grammostola spatulata mechano-toxin 4 |
| HMSCs | Human mesenchymal stem cells |
| HSA | Human serum albumin |

| | |
|---|---|
| $IC_{50}$ | Half maximal inhibitory concentration |
| Ig | Immunoglobulins |
| IK | Intermediate conductance calcium activated potassium channels |
| $IP_3$ | Inositol-1,4,5-trisphosphate |
| $K_{2p}$ | Two pore domain potassium channels |
| KCa | Calcium activated potassium channels |
| KO | Knockout |
| LPS | Lipopolysaccharide |
| Min | Minute |
| MS | Mechanosensitive ion channels |
| MW | Molecular weight |
| NA | Numerical aperture |
| PARs | Protease activated receptors |
| PF4 | Platelet factor 4 |
| PH | Phase contrast |
| $PIP_2$ | Phosphoinositide-4,5-bisphosphate |
| PLC | Phospholipase C |
| PLL | Poly-L-lysine |
| PLL-g-PEG | Poly (L-lysine)-graft-poly (ethylene glycol) |
| PMCA | Plasma membrane $Ca^{2+}$-ATPase |
| PPP | Platelet poor plasma |
| $PGI_2$ | Prostaglandin $I_2$ sodium salt |
| PRP | Platelet rich plasma |
| PS | Phosphatidylserine |
| RAG | Recombination activation gene |
| ROI | Area of interest |
| RT | Room temperature |
| SD | Standard deviation |
| SDF-1 | Stromal cell-derived factor 1 |
| Sec | Second |
| SEM | Standard error of mean |
| SERCA | Sarco/endoplasmic reticulum $Ca^{2+}$-ATPase |

| | |
|---|---|
| SK | Small conductance calcium activated potassium channels |
| SOCE | Store-operated calcium entry |
| STIM1 | Stromal interaction molecule 1 |
| TRAAK | TWIK open by arachidonic acid |
| TREK-1 | TWIK-1 related K channel |
| TRP | Transient receptor potential channels |
| TRPC | Transient receptor potential canonical |
| TRPM | Transient receptor potential melastatin |
| TRPV | Transient receptor potential vanilloid |
| TWIK | Tandem of P domains in a weakly inward-rectifying potassium channel |
| vWF | Von Willebrand Factor |
| WT | Wild type |

# List of tables

# List of figures

# Abstract

Platelets are anucleated cell fragments, generated from megakaryocytes in the bone marrow and cleared in the spleen and liver. They are central players in haemostasis and thrombosis. Once the vessel wall is injured, circulating platelets are attached to the exposed sub endothelial matrix via glycoproteins receptors on the membrane, aggregate and form a hemostatic plug. Although the role of platelets in haemostasis and thrombosis has been intensely investigated, the cellular biology of platelets motility remains elusive. More recently, Gaertner et al. in our group provided the first in depth analysis of the biomechanical principles underlying platelet migration. Although these studies show that mouse platelets migrate autonomously in vivo and reveal the characteristics of human platelets motility in vitro, how mouse platelets migrate in vitro has not been addressed. On the basis of this work, my experiments demonstrate that similar to human platelets, mouse platelets are able to migrate within mouse plasma in vitro. Furthermore, Gaertner et al. identified that albumin in the plasma was an essential factor for human platelet migration on fibrinogen substrate by reducing substrate adhesiveness. However, my studies show that unlike human platelets, albumin alone is not capable of supporting mouse platelets motility. Instead, casein, poly lysine-albumin conjugates and pluronic trigger mouse platelet migration by regulating calcium oscillations with the involvement of mechanosensitive ion channels. Interestingly, these proteins -- casein, PLL-g-PEG, and pluroinc -- do not affect platelets aggregation or secretion. Then by investigating the role of calcium ion channels in platelets function, my results reveal that platelet migration and aggregation are dependent on distinct calcium entry channels on plasma membrane. STIM-mediated SOCE is important in platelet aggregation and secretion but not in platelet migration; on the contrary, mechanosensitive ion channels are critically implicated in platelet motility but not in platelet aggregation or secretion. In summary, my studies demonstrate that mouse and human platelets need different requirements for migration, which shed light to disparities between human and mouse platelets. Then my results reveal that platelets aggregation and migration are mediated by different calcium ion channels. These findings provide a deeper understanding of the cellular mechanisms involved in platelets function.

# 1 Introduction

## 1.1 Platelets in haemostasis and thrombosis

Human platelets are anucleated cell fragments with 2-3 µm diameters in circulating blood, generated from megakaryocytes in the bone marrow with an average life span of 10 days and cleared in the spleen and liver (Kaplan and Saba 1978, Patel, Hartwig et al. 2005). Platelets are central players in haemostasis and thrombosis. Resting disc-like platelets are rolling in the direction of blood flow in the vessel. Once the vessel wall is injured, circulating platelets are attached to the exposed sub endothelial matrix via glycoproteins receptors on the membrane. Ligands binding initially trigger platelets activation, which cause cytoskeleton re-arrangement and extensive formation of pseudopodia originating from the plasma membrane (Jurk and Kehrel 2005, Ghoshal and Bhattacharyya 2014). Activated platelets release components from granules in a process called platelet degranulation. Platelets have two major granules, namely $\alpha$ granules and dense granules that are comprised of biologically active molecules (Rendu and Brohard-Bohn 2001). The more prevalent $\alpha$ granules secret proteins like P selectin, integrin, vWF, fibrinogen, fibronectin, platelet factor that are involved in the coagulation cascades (Blair and Flaumenhaft 2009). Dense granules release serotonin, calcium, ADP and ATP (McNicol and Israels 1999). It is notable that although ADP is a weak agonist, it triggers platelets shape change, granule release and amplifies the activation cascade (Kahner, Shankar et al. 2006). These secretion events in turn attract more circulating platelets for activation and adhesion at the site of injury.

Following platelets stimulation, the major platelets receptor GPIIbIIIa (integrin $\alpha_{IIb}\beta_3$) binds to fibrinogen in plasma, which leads to platelets aggregation and form a hemostatic plug. For quite a long time fibrinogen was considered to be essential for platelets aggregation. However, more substantial studies demonstrate fibrinogen-independent way of platelets aggregation exists (Ni, Denis et al. 2000, Jackson 2007, Hou, Carrim et al. 2015). Besides their critical roles in aggregation, platelets contribute to coagulation pathway. Activated platelets express a negatively charged surface phosphatidylserine (PS), which increases thrombin generation and harbours the coagulation factors (Lentz 2003). Thrombin is a serine protease that converts fibrinogen

to fibrin that is the end product of coagulation. At the same time, thrombin robustly stimulates platelets via protease activated receptors (PARs) and provides a positive feedback for blood coagulation (Coughlin 2000).

The process of haemostasis is beneficial for the body to prevent blood loss. However, when it occurs in ruptured arterial lesion, platelets aggregation and blood coagulation cause thrombus formation and subsequent vessel occlusion that is the main reason of heart attack and stroke (Packham 1994).

## 1.2 Platelet migration

Although platelets have been demonstrated to play crucial roles in haemostasis and thrombosis, platelets function on a cellular level are still not completely understood. For example the fundamental question that whether platelets have the ability to migrate autonomously still needs to be solved in detail. In 1970s, Lowenhaupt et al. first discovered that human platelet motility in vitro was an active biological process instead of passive diffusion or Brownian movement. Within autologous platelet poor plasma as medium platelets were capable of migrating from packed capillary tube toward collagen, indicating the chemotactic response of platelets (Lowenhaupt, Miller et al. 1973). Later on, the authors revealed that anticoagulants, temperature and pH could modulate platelet motility. Furthermore, platelet migration was impaired by metabolic inhibitor and actin polymerization inhibitor, providing more convincing evidence that platelet movement was an autonomous process (Lowenhaupt, Glueck et al. 1977). Duquesnoy et al. and Kakoma et al. reported that platelet migration from capillary tube was inhibited by sera from patients of idiopathic thrombocytopenic purpura (ITP) and sera from dog infected with Ehrlichia, respectively (Duquesnoy, Lorentsen et al. 1975, Kakoma, Carson et al. 1978). In addition, Valone et al. investigated the optimal conditions for random platelet locomotion using a Boden chamber and demonstrated that platelet spontaneous mobility was increased by the cholinomimetic agent carbachol, but inhibited by the adrenergic agonist-epinephrine (Valone, Austen et al. 1974).

In 1990s, the physiological significance of platelet migration in vivo was emerging. Feng et al. reported that after injection of N-formyl-methionyl-leucyl-phenylalanine (fMLP) in guinea pig skin, individual intact platelets crossed undamaged vascular endothelium by engulfing into large endothelial vacuoles. Subsequently, these platelets

moved across the basal lamina toward dermal connective tissue without displaying secretory morphological changes (Feng, Nagy et al. 1998). Nakamura et al. found that in mice treated with lipopolysaccharide (LPS) platelets translocated from sinusoidal into Disse spaces and some of them entered into hepatocytes with the aid of Kupffer cells (Endo and Nakamura 1992, Nakamura, Shibazaki et al. 1998). Later, Pitchford et al. discovered that platelets migrated extravascularly into lung parenchyma in allergen-sensitized mice via IgE--FcεRIγ receptor mediated mechanism in vivo. Moreover, platelets from allergen-sensitized mice or patients with allergic asthma migrated in vitro toward the relevant allergen or anti-IgE antibodies (Pitchford, Momi et al. 2008). Czapiga et al. reported that human platelets expressed functional formyl peptide receptor (FPR), and exhibited chemotactic migration toward formyl peptides released from necrotic cells (Czapiga, Gao et al. 2005). Recently, Kraemer et al. discovered stromal cell-derived factor 1 (SDF-1) induced chemotaxis of platelets via C-X-C chemokine receptor type 4 (CXCR4), a process dependent on phosphoinositol 3-kinase ($PI_3$ kinase) (Kraemer, Borst et al. 2010). Schmidt et al. identified that Oria1 and $Ca^{2+}$ activated $K^+$ channels were involved in SDF-induced platelet chemotaxis (Schmidt, Munzer et al. 2011). More recently, Gaertner et al. in our group provided the first in depth analysis of the biomechanical principles underlying platelet migration. By using two-photon microscopy, Gaertner et al. established a novel approach to visualize individual platelets during thrombus formation and discovered autonomous platelets locomotion in vivo. Then in vitro studies reveal that platelets polarize, form half-moon like shape and adapt the typical pattern of cell migration. Furthermore, Gaertner et al. found that platelet migration was integrin $\alpha_{IIb}\beta_3$ dependent and identified that albumin and calcium in the plasma were essential factors for human platelet migration on fibrinogen substrate (Gärtner, Engelhardt et al. 2015).

## 1.3 Calcium homeostasis in platelets

Calcium as a ubiquitous second messenger play crucial roles in a large variety of biological activities (Berridge, Bootman et al. 2003, Clapham 2007). Although various agonists, such as collagen (Roberts, McNicol et al. 2004), thrombin (Heemskerk, Feijge et al. 1997), ADP (Daniel, Dangelmaier et al. 1998), thromboxane $A_2$ (Paul, Jin

et al. 1999) evoke platelets activation through different pathways, they all cause elevation of cytoplasmic calcium ( $[Ca^{2+}]_i$). The increase of intracellular calcium derives from two sources, the release of calcium from internal pools, and calcium influx from extracellular department (Rink and Sage 1990, Varga-Szabo, Braun et al. 2009). Agonist-induced stimulation of various receptors results in activation of phospholipase C (PLC) isoforms, which hydrolyse phosphoinositide-4, 5-bisphosphate ($PIP_2$) to inositol-1, 4, 5-trisphosphate ($IP_3$) and 1, 2-idacyl-glycerol (DAG). $IP_3$ directly activates $IP_3$ receptors ($IP_3R$) in the endoplasmic reticulum (ER), which triggers calcium release from ER. Meanwhile, exogenous calcium influx into platelets is mediated by calcium ion channels on the plasma membrane. To maintain the equilibrium of cytoplasmic calcium, $[Ca^{2+}]_i$ is pumped back by the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) (Enouf, Bredoux et al. 1992, Cavallini, Coassin et al. 1995) to the ER, and by plasma membrane $Ca^{2+}$-ATPase (PMCA) (Dean, Chen et al. 1997, Paszty, Kovacs et al. 1998) to the extracellular space (Fig.1.1).

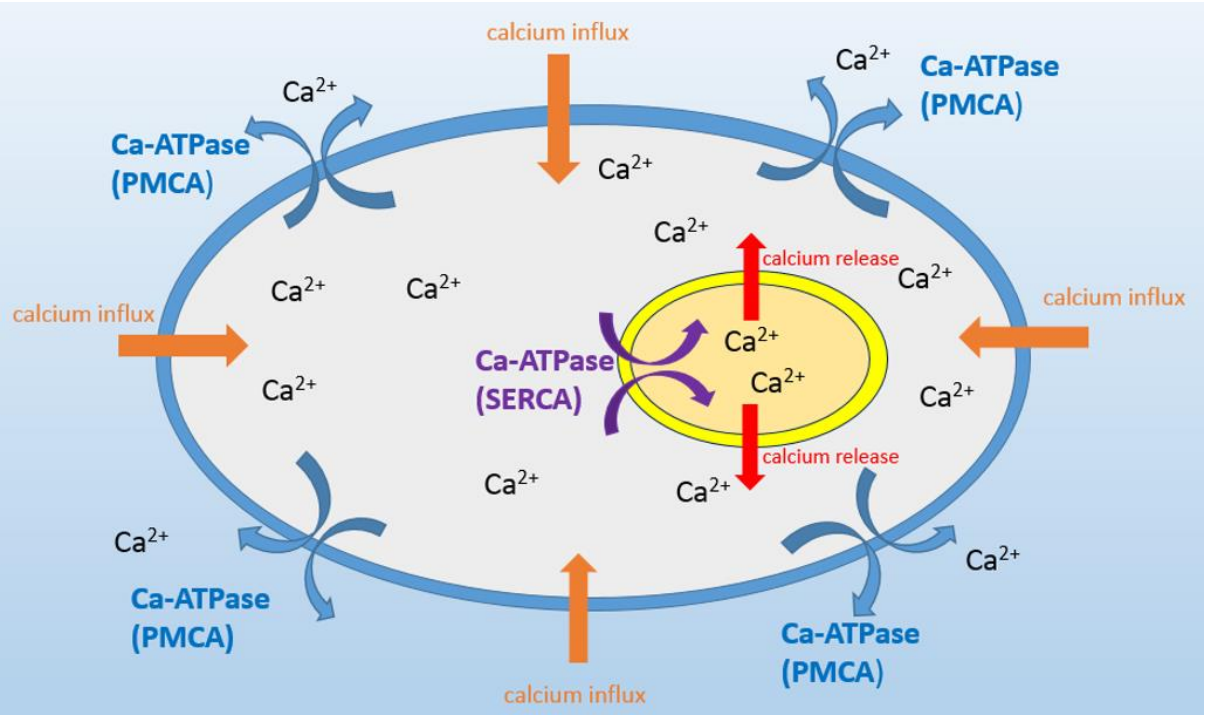


**Fig. 1.1 Schematic of calcium homeostasis in platelets.** The release of calcium from internal pools and calcium influx from outside department cause the elevation of cytoplasmic calcium. The elevated cytoplasmic calcium is pumped out by SERCA and PMCA to keep the equilibrium of intracellular calcium.

## 1.4 Calcium channels in platelets membrane

Extracellular calcium enters into platelets via calcium ion channels on the plasma membrane. A variety of ion channels are reported to be expressed in platelets, although the evidence for some of these channels are relied upon a small number of studies (Mahaut-Smith 2012, Wright, Amisten et al. 2016). In this introduction, a few of these channels that have been demonstrated to be crucial in platelets function are summarized (Fig.1.2).

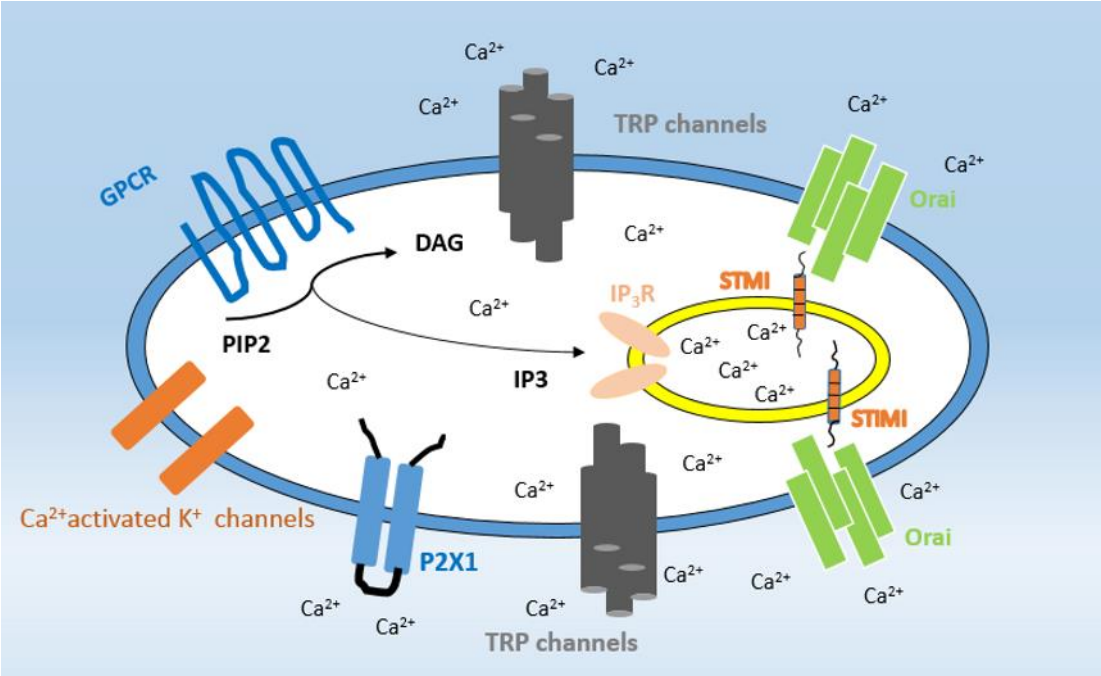


**Fig. 1.2 Ion channels on platelets membrane.** Platelets activation by GPCR agonists results in the calcium release from ER via $IP_3$ receptor ($IP_3R$). The depletion of calcium in ER is sensed by STIM on the ER membrane that transfers the signal to Orai channels on the plasma membrane, then extracellular calcium enters into platelets from Orai channels, which is referred to as SOCE. $Ca^{2+}$ activated $K^+$ channels are activated by the elevation of cytosolic free calcium; $P2X_1$ channels are gated directly by ATP; TRP channels can be activated by both physical and chemical stimuli.

### $P2X_1$ channels

$P2X_1$ channels belong to the large P2 purinoreceptors for extracellular nucleotides that include two subgroups: P2X receptors and P2Y receptors (Gachet 2008). Although several subtypes of P2X and P2Y receptor have been identified, only $P2X_1$, $P2Y_1$ and $P2Y_{12}$ are expressed in significant level in platelets (Wang, Ostberg et al. 2003). Dense granules of platelets secret both ADP and ATP: ADP binds to $P2Y_1$ and $P2Y_{12}$ receptors that are G protein-coupled receptors (GPCR) (Gachet 2001); ATP directly activates $P2X_1$ channels, which results in rapid calcium influx (Mahaut-Smith,

Jones et al. 2011). The generation of $P2X_1$ transgenic mice provides convincing evidence for an important role of $P2X_1$ channels in platelets function. $P2X_1$ deficient platelets display impaired aggregation upon to lower dose of GPVI agonist collagen, while they respond to GPCR agonists and higher dose of collagen as well as wild type platelets. Furthermore, $P2X_1$ knockout mice exhibit reduced thrombus formation at higher arterial shear rates (Hechler, Lenain et al. 2003). Conversely, overexpression of $P2X_1$ in mice results in enhanced platelets aggregation stimulated by lower dose of collagen and thromboxane $A_2$ mimetic-- U46619 in vitro and increased thrombotic tendency in vivo (Oury, Kuijpers et al. 2003).

## Store-operated calcium entry (SOCE)

In non-excitable cells, calcium release from intracellular stores triggers calcium entry from the extracellular department, a process referred to as store-operated calcium entry (SOCE) (Patterson, van Rossum et al. 1999, Parekh and Putney 2005, Smyth, Hwang et al. 2010). Recent studies have established stromal interaction molecule 1 (STIM1) and Orai1 as the key players of SOCE in platelets, with STIM1 as the senor of calcium release from internal stores and Oria1 as the major SOC channels on platelets membrane (Bergmeier and Stefanini 2009, Varga-Szabo, Braun et al. 2011). STIM1 is a single transmembrane protein containing two N-terminal EF hand domains that are situated in the ER lumen and detecting calcium levels. Consequently, EF hand mutants lead to continuous calcium influx from the extracellular space (Grosse, Braun et al. 2007). Platelets lacking STIM1 aggregate normally to GPCR agonists, such as thrombin, ADP and thromboxane $A_2$, but exhibit diminished aggregation in response to GPVI agonists collagen, or convulxin. In vivo, STIM1 deficient mice display unstable arterial thrombi and are protected from cerebral ischemia (Varga-Szabo, Braun et al. 2008, Ahmad, Boulaftali et al. 2011). Similar to SITM1, STIM1 isoform STIM2 is able to trigger Orial1 mediated calcium influx on plasma membrane but is activated by small reduction of $Ca^{2+}$ in ER. Moreover, SITM2 functions as primary regulator of basal cytosolic calcium (Brandman, Liou et al. 2007, Gruszczynska-Biegala, Pomorski et al. 2011). Orai1, also called *calcium-release activated calcium modulator 1* (CRACM1) was first discovered to be the SOC channels in T cells and mast cell (Prakriya, Feske et al. 2006, Vig, Peinelt et al. 2006, Vig,

DeHaven et al. 2008). Then Orai1 was found to be expressed and identified as the principle SOC channel in platelets plasma membrane. Orai1 deficient platelets show similar defects to platelets devoid of STIM1 (Braun, Varga-Szabo et al. 2009) .

## Transient receptor potential (TRP) channels

TRP channels are six transmembrane non-selective cation channels permeable for both monovalent and divalent ions that are divided into six subfamilies, TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin) and TRPA (ankyrin) (Clapham, Runnels et al. 2001, Clapham 2003). TRP channels are able to be activated by both chemical and physical stimuli, such as $PIP_2$, cyclin nucleotides, pH, heat, temperature (Clapham, Runnels et al. 2001, Zheng 2013). Human platelets have been reported to express TRPC1, TRPC3, TRPC4, TRPC5 and TRPC6 (Brownlow and Sage 2005). TRPC1 is proposed to mediate SOCE in platelets based on the evidence that inhibiting TRPC1 reduces SOCE response to thrombin and thapsigargin (Rosado and Sage 2000, Rosado and Sage 2001, Rosado, Brownlow et al. 2002). However, whether TRPC1 is SOCE channel still remains controversial. Some studies reveal that SOCE is independent of TRPC1, since TRPC1 is only expressed in ER membrane (Hassock, Zhu et al. 2002), and TRPC1 deficient platelets exhibit completely intact SOCE (Varga-Szabo, Authi et al. 2008). TRPC6 is expressed at high levels on platelet plasma membrane and is regarded to be a non-SOCE channel on platelet membrane (Hassock, Zhu et al. 2002). Platelets in the absence of TRPC6 do not show an altered calcium response and granule release in agonist-evoked activation. Furthermore, TRPC6 deficient mice form arterial thrombus normally, which indicates a minor role of TRPC6 in platelets function (Ramanathan, Gupta et al. 2012).

## Calcium activated potassium (KCa) channels

KCa channels are a large family of potassium channels activated by the elevation of cytosolic free calcium. According to the single channel conductance, KCa are divided into small conductance KCa (SK), intermediate conductance KCa (IK) and big conductance KCa (BK) (McManus 1991, Vergara, Latorre et al. 1998). Platelets have

been shown to express a small number of IKs, which determine the membrane potential based on the evidence that platelets are sensitive to IK blocker-carybdotoxin, but not to SK inhibitor- apamin (Fine, Hansen et al. 1989, Mahaut-Smith 1995). Recently, Wright et al. used quantitative polymerase chain reaction (PCR) to analyse the expression of ion channels on human platelets and reported that BK also existed in platelets (Wright, Amisten et al. 2016). KCa in platelets is suggested to regulate cell volume (Sullivan, Koliwad et al. 1998), migration (Schmidt, Munzer et al. 2011), and procoagulant activity (Wolfs, Wielders et al. 2006).

## 1.5 Mechanosensitive (MS) ion channels

MS channels are transmembrane proteins that are involved in mechanotransduction - the conversion of mechanical stimuli to biological signals. Mechanotransduction is essential for a large variety of biological processes in humans, such as hearing, touching, temperature and pain sensation. The question how MS channels respond to mechanical stimuli is still under debate, but two models have been proposed so far (Hamill and Martinac 2001, Martinac 2004). One is the lipid bilayer model. In this model, it is perceived that mechanical deformation of the bilayer membrane triggers the conformational change of the channels, which results in activation of MS channels. A number of studies support this model. For instance, amphiphiles are reported to gate MS by inserting into the membrane bilayer and changing the membrane curvature (Martinac, Adler et al. 1990, Perozo, Kloda et al. 2002, Qi, Chi et al. 2005). Furthermore, MS channels are able to be activated by membrane tension in cytoskeleton free membrane blebs or liposomes, which provide more evidence to this model (Zhang, Gao et al. 2000, Kloda and Martinac 2001, Perozo and Rees 2003). The other model is the tethered model. In this model, MS channels are connected extracellular matrix and/or the cytoplasmic skeleton. These tethers convey the exogenous or endogenous mechanical forces and then trigger opening of the MS channels (Gillespie and Walker 2001). For example, in auditory and vestibular hair cells MS channels are connected to extracellular tip links and to intracellular cytoskeleton through the adaptation motor, thereby transduce the mechanical stimuli (Pickles, Comis et al. 1984, Corey 2003, Chalfie 2009). Till now, a large amount of ion channels have been proposed to be candidates of MS channels.

## Epithelial sodium channel/degenerin (eNaC/DEG)

The eNaC/DEG gene family represents a class of sodium channels comprised of two transmembrane domains. ENaC is located on the apical surface of epithelia and contributes to salt and water homeostasis (Butterworth 2010). DEG family are able to be activated by mechanical forces like shear stress, and are involved in touch sensation, blood pressure regulation and hypertension (Kellenberger and Schild 2002).

## TRP channels

TRP family of ion channels are implicated in a wide variety of mechanical transduction processes, like taste, vision, olfaction, and heat sensation (Clapham 2003, Corey 2003, Christensen and Corey 2007). A number of TRP channels are implicated in mechanotransduction. To name only a few. TRPC1 is reported to be the component of MS channels in Xenopus oocytes and gated by lipid bilayer tension (Maroto, Raso et al. 2005). TRPC6 functions as a direct sensor of mechanically and osmotically induced membrane stretch (Spassova, Hewavitharana et al. 2006).TRPV1 was originally regarded as the receptor of capsaicin, the component of hot chili pepper, and later was demonstrated to be gated by mechanical stimuli, like noxious heat (Pingle, Matta et al. 2007). TRPM3 and TRPM7, members of melastatin TRP subgroup are both mechanically activated ion channels in that TRPM3 can be evoked by osmotic swelling (Grimm, Kraft et al. 2003) and TRPM7 is activated by membrane stretch as well as osmotic swelling (Numata, Shimizu et al. 2007).

## Two pore domain potassium channels

Two-pore domain potassium channels are a novel class of potassium channels that comprise four transmembrane segments (Lesage and Lazdunski 2000). The first identified $K_{2p}$ is tandem of P domains in a weakly inward-rectifying K channel (TWIK) by gene cloning (Lesage, Guillemare et al. 1996). Subsequently, more mammalians $K_{2p}$ channels have been discovered, but only TREK-1 (TWIK-1 related K channel) (Fink, Duprat et al. 1996), TREK-2 (Lesage, Terrenoire et al. 2000) and TRAAK (TWIK open by arachidonic acid) (Fink, Lesage et al. 1998) are shown to be mechanically

gated. TREK and TRAAK channels are polymodal potassium channels that are opened by both physical stimuli (stretch, cell swelling, heat, voltage) and chemical stimuli (poly unsaturated fatty acid, lysophosholipids) (Dedman, Sharif-Naeini et al. 2009, Brohawn, Su et al. 2014).

### PIEZO

PIEZO1 and PIEZO2 are more recently identified mechanically activated ion channels permeant to $Na^+$, $K^+$, $Ca^{2+}$ , and $Mg^{2+}$ with a preference to $Ca^{2+}$ (Coste, Mathur et al. 2010, Coste, Xiao et al. 2012). Different from other MS channels containing two, four or six transmembrane domains, PIEZO channels are assumed to consist of 24-40 transmembrane domains and the largest ion channels on plasma membrane identified so far (Bagriantsev, Gracheva et al. 2014, Ranade, Syeda et al. 2015, Volkers, Mechioukhi et al. 2015). PIEZO1 is widely distributed in bladder, skin, lung, kidney, and colon and plays important roles in cellular biology and physiology (Bagriantsev, Gracheva et al. 2014). To name only a few. PEIZO1 on bladder urothelium is activated by stretch stimulus, resulting in potent ATP release (Miyamoto, Mochizuki et al. 2014). PIEZO1 on embryonic endothelia, gated by fluid shear stress is responsible for vascular development (Ranade, Qiu et al. 2014). PIEZO1 on smooth muscle cell is involved in remodelling of arterial walls upon hypertension (Retailleau, Duprat et al. 2015). PIEZO2 is abundantly expressed in dorsal root ganglion and trigeminal ganglia sensory neurons, Merkel cells and is involved in mechanosensory transduction (Ranade, Woo et al. 2014, Woo, Ranade et al. 2014).

## 1.6 Aims of the thesis

1. Our previous studies show that mouse platelets migrate autonomously in vivo and reveal the characteristics of human platelets motility in vitro, but how mouse platelets migrate in vitro has not been addressed. Therefore, I will investigate the requirements for mouse platelet migration in vitro and explore the underlying molecular mechanism.

2. The above introduction suggest that calcium signaling is crucial in platelets activation. Our previous studies reveal that extracellular calcium is required for platelet

migration, therefore I will work on identifying the calcium ion channels that mediate platelet migration. Based on the identified channels involved in platelets mobility, I will compare the role of MS channels and SOCE channels in different platelet functions and reveal the molecular differences among platelet aggregation, secretion and migration.

# 2 Materials and methods

## 2.1 Mouse strains

*C57/Bl6* mice, *Pf4-Cre* mice (Tiedt, Schomber et al. 2007), albumin deficient mice (Roopenian, Low et al. 2015), RAG1-deficient mice (Mombaerts, Iacomini et al. 1992) , GPIbα/IL4R transgenic mice (Bergmeier, Piffath et al. 2006) , PC::G5-tdT mice (Gee, Smith et al. 2014), $P2X_1$ knockout mice (Hechler, Lenain et al. 2003) were obtained from the Jackson laboratory. *Stim1*$^{floxl/flox}$ mice (Ahmad, Boulaftali et al. 2011), *Stim1/2*$^{flox/flox}$ mice, TRPC1/6 knockout mice (Ramanathan, Gupta et al. 2012) were gifts of Prof. Michael Mederos y Schnitzler and Prof. Alexander Dietrich. *Stim1*$^{floxl/flox}$, *Stim1/2*$^{flox/flox}$ mice, or PC::G5-tdT mice were crossed with *Pf4-Cre* mice to obtain the magakaryocyte-platelets specific gene knockout mice. Experiments were performed on 8-10 weeks old mice and approved by the local animal protection law.

## 2.2 Materials

ACD buffer: 85 mM $Na_3$Citrate, 65 mM Citric acid monohydrate, and 111 mM glucose.

Modified Tyrode's buffer: 136.9 mM NaCl, 12.1 mM $NaHCO_3$, 2.6 mM KCl, 5.5 mM glucose, and 10 mM HEPES.

Factors tested in mouse platelet migration are listed in table 2-1 and table 2-2, calcium channel inhibitors are summarized in table 2-3.

**Table 2-1 factors tested in mouse platelet migration**

| Reagents | Solvent | Company |
|---|---|---|
| Albumin from mouse serum | $H_2O$ | Sigma |
| Albumin human, recombinant | $H_2O$ | Sigma |
| Annexin V, unlabeled recombinant protein | $H_2O$ | Ebioscience |
| Bovine albumin fraction V | $H_2O$ | Roche |
| Casein fluorescein isothiocyanate from bovine milk | $H_2O$ | Sigma |
| Casein from bovine milk | PBS | Sigma |
| Casein, bovine milk, carbohydrate and fatty acid free | PBS | Millipore |
| Casein, dephosphorylated from bovine milk | PBS | Sigma |
| Chondroitinase ABC from Proteus vulgaris | $H_2O$ | Sigma |
| Complement C1, human | NaCl | Millpore |
| Complement C1q, human | NaCl | Millpore |
| Complement C3 from human serum | PBS | Sigma |
| Complement Factor H from human plasma | PBS | Sigma |
| C-reactive protein, human | NaCl | Millpore |
| Epsilon-aminocaproic acid | $H_2O$ | Sigma |
| Heparinase II from Flavobacterium heparinum | $H_2O$ | Sigma |
| Neuraminidase (Sialidase) from Clostridium perfringens | $H_2O$ | Roche |
| Plasminogen human | NaCl | Biopur |
| PLL-g-PEG | $H_2O$ | Susos |
| Pluronic F127 | $H_2O$ | Thermofisher |
| Poly L arginine, MW 15,000-70,000 | $H_2O$ | Sigma |
| Poly L lysine, MW 30,000-70,000 | $H_2O$ | Sigma |
| Poly L lysine, MW 1,000-5,000 | $H_2O$ | Sigma |
| Poly L ornithine, MW 30,000-70,000 | $H_2O$ | Sigma |
| Protamine from salmon | $H_2O$ | Sigma |
| Recombinant Human Transforming Growth Factor-beta 1 (rh TGF-β 1) | $H_2O$ | Immunotools |
| Recombinant Mouse Beta Defensin-1 (rm BD-1) | $H_2O$ | Immunotools |
| Recombinant Mouse Beta Defensin-2 (rm BD-2) | $H_2O$ | Immunotools |
| Recombinant Mouse Epidermal Growth Factor (rm EGF) | $H_2O$ | Immunotools |
| Recombinant Mouse Fibroblast Growth Factor-basic (rm FGF-b) | $H_2O$ | Immunotools |
| Recombinant Mouse Heparin Binding EGF-like Growth Factor (rm HB-EGF) | $H_2O$ | Immunotools |
| Recombinant Mouse Midkine (rm MK) | $H_2O$ | Immunotools |
| Recombinant Mouse Platelet-derived Growth Factor AA (rm PDGF-AA) | $H_2O$ | Immunotools |
| Recombinant Mouse Platelet-derived Growth Factor BB (rm PDGF-BB) | $H_2O$ | Immunotools |

**Table 2-2 factors tested in mouse platelet migration**

| Reagents | Solvent | Company |
|---|---|---|
| Recombinant Mouse Vascular Endothelial Growth Factor A (rm VEGF-A) | $H_2O$ | Immunotools |
| Recombinant Murine Insulin-Like Growth Factor 1 (rm IGF-1) | $H_2O$ | Immunotools |
| Spermine | $H_2O$ | Sigma |
| α-Casein from bovine milk | $H_2O$ | Sigma |
| β-Casein from bovine milk | PBS | Sigma |
| κ-Casein from bovine milk | $H_2O$ | Sigma |

**Table 2-3 list of calcium channel inhibitors**

| Inhibitor | Company | Solvent | Description |
|---|---|---|---|
| Thapsigargin | Sigma | DMSO | SERCA inhibitor |
| Caloxin2A1 | Anaspec peptide | DMSO | PMCA inhibitor |
| A23187 | Sigma | DMSO | Calcium ionophore |
| EGTA | Bioworld | $H_2O$ | Extracellular calcium chelator |
| LOE908 | Tocris | $H_2O$ | Broad spectrum cation inhibitor |
| NF449 | Tocris | $H_2O$ | $P2X_1$ inhibitor |
| BTP2 | Tocris | DMSO | SOC inhibitor |
| $LaCl_3$ | Sigma | $H_2O$ | Mechanosensitive ion channel inhibitor |
| GsMTx4 | Abcam | $H_2O$ | Mechanosensitive ion channel inhibitor |
| Amiloride | Sigma | $H_2O$ | eNaC, TREK, TRAAK inhibitor |
| Larixol | Sequoia research | DMSO | TRPC6 inhibitor |
| NS8593 | Gift of Prof. Schnitzler | DMSO | TRPM7 inhibitor |

## 2.3 Isolation of washed human platelets

Human blood was drawn from cubital vein by safety multifly-needle (Sarstedt) on volunteers who did not take any drugs in the last two weeks. The first 1 ml blood was discarded and the next 5 ml was taken into a syringe with one seventh volume of ACD inside. Then the blood was mixed with equal volume of modified Tyrode's buffer (pH6.5) in 15 ml falcon. Platelet rich plasma (PRP) was obtained by centrifuging the mixture of blood and Tyrodes buffer with 70 g (Eppendorf 5804) for 35 min without brake at room temperature. Then 3 ml PRP was mingled with 7 ml Tyrode's buffer (pH6.5) in the presence of 0.1% HSA (w/v) and 100 ng/mg prostaglandin $I_2$ sodium salt ($PGI_2$, Abcam), and centrifuged at 1200 g for 10 min. Finally, the supernatant was

discarded and the pellet was carefully suspended in 1 ml Tyrode's buffer (pH6.5). Platelet concentration was measured on an automated hematology counter (ABX Micros ES60, Horiba Medical).

## 2.4 Isolation of washed mouse platelets

Mouse was anesthetized by MMF (fentanyl 0.5 mg/kg, midazolam 5 mg/kg, medetomidine 0.05 mg/kg). Thoracic skin was removed by scissors and blood was drawn intra caridially by a syringe in the presence of 145 ul ACD. Then mouse blood was mixed with 1 ml Tyrode's buffer (pH6.5) and centrifuged at 70 g for 20 min without brake. Then PRP was blended with 3 ml of Tyrode's buffer in presence of 100 ng/ml $PGI_2$ and centrifuged 1200 g for 10 min. The supernatant was discarded and pellet was suspended in 500 ul modified Tyrodes buffer (pH6.5).

## 2.5 Preparation of mouse plasma and serum

Mouse blood was obtained as above, diluted with equal volume of modified Tyrodes buffer (pH6.5) and centrifuged at 1500 g for 15 min at RT. The supernatant was taken as mouse plasma.

Mouse plasma was incubated with 1 U/ml bovine thrombin (Sigma) for 20 min to initiate coagulation, then thrombin activity was ceased by 40 µM PPACK (D-Phe-Pro-Arg-chloromethylketone, Enzo Life Science). Serum was obtained by centrifugation of the plasma at 2000 g for 15 min.

## 2.6 Platelet migration assay

Glass coverslips (24 mmX24 mm, Schott nexterion) were washed with 20% $HNO_3$ for 1 hour, then rinsed with distilled water ($ddH_2O$) for 1 hour at RT. Freshly cleaned coverslips were air-dried at 90 rps for 10 sec and silanized with hexamethydisilazane (HDMS, Sigma) at 80 rps for 30 sec by a KLM spin coater (Schaefer) to ensure homogeneous silanization.

In some experiments, the original glass bottom (14 mM diameter) of the dish (MatTek) was removed and replaced with silanized coverslips via melted paraffin. If

not otherwise stated, $10\times10^3$/µl human platelets were reconstituted with 1500 µg/ml HSA, while $10\times10^3$/µl mouse platelets were reconstituted with 30 µg/ml casein, along with 38 µg/ml human fibrinogen (Sigma), 200 µM $CaCl_2$, 2 µM U46619 (Enzo life science) and 4 µM ADP (Sigma) in a total volume of 240 µl modified Tyrodes buffer (pH7.2) in the MatTek dish. Then the dish was mounted on an inverted microscope described below to record the motility of platelets.

In some experiments, the silanized coverslip was sticky to the chamber (Ibidi sticky slides VI$^{0.4}$). Then chamber was filled with 37.5 µg/ml Alexa Fluor® 488 Conjugate fibrinogen (Thermofisher) and 2000 µg/ml HSA in modified Tyrodes buffer for 15 min, then washed with Tyrodes buffer. If not otherwise stated, $10\times10^3$/µl human platelets were reconstituted with 1000 µg/ml HSA, while $10\times10^3$/µl mouse platelets were reconstituted with 30 µg/ml casein, together with 200 µM $CaCl_2$, 2 µM U46619 and 4 µM ADP in a total volume of 240 µl Tyrodes buffer (pH7.2) in the chamber. The Ibidi chamber was incubated at 37℃ for 1 hour and then images were taken at fluorescent microscope described below.

## 2.7 Time-lapse microscope

Differential interference contrast (DIC) movies (1 frame/ 12 sec) were recorded on an inverted microscope (Olympus IX83) with an 40x/NA1.00 oil objective or 60x/ NA1.35 oil objective with cooled CCD (charged couple device) camera (Olympus XM10). Phase contrast (PH) (1 frame / 20 sec) movies were recorded on the same microscope with 100x/NA1.40 oil objective**.** The microscope was equipped with a stage incubator (37 ℃, humidified) (Tokai Hit) and objective heater. Images were acquired by Cellsense software (Olympus) installed in a computer connected to the microscope.

## 2.8 Quantification of the velocity and percentage of migrating platelets

To quantify cell velocity from DIC or PH movies, frames were extracted every 2 min in Cellsense software, then stacks were imported to ImageJ. The central area of the movie was taken as area of interest (ROI). The platelets whose migrating paths

are more than one of their diameters were defined as migrating cells. The number of spreading platelets in the ROI which include both the non-migrating and migrating platelets were manually counted. The trajectories of mobile platelets was manaully tracked using the ‘‘ Manual tracking’’ plugin in ImageJ with the pseudonucleus of the platelets as the morphologcial tracking marker (Fig.2.1). Cell velocity was obtained by the accumulating distances divided by the migrating time. The percentage of migrating platelets was calculated by the total number of spreading platelets divided by the number of migrating platelets.

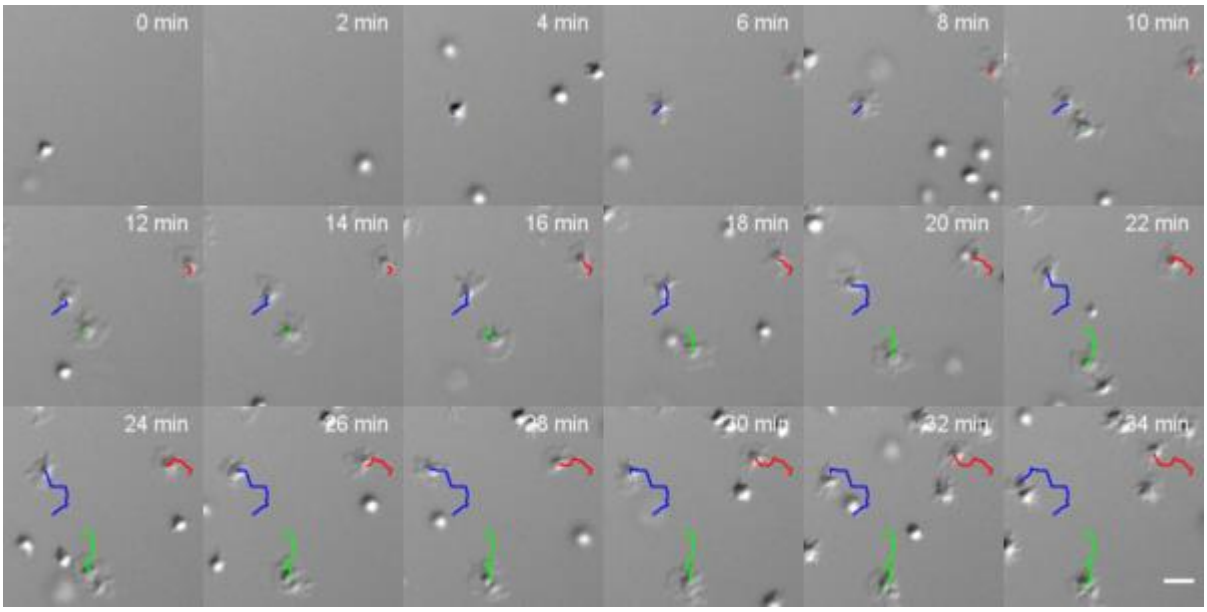


**Fig. 2.1 Quantification of the velocity of migrating platelets in DIC stacks**. The trajectories were manaully tracked using the’’ Manual tracking’’ Plugin in ImageJ with the pseudonucleus of the platelets as the morphologcial tracking marker. Cell velocity was obtained by the accumulating distances divided by the migrating time. The colorful lines indicate the the trajectories manaually tracked. Scale bar=5 µm.

Our previous studies revealed that platelets removed fibrinogen from the substrate during migration (Gärtner, Engelhardt et al. 2015). Because of this feature, platelets migrating paths in Alexa488-conjugated fibrinogen coated surface were manually drawn through ‘‘freehand line’’ in Fiji (Fig.2.2). The velocity of the cell was calculated by the total length measured divided by the migrating time. The percentage of migrating platelets was calculated in the same way as above.

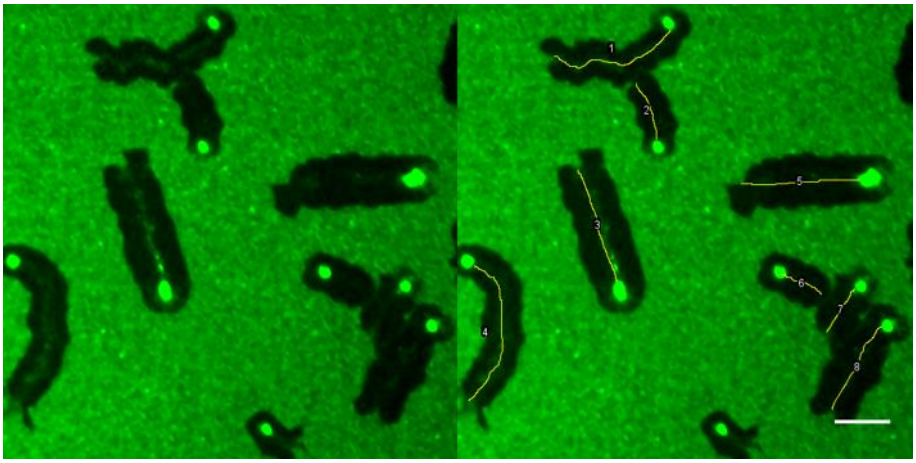

**Fig. 2.2 Velocity calculation of migrating platelets on Alexa488-conjugated fibrinogen coated substrate**. Left: fluorescent images taken after platelet migration in the Ibidi chamber with Alexa488-conjugated fibrinogen coated surface. The back area indicates that fibrinogen was removed away by mobile platelets. Right: The migrating paths (yellow lines) was manually drawn through ''freehand line'' in Fiji, scale bar= 5 µm.

## 2.9 Intracellular calcium measurement by flow cytometry

Fluo4-AM (Thermofisher) powder was dissolved in DMSO as 5 mM stock solution at -20℃. Washed platelets prepared as above were loaded Fluo4-AM with 5 µM final concentration in an Eppendorf tube, then the tube was inverted a few times to thoroughly mix the suspension. Platelets were incubated with Fluo4-AM for 20 min at RT in the dark, and then centrifuged at 1200 g for 3 min in the presence of 100 ng/ml $PGI_2$. The supernatant was removed and the pellet was gently suspended in modified Tyrodes buffer (pH6.5). Then Fluo4-AM loaded platelets remained for 20 min at RT for complete de-esterification of AM esters before experiments and were used within up to 2 hours.

Fluo4-AM loaded platelets were reconstituted in Tyrodes buffer (pH7.2) at the final concentration of $10\times10^3$/µl in the presence of 1 mM $CaCl_2$ in the FACS tube. Fluo4 intensity was recorded in FL1 channel on the flow cytometer (BD LSRFortessa) for 30 sec to obtain the resting calcium level. Then the FACS tube was moved away from the flow cytometer, agonist was quickly added and mixed. Finally the tube was put back to flow cytometer and Fluo4 intensity was recorded for another 150 sec.

Data from flow cytometry were analysed on Flowjo. The basal calcium level was quantified as the MFI of 30 sec before activation. The cytosolic calcium after agonist-induced stimulation was calculated as the MFI of the 30 sec after activation. Elevated cytosolic calcium was calculated as the intracellular calcium after activation subtracted by basal calcium level.

## 2.10 Calcium oscillations recorded at fluorescent microscope

5uM Fluo4-AM loaded washed platelets were supplemented with the migration buffer described above in the MatTek dish. The fluorescent light engine Lumencor SOLAR SE II was connected to the microscope (Olmpus IX83) with CCD camera (Olympus XM10). The MatTek dish was mounted on 60x/NA1.35 oil objective at the microscope equipped with an autofocus function of ZDC ( Z-drift compensation). The lumencor intensity was set properly to minimize the phototoxic to platelets meanwhile to acquire strong enough fluorescent intensity. Frames from the green fluorescent channel (excitation 488 nM) were recorded every 5 sec to record the calcium intensities of platelets.

## 2.11 Quantification of calcium oscillations

Fluorescent stacks recorded at florescent microscope were imported to Fiji (Schindelin, Arganda-Carreras et al. 2012). Singe platelet migration area was carefully selected by ‘‘rectangular selection’’ that did not include any other cells, and added to the ROI manager (Kardash, Bandemer et al. 2011). Then the background of the stack was subtracted with rolling ball method. Image type was changed to 8 bit and platelets in the stacks were identified by auto threshed with black object on white background (Fig.2.3). Platelets fluorescent intensity was obtained by subtracting the auto-threshold stacks from background corrected stacks by ‘‘image calculator’’. Finally mean fluorescent intensity over time was calculated by ‘‘stack-plot Z profile’’ in Fiji. The schematic steps for quantifying calcium oscillations was shown in Fig.2.4.

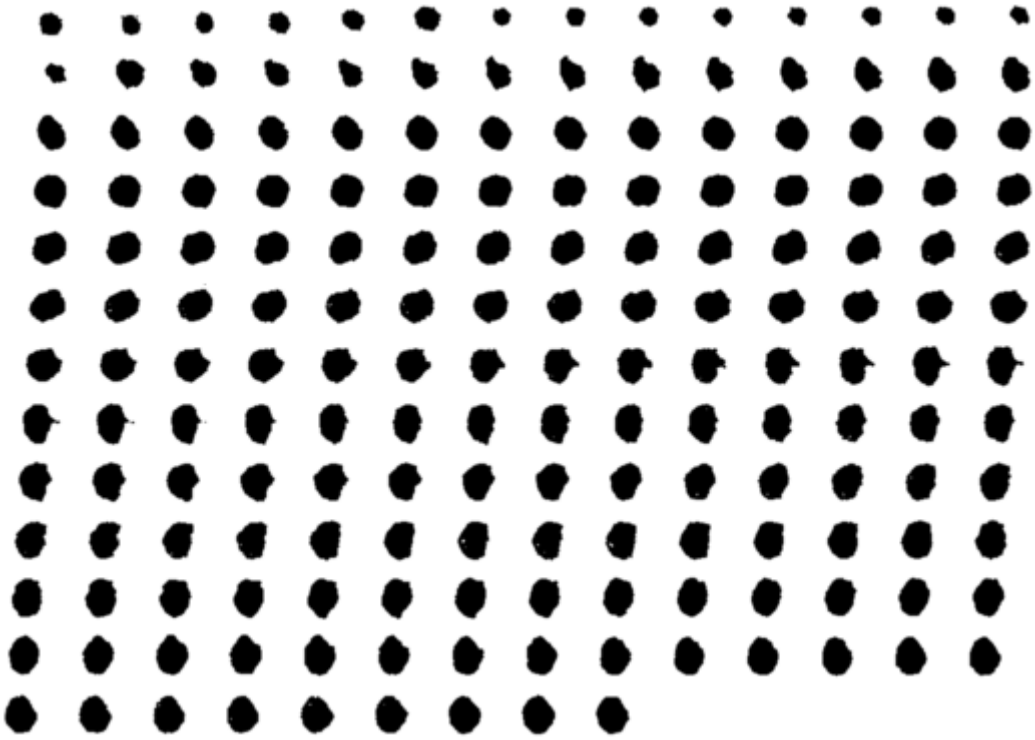

**Fig. 2.3 Motile platelets were identified by auto threshold in Fiji.** The black objects in each frame represents the mobile platelets.

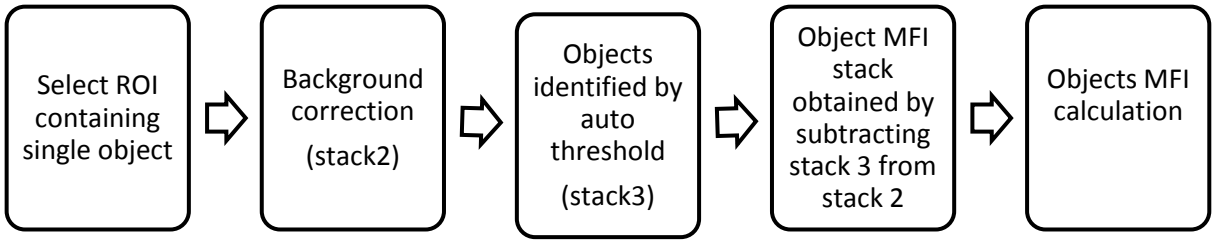


**Fig. 2.4 Steps for quantification of calcium oscillations in platelets.**

## 2.12 Platelets aggregation and ATP release measurement

Washed platelets were prepared as above and adjusted to 200 $\times 10^3$/µl. The Lumi-aggregometer (Chrono-log 700 Series) with AGGRO/LINK8 software was turned on and warmed up to 37℃.

Luciferin-luciferase kit (CHRONO-LUME™) was used to measure ATP released by platelets dense granules (Beigi, Kobatake et al. 1999). Tyrodes buffer 250µl in glass cuvette was prewarmed for 5 min in the incubation well, then placed in PPP well of the Lumi-aggregometer. 225 µl washed platelets was pipetted in another cuvette with a stir bar inside and prewarmed for 5 min, then 25 µl CHRONO-LUME was

added, the glass cuvette was transferred to the PRP well. After incubation for 2 min, 5 µl of ATP standard that contains 2 nM ATP was added. The curve whose peak signal is between 20%-60% was saved as the standard ATP curve.

The aggregation of washed platelets was performed with optical mode on aggregometer (Fig.2.5). Washed platelets along with 38 µg/ml fibrinogen, 200 µM $CaCl_2$ in a total volume of 225 µl in a glass cuvette with a stir bar inside was incubated for 5 min in the incubation well, then 25 µl of CHRONO-LUME was added into the cuvette that was transferred to the PRP well. After incubation for 2 min, agonists were added to induce platelets aggregation and granule release. Aggregation and ATP release curve were recorded simultaneously for 6 min. Aggregation amplitude and ATP release were calculated by ‘‘calculate results’’ in AGGRO/LINK 8 software.

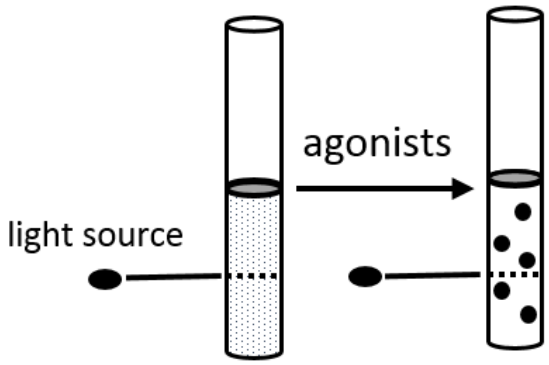


**Fig. 2.5 Principe of platelets aggregation by light transmission.** Before activation, resting washed platelets were distributed homogenously in the suspension. After addition of agonists, platelets become aggregated, resulting in the changes of light transmission that was recorded by the aggregometer.

## 2.13 $\alpha_{IIb}\beta_3$ activation and P selectin measurement by flow cytometry

Mouse platelets were obtained as previously. Equal volume of PE-labeled JON/A antibody (Emfret) that selectively binds to high affinity of mouse $\alpha_{IIb}\beta_3$ (Ingerman-Wojenski and Silver 1984) and FITC-labeled Wug.E9 antibody that reacts with P-selectin on mouse platelets (Emfret) were mixed and stored on ice before use. Agonists were added along with 5 µl of the antibody mixture in 240 µl Tyrodes buffer (pH7.2) which contains $10\times10^3$/µl platelets and 1 mM $CaCl_2$ in an Eppendorf tube. The

suspension was incubated for 20 min at RT in the dark, then transferred to FACS tube and immediately analysed on flow cytometer.

Voltages of forward light scatter (FSC) and side light scatter (SSC) were set properly to bring the platelets population toward the centre of the plot on the flow cytometer (BD LSRFortessa). Cell debris were removed by adjusting the threshold. Non-stained platelets were served as negative control in the lower left of the plot and single stain of JON/A-PE and Wug.E9-FITC to activated platelets were used to adjust compensation. Flow rate was set as low to minimize coincident events. 30 000 individual platelets were collected in each experiment. Acquired data were analysed via Flowjo.

## 2.14 Statistics

T-test or one way ANOVA were performed if data fulfil normal distribution with Kolmogorov-Smirnov-test, otherwise Mann-Whitney test or Kruskal-Wallis tests were applied. $P<0.05$ was defined as statistical significance. If overall ANOVA or Kruskal-Wallis tests were significant, post hoc test were performed. All data were presented as mean ± SEM, unless otherwise stated. Analysis were performed within Prism6.

# 3 Results

## 3.1 Casein proteins trigger platelet migration by regulating calcium oscillations with the involvement of mechano-sensitive ion channels.

### 3.1.1 Mouse platelets are able to migrate in the presence of mouse plasma or serum in vitro.

Very recently, Gaertner et al. in our group observed autonomous mouse platelets locomotion in thrombus formation in vivo and identified the requirements for human platelet migration in vitro (Gärtner, Engelhardt et al. 2015). However, whether mouse platelets are motile or not in vitro is still unknown. To address this question, mouse platelets were reconstituted in plasma along with fibrinogen and were activated by both U46619 and ADP, as a results, 50.18±3.00% of spreading mouse platelets migrate (Fig.3.1). To further characterize the plasma components that facilitate platelet movement, mouse serum that does not contain fibrinogen and clotting factors was substituted for plasma. Consequently, 42.14±8.54% of spreading mouse platelets are motile (Fig.3.1). The percentage of mobile platelets in plasma and serum are not different ($p=0.68$), but the velocity of migrating platelets are higher in serum than in plasma (0.43±0.06 vs18±0.02, $p<0.001$) (Fig.3.1), which may be explained by the stronger substrate adhesiveness resulting from fibrinogen content in mouse plasma. Overall, these experiments demonstrate that mouse platelets are able to migrate in vitro within plasma or serum.

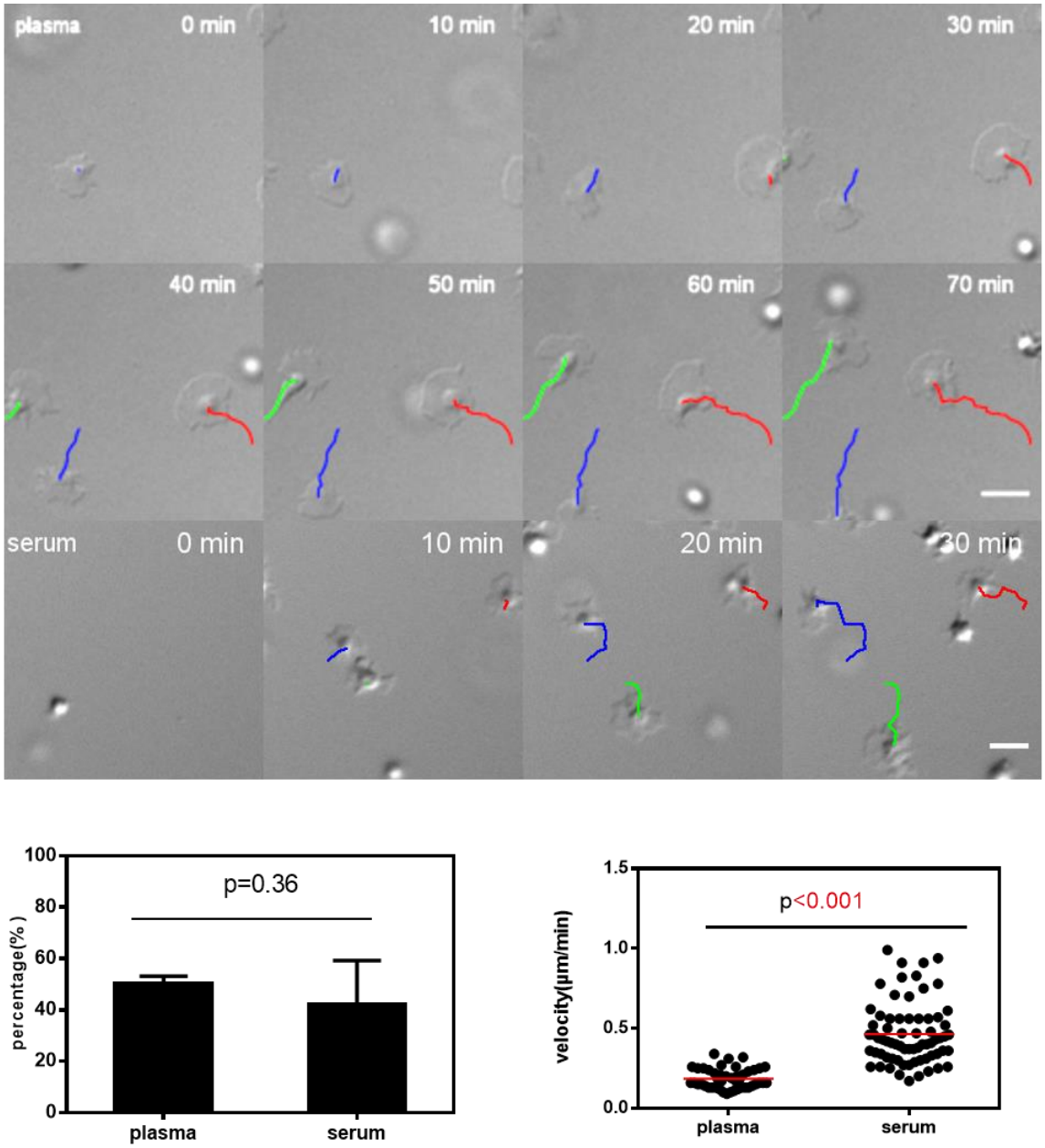


**Fig. 3.1 Mouse platelets are able to migrate within mouse plasma or serum in vitro**. Mouse platelets were reconstituted in plasma or serum along with 38 µg/ml fibrinogen, activated by 2 µM U46619 and 4 µM ADP, and observed at time-lapse DIC microscope. Upper panel: representative time series of migrating mouse platelets within mouse plasma or serum. The colourful lines indicate accumulated migrating paths of platelets at different time points. Scale bar= 5 µm. Lower panel: quantification of the fraction (left) and velocity (right) of migrating platelets. Platelets were pooled from n=4 experiments; red bars indicate the mean velocity; error bars=SEM; t-test.

## 3.1.2 Casein but not albumin triggers mouse platelet migration.

Previous study in our group showed that human platelets were capable of migrating in serum. After systematic analysis of serum components, albumin and calcium

were identified as two factors that promote human platelet motility on fibrinogen substrate with the underlying mechanism that albumin reduced the adhesiveness of fibrinogen on the surface and extracellular calcium was required for platelet polarization (Gärtner, Engelhardt et al. 2015). Nevertheless, when human albumin and extracellular calcium were applied on mouse platelets in the same manner as human platelets, mouse platelets are not mobile (Fig.3.2). Because of distinctive species which human and mouse belong to, mouse albumin instead of human albumin could be physiological for mouse platelets. However, mouse platelets are still stationary after human albumin replacement with mouse albumin (Fig.3.2). Bovine albumin is widely used to block unspecific area and thereby reduces substrate adhesiveness, but it does not facilitate mouse platelet movement (Fig.3.2). Since extracellular calcium is required for platelet migration and the percentage of migrating platelets rises as the concentration of extracellular calcium is increased (Gärtner, Engelhardt et al. 2015), higher extracellular calcium concentration was applied on mouse platelets in the presence of various kinds of albumin, but mouse platelets remain immobile in each condition (Fig.3.2). At the same concentration of albumin along with 200 µM calcium, the number of spreading platelets in the presence of human albumin is lowest among of human albumin, bovine albumin and mouse albumin, which indicates that human albumin exhibits stronger anti-adhesiveness effect than others, although it could not promote mouse platelet migration. Taken together, none of the human albumin, bovine albumin, and mouse albumin are able to promote mouse platelet locomotion.

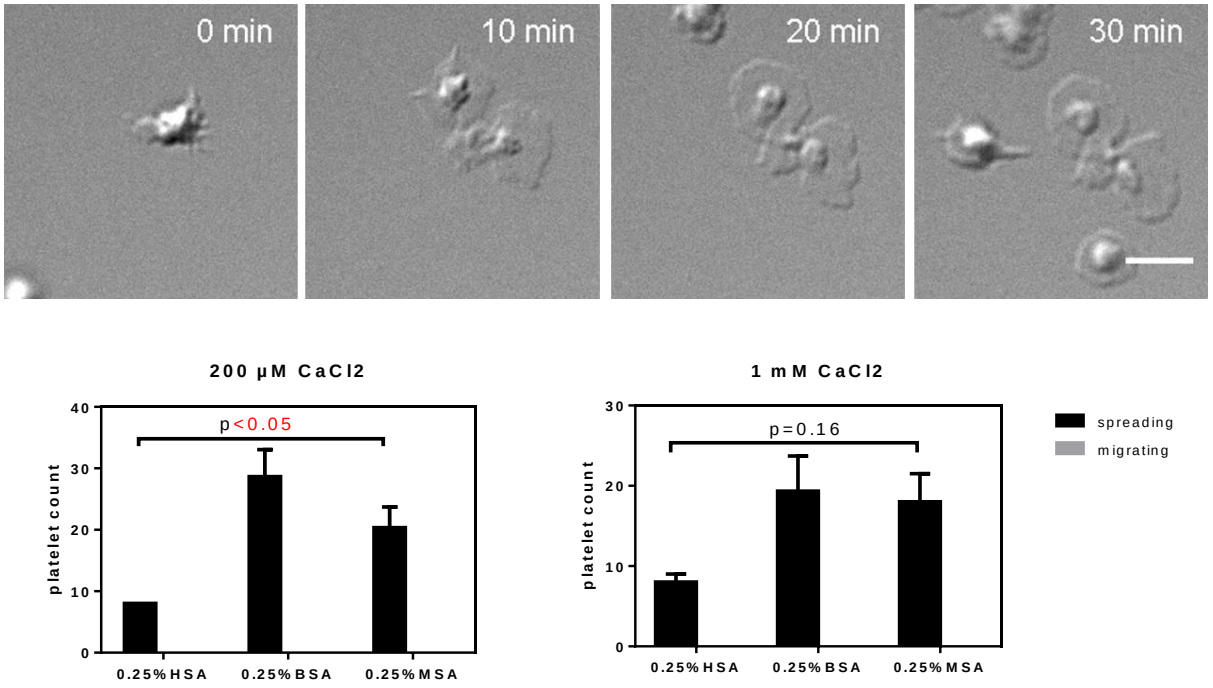


**Fig. 3.2 Albumin alone does not facilitate mouse platelet migration**. Mouse platelets were reconstituted with indicated albumin, $CaCl_2$, fibrinogen and U46619/ADP in modified Tyrode's buffer, and observed at time-lapse DIC microscope. Upper panel: representative time series at DIC microscope of mouse platelets in the presence of 0.25% MSA and 200 µM $CaCl_2$. Note, during 30 min spreading mouse platelets do not move for more than one of their diameters. Scale bar=5 µm. Lower panel: quantification of the number of spreading and migrating platelets in each experiment. Note, the number of migrating platelets are zero in each experiment. Error bars=SEM, n=3, ANOVA. HSA: human serum albumin; BSA: bovine serum albumin; MSA: mouse serum albumin.

Although albumin is able to facilitate human platelet migration, it is not the only anti-adhesive protein that triggers platelet migration. Casein, a common blocking agent in immunochemistry was capable of being substituted for albumin to promote human platelet motility (Gärtner, Engelhardt et al. 2015). Thus, casein was replaced of albumin on mouse platelets. Surprisingly, mouse platelets are able to migrate in the presence of casein in a concentration dependent manner (Fig.3.3). At 15 µg/ml of casein, mouse platelets spread, but only 4.98±3.63% of spreading platelets migrate. With 30 µg/ml casein, 54.23±4.49% of spreading mouse platelets are motile with the velocity of 0.42±0.02 µM/min. When casein concentration is increased to 60 µg/ml, the percentage of migrating cell rises to 63.24±2.94%, and the cell velocity grows up to 0.70±0.05 µm/min. Overall, the results demonstrate that casein is able to trigger mouse platelet migration and with the elevation of casein concentration, the fraction and velocity of migrating mouse platelets grow up.

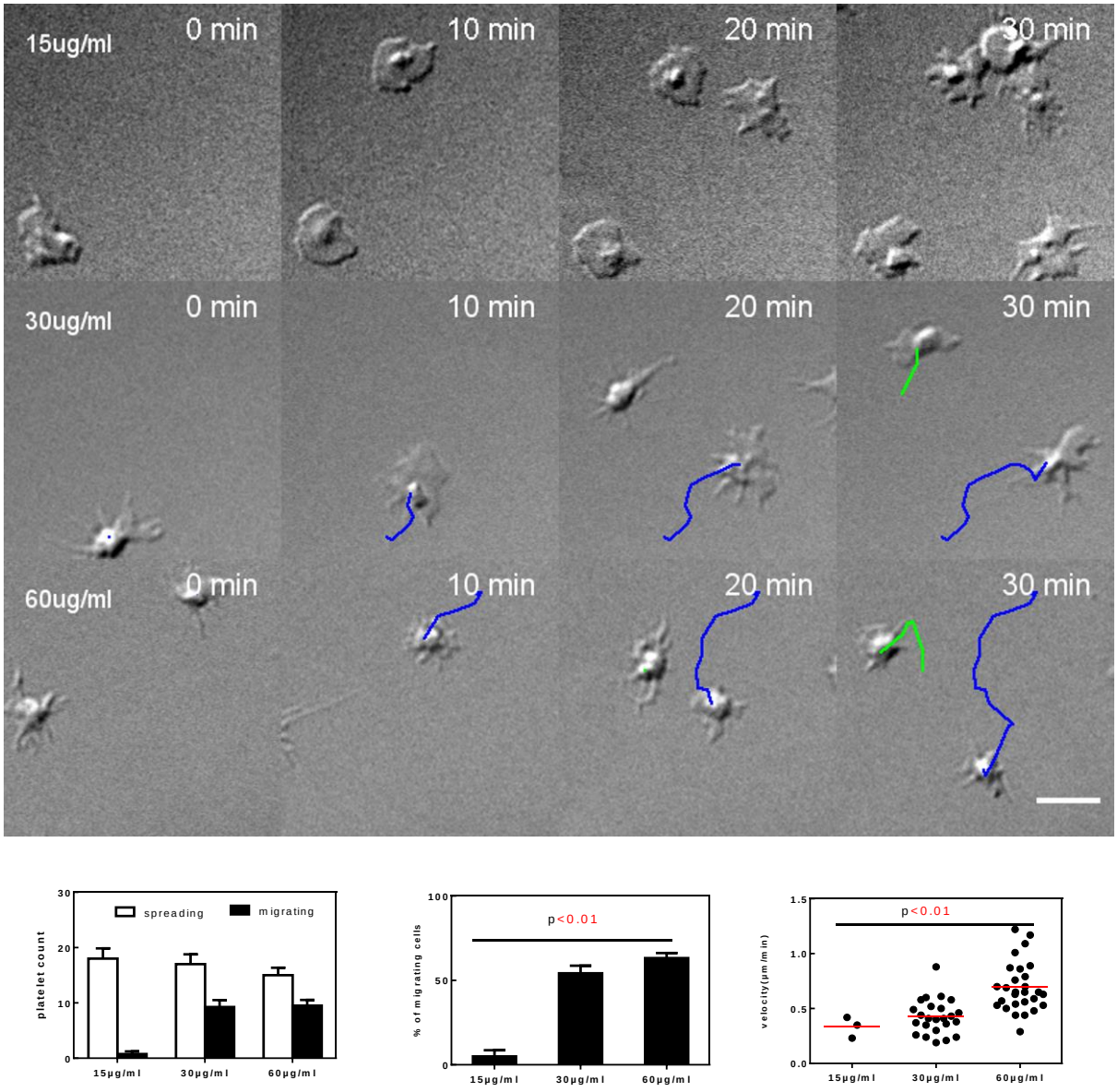


**Fig. 3.3 Casein is able to trigger mouse platelet migration.** Mouse platelets were reconstituted with indicated concentration of casein, 200 µM $CaCl_2$, 38 µg/ml fibrinogen and U46619/ADP, and observed at time-lapse DIC microscope. The upper panel: representative time series of mouse platelets at indicated casein concentration. The colourful lines indicate the accumulated migrating paths of mouse platelets at different time points. Note, at 15 µg/ml casein, platelets spread but do not migrate. Scar bar=5 µm. The lower panel: quantification of the total number of spreading and migrating platelets middle (left), the percentage of migrating platelets (middle), the velocity of single migrating platelets (right). Platelets were pooled from n=4 experiments; red bars indicate the mean velocity; error bars= SEM; one way ANOVA.

Casein proteins constitute approximately 80% of total proteins in bovine milk and exist as large colloidal particles called casein micelles. Casein micelles are comprised mainly of α-casein ($\alpha_{s1}$-casein and $\alpha_{s2}$- casein), β-casein, and κ-casein (Bhat, Dar et al. 2016). Which type of casein is the essential part of casein micelles for platelet migration? To elucidate this question, α- casein, β-casein or κ-caseins was applied on mouse platelets separately. Interestingly, each kind of casein is capable of promoting

mouse platelet migration. The minimal concentration to facilitate mouse platelet migration of α-, β-and κ-casein is 30 µg/ml, 30 µg/ml and 120 µg/ml, respectively; whereas the percentages of migrating platelets in the presence of them are not different (p=0.47, Fig.3.4). Compared with α casein monomer which possesses 8 – 12 phosphate residues, or β-casein monomer with five phosphate residues, κ-casein contains one phosphate residues per monomer (Horne 2006). Furthermore, k-casein is the only casein type that is glycosylated (Eigel, Butler et al. 1984). Therefore, the higher concentration of κ-casein to promote migration is possibly due to its different properties from other caseins.

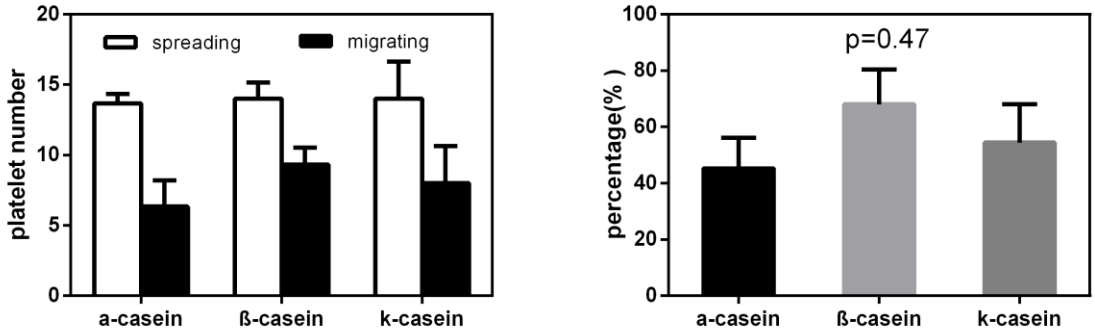


**Fig. 3.4 α-, β- and κ-casein trigger mouse platelet migration.** 30 µg/ml α-casein, 30 µg/ml β-casein, or 120 µg/ml κ-casein was applied on mouse platelets along with 38 µg/ml fibrinogen, 200 µM $CaCl_2$, U46619/ADP. Left: quantification of the number of spreading and migrating platelets in each experiment. Right: quantification of the percentage of migrating platelets in each experiment. Error bars=SEM, n=3, one way ANOVA.

Since casein micelles are phosphoproteins and glycosylated (Bhat, Dar et al. 2016), it is interesting to know whether the carbohydrate chains or the phosphate residues are the functional parts of caseins to trigger platelet migration. To that end, either dephosphorylated casein or fatty acid/carbohydrate free casein was applied on mouse platelets, consequently both of them trigger mouse platelet migration and the minimal concentration for them to facilitate motility are both 30 µg/ml, which are the same concentration as casein (Fig.3.5). The percentage of migrating platelets in the presence of dephosphorylated casein and fatty acid/carbohydrate free casein are 83.09±2.76%, 72.26±1.03%, respectively (Fig.3.5). Together, the results indicate that the phosphorylation residues or carbohydrate chain are not essential parts of caseins that trigger platelet migration.

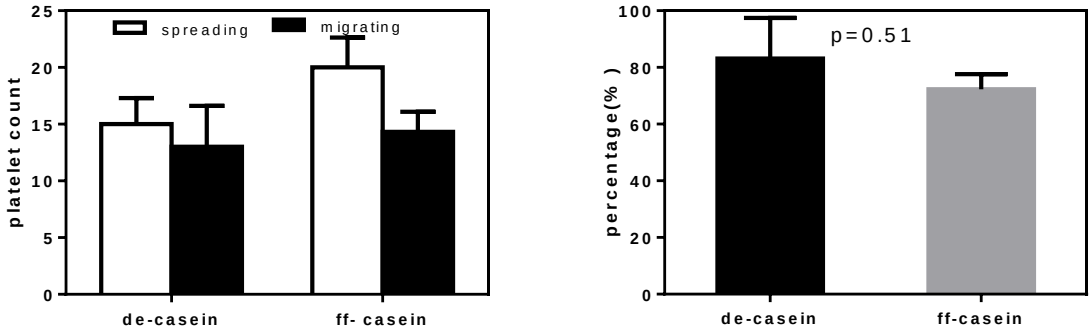


**Fig. 3.5 Dephosphorylated casein, fatty acid/carbohydrate free casein trigger mouse platelet migration**. 30 µg/ml dephosphorylated casein, 30 µg/ml fatty acid/carbohydrate free casein were applied on mouse platelets in the presence of 38 µg/ml fibrinogen, 200 µM calcium and U46619/ADP. Left: quantification of the number of spreading and migrating platelet in each experiment. Right: quantification of the percentage of migrating platelets in each experiment. Error bars=SEM; n=3; t-test. De-casein: dephosphorylated casein; ff-casein: fatty acid/carbohydrate free casein.

Our previous study on human platelets revealed that human platelet migration is integrin $\alpha_{IIb}\beta_3$ dependent, moreover, fibrinogen was removed from the substrate by motile platelets and accumulated within invaginations of the open canalicular system (OCS) on platelets surface (Gärtner, Engelhardt et al. 2015). To examine whether mouse platelets also remove fibrinogen during migration, Alexa488-conjugate fibrinogen was used. The results show that mouse platelets also ripped away fibrinogen from the substrate during migration, and we use this feature for tracking migrating platelets (Fig.3.6).

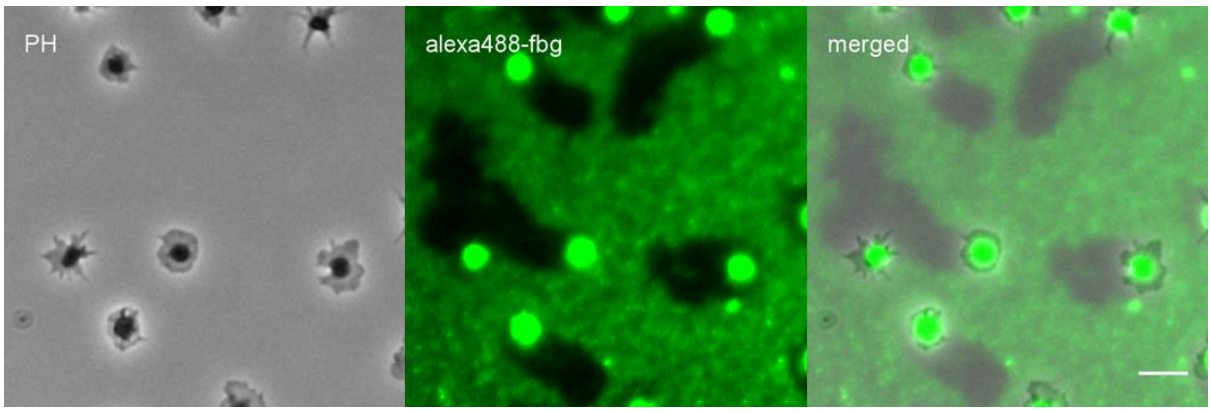


**Fig. 3.6 Mouse platelets remove fibrinogen from the substrate during migration.** Washed mouse platelets was reconstituted with 30 µg/ml casein, 200 µM $CaCl_2$ and U46619/ADP in the chamber with HSA/Alexa488-fbg coated surface. The figure shows images of HSA/Alexa488-fbg coated surface after washed platelets incubation for 1 hour. The black area in Alexa488-fbg images indicate the area where Alexa488-fbg was removed from the substrate. Scale bar=5 µm. Fbg: fibrinogen.

How do casein proteins facilitate platelet migration? To investigate whether casein proteins are merely working on the substrate or interacting with platelets, FITC

(fluorescein-isothiocyate)-casein is substituted for casein. The results show that FITC-caseins are distributed both on the substrate and on platelets membrane (Fig.3.7), which indicate that casein proteins function on the glass surface as well as on platelets plasma membrane. Surprisingly, mouse platelets do not migrate in the presence of FITC-casein (Fig.3.7), which implies that interaction points between caseins and platelets are occupied by FITC.

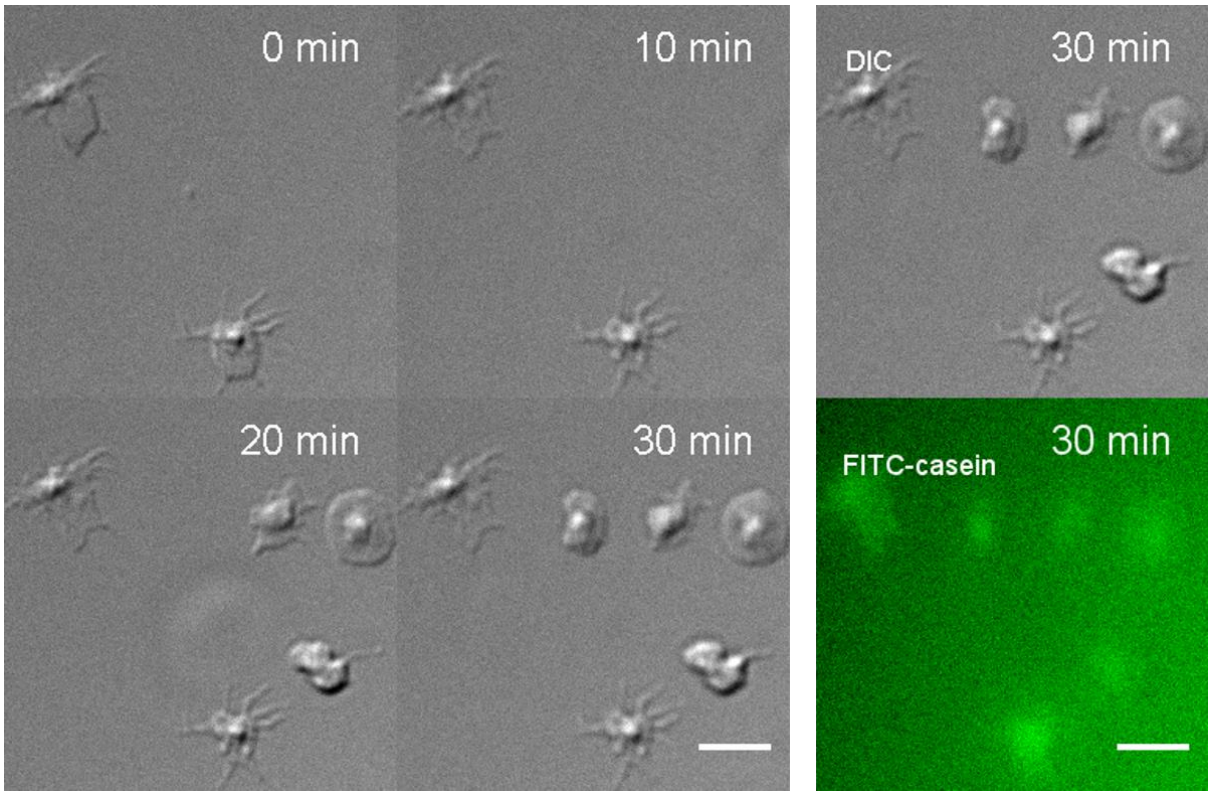


**Fig. 3.7 FITC-casein does not trigger mouse platelet migration**. 300 µg/ml FITC-casein was applied on mouse platelets in the presence of 38 µg/ml fibrinogen, 200 µM $CaCl_2$ and U46619/ADP. Left: representative time series of mouse platelets at indicated time points. Note, platelets spread but do not migrate. Right: representative images of platelets and FITC-casein after 30 min. Scale bar=5 µm.

To further confirm that casein is functioning both on the substrate and interacting with platelets, the glass surface was coated with Alexa488-conjugated fibrinogen alone or with casein proteins, then washed platelets were reconstituted in the supernatant in the presence or absence of casein. Our results show that when casein is present both on the substrate and supernatant, 81.61±1.76% of spreading platelets are mobile (Fig.3.8). When casein is only in the supernatant but not on the substrate, mouse platelets are immobile, indicating that the effect of casein on the substrate is necessary for platelet migration. Conversely, when casein is only on the surface but not in the supernatant, 81.11± 7.78% of spreading platelets are migrating (Fig.3.8).

However, when casein is present on the substrate, it is still able to interact with platelets. Next, casein on the substrate is substituted by albumin, as a results, mouse platelets are motile with casein in the supernatant. Nevertheless, when casein on the substrate and the supernatant are both replaced by albumin, mouse platelet are not mobile (Fig.3.8). Together, these data suggest that casein proteins play two roles in platelet migration. One is that casein is working on the substrate which can be replaced by albumin, suggesting its effect of reducing the fibrinogen adhesiveness on the substrate. The other role is the interaction with platelets, but the reaction between casein and platelets cannot be substituted by albumin on mouse platelets.

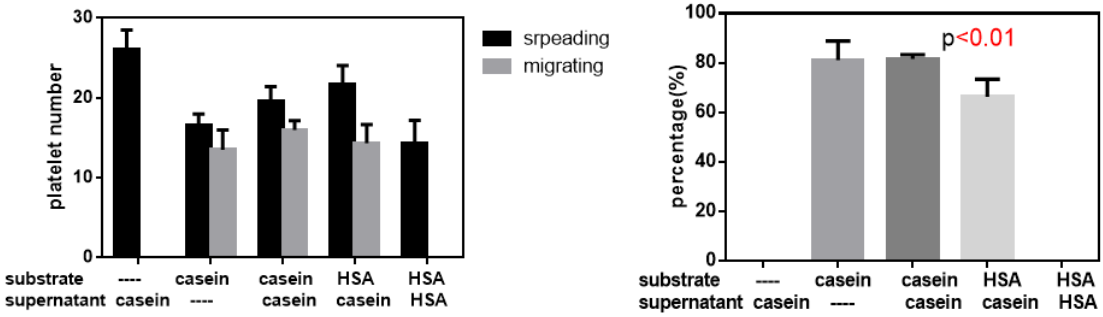


**Fig. 3.8 Casein proteins are functioning both on the substrate and interacting with platelets.** The glass surface was coated with 38 µg/ml Alexa488-fibrinogen alone, with 30 µg/ml casein or with 1000 µg/ml HSA, washed platelets were constituted in modified Tyrodes buffer in the presence or absence of 30 µg/ml casein, or 1000 µg/ml HSA, along with 200 µM $CaCl_2$, 2 µM U46619/ 4 µM ADP and incubated for 1 hour. The figure shows the quantification of the number of spreading and migrating platelets (left) and the percentage of migrating platelets (right). Error bars= SEM, n=3, Kruskal-Wallis test. --- indicates no casein and no HSA.

### 3.1.3 Poly lysine-albumin conjugates promote mouse platelet migration.

FITC is a fluorescein molecular with functional group of isothiocyanate, which reacts towards free amines in protein molecules (Hermanson 2013) (Fig.3.9). Since casein proteins are not able to trigger platelet motility when their amine groups interact with isothiocyanate, amine groups of amino acids that contain positive charges might be the essential parts for facilitating platelet migration. To demonstrate this assumption, poly-L-arginine (MW 15,000-70,000), poly-L-lysine (MW 30,000-70,000), or poly-L-ornithine (MW 30,000-70,000) that possess free amine groups (Fig.3.10) was replaced of casein, however, none of them promote platelets motility (Fig.3.11). Nevertheless, when these cationic polyamino acids were applied in combination with human albumin in the supernatant, mouse platelets are able to migrate. The percentage of

migrating platelets in the presence of poly-L-arginine, poly-L-lysine or poly-L-ornithine along albumin are 14.02±0.61%, 57.25±1.15%, 50.98±0.97%, respectively (Fig.3.12). Taken together, our results show that the conjugations of poly-arginine, poly-lysine, or poly- ornithine and albumin trigger mouse platelet migration, which indicate that the positive charges in casein proteins are functional parts for their roles in promoting platelet motility.

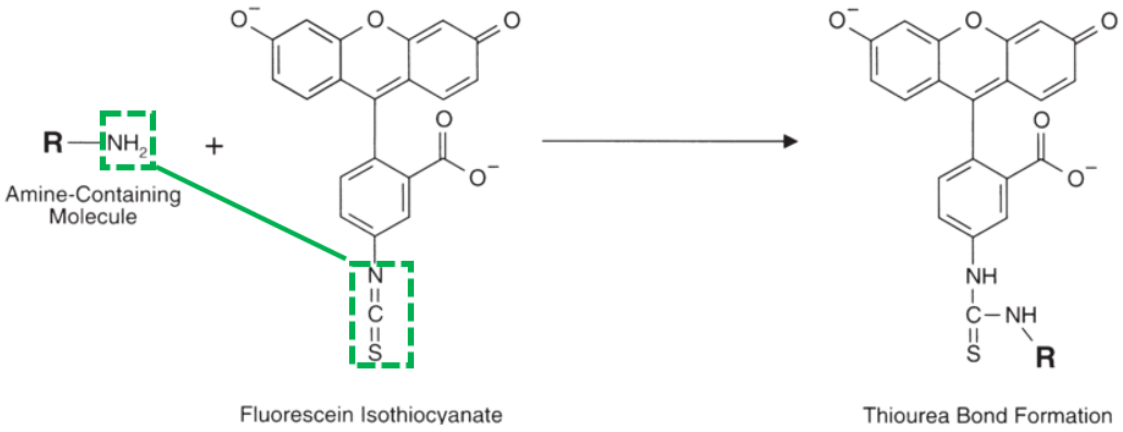


**Fig. 3.9 Fluorescein isothiocyanate reacts with amine groups in proteins to produce an isothiourea linkage**. Adaped from (Hermanson 2013). The green dashed rectangles show reacting groups.

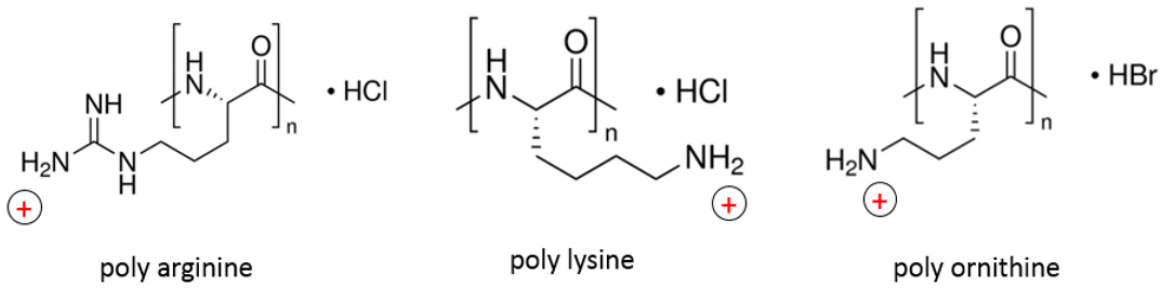


**Fig. 3.10 Structure of poly-arginine, poly-lysine, and poly-ornithine.** The free amine group in pH neutral solution contain postive charges.

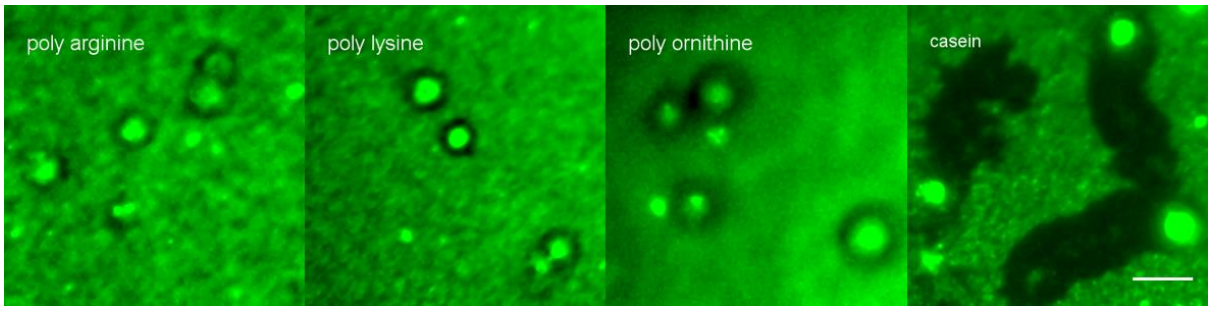


**Fig. 3.11 Ploy-arginine, poly-lysine or poly-ornithine in the absence of albumin in the supernatant do not trigger mouse platelet migration.** 50 µg/ml ploy-arginine, 50 µg/ml poly-lysine, 50 µg/ml poly-ornithine, or 30 µg/ml casein was applied in the presence of 200 µM $CaCl_2$ and U46619/ADP on washed mouse platelets in the chamber with HSA/Alexa488-fbg coated surface. The figure shows representative images of HSA/Alexa488-fibrinogen coated surface after washed platelets incubation for 1 hour in the presence of indicated substances. Note, in the presence of casein, the migrating area of platelets is black. Scale bar=5 µm.

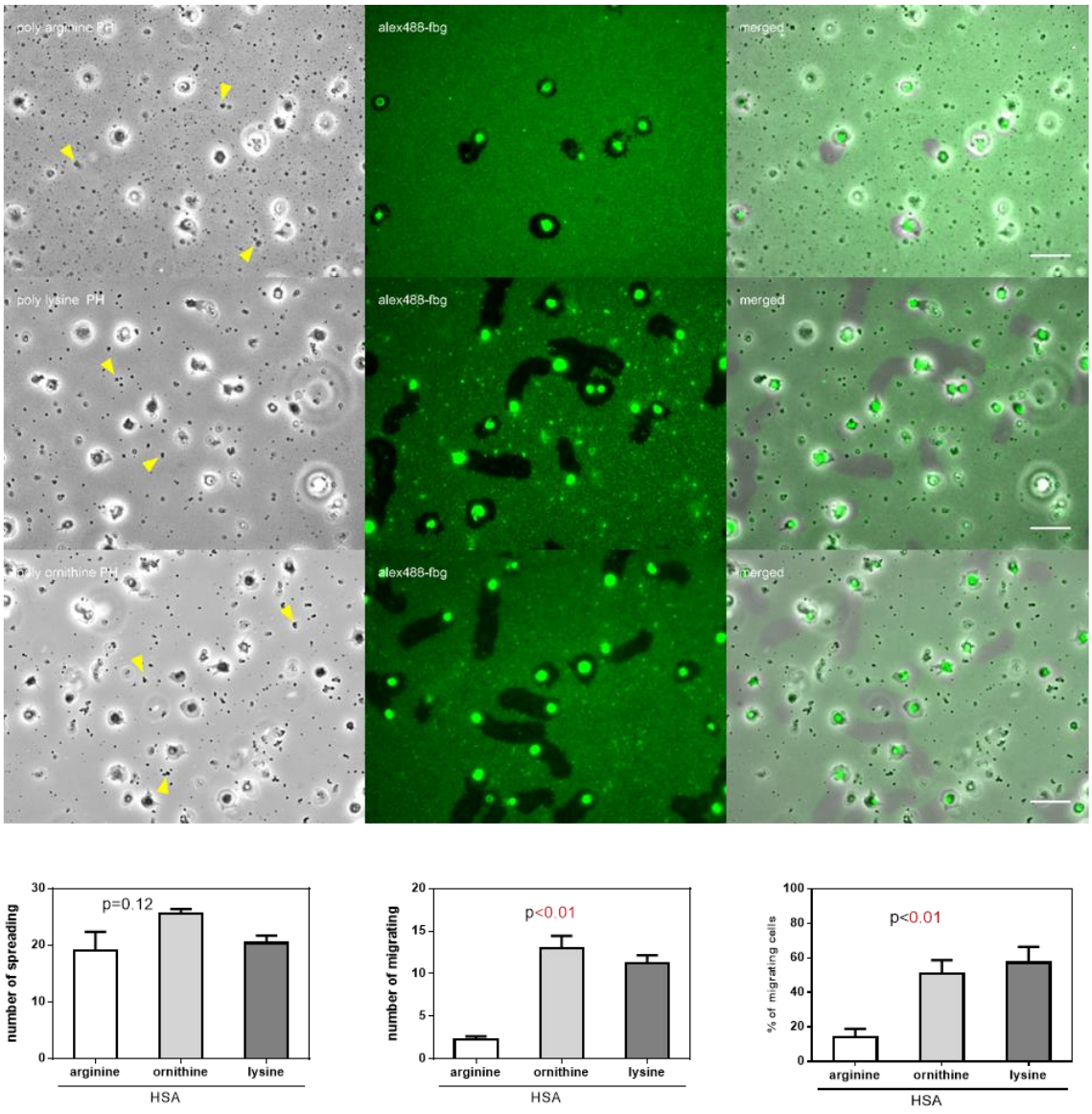


**Fig. 3.12 Conjugations of ploy-arginine, poly-lysine, poly-ornithine and albumin promote mouse platelet migration.** 25 µg/ml ploy-arginine, 25 µg/ml poly-lysine, or 25 µg/ml poly-ornithine was applied in the presence of 1000 µg/ml albumin, 200 µM $CaCl_2$, U46619/ADP on washed mouse platelets in the chamber with HSA/Alexa488-fbg coated surface. Upper panel: representative images of HSA/Alexa488-fbg coated surface after platelets migration in the presence of indicated substances. The yellow arrows in PH images indicate the aggregates formed by positively charged amino acids and HSA. The green and black area in Alexa488- fbg images indicate non-migrating and migrating area, respectively. Scale bar=10 µm. Lower panel: quantification of the number spreading (left), the number of migrating (middle) and the percentage of migrating platelets (right) in each experiment. Error bars=SEM, n=4, ANOVA. Fbg: fibrinogen. Arginine: poly-arginine; ornithine: poly-ornithine; lysine: poly-lysine.

Cationic polypeptides react with albumin to form conjugates and thus trigger platelet migration, raising the possibility that similar positively charged polymers could have the same effect on platelet locomotion. PEG (poly ethylene glycol)-peptides conjugates are the combination of PEG to peptides or proteins (Canalle, Lowik et al. 2010, Hamley 2014), and poly (L-lysine)-graft-poly (ethylene glycol) (PLL-g-PEG), is a graft

copolymer with positively charged PLL backbone and PEG side chains (Fig.3.13) (Lee and Spencer 2008, Bergstrand, Rahmani-Monfared et al. 2009). Interestingly, 51.77±3.10% of spreading mouse platelets are motile in the presence of PLL-g- PEG (Fig.3.14).

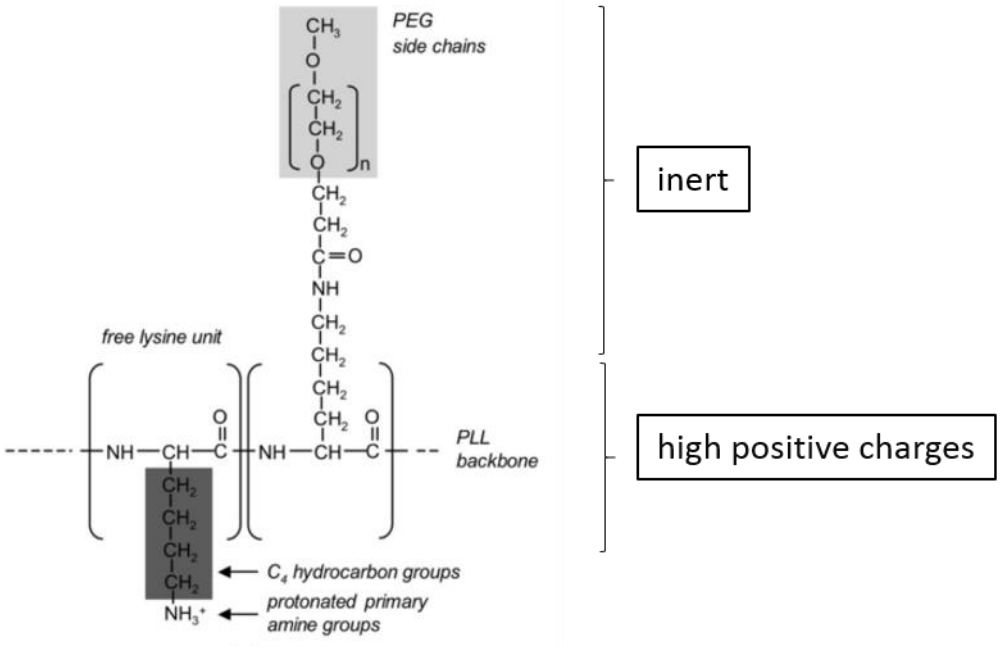


**Fig. 3.13 Structure of PLL-g-PEG**. Adapted from (Lee and Spencer 2008).Inert PEG side chains are attached positively charged PLL backbone.

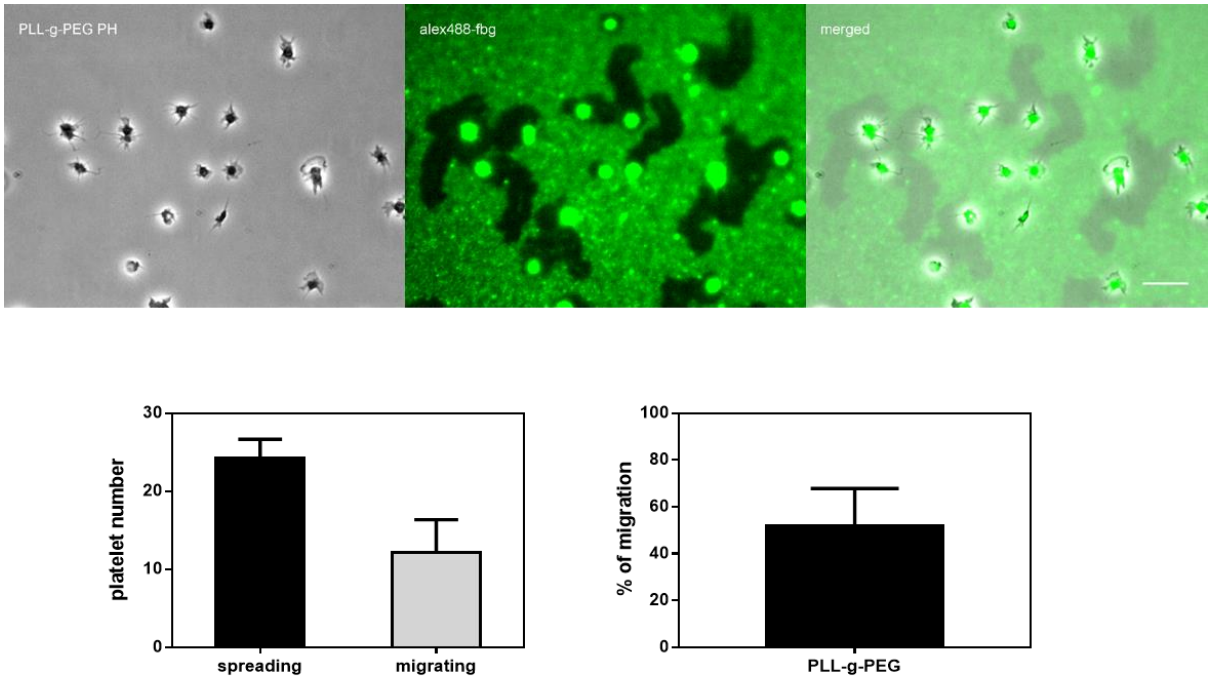


**Fig. 3.14 Mouse platelets migrate in the presence of PLL-g-PEG**. 25 µg/ml PLL-g-PEG was applied in the presence of 200 µM $CaCl_2$, U46619/ADP on mouse platelets in the chamber with HSA/Alexa488-fbg coated surface. Upper panel: representative images of HSA/Alexa488-fbg coated surface after platelets migration. The green and black area in Alexa488-fbg images indicate non-migrating and migrating area, respectively. Scale bar=10 µm. Lower panel: (left) quantification of the number of spreading and migrating platelet in each experiment. (Right) quantification of the percentage of migrating platelet. Error bars=SEM, n=3. Fbg: fibrinogen.

Since positively charged polypeptides-albumin conjugates are able to promote platelet migration, we speculate that similar cationic proteins might have the same effect. 6-aminocaproic acid (EACA) is a lysine analogue that inhibits plasminogen activation and subsequent fibrolysis (Griffin and Ellman 1978, Krishnamurti, Vukelja et al. 1994). However, when EACA was applied along with albumin, mouse platelets are not able to migrate (Fig.3.15). Protamine is in high content of arginine amino acids and present in the form of nuclear protein by binding to DNA (Aoki and Carrell 2003, Balhorn 2007), but it was unable to promote mouse platelet motility either (Fig.3.15). Spermine is a polyamine that plays crucial roles in a variety of cell physiology (Pegg 2009, Pegg 2014), but it does not facilitate mouse platelet migration (Fig.3.15). Similarly, growth factors (Schlessinger and Ullrich 1992) do not induce mouse platelet migration (Table 3-1). Since those cationic proteins have lower molecular weight than poly-L-lysine (PLL) we used, we postulate that the molecular weight affects the function of PLL on platelet locomotion. For that reason PLL with MW 1,000 to 5,000 was applied, the results show that low MW PLL is unable to trigger platelets migration along with albumin (Fig.3.16). Furthermore, the number of spreading platelets is lower in the presence of low MW PLL than high MW PLL (Fig.3.17), which is due to less positive

charges in each lower MW PLL molecular. Taken together, EACA, protamine, spermine, growth factors and low MW PLL are not able to promote mouse platelet migration.

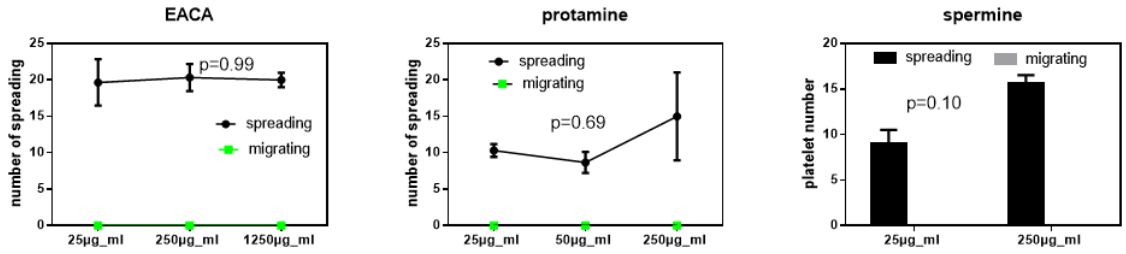


**Fig. 3.15 EACA, protamine, spermine are not able to promote platelet migration.** EACA, protamine, or spermine at indicated concentration was applied in the presence of 1000 µg/ml albumin, 200 µM $CaCl_2$, U46619/ADP on washed mouse platelets in the chamber with HSA/Alexa488-fbg coated surface. The figure shows the quantification of the number of spreading and migrating platelet in each experiment. Note, the number of migrating platelets in each experiment is zero. Error bars=SEM, n=3. Kruskal-Wallis test on the spreading platelets in the presence of EACA and protamine, Mann-Whitney test on the spreading platelets in the presence of spermine.

**Table 3-1 growth factors do not promote mouse platelet migration**

| Growth factors | Spreading | Migrating |
|---|---|---|
| BD-1(1 µg/ml) +BD-2+(100 ng/ml) | 23.67±0.23 | 0 |
| VEGF(10 ng/ml )+ PDGF-BB(100 ng/ml) | 21.00±0.51 | 0 |
| rmFGF-b(100 ng/ml) | 18.50±0.18 | 0 |
| rmIGF-1(100 ng/ml) + rhTGF-ß(10 ng/ml) | 21.33±0.45 | 0 |
| MK(100 ng/ml) | 21.00±0.22 | 0 |
| HB-EGF(100 ng/ml) + rmEGF-1(10 ng/ml) | 23.33±0.61 | 0 |

Growth factors at indicated concentration were applied on washed mouse platelets in the presence of 1000 µg/ml HSA, 200 µM $CaCl_2$, U46619/ADP in the chamber with HSA/Alexa488-fbg coated surface. Then the number of spreading and migrating platelet in each experiment were quantified. BD-1: recombinant mouse beta defensin-1; BD-2:recombinant mouse beta defensin-2; VEGF: recombinant mouse vascular endothelial growth factor A; PDGF-BB: recombinant mouse platelet-derived growth factor-BB; rmFGF-b: recombinant mouse fibroblast growth factor-basic; rmIGF-1: recombinant murine insulin-like growth factor1; rhTGF-ß: recombinant human transforming growth factor-beta1; MK: recombinant mouse midkine; HB-EGF: recombinant mouse heparin binding EGF-like growth factor; rmEGF-1: recombinant mouse epidermal growth factor-1

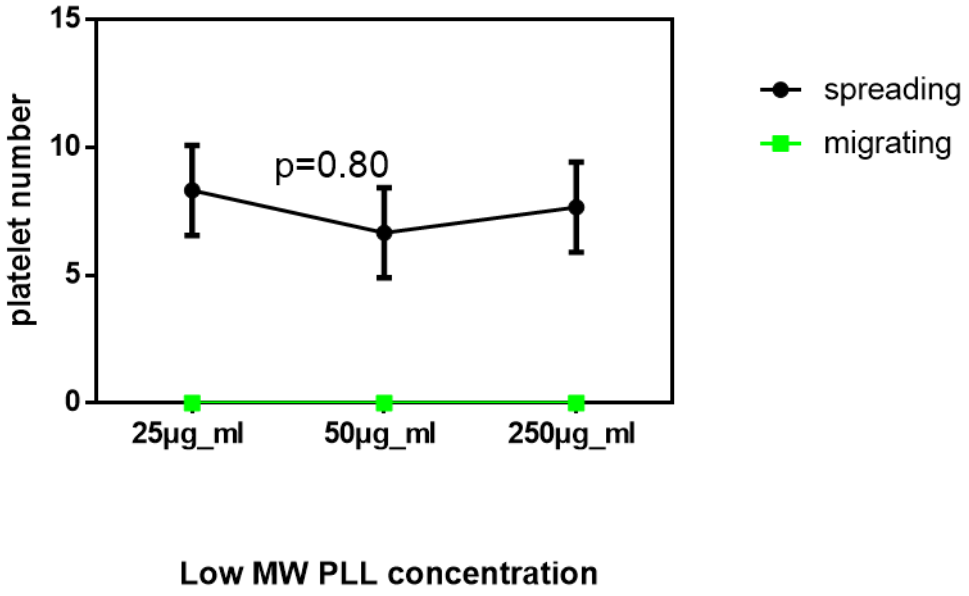


**Fig. 3.16 Low MW PLL does not trigger platelet migration.** PLL with MW 1,000 to 5,000 was applied in the presence of 1000 µg/ml albumin, 200µM $CaCl_2$, U46619/ADP on washed mouse platelets in the chamber with HSA/Alexa488-fbg coated surface. The figure shows the quantification of the number of spreading and migrating platelet. Note, the number of migrating platelets in each experiment is zero. Error bars=SEM, n=3, ANOVA on the spreading platelets.

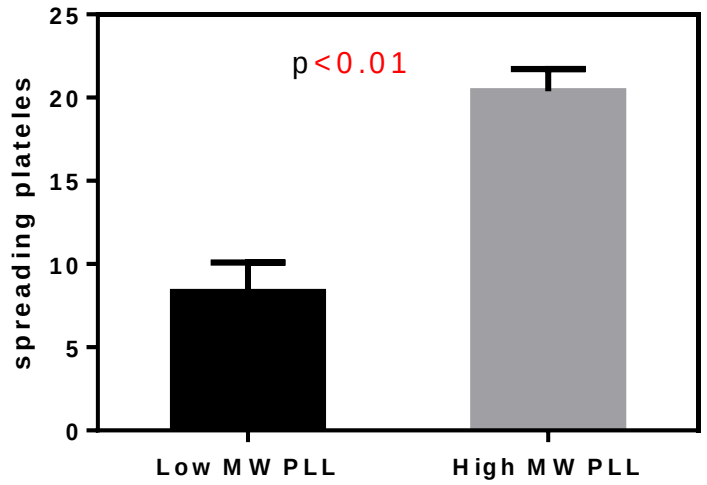


**Fig. 3.17 The number of spreading platelets is less in the presence of low MW PLL than that of high MW PLL**. 25 µg/ml PLL with MW 1,000 to 5,000 (low MW), or MW 30, 000 to 70, 000 (high MW) was applied in the presence of 1000 µg/ml HSA, 200 µM $CaCl_2$, U46619/ADP on washed mouse platelets in the chamber with HSA/Alexa488-fbg coated surface. The figure shows the quantification of the number of spreading platelets. Error bars=SEM, n=3, t-test.

### 3.1.4 Reducing negative charges on plasma membrane does not initiate platelet migration.

How are polycationic proteins interacting with platelets? Basic polyamino acids, like poly-lysine are usually treated on glass surfaces for cell attachment because of the electrostatic attraction between cationic charges on the peptides and anionic charges on the cell membrane (Nevo, De Vries et al. 1955, Yavin and Yavin 1974). Thus, one possible mechanism by which cationic polypeptides facilitate platelet migration is that they neutralize anionic charges on platelets surfaces. Therefore, we assume that removing the negative charges on platelet membrane might promote migration (Fig.3.18).

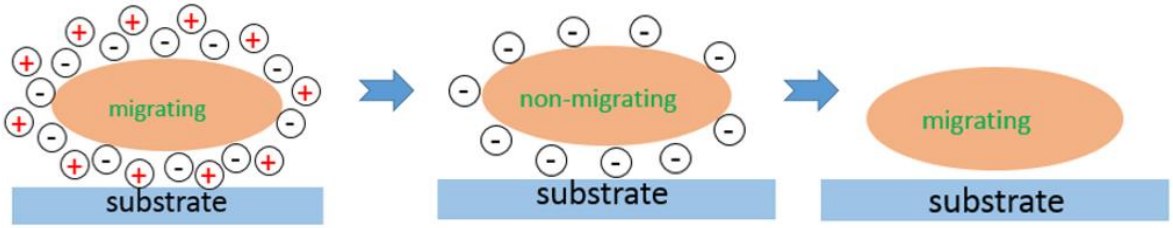


**Fig. 3.18 Hypothesis of the mechanism by which cationic proteins trigger platelet migration**. We hypothesize that cationic polypeptides facilitate platelet migration by neutralizing anionic charges on platelets surfaces. And negative charges on platelets membrane impede platelet motility, therefore, removing the negative charges on platelet membrane might promote migration.

The anionic charges on the cell surface are conveyed by the extracellular carbohydrate chains of glycoproteins on the plasma membrane. Salic acids, also named neuraminic acids attached to glycoproteins are largely responsible for the negative charges on the cell membrane (Eylar, Madoff et al. 1962, Traving and Schauer 1998). However, enzymatic removal of sialic acid by neuraminidase does not initiate platelet locomotion (Fig.3.19). Besides sialic acids, heparan sulfate and chondroitin sulfate contribute to the anionic charges on cell membrane due to the sulfate group (Honke and Taniguchi 2002, Tan, Poh et al. 2013). Nevertheless, eliminating the sulfate group by heparinase and chondroitinase does not trigger platelet migration (Fig.3.19). Surprisingly, we found that the number of spreading platelets was increased, and the percentage of platelet migration was diminished in the presence of neuraminidase (Fig.3.20), which indicates that sialic acids on glycoproteins affect platelets spreading and migrating.

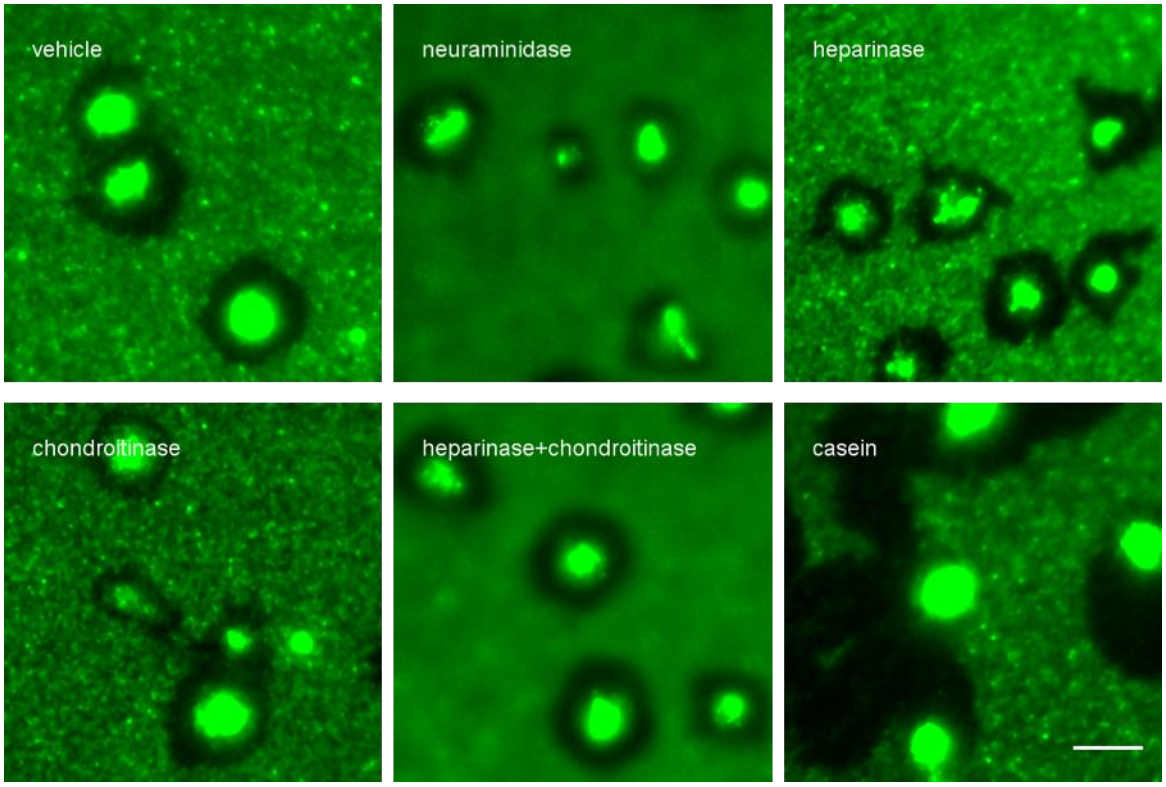


**Fig. 3.19 Enzymatic removal of negative charges on platelet membrane do not initiate migration.** Vehicle, 100 mU/ml neuraminidase, 25 mU/ml heparinise II, 50 mU/ml chondroitinase ABC, the combination of 25 mU/ml heparinise II and 50 mU/ml chondroitinase ABC, or 30 µg/ml casein was applied on mouse platelets in the presence of 1000 µg/ml HSA, 200 µM $CaCl_2$ and U46619/ADP in the chamber with HSA/Alexa488-fbg coated surface. The figure shows representative images of HSA/Alexa488-fbg coated surface after washed platelets incubation for 1 hour in the presence of indicated substances. Scale bar=5 µm.

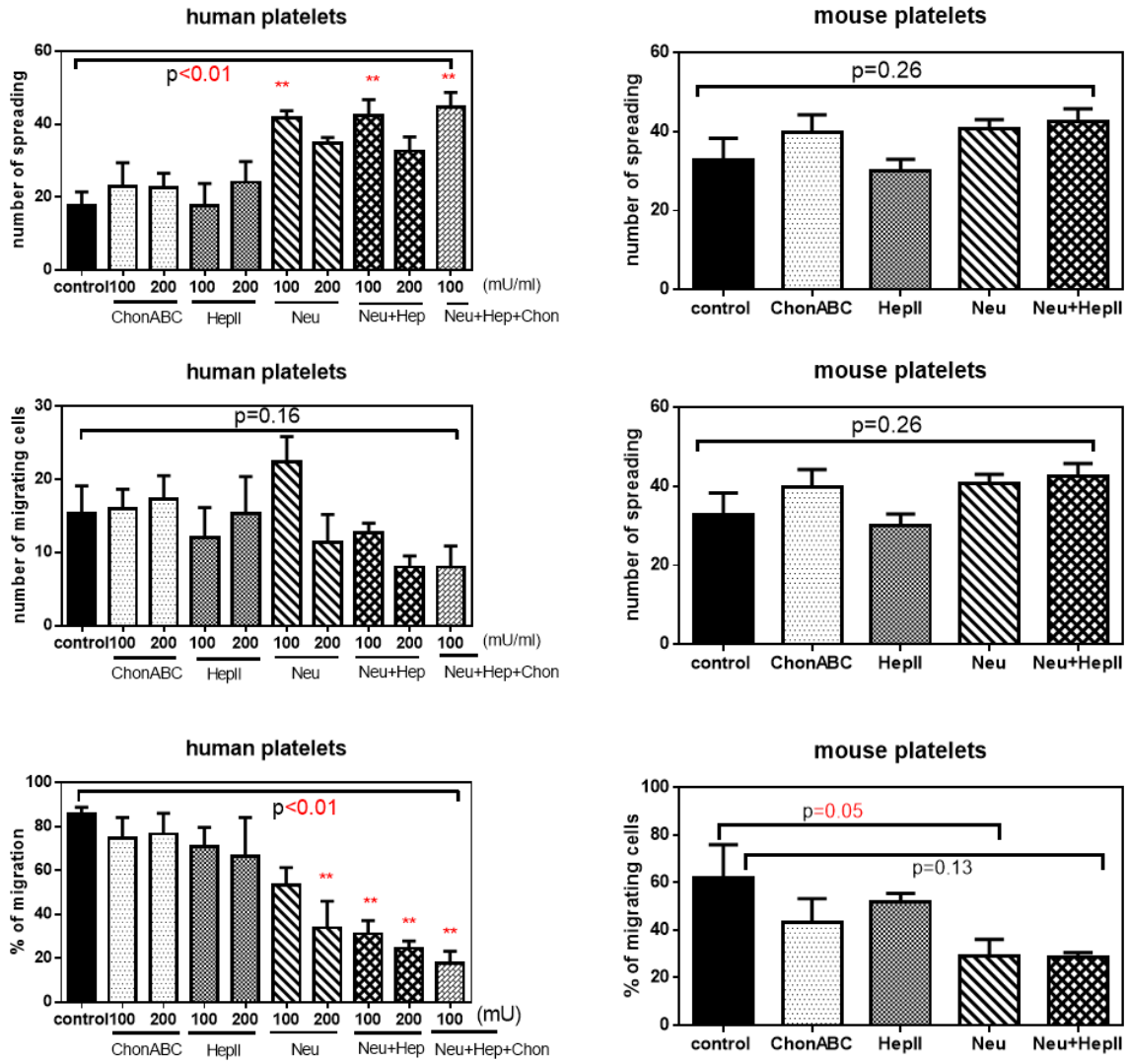


**Fig. 3.20 Enzyme treatment impairs platelet spreading and migrating in the presence of calcium-.** 200 mU/ml chondroitinase ABC, 200 mU/ml heparinise II, or 200 mU/ml neuraminidase was applied on mouse platelets in the presence of plasma, 38 µg/ml fibrinogen and U46619/ADP. Vehicle or indicated concentration of enzymes were applied on human platelets in the presence of 1000 µg/ml HSA, 200 µM $CaCl_2$ and U46619/ADP on HAS/Alexa488-coated surface. The figure shows the quantification of the number of spreading (upper), the number of migrating (middle) and the percentage of migrating platelets (lower) on human and mouse platelets. Error bars=SEM, n=3, ANOVA. **indicate p<0.01 (compared with control group). ChonABC: chondroitinase ABC; HepII: heparinise II; Neu: neuraminidase.

GPIbα are transmembrane glycoproteins whose N- and O-linked carbohydrate chains are decorated with sialic acid, and constituted major sialic acid content on platelet membrane (Solum, Hagen et al. 1980, Li, van der Wal et al. 2015). GPIbα/IL4R mice are transgenic mice in which the extracellular domain of GPIb on platelets membrane is replaced by human IL-4 receptor (Bergmeier, Piffath et al. 2006). Our results show that in the absence of casein, GPIbα/IL4R platelets are not mobile (Fig.3.21). Therefore, reducing negative charges by decreasing the sialic acids on platelet surface does not initiate platelet migration.

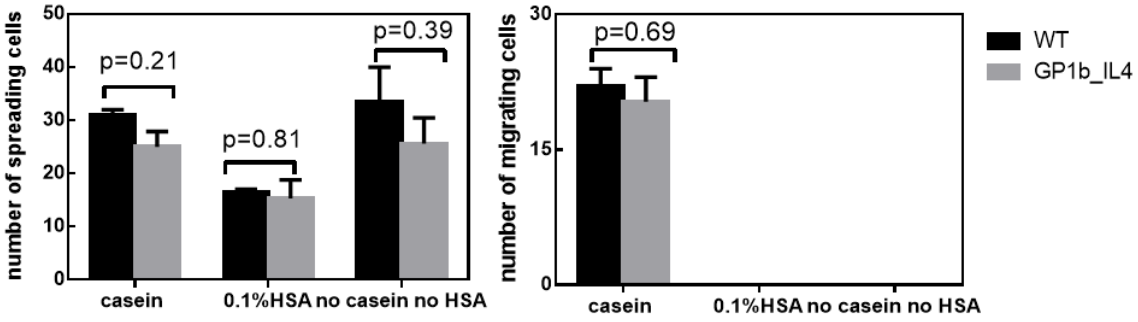


**Fig. 3.21 Decreasing the sialic acids on platelet surface does not initiate platelet migration**. Platelets from control or GPIbα/IL4R mice were reconstituted with 30 µg/ml casein, 0.1% HSA, or neither of them in the presence of 200 µM $CaCl_2$, U46619/ADP in the chamber with HSA/Alexa-488fbg coated surface. The figure shows the quantification of the number of spreading (left) and migrating (right) platelets in each experiment. Note: the number of migrating platelets in the presence of 0.1% HSA or no casein no HSA are zero. Error bars=SEM, n=3, t-test.

Agonist-induced procoagulant platelets expose negatively charged phosphatidylserine (PS) which is a key event in blood coagulation (Lentz 2003, Schoenwaelder, Yuan et al. 2009). Annexin V preferentially binds to PS and abolishes the negative charges on platelets membrane (Thiagarajan and Tait 1990, Sun, Bird et al. 1993). However, when poly-L- lysine is replaced by annexin V, mouse platelets are not motile (Fig.3.22). Overall, these experiments demonstrate that reduction of negative charges on platelets membrane do not trigger platelet migration, which implies that cationic proteins do not neutralize anionic charges on platelet surface to facilitate locomotion.

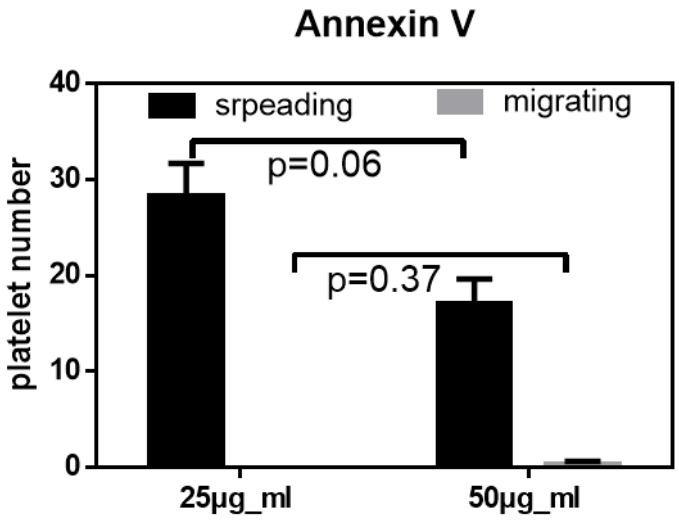


**Fig. 3.22 Annexin V is unable to facilitate platelet migration**. Annexin V was applied in the presence of 1000 µg/ml HSA, 200 µM $CaCl_2$, U46619/ADP on washed mouse platelets in the chamber with HSA/Alexa488-fbg coated surface. The figure shows the quantification of the number of spreading and migrating platelet in each experiment. Error bars=SEM, n=3, t-test.

### 3.1.5 Casein proteins trigger platelet migration by regulating calcium oscillations.

In the presence of casein proteins, we observed blebs formation prior to platelet death (Fig.3.23). The percentage of blebbing platelets in the presence of α-, β-, κ-casein, fatty acid and carbohydrate free casein is 32.31±1.18%, 11.71±0.34%, 24.81±1.15%, 9.37±0.71%, respectively (Fig.3.23). However, platelets do not form the blebs in the presence of dephosphorylated casein (Fig.3.23), which suggests that the phosphorylation level of casein influences platelets viability.

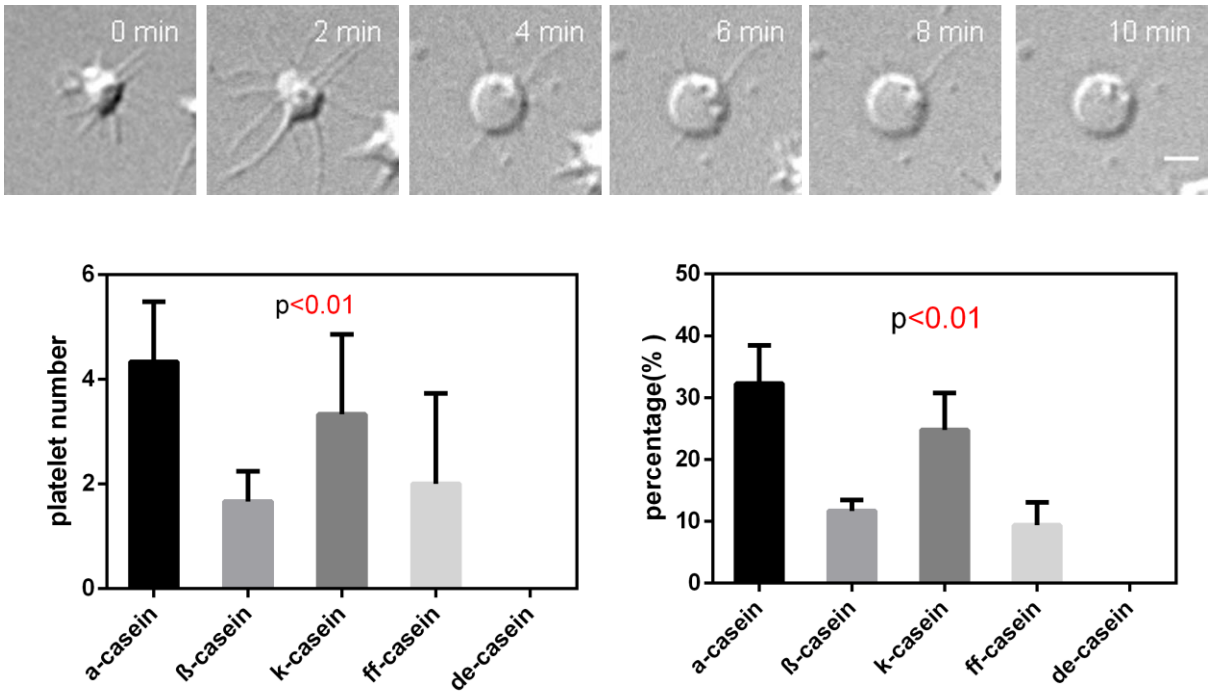


**Fig. 3.23 Mouse platelets form blebs in the presence of casein proteins.** Upper panel: representative time series of one platelet that forms blebs in the presences of casein. Scale bar=2 µm. Lower panel: Left: quantification of the number of blebbing platelets in each experiment. Right: quantification of percentage of blebbing platelet in each experiment. Note: the number and percentage of blebbing platelets in the presence of de-casein is zero. Error bars=SEM, n=3, ANOVA. De-casein: dephosphorylated casein; ff-casein: fatty acid and carbohydrate free casein

Cell blebbing is balloon-like membrane protrusion that occurs in cell movement, cytokinesis, and apoptosis (Charras 2008, Bovellan, Fritzsche et al. 2010). In zebrafish germ cells, blebs form at the site of high intracellular calcium that depends on the activation of SDF receptor (Blaser, Reichman-Fried et al. 2006). To examine whether blebs of platelets have increased cytosolic calcium in the presence of casein proteins, platelets were loaded with calcium sensitive dye-Fluo4 AM and observed at fluorescent microscope. Our results show that platelets with blebs formation exhibit sustained elevated cytoplasmic calcium (Fig.3.24), which implies that casein proteins influence

the calcium signaling in platelets. Previous study in our group revealed that platelets migrate with calcium oscillations, giving a cue that casein may regulate calcium fluctuations in mobile platelets. We first examined calcium oscillations of platelets in the absence of casein proteins or albumin. Although platelets are stationary, they show frequent and transient calcium spikes (Fig.3.25). In the presence of albumin, platelets exhibit less frequent calcium oscillation without locomotion (Fig.3.26). In contrast, in the presence of casein proteins platelets migrate with less frequency but prolonged duration of calcium oscillations. More importantly, the elevated calcium level sustains for longer time at the initial phase of migration (Fig.3.26). Taken together, these findings suggest that casein proteins trigger platelet motility by modulating calcium signals in platelets.

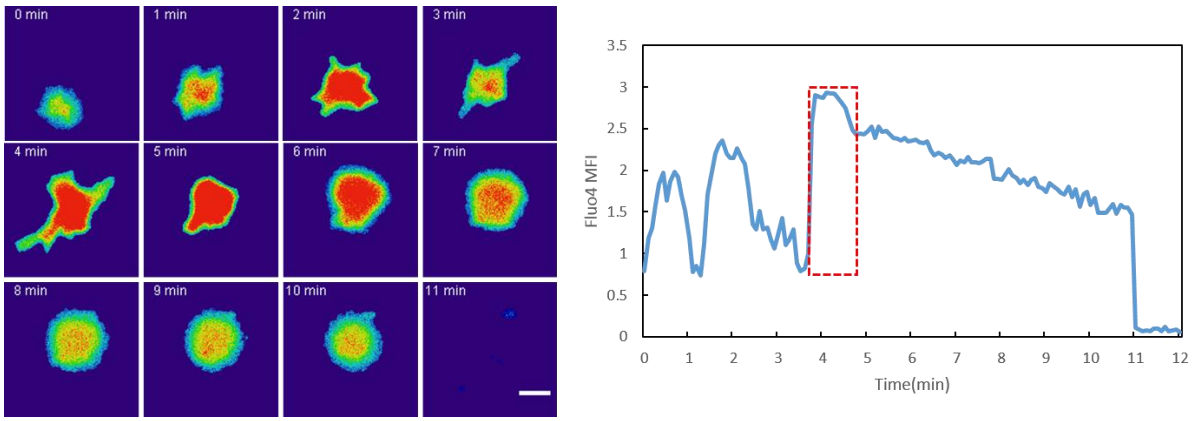


**Fig. 3.24 Platelets with blebs formation exhibit sustained elevated intracellular calcium.** Washed platelets were loaded with 5 uM Fluo4-AM, and then reconstituted in 30 μg/ml casein, 200 μM $CaCl_2$, 38 μg/ml fibrinogen and U46619/ADP, then Fluo4 intensity was recorded at time-lapse fluorescent microscope with 5 sec interval. Left panel shows the pseudocolor frame images of Fluo4 intensity of one platelet that forms bleb at different time points (blue→red, low calcium level→ high calcium level), scale bar= 2 μm. Right panel shows the quantification of Fluo4 MFI in time course. Red dashed rectangle indicate the elevated cytosolic calcium level when bleb forms in platelets.

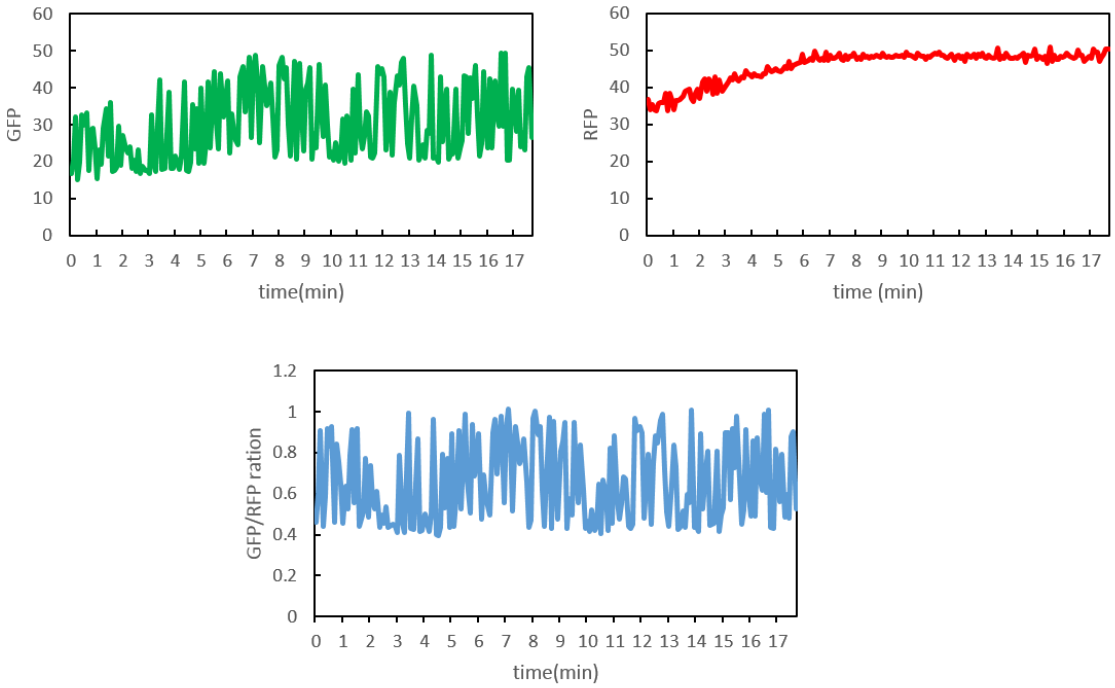


**Fig. 3.25 Platelets show frequent calcium oscillations in the absence of proteins.** Washed platelets from PC::G5-tdT; Pf4 cre mouse were reconstituted in tyrodes buffer with 200 µM $CaCl_2$, 38 µg/ml fibrinogen and U46619/ADP, then observed at time-lapse fluorescent microscope with 5 sec interval. The images show the quantification of fluorescent changes from one representative platelet.

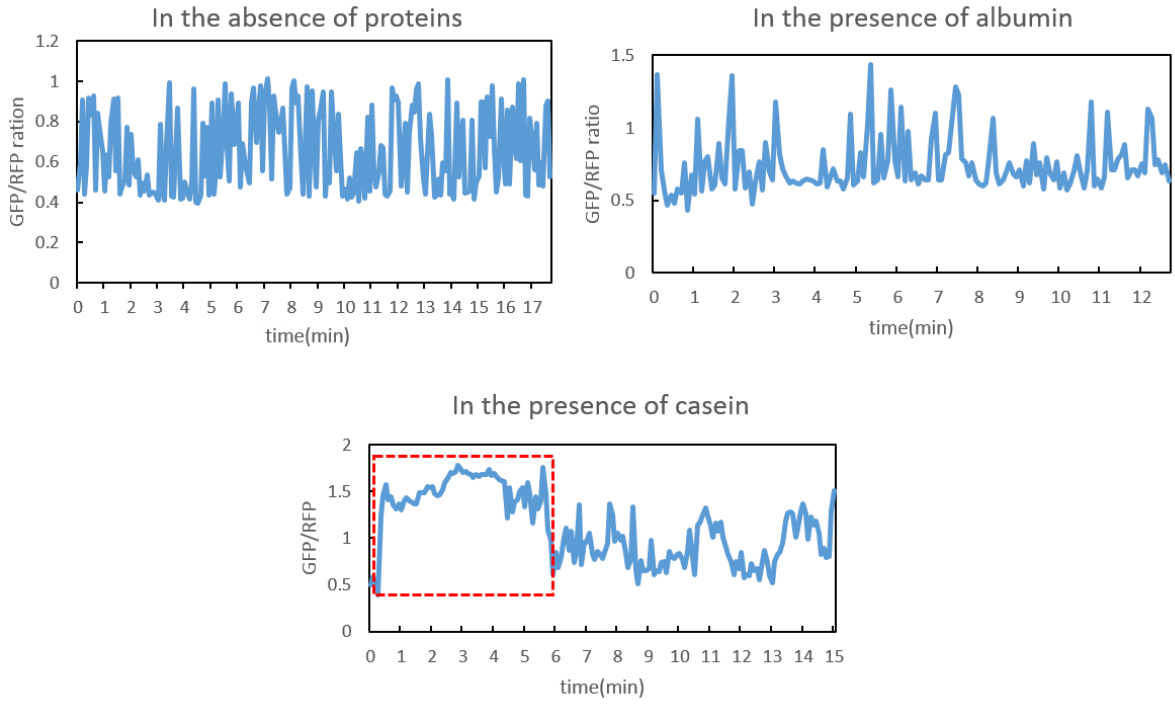


**Fig. 3.26 Casein and albumin proteins modulate calcium oscillations of platelets on fibrinogen substrate.** Washed platelets from PC::G5-tdT; Pf4 cre mouse were reconstituted with 200 μM $CaCl_2$, 38 μg/ml fibrinogen, U46619/ADP and 1000 μg/ml HSA or 30 μg/ml casein, then observed at time-lapse fluorescent microscope with 5 sec interval. The figures show the quantification of the calcium signals in representative platelets in each condition. The red dashed rectangle indicate the sustained cytosolic calcium in the initial phase of migrating platelets in the presence of casein, which is different from calcium signals in the absence of proteins and in the presence of albumin.

Our experiments reveal that albumin is able to trigger human platelet not mouse platelet migration, suggesting the different properties of human and mouse platelets. To examine whether human platelets show different calcium oscillations from mouse platelets in the presence of casein, human platelets were loaded with Fluo4-AM and then observed at fluorescent microscope. Consistent with previous results, human platelets exhibit different patterns of calcium oscillations from mouse platelets in the presence of casein (Fig.3.27). Particularly, at the initial phase of migration, human platelets do not exhibit sustained elevated cytoplasmic calcium, which further confirms that human and mouse platelets behave differently in migration.

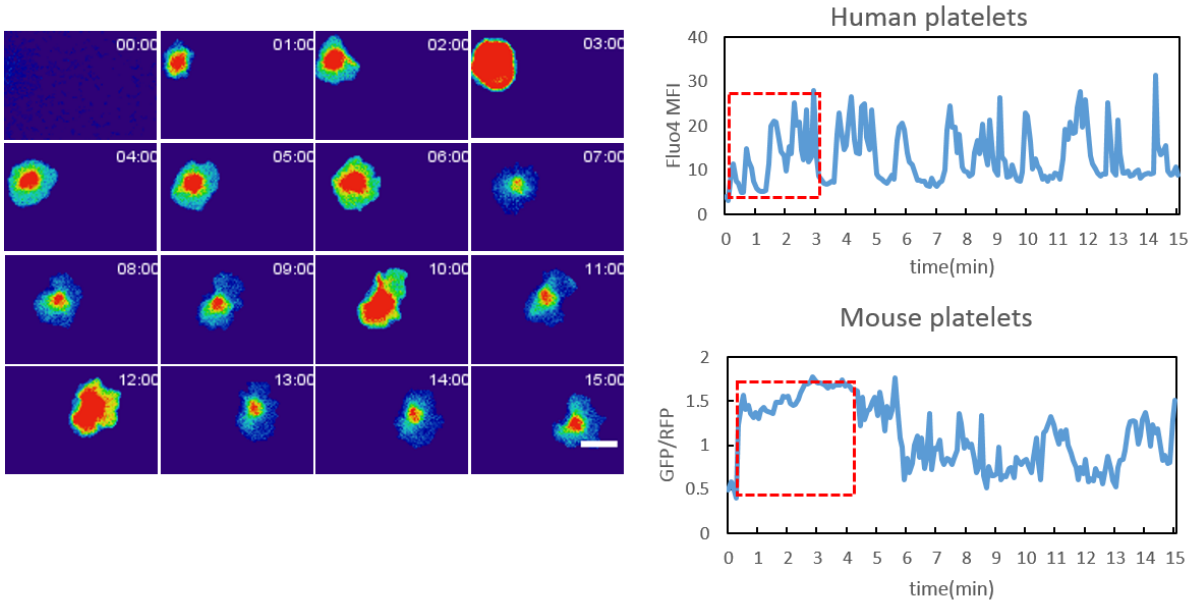


**Fig. 3.27 Human platelets exhibit different patterns of calcium oscillations from mouse platelets in the presence of casein.** Human platelets were loaded with 5 µM Fluo4-AM and reconstituted in modified tyrodes buffer with 200 µM $CaCl_2$, 38 µg/ml fibrinogen and U46619/ADP, then observed at time-lapse fluorescent microscope with 5 sec interval. Left panel shows the pseudocolorful frame images of Fluo4 intensity of one representative migrating human platelet in the presence of casein at different time points (blue→red, low calcium level→ high calcium level), scale bar= 5 µm. Right panel (upper) shows the quantification of Fluo4 MFI of migrating human platelet shown in the left. Right panel (lower) shows the quantification of Fluo4 MFI of one representative migrating mouse platelet in the presence of casein. The red dashed rectangles indicate the initial phase of migrating platelets, which are different between human and mouse platelets.

Since caseins proteins regulate calcium oscillations in platelets, we speculate that compounds that affect calcium signals could have the same targets as casein on platelets. A23187, a calcium ionophore, disturbs the calcium gradient by forming complex with divalent cation ions and crossing cell membranes (Reed and Lardy 1972). However, A23187 does not facilitate platelet migration (Fig.3.28), which suggests that caseins do not function in the same way as calcium ionophore.

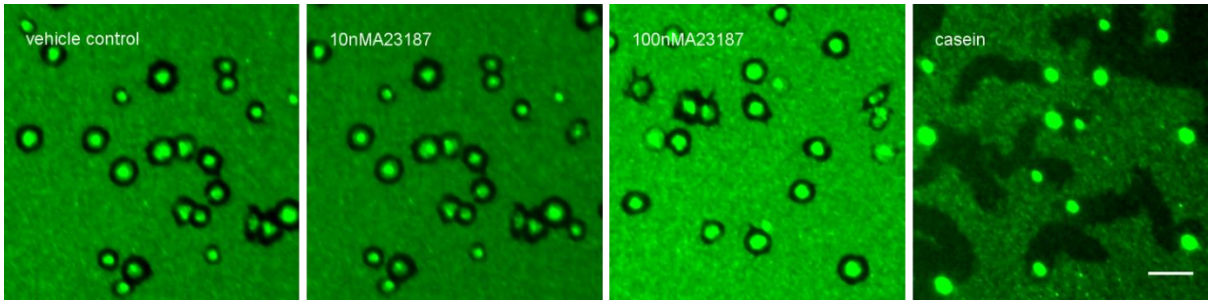


**Fig. 3.28 A23187 does not trigger platelet migration.** Vehicle control, 10 nM A23187, 100 nM A23187, or 30 µg/ml casein was applied in the presence of 200 µM $CaCl_2$, U46619/ADP on washed mouse platelets in the chamber with HSA/Alexa488-fbg coated surface. The figure shows representative images of HSA/Alexa488-fbg coated surface after washed platelets incubation for 1 h in the presence of indicated substances. Note, platelets do not migrate in the presence of vehicle control or A23187, but platelets are migrating in the presence of casein, the migrating area of platelets is black. Scale bar=10 µm.

Pluronic F127 is nonionic surfactant that is used to increase the loading efficiency of the calcium acetoxymethyl (AM) ester dyes into live cells (Hamad, Krause et al. 2015). Surprisingly, pluronic F127 triggers platelet locomotion with minimal concentration of 5 µg/ml. In the presence of 5 µg/ml pluronic F127, 39.91± 2.11% of spreading platelets are mobile (Fig.3.29), which implies that the functional target of pluronic could be the interaction part of caseins on platelets.

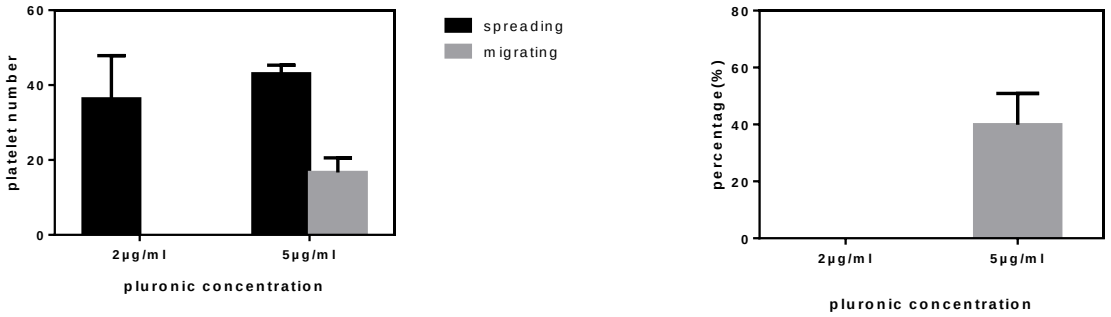


**Fig. 3.29 Pluronic F127 triggers platelet migration.** Pluronic F127 was applied at indicated concentrations in the presence of 200 µM $CaCl_2$, U46619/ADP on washed mouse platelets in the chamber with HSA/Alexa488-fbg coated surface. The figure shows the quantification of the number of spreading and migrating platelet (left) and the percentage of migrating platelets (right) in each experiment. Note, the number and percentage of migrating platelets at 2 µg/ml pluronic F127 are zero. Error bars=SEM, n=3.

Given that pluronic F127 is an amphiliphic triblock copolymer (Kabanov, Lemieux et al. 2002), we are searching for the possible targets of amphilphilies on cell membrane. Due to hydrophilic character, amphiphilies are able to insert into plasma membrane, modulate the membrane stretch and activate the mechanosensitive (MS) ion channels (Martinac, Adler et al. 1990, Qi, Chi et al. 2005). We speculate that if pluronic F127 promotes platelet locomotion by activating MS ion channels, blocking these channels could inhibit platelet migration. As expected, MS ion channel inhibitor GsMTx4 (Bowman, Gottlieb et al. 2007, Gnanasambandam, Ghatak et al. 2017) abolishes platelet motility in the presence of pluronic (Fig.3.30). In line with these results, GsMTx4 inhibits platelet migration in the presence of casein, PLL-g-PEG or plasma (Fig.3.30. Taken together, these results indicate that pluronic, casein, PLL-g-PEG are likely to function on the MS ion channels on platelets.

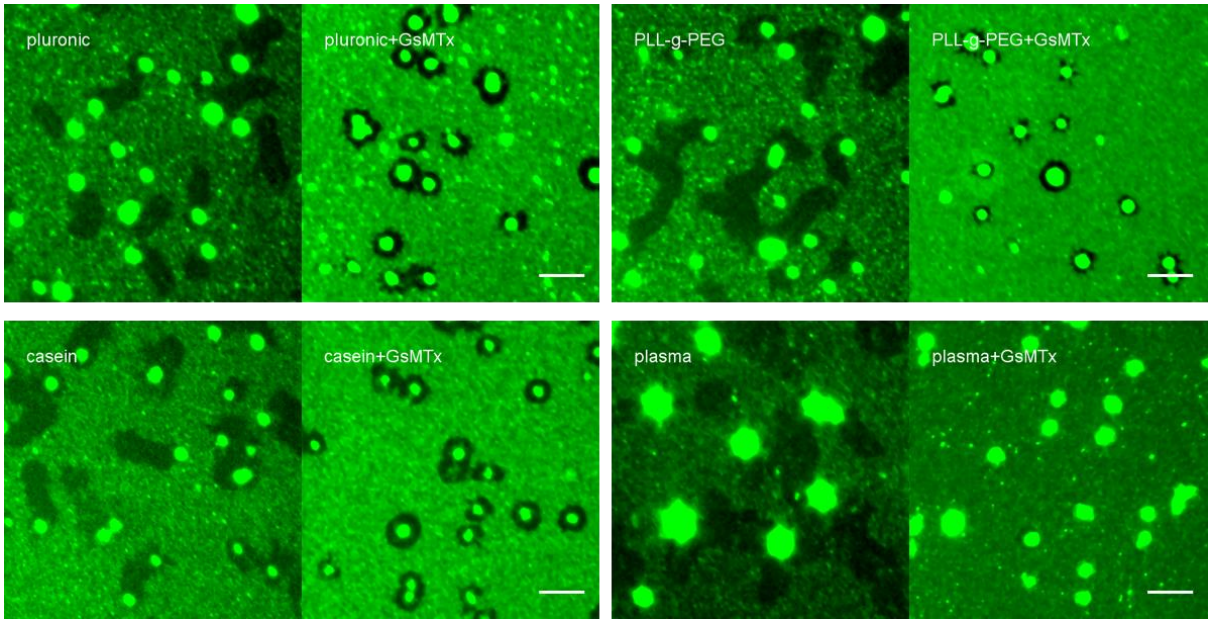


**Fig. 3.30 GsMTx4 inhibits platelet migration in the presence of pluronic, PLL-g-PEG, casein or plasma.** 5 µg/ml pluornic F127, 25 µg/ml PLL-g-PEG, 30 µg/ml casein or plasma from wild type mouse was applied in the presence of 10 µM GsMTx4 or vehicle control ($ddH_2O$) along with $CaCl_2$ and U46619/ADP on washed mouse platelets in the chamber with HSA/Alexa488-fbg coated surface. The figure shows representative images of HSA/Alexa488-fbg coated surface after washed platelets incubation for 1 hour in the presence of indicated substances, scale bar=10 µm.

The present study demonstrate that casein proteins, polycation peptides-albumin conjugates and pluronic are able to trigger platelet migration, then we are interested whether they influence other platelets functions, such as platelets aggregation and secretion. Intriguingly, none of casein proteins, PLL-g-PEG and pluronic trigger platelets aggregation and ATP secretion in the absence of agonists (Fig.3.31). Furthermore, these proteins do not affect platelets aggregation or secretion upon agonist-induced activation (Fig.3.32).

The above findings also suggest that casein proteins regulate calcium signal with the involvement of mechanical force in platelet locomotion. To investigate whether casein proteins, pluornic, or PLL-g-PEG influence the calcium response in platelets in the absence of mechanical forces, we measured the intracellular calcium in platelets upon activation by flow cytometry. The results reveal that none of these proteins influence the calcium level of platelets after stimulation (Fig.3.33). Taken together, these data show that although casein proteins, PLL-g-PEG and pluronic trigger platelet migration, they do not influence platelets aggregation or secretion.

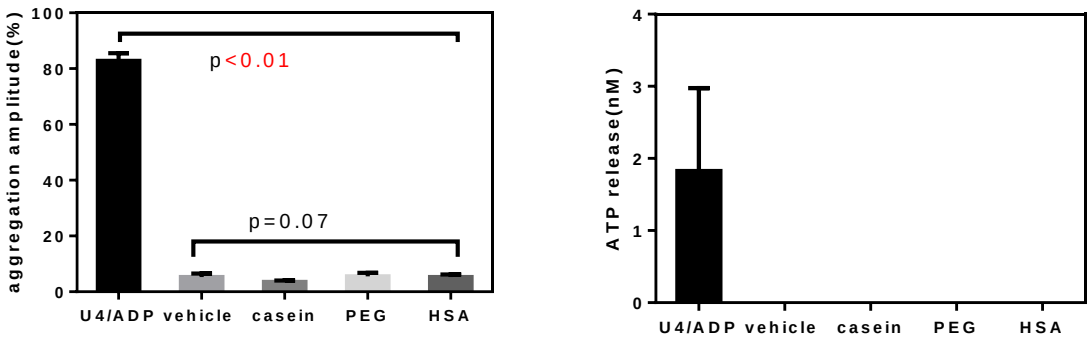


**Fig. 3.31 Casein, PLL-g-PEG, albumin do not induce platelets aggregation or ATP release in the absence of activators.** Washed platelets (200 ×$10^3$/μl) were stimulated by 2 μM U46619/4 μM ADP, vehicle control, 30 μg/ml casein, 25 μg/ml PLL-g-PEG or 1500 μg/ml HSA in the presence of 38 μg/ml fibrinogen and 200 μM $CaCl_2$, then aggregation transmission was recorded by Lumi-aggregometer, meanwhile ATP release were measured by firefly luciferin- luciferase. Note, ATP release of platelets stimulated by vehicle, casein, PLL-g-PEG, or HSA is zero. Error bars= SD, n=4, ANONA. U4: U46619; PEG: PLL-g-PEG.

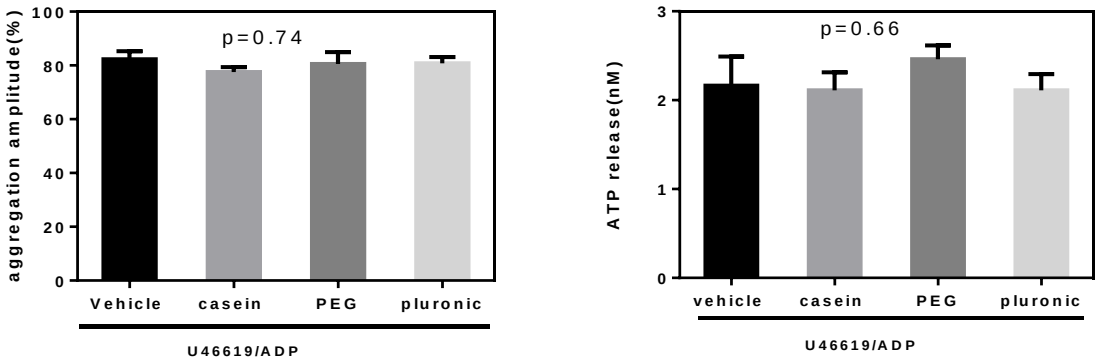


**Fig. 3.32 Casein, PLL-g-PEG, pluronic127 do not affect platelets aggregation or ATP release after activation.** Washed mouse platelets (200 ×$10^3$/μl) were stimulated by 2 μM U46619/ 4 μM ADP in the presence vehicle control, 30 μg/ml casein, 25 μg/ml PLL-g-PEG, or 5 μg/ml pluronic F127 along with 38 μg/ml fibrinogen and 200 μM CaCl2, then aggregation transmission was recorded by Lumi-aggregometer, meanwhile ATP release were measured by firefly luciferin- luciferase. Error bars= SD, n=4, ANONA. PEG: PLL-g-PEG.

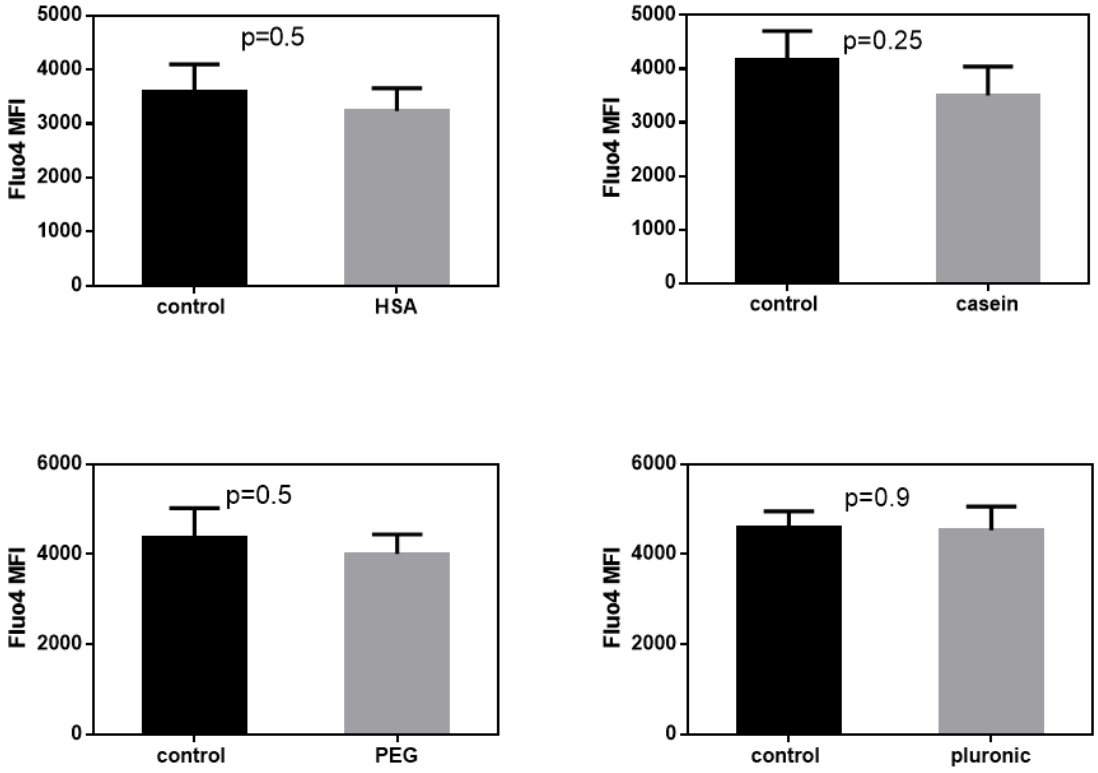


**Fig. 3.33 Albumin, casein, PLL-g-PEG, Pluornic F127 do not affect calcium influx in platelets after activation in absence of mechanical forces.** 5 µM Fluo4-AM loaded platelets ($10\times10^3$/µl) were stimulated by 2 µMU46619/4 µM ADP in the presence of 1 mM$CaCl_2$ along with vehicle control,1500 µg/ml HSA, 30 µg/ml casein, 25 µg/ml PLL-g-PEG, or 5 µg/ml pluronic F127. Fluo4 intensity was recorded on the flow cytometer before and after addition of the activators. The graphs show the increased Fluo4 intensity after activation by U46619/ADP. Error bars= SEM, n=3, t-test. PEG: PLL-g-PEG.

### 3.1.6 Mouse albumin and plasminogen in the plasma are involved in promoting platelet migration.

Although casein proteins, cationic polypeptides-albumin conjugates, and pluronic are able to trigger platelet migration, they do not physiologically exist in plasma. What are the essential factors in plasma that promote mouse platelet motility? The above experiments show that mouse platelets migrate within plasma, but mouse albumin alone did not trigger platelet migration. To further examine whether albumin in plasma plays a role in platelet motility, albumin deficient mouse was employed. Albumin deficient mice are lack of serum albumin, but they have increased level of total bilirubin, high density lipoprotein (HDL), low density lipoprotein (LDL), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which are similar to human analbuminermia (Roopenian, Low et al. 2015). Our results show that platelets show strikingly impaired mobility within albumin deficient plasma and if albumin was

reconstituted in albumin deficient plasma, the reduction of platelets migration was recovered (Fig.3.34). These data indicate that mouse albumin is one of the critical factors in plasma involved in platelet migration.

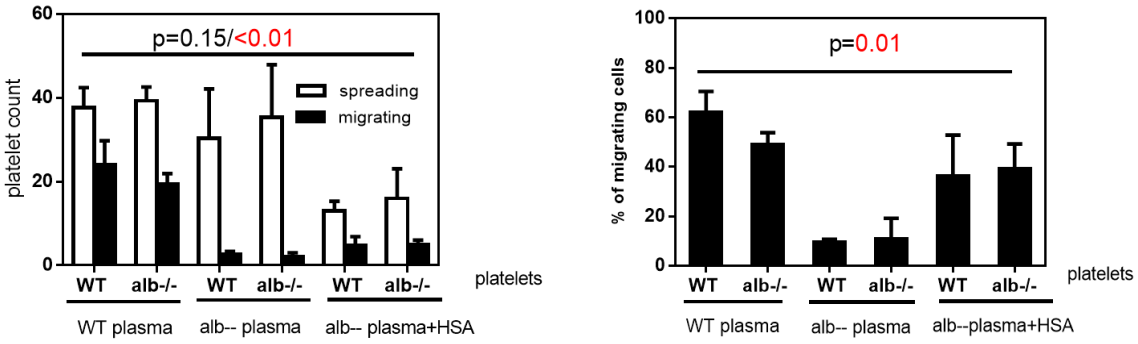


**Fig. 3.34 Platelets show impaired migration within albumin deficient plasma.** Mouse platelets were reconstituted within plasma along with fibrinogen and U46619/ADP. Left: quantification of the number of spreading and migrating platelet in each experiment. Right: quantification of percentage of migrating platelets in each experiment. Error bars=SEM, n=3, ANOVA.

In addition to albumin, plasma contains immunoglobins and a wide variety of regulatory proteins. Do immunoglobins play a role in platelet locomotion? RAG 1 deficient mice do not have mature B and T lymphocytes and thereby are lack of immunoglobins in plasma (Mombaerts, Iacomini et al. 1992). Our results show that platelets display enhanced motility within RAG1 deficient mouse plasma than wild type mouse plasma, which implies that instead of promoting migration, immunoglobins could hinder platelets locomotion (Fig.3.35).

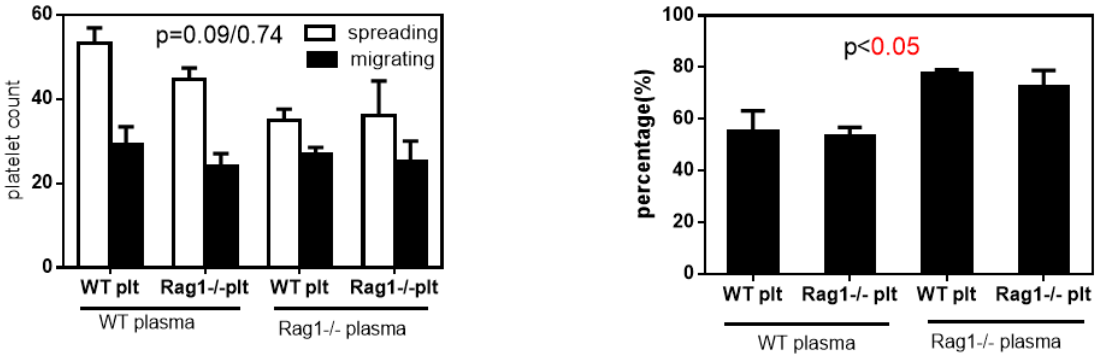


**Fig. 3.35 Mouse platelets display enhanced migration in RAG1 deficient mouse plasma.** Mouse platelets were reconstituted within plasma from wild type or RAG1 deficient mice in the presence of fibrinogen and U46619/ADP. Left: quantification of the number of spreading and migrating platelet in each experiment. Right: quantification of percentage of migrating platelets in each experiment. Error bars=SEM, n=3, ANOVA, plt: platelets; WT: wild type.

Platelets are capable of migrating within the wild type mouse plasma, but albumin alone is not sufficient to support migration, which indicate that other factors in

plasma are required for platelet motility. The above experiments suggest that conjugation of cationic proteins and albumin are able to promote migration, raising the possibility that positively charged factors in plasma could be the candidates. Plasminogen, the precursor of plasma fibrinolytic enzyme-plasmin is activated by tissue-type plasminogen activator (t-PA), or urokinase-type plasminogen activator(u-PA) and involved in degradation of fibrin and fibrinogen during thrombolysis (Castellino and Ploplis 2005). The results show that plasminogen-albumin conjugates are able to promote platelet migration. With 10 µM plasminogen, the percentage of migrating platelets is 31.44± 1.43% (Fig.3.36). Plasminogen activation can be inhibited by EACA through interacting with the kringle domains of plasminogen (Castellino and Ploplis 2005). Interestingly, EACA also abolishes plasminogen induced platelet migration (Fig.33.37).These data suggest plasminogen are able to induce platelets motility, however, the effect of plasminogen in platelet migration can be abolished by EACA.

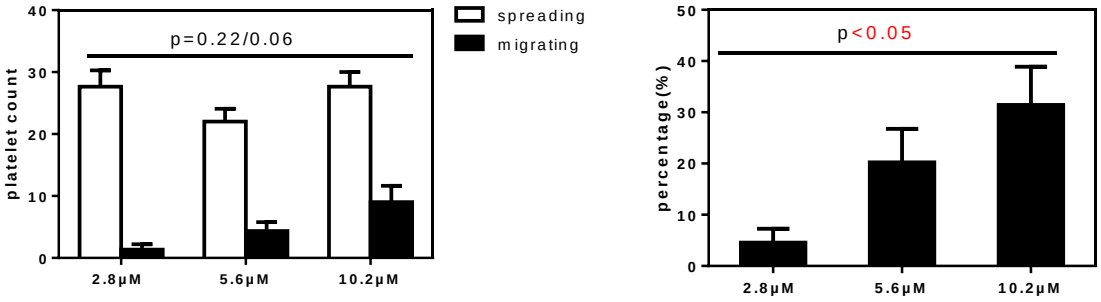


**Fig. 3.36 Plasminogen-albumin conjugates promote mouse platelet migration.** Plasminogen along with 1000 µg/ml HSA was applied on washed mouse platelets in the presence of 200 µM $CaCl_2$ and U46619/ADP in the chamber with HSA/Alexa488-fbg coated surface. Left: quantification of the number of spreading and migrating platelet in each experiments. Right: quantification of the percentage of migrating platelet. Error bars=SEM, n=3, ANOVA.

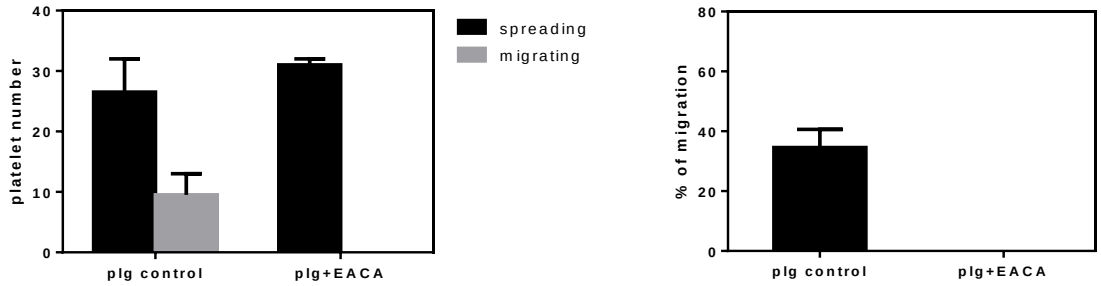


**Fig. 3.37 EACA inhibits plasminogen induced platelet migration.** 40 mM EACA or vehicle control was applied on mouse platelets in the presence of 10 µM plasminogen, 1000 µg/ml HSA, 200 µM $CaCl_2$, U46619/ADP in the chamber with HSA/Alexa488-fbg coated surface. Left: quantification of the number of spreading and migrating platelet in each experiment. Right: quantification of the percentage of migrating platelet in each experiment. Error bars=SEM, n=2. Plg: plasminogen.

Does EACA also inhibit platelet motility triggered by casein or plasma? Our results show that EACA does not diminish platelet motility in the presence of casein (Fig.3.38). Furthermore, EACA does not inhibit platelet migration within plasma (Fig.3.38), indicating that plasminogen is not the only factor in plasma that promotes migration. Therefore, some factors in plasma were examined on mouse platelets, but none of them trigger platelet migration (Table 3-2). Together, these results suggest that plasminogen are capable of facilitating platelet migration, but does not function the same way as casein or plasma.

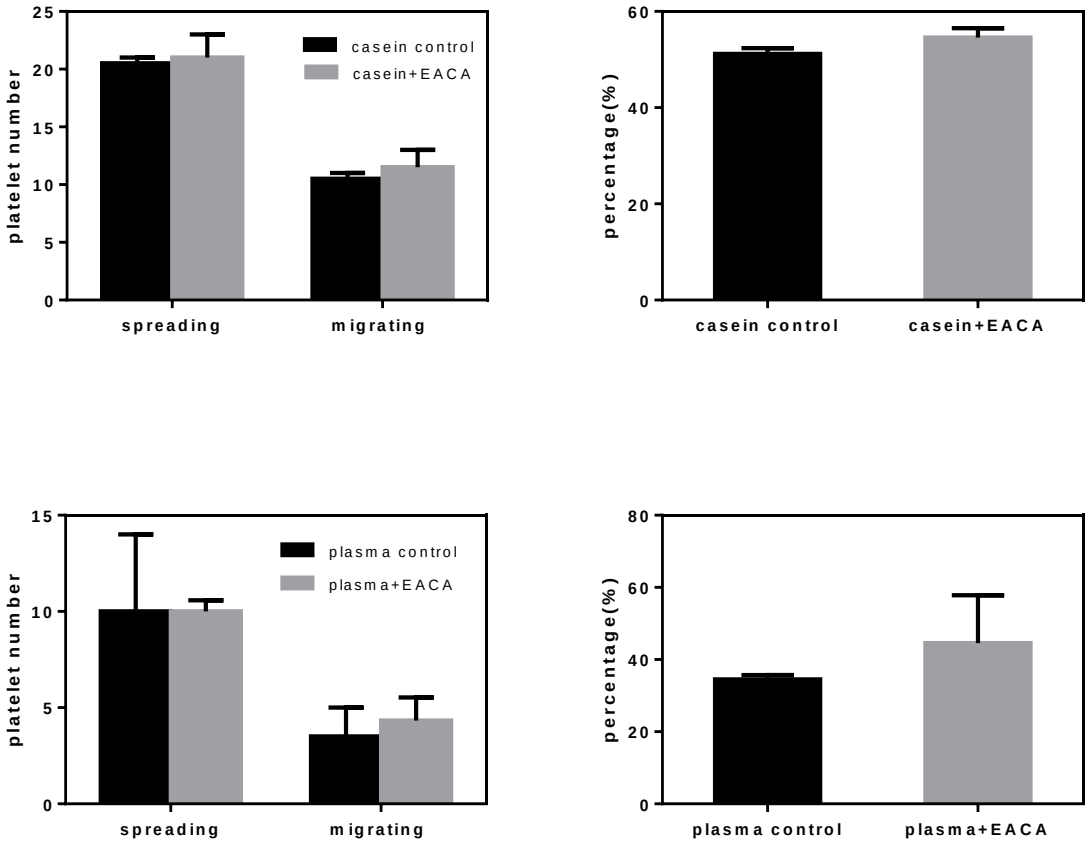


**Fig. 3.38 EACA does not inhibit platelet migration in the presence of casein or plasma.** 40 mM EACA or vehicle control was applied on mouse platelets in the presence of plasma from wild type mice or 30 µg/ml casein, 200 µM$CaCl_2$, U46619/ADP in chamber with HSA/Alexa488-fbg coated surface. Left: quantification of the number of spreading and migrating platelet in each experiment. Right: quantification of the percentage of migrating platelet in each experiment. Error bars=SEM, n=2.

**Table 3-2 factors in plasma that do not facilitate platelet migration**

| Factors | Concentration( μg/ml) | Spreading | Migrating |
|---|---|---|---|
| Complement C3 | 150 | + | - |
| Complement C3 | 250 | + | - |
| Complement C3 | 500 | + | - |
| Complement C1q | 50 | + | - |
| Complement C1q | 100 | + | - |
| Complement C1q | 200 | + | - |
| Complement C1 | 10 | - | - |
| Complement C1 | 20 | - | - |
| Complement C1 | 40 | - | - |
| C-reactive protein | 25 | + | - |
| C-reactive protein | 50 | + | - |
| C-reactive protein | 100 | + | - |
| Complement factor H | 25 | + | - |
| Complement factor H | 50 | + | - |
| Complement factor H | 100 | + | - |
| Complement factor H | 500 | + | - |

Plasma factors at indicated concentrations were applied on washed mouse platelets in the presence of1000 μg/ml HSA, 200 μM $CaCl_2$, U46619/ADP in the chamber with HSA/Alexa488-fbg coated surface. + indicates there are spreading platelets, - indicates there is not migrating platelets.

## 3.2 Comparison of the role of SOC and MS channels in platelet migration and aggregation

### 3.2.1 Calcium release from internal stores is insufficient to support platelet migration.

Previous study in our group showed that human platelets migrated with calcium oscillations. The elevation of cytosolic free $Ca^{2+}$ ($[Ca^{2+}]_i$) in platelets upon agonist-induced activation derives from two sources: $Ca^{2+}$ release from intracellular stores (in platelets they are referred as dense tubular system (DTS), and $Ca^{2+}$ influx from outside via plasma membrane. Meanwhile, the $[Ca^{2+}]_i$ are pumped out by the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) and plasma membrane $Ca^{2+}$-ATPase (PMCA) (Varga-Szabo, Braun et al. 2009). Although external calcium was demonstrated to increase $[Ca^{2+}]_i$ and then initiate platelet locomotion (Gärtner, Engelhardt et al. 2015), it is still a question whether calcium release from internal pools is sufficient for platelet locomotion. Thapsigargin specifically inhibits the ubiquitous SERCA, leading to the rise of $[Ca^{2+}]_I$ (Thastrup, Cullen et al. 1990, Lytton, Westlin et al. 1991). However, our results show that in the absence of extracellular calcium thapsigargin increases the number of spreading platelets, but is not able to initiate platelet migration (Fig.3.39). Caloxin 2A1 is the PMCA inhibitor which selectively binds to the extracellular domains of PMCA (Chaudhary, Walia et al. 2001, Szewczyk, Pande et al. 2008). When caloxin2A1 was applied without extracellular calcium, it does not affect the number of spreading platelets, moreover, it does not promote platelet migration (Fig.3.39). A23187, a calcium ionophore that facilitates the transportation of calcium ions across biological membranes, disturbs the intracellular calcium gradient which results in the elevation of $[Ca^{2+}]_I$ (Reed and Lardy 1972). Nevertheless, A23187 modulates neither the number of spreading platelets nor the number of migrating platelets (Fig.3.39). Taken together, these results reveal that intracellular calcium is insufficient for platelet migration, which suggests the indispensable role of external calcium.

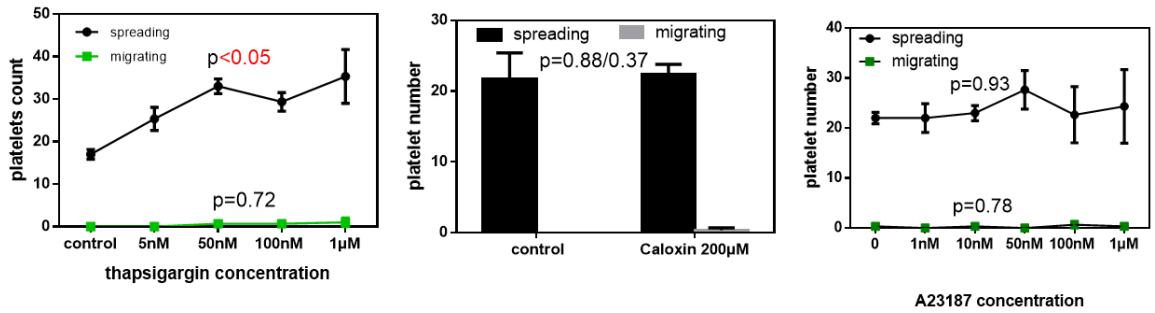


**Fig. 3.39 Calcium release from internal stores is insufficient for supporting platelet migration.** Thapsigargin, caloxin, or A23187 at indicated concentrations was applied on human platelets in the absence of external calcium in chamber with HSA/Alexa488-fbg coated surface. The figure shows quantification of the total number of spreading and migrating platelet in each experiment. Error bars=SEM, n=3, ANOVA on thaspigargin and A23187, t-test on caloxin.

To examine whether external calcium is required for the whole process of platelet motility, we first applied external calcium to initiate platelet migration, then added EGTA to chelate extracellular calcium. Subsequently, platelet motility was immediately abolished (Fig.3.40). The results further demonstrate that extracellular calcium is essential for initiation and maintenance of platelet locomotion.

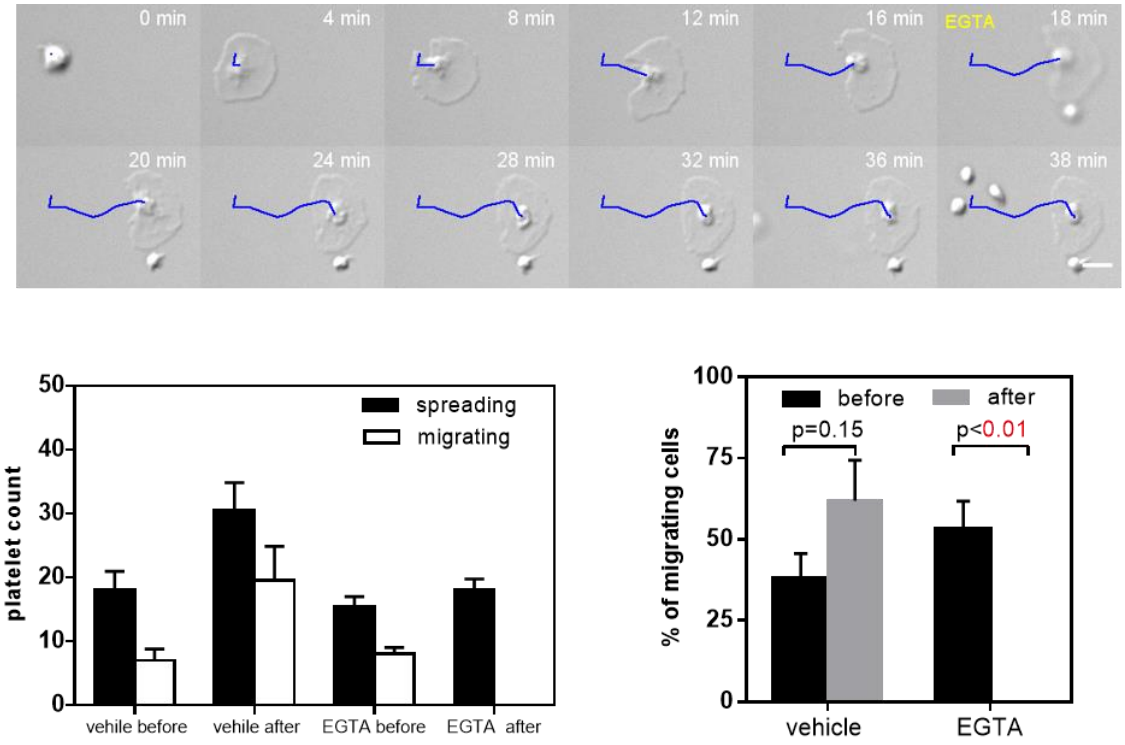


**Fig. 3.40 Extracellular calcium is required for platelet migration.** Human platelets are migrating in the presence of 200 µM $CaCl_2$, then 3 mM EGTA or vehicle control ($H_2O$) is added. The upper panel: representative time series of one migrating platelet before and after the addition of EGTA at 18 min. The blue line indicates the accumulating migrating path. Scale bar= 5 µm. The lower panel: quantification of the spreading and migrating platelets number (left) and the percentage of migrating platelets (right) before and after addition of vehicle or EGTA. Note: the number and percentage of migrating platelets after EGTA is zero. Error bar=SEM, n=4, t-test.

### 3.2.2 $P2X_1$ channels, SOC channels do not play major roles in platelet migration.

External calcium enters into platelets via calcium ion channels on plasma membrane. Since extracellular calcium is indispensable for platelet mobility, blocking calcium ion channels on plasma membrane is supposed to diminish platelet migration. Pinokalant (LOE908) is a broad spectral cation channel blocker, which inhibits both the monovalent and divalent cation channels, including $Na^+$ channels, $K^+$ channels, SOC channels, and voltage-operated calcium channels (Christensen, Wienrich et al. 2005). Our results show that LOE908 diminishes both mouse and human platelet migration, with $IC_{50}$ of 10.71 µM on mouse platelets and $IC_{50}$ of 9.96 µM on human platelets (Fig.3.41). However, LOE908 reduces human platelet migration in a different manner from mouse platelets: it inhibits mouse platelet migration gradually, but human platelets abruptly. LOE908 does not affect human platelet motility below the concentration of 10 µM, whereas it abolishes platelet migration at 25 µM.

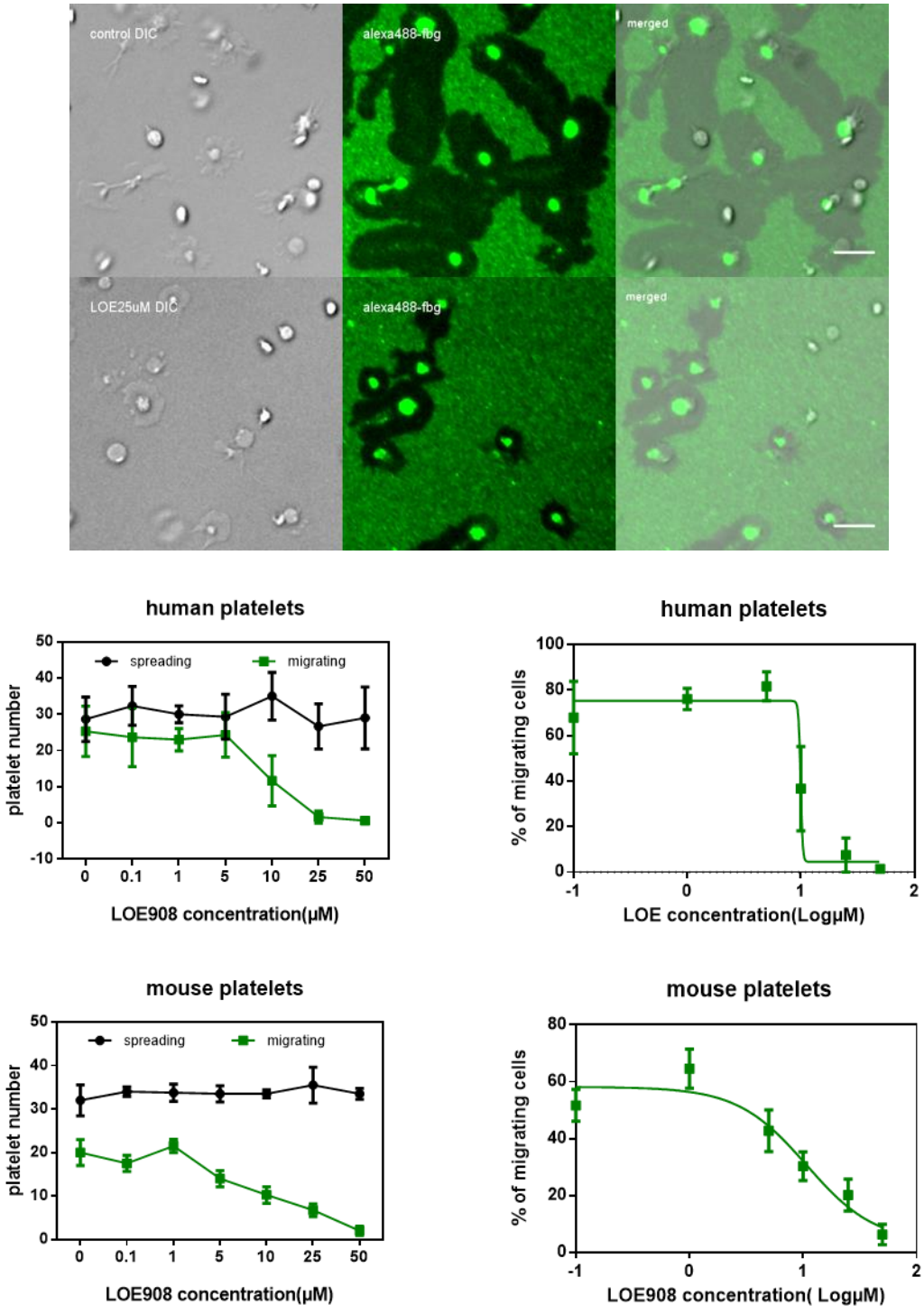


**Fig. 3.41 Broad spectrum cation channel blocker LOE908 inhibits platelet migration.** LOE908 was applied on human or mouse platelets in the chamber with HSA/Alexa488-fibrinogen coated surface. Upper panel: the representative images of HSA/Alexa488-fbg coated surface after human platelets migration in the presence of vehicle control or LOE908.The green and black area on Alexa488-fbg image indicate the non-migrating and migrating area, respectively. Scale bar=10 μm. Middle and lower panel: quantification of platelet count and the percentage of migrating platelets in each experiment. Error bars=SEM, n=4.

A wide variety of cation ion channels have been identified on platelet plasma membrane (Rink and Sage 1990,Varga-Szabo, Braun et al. 2009, Mahaut-Smith 2012), which of them are involved in calcium entry into platelets that mediate mobility remains elusive. To address this question, calcium channel blockers and genetic knockout mouse platelets were employed to examine the role of ion channels in platelet migration.

$P2X_1$, a receptor-gated ion channel is the only P2X family expressed on platelets and exclusively evoked by ATP (Mahaut-Smith, Jones et al. 2011, Oury, Lecut et al. 2015). Upon stimulation calcium from outside rapidly enters into platelets via $P2X_1$ channels, which leads to the rise of $[Ca^{2+}]_i$. $P2X_1$ receptors play important roles in platelets aggregation and secretion to low concentration of collagen and protease activated receptor activation (Oury, Kuijpers et al. 2003, Erhardt, Toomey et al. 2006). However, our experiments reveal that $P2X_1$ ion channel inhibitor NF449 does not decrease human platelet migration (Fig.36). Furthermore, platelets from $P2X_1$ knockout mice do not exhibit lower percentage of locomotion than platelets from wild type mice (Fig.3.42). Overall, these results suggest that $P2X_1$ channels do not play a significant role in platelet migration.

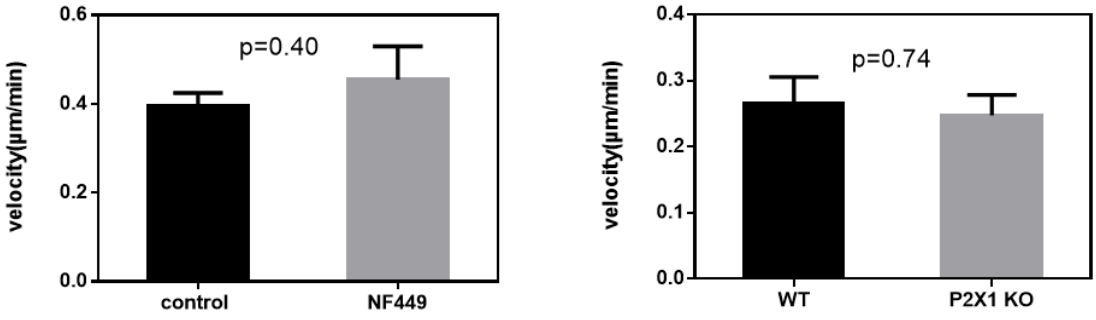


**Fig. 3.42 $P2X_1$ channels do not play significant roles in platelet migration[1].** Left: quantification of the percentage of migrating platelets in the presence of 10 µM NF449 or vehicle control on human platelets. n=3. Right: quantification of the percentage of migrating platelets from $P2X_1$ knockout and control mice. n=4, Error bars= SEM, t-test.

Stored operated calcium entry (SOCE) is recently of great interest due to its crucial roles in platelets function in vitro and in vivo (Grosse, Braun et al. 2007, Varga-Szabo, Braun et al. 2011). Agonist-evoked activation on platelets results in the activation of phospholipase C (PLC), and subsequently the production of diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate ($IP_3$). $IP_3$ induces the release of $Ca^{2+}$ from the

[1] Experiments were performed by Zerkah Ahmad.

sarcoplasmatic reticulum, thus triggers the influx of extracellular $Ca^{2+}$ known as SOCE (Parekh and Putney 2005). Pyrazole derivative BTP2, a potent SOC channel blocker (Ishikawa, Ohga et al. 2003, Zitt, Strauss et al. 2004) inhibits SOC mediated calcium influx in platelets with $IC_{50}$ of 0.5 µM (Harper and Poole 2011). Our results reveal that BTP2 slightly diminishes the percentage and velocity of migrating platelets (Fig.3.43).

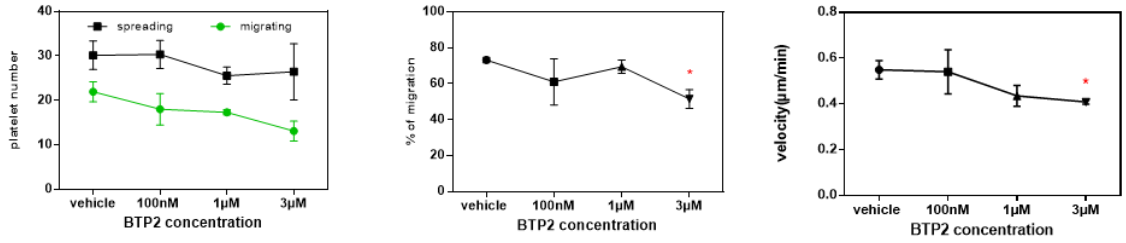


**Fig. 3.43 BTP2 diminishes human platelet migration.** Human platelets were incubated with indicated concentrations of BTP2 or vehicle control, then reconstituted with 1000 µg/ml HSA, 200 µM $CaCl_2$, U46619/ADP in the chamber with HSA/Alexa488 fibrinogen coated surface. The figure shows quantification of the number of spreading and migrating platelets (left), the percentage (middle) and velocity of migrating platelets (right) in each experiment. Error bars= SEM, n=3, red stars indicate p<0.05, t-test (compared with vehicle control).

Stromal interaction molecule 1 (STIM1) is a SR/ ER resident protein identified as the sensor for detecting $Ca^{2+}$ depletion that activates SOC mediated calcium entry in platelets(Ahmad, Boulaftali et al. 2011). STIM1-deficient platelets are defective in agonist-induced $Ca^{2+}$ response and STIM1 knockout mice exhibit unstable arterial thrombi (Varga-Szabo, Braun et al. 2008). However, our results show that STIM1 deficient platelets do not have any defect in migration compared with platelets from control mice (67.83 ± 5.073 vs72.24± 2.61, p=0.46) (Fig.3.44). SITM2, the isoform of STIM1 is also able to trigger SOC mediated calcium influx on plasma membrane. Moreover, SITM2 is crucial in regulating basal cytosolic calcium concentration (Brandman, Liou et al. 2007, Gruszczynska-Biegala, Pomorski et al. 2011). To deplete both STIM1 and STIM2 in platelets, STIM1/2 $^{flox/flox}$ mice were crossed with *PF4cre* mice. In line with the results from platelets lacking STIM1, the percentage of migrating STIM1/2 deficient platelets are not different from control wild type platelets (66.31 ±3.91% vs 65.78± 7.93%, p=0.94) (Fig.3.44).

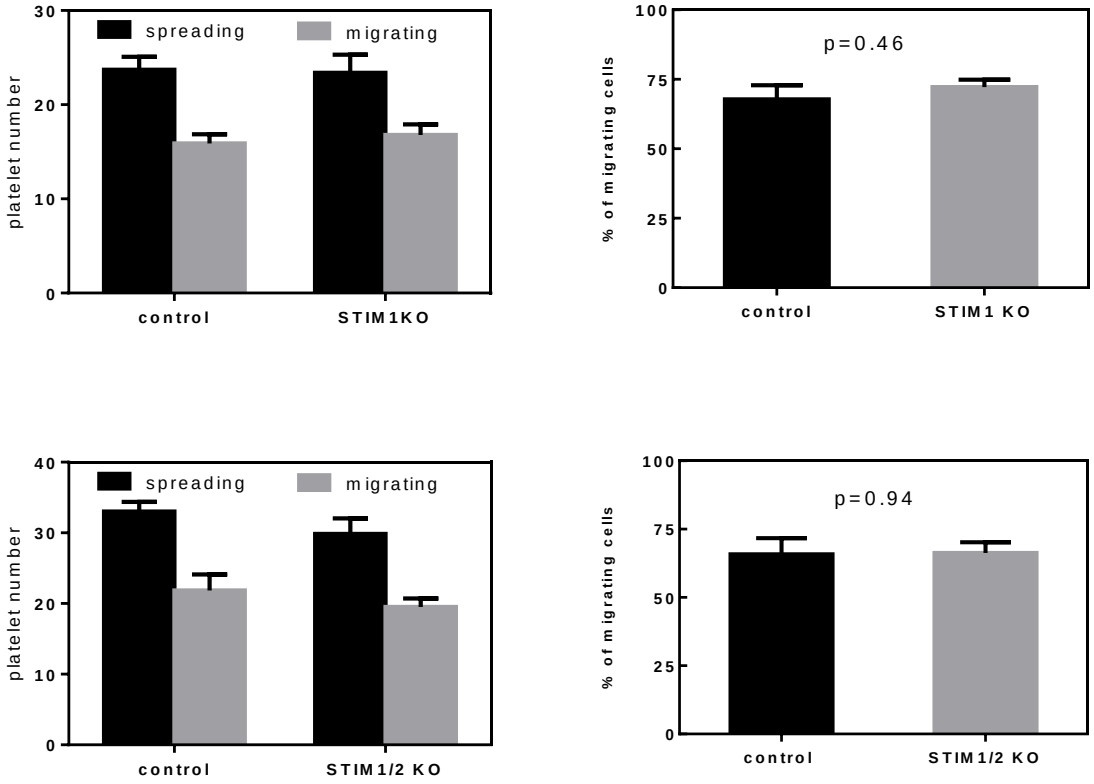


**Fig. 3.44 STIM1 deficient and STIM1/2 deficient platelets do not have defects in migration.** Washed platelets were reconstituted in Tyrodes buffer with 30 µg/ml casein, 200 µM $CaCl_2$, U46619/ADP in the chamber with HSA/Alexa488 fibrinogen coated surface. The figure shows quantification of the number of spreading and migrating platelets (left) and the percentage of migrating platelets (right) in each experiment. n=6, error bar=SEM, t-test.

### 3.2.3 STIM1/2 deficient platelets exhibit impaired aggregation and secretion upon activation.

To determine the significance of STIM1/2 in calcium response upon activation in platelets, platelets devoid of STIM1/2 were labeled with Fluo4-AM and the intensity of Fluo4 were recorded before and after stimulation by flow cytometry. Interestingly, the STIM1/2 deficient platelets display lower basal intracellular calcium than wild type control platelets (Fig.3.45), furthermore after activation by agonists, STIM1/2 deficient platelets show dramatically impaired intracellular calcium elevation (Fig.3.46). Together, these results indicate the crucial role of STIM1 and STIM2 in regulating the basal intracellular calcium and the calcium influx after activation in platelets.

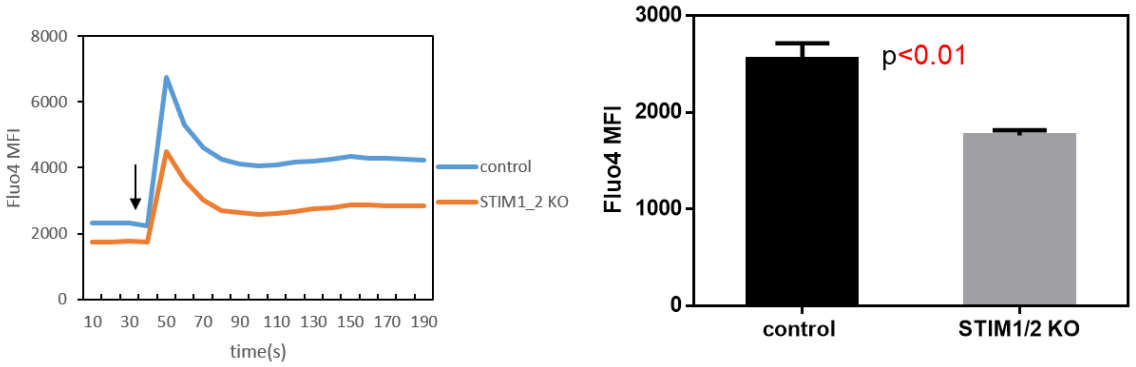


**Fig. 3.45 STIM1/2 deficient platelets show lower basal intracellular calcium.** Washed platelets were loaded with 5 µM Fluo4-AM, and Fluo4 intensity was recorded before and after the addition of U46619/ADP in real time on flow cytometer in the presence of 1 mM $CaCl_2$. Left: the representative Fluo4 MFI changes in real time. Arrow indicates the addition of agonists. Right: quantification the MFI of Fluo4 before adding agonists. n=5, error bars=SEM, t-test.

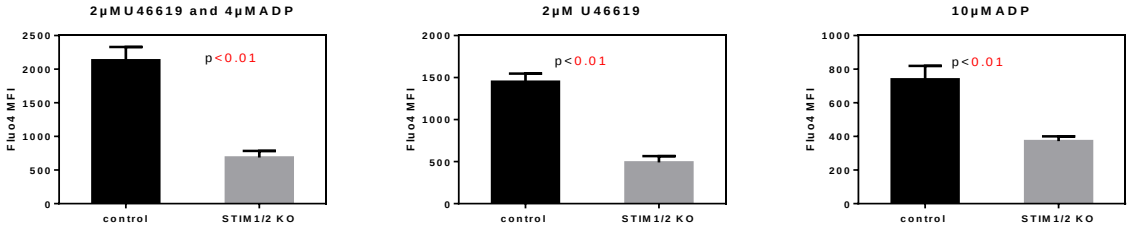


**Fig. 3.46 STIM1/2 deficient platelets are defective in intracellular calcium elevation upon activation.** 5 µM Fluo4 loaded platelets were activated by indicated agonists in the presence of 1 mM $CaCl_2$. Fluo4 intensity was recorded before and after the addition of agonists in real time on flow cytometer. The graph shows the increased Fluo4 intensity after activation by indicated agonists. n=5, error bars=SEM, t-test.

To investigate the functional consequence of defective calcium response in STIM/2 deficient platelets, in vitro platelets aggreation experiments were performed. Interestingly, upon activation by U46619 or ADP, STIM1/2 deficient platelets show strkingly impaired aggregtion compared with control platelets (Fig.3.47). Upon activation by the combination of U46619 and ADP, platelets lack of STIM1/2 display slight but signficiant reduction of platelets aggreagtion (84.75±4.65% vs 73.00±5.42%, p=0.01). In contrast, they exhibit dramatically diminished aggregation after stiumation by collagen (Fig.3.47). Platelets aggregate via fibrinogen and $\alpha_{2b}\beta_3$ integrin on the plasma membrane, to confirm the defects of STIM1/2 deficient platelets in aggregation, the level of integrin $\alpha_{2b}\beta_3$ activation was assesed by flow cytometry. Consistent with the results from platelets aggregation, STIM1/2 deficient platelets display impaired activation of $\alpha_{2b}\beta_3$ (Fig.3.47).

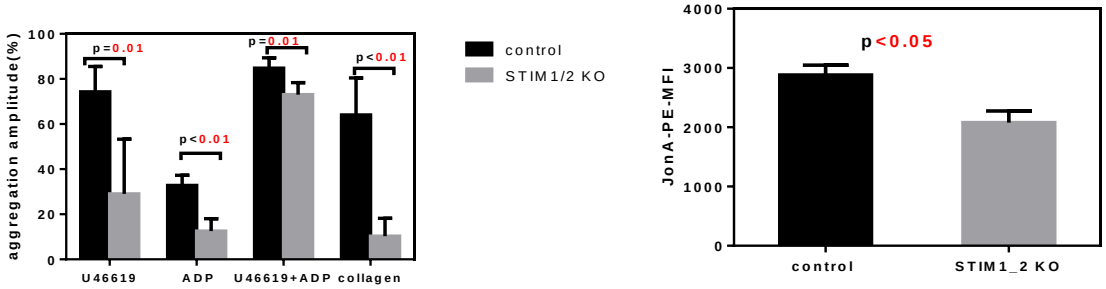


**Fig. 3.47 STIM1/2 deficient platelets show impaired aggregation and $\alpha_{2b}\beta_3$ activation upon stimulation.** Left: washed platelets aggregation were induced by 2 µM U46619, 4 µM ADP, 2 µM U46619/ 4 µM ADP, or 2 µg/ml collagen in the presence of 38ug/ml fibrinogen and 200 µM $CaCl_2$. Light transmission of platelets aggregation was recorded by Lumi-aggregomteter. Scale bars=SD, n=4 per group, t-test. Right: Flow cytometric analysis of $\alpha_{2b}\beta_3$ activation (binding by JonA-PE) on platelets in response to 2 µM U46619/ 4 µM ADP in the presence of 1 mM $CaCl_2$. Scale bar=SD, n=5, t-test.

Platelets contain two major storage graunles, namely α granules and dense granules (McNicol and Israels 1999,Blair and Flaumenhaft 2009,Ghoshal and Bhattacharyya 2014). P selectin, a transmembrane protein contained in α granules is translocated to plasma membrane after platelets activation which is able to be measured by fluorescent-labeled antibody (Koedam, Cramer et al. 1992). Beside, upon activation dense granules of platelets release ATP that can be detected by surface attached-firefly luciferase (Beigi, Kobatake et al. 1999). Our results reveal that STIM1/2 deficient platelets exhibit marked defects in P selectin expression and ATP release upon stimulation (Fig.3.48). Taken together, these findings demonstate the crucial contribution of STIM1/2 to platelets aggregation and degranulation upon activation, although STIM1/2 is not important in platelet migration.

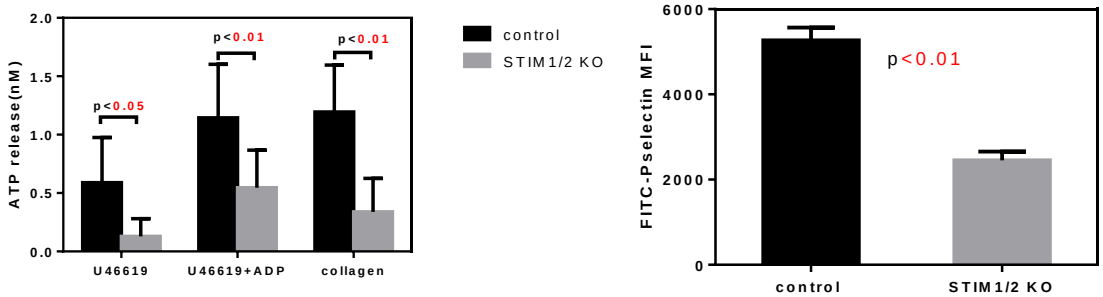


**Fig. 3.48 STIM1/2 deficient platelets display impaired P selectin expression and ATP release after agonist-induced activation.** Left: washed platelets were stimulated by 2µM U46619, 2 µM U46619 and 4 µM ADP, or 2 µg/ml collagen in the presence of 38 µg/ml fibrinogen and 200 µM $CaCl_2$, then ATP release were measured by firefly luciferin- luciferase. Scale bar=SD, n=4 per group, t-test. Right: flow cytometric analysis of P selectin expression (binding by Wug.E9-FITC) on washed platelets in response to 2 µM U46619 and 4 µM ADP in the presence of 1 mM $CaCl_2$. Scale bars=SD, n=5, t-test.

### 3.2.4 MS ion channels are critical, whereas eNaC, TREK, TRAAK, TRPC1/6, TRPM7 channels are not important in platelet migration.

Stretch activated ion channels are implicated in cell migration (Lee, Ishihara et al. 1999, Munevar, Wang et al. 2004). $LaCl_3$, a general stretch activated ion inhibitor abolishes mouse platelets migration at 10 µM (Fig.3.49), however, it does not supress human platelet migration even when the concentration is increased to 50 µM (Fig.3.49). GsMTx4, a peptide MS channels blocker extracted from spider venom (Bowman, Gottlieb et al. 2007, Gnanasambandam, Ghatak et al. 2017) diminishes platelet migration in a concentration dependent manner, with $IC_{50}$ of 4.89 µM (Fig.3.50).

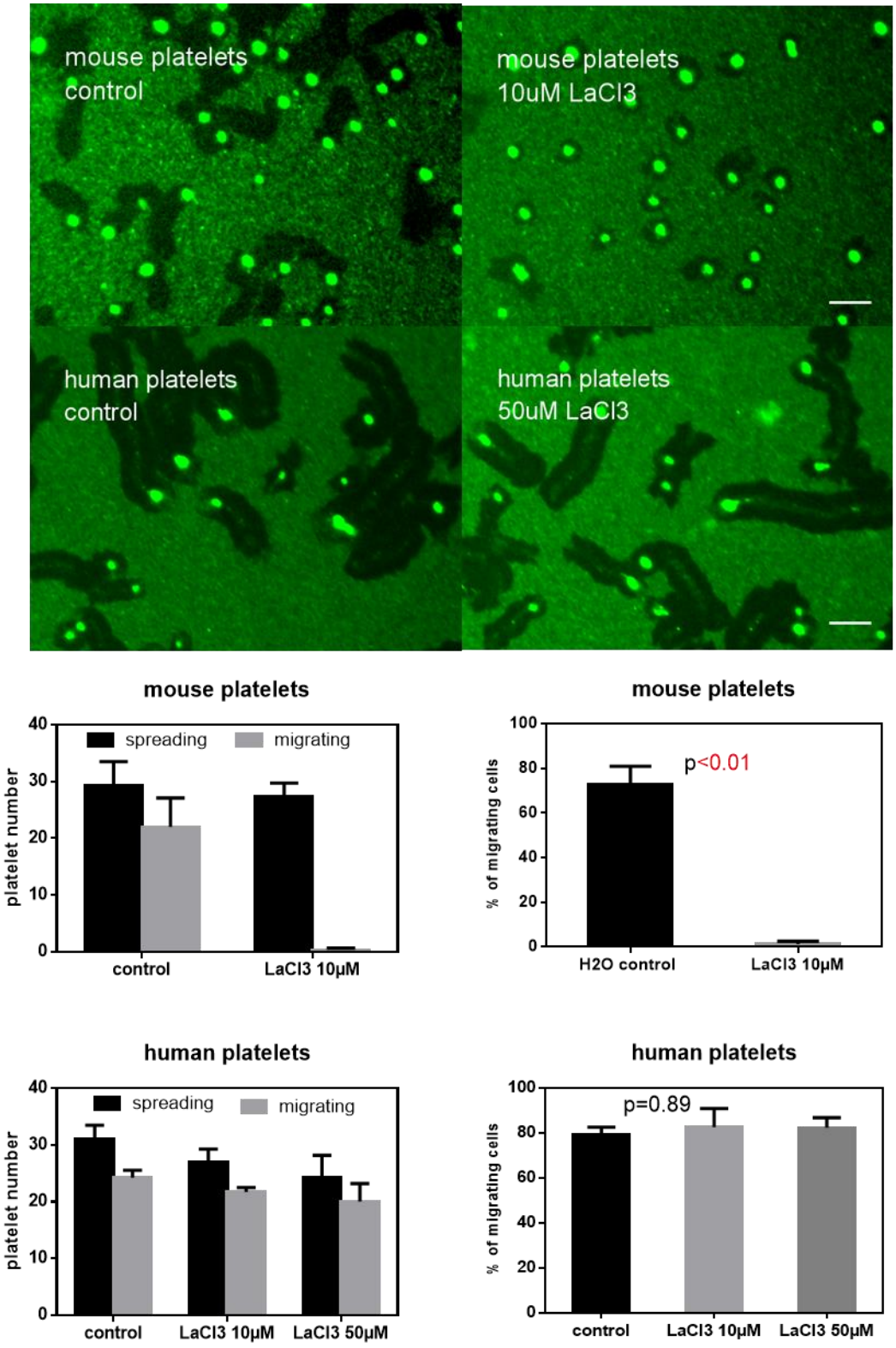


**Fig. 3.49 $LaCl_3$ inhibits mouse platelet but not human platelet migration.** $LaCl_3$ or vehicle was applied on mouse platelets in the presence of 30 µg/ml casein (or on human platelets in the presence of 1000 µg/ml HSA), 200 µM $CaCl_2$, U46619/ADP in the chamber with HSA/Alexa488-fbg coated surface. Upper panel: representative images of HSA/Alexa488-fbg coated surface after platelets migration in the presence of vehicle or $LaCl_3$ on mouse platelets and human platelets. The green and black area indicate the non-migrating and migrating area of platelets, respectively. Lower panel: quantification of the number the spreading, migrating and the percentage of migrating platelets in each experiment. Scale bar= 10 µm, error bars=SEM, n=3, t-test on mouse platelets, one way ANOVA on human platelets.

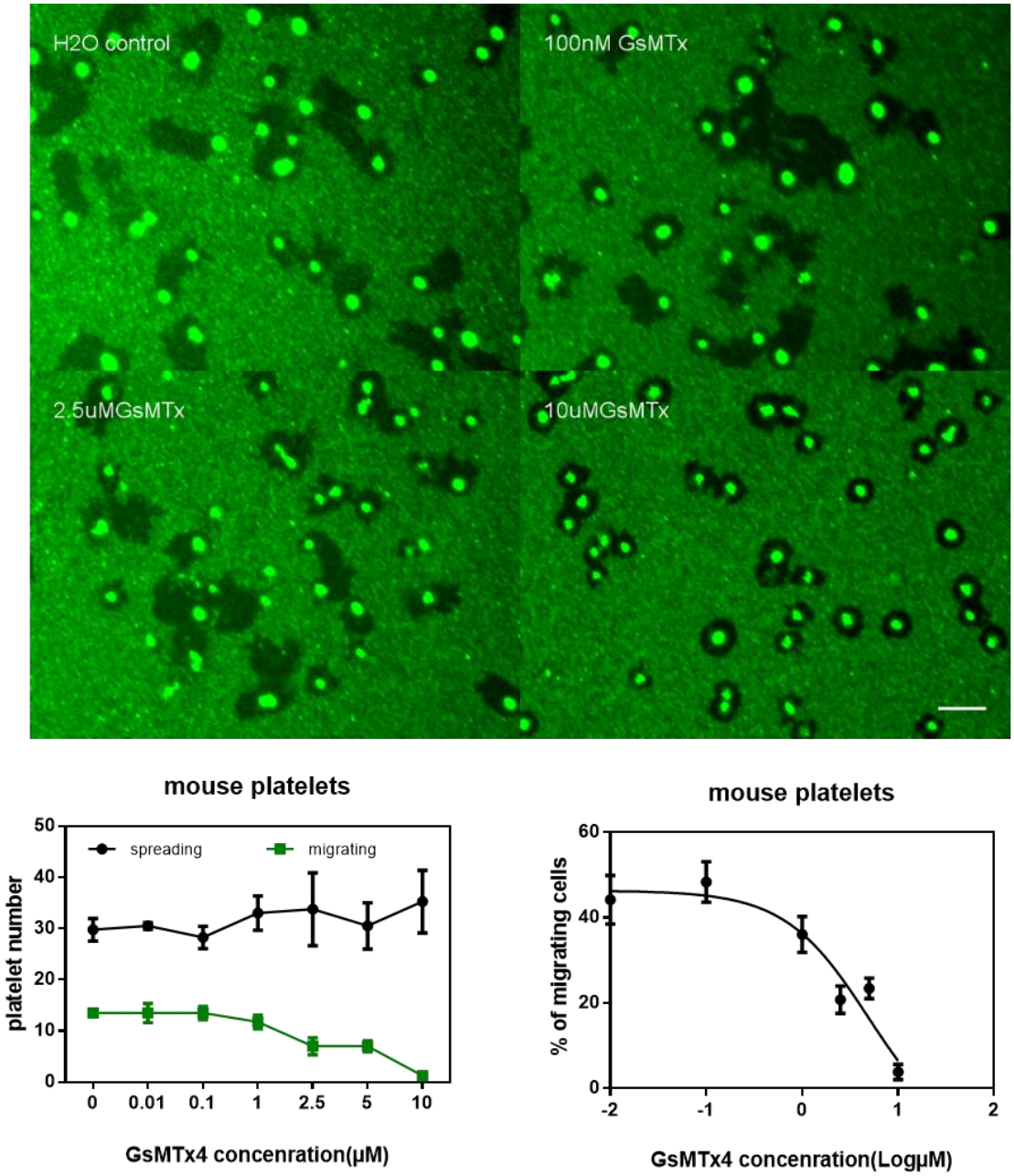


**Fig. 3.50 GsMTx4 inhibits platelet migration.** Different concentrations of GsMTx4 was applied on mouse platelets in the presence of 30 μg/ml casein, 200 μM $CaCl_2$, U46619/ADP in the chamber with HSA/Alexa488-fbg coated surface. Upper panel: representative images of HAS/Alexa488-fbg coated surface after platelets migration in the presence of indicated concentration of GsMTx4. The green and black area indicate the non-migrating and migrating area of platelets, respectively. Scale bar=10 μM. Lower panel: quantification of the number of spreading, migrating platelets, the percentage of migrating platelets. n=4, error bars= SEM.

MS ion channels are non-selective cation channels that are involved in mechanotransduction - the conversion of mechanical stimuli to biological signals. Till now, a large number of ion channels have been proposed to be MS ion channels, which of these channels medicate platelet migration is a question. Epithelium sodium channels (eNaC) are highly selective $Na^+$ channels that are involved in mechanotransduction and associated with collagen activation in platelets (Cerecedo, Martinez-Vieyra et al.

2014). TREK, TRAAK are two pore (2P) domain mechano-gated K channels (Patel, Honore et al. 1998). Amiloride inhibits eNaC as well as TREK channels (Maingret, Patel et al. 2000), but does not diminish platelet migration (Fig.3.51). Overall, these results suggest that eNaC and TREK, TRAA channels do not play major roles in platelet motility.

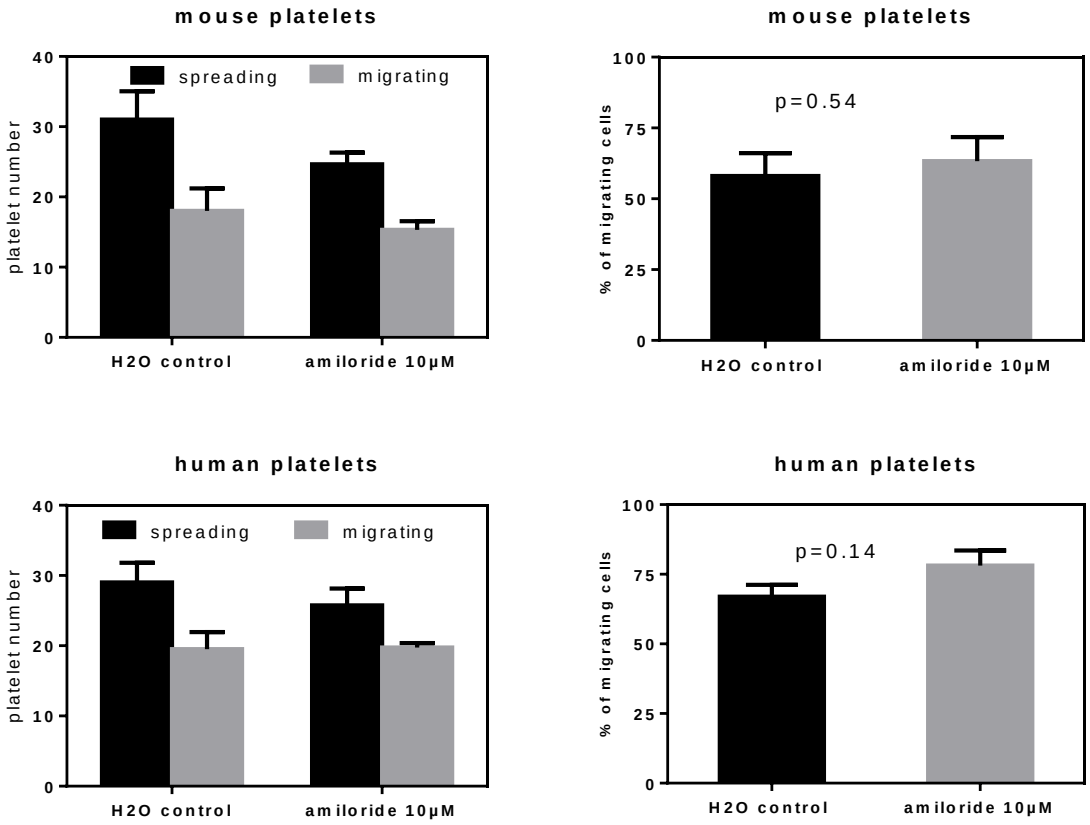


**Fig. 3.51 Amiloride does not inhibit mouse or human platelet migration.** 10 µM amiloride or vehicle ($ddH_2O$) was applied on mouse platelets in the presence of 30 µg/ml casein (or on human platelets in the presence of 1000 µg/ml HSA), 200 µM $CaCl_2$, U46619/ADP in the chamber with HSA/Alexa488-fbg coated surface. Then the number of spreading, migrating platelets, the fraction of migrating platelets were quantified. Error bars =SEM, n=3, t –test.

Transient receptor potential (TRP) superfamily are subdivided into several subfamilies on the basis of sequence similarity (Zheng 2013). TRP channels are widely expressed on platelets (Brownlow and Sage 2005). TRPC6 is reported to be a direct sensor of mechanically and osmotically induced membrane stretch (Spassova, Hewavitharana et al. 2006). Our results show that Larixol, an inhibitor of TRPC 6 channels does not supress platelet migration (Fig.3.52). Furthermore, TPRC1/6 deficient mouse platelets do not display impaired motility compared with control platelets

(Fig.3.52). TRPM7, a $Ca^{2+}$ and $Mg^{2+}$ permeant cation channel is implicated in actomyosin contractility and cell migration (Clark, Langeslag et al. 2006, Gao, Chen et al. 2011), however, the TRPM7 inhibitor NS8593 does not reduce human platelet mobility (Fig.3.53). Taken together, these results suggest that TRPC1/6, TRPM7 are not important in platelet migration.

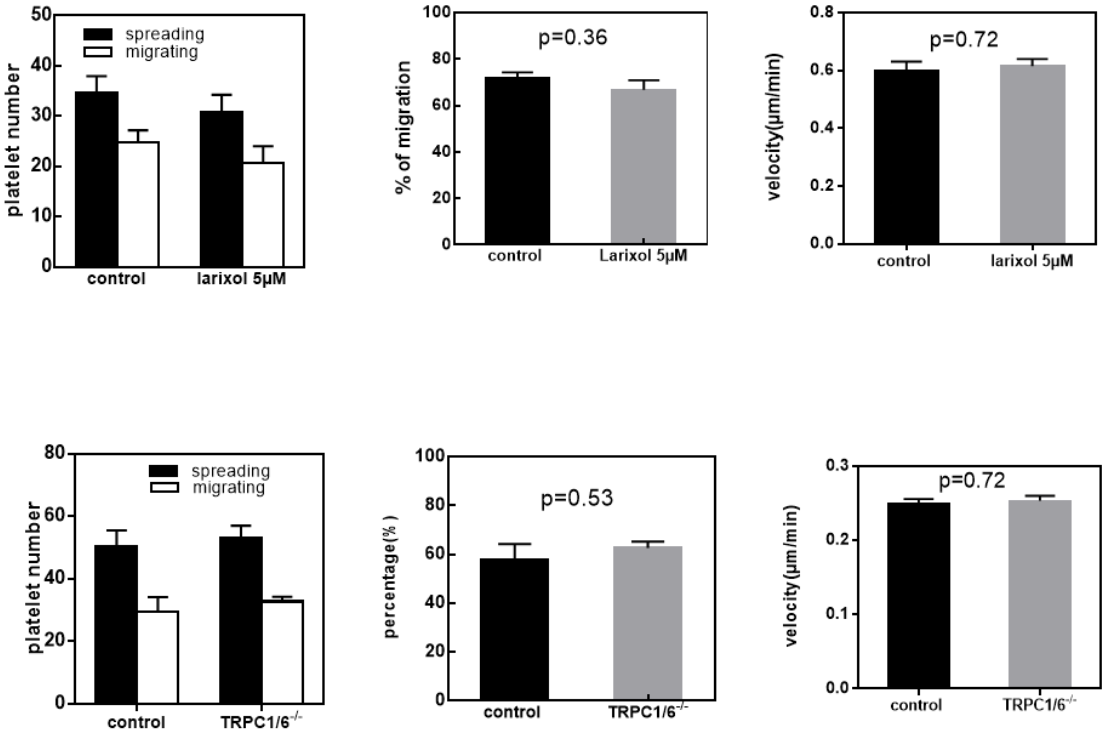


**Fig. 3.52 TRPC1/6 channels are not important in platelet migration.** 5 µM Larixol or vehicle control was applied on human platelets in the presence of 1000 µg/ml HSA, 200 µM $CaCl_2$, U46619/ADP in the chamber with hsa/Alexa488-fbg coated surface. Control or TRPC1/6 deficient platelets were reconstituted with 30 µg/ml casein, 200 µM $CaCl_2$, U46619/ADP in the chamber with HSA/Alexa488-fbg coated surface. The number of spreading, migrating platelets (left), the fraction (middle) and the velocity (right) of migrating platelets were quantified. Error bars =SEM, n=3, t –test.

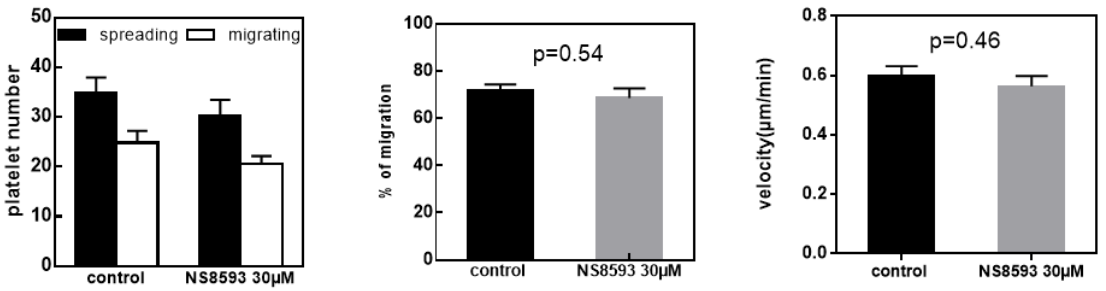


**Fig. 3.53 TRPM7 inhibitor NS8593 does not diminish human platelet migration.** 30 µM NS8593 or vehicle was applied on human platelets in the presence of 1000 µg/ml HSA, 200 µM $CaCl_2$, U46619/ADP in the chamber with HSA/Alexa488-fbg coated surface. The number of spreading, migrating platelets and the fraction of migrating platelets were quantified. Error bars =SEM, n=4, t-test.

### 3.2.5 GsTMx4 does not inhibit platelets aggregation or secretion upon activation.

Till now our results show that eNaC, TREK, TRAAK, TRPM7 and TRPC1/6 channels do not play major roles in platelet locomotion, but the general MS ion channel blocker GsMTx4 completely inhibit platelet migration. To examine the effect of MS ion channel blockers on other platelets function, in vitro platelets aggregation and secretion experiments were performed. Surprisingly, GsMTx4 but not $LaCl_3$ induces platelets aggregation in the absence of agonists, however, neither $LaCl_3$ nor GsMTx4 trigger ATP release without agonist-evoked activation (Fig.3.54). Upon activation by U46619/ADP, $LaCl_3$ or GsMTx4 do not affect platelets aggregation or ATP release (Fig.3.55). Then to further investigate whether GsMTx4 influence calcium response in platelets in the absence of mechanical forces, washed platelets were loaded with Fluo4-AM and the Fluo4 intensity was recorded in flow cytometer. Interestingly, GsMTx4 does not affect calcium response in platelets in the absence of mechanical force (Fig.3.56). Together, although GsMTx4 abolishes platelet motility, it does not inhibit platelets aggregation or secretion upon agonist-evoked activation.

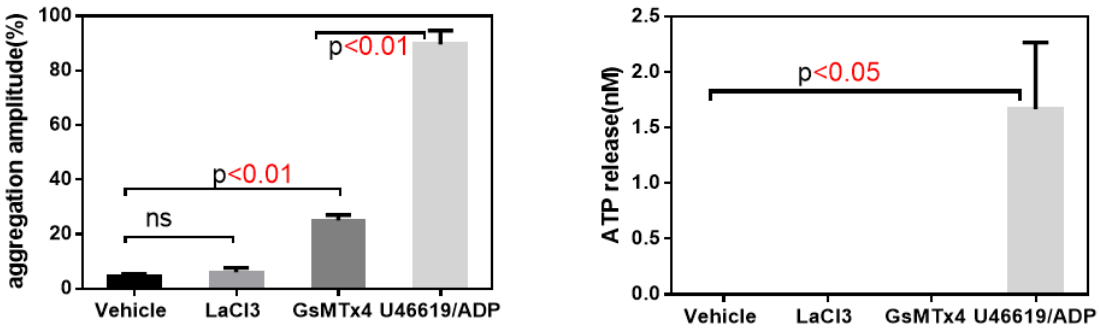


**Fig. 3.54 GsMTx4 but not LaCl3 induces platelets aggregation, but neither LaCl3 nor GsMTx4 induces ATP release.** Washed mouse platelets ($200x10^3$/µl) were activated by vehicle control ($ddH_2O$), 10 µM $LaCl_3$, 5 µM GsMTx4 or U46619/ADP in the presence of 38 µg/ml fibrinogen and 200 µM $CaCl_2$, then aggregation transmission was recorded by Lumi-aggregaometer, meanwhile ATP release were measured by firefly luciferin- luciferase. Error bars= SD, n=3, ANOVA on aggregation, Kruskal-Wails test on ATP release.

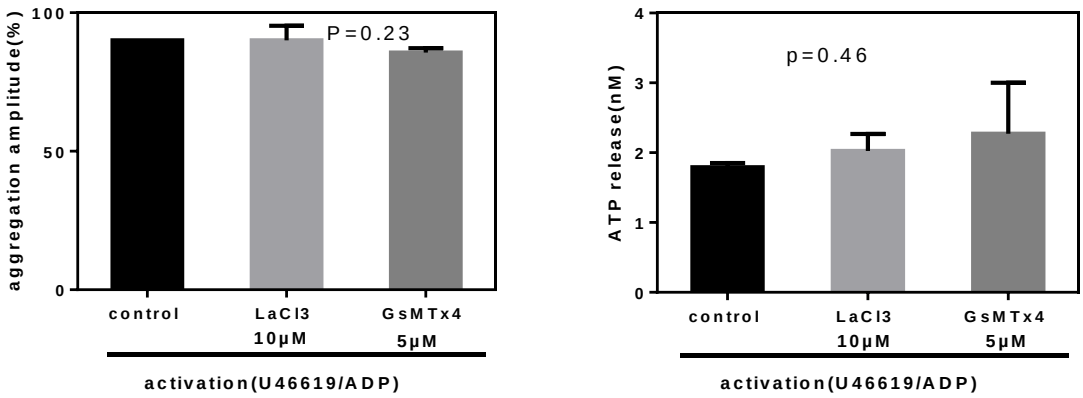


**Fig. 3.55 $LaCl_3$ and GsMTx4 do not inhibit platelet aggregation or ATP release after U46619/ADP activation.** Washed mouse platelets ($200x10^3$/μl) were stimulated by 2 μM U46619 and 4 μM ADP in the presence of 38 μg/ml fibrinogen and 200 μM $CaCl_2$, then aggregation light transmission was recorded by Lumi-aggregometer, meanwhile ATP release were measured by firefly luciferin- luciferase. Error bars= SD, n=3, ANONA.

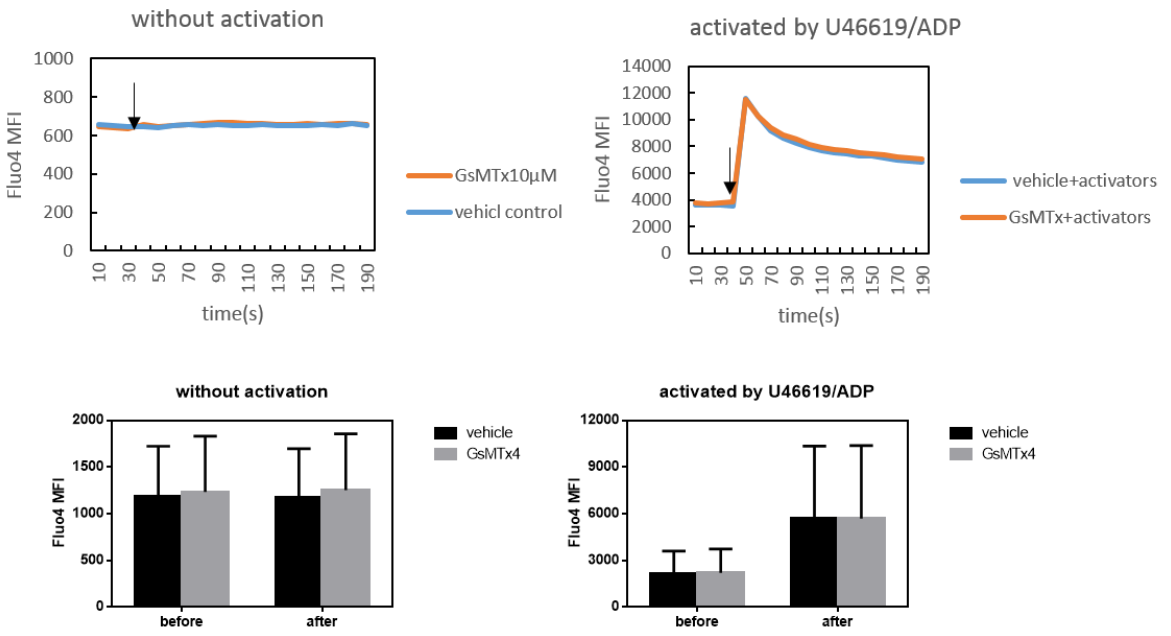


**Fig. 3.56 GsMTx4 does not affect calcium response of platelets in the absence of mechanical force.** 5 μM Fluo4-AM loaded platelets ($10×10^3$/μl) in the presence of 1 mM $CaCl_2$ were stimulated by GsMTx4 or vehicle ($ddH_2O$), or by the combination of U46619/ADP and vehicle or 10 μM GsMTx4. Fluo4 intensity was recorded on the flow cytometer before and after addition of the activators. The upper panel shows the representative flou4 intensity changes in real-time. The arrows indicate the addition of activators. The lower panel shows the quantification of the Fluo4 intensity before or after activation. Error bars= SEM, n=2.

# 4 Discussion

Although the role of platelets in haemostasis and thrombosis has been intensely investigated, the cellular biology of platelets motility remains elusive. More recently, our group has discovered a yet undefined manner of platelet locomotion and further revealed that human platelets migrate in the presence of plasma on fibrinogen coated surfaces with albumin and calcium as essential factors that facilitate migration (Gärtner, Engelhardt et al. 2015). On the basis of this work, my experiments demonstrate that similar to human platelets, mouse platelets are able to migrate within mouse plasma in vitro. However, albumin alone is not capable of supporting mouse platelets motility. Instead, casein, poly lysine-albumin conjugates and pluronic trigger mouse platelet migration by regulating calcium oscillations with the involvement of MS ion channels. Interestingly, these proteins -- casein, PLL-g-PEG, and pluroinc -- do not affect platelets aggregation or secretion. Then by investigating the role of calcium ion channels in platelets function, my results reveal that platelet migration and aggregation are dependent on distinct calcium entry channels on plasma membrane. STIM-mediated SOCE is crucial in platelet aggregation and secretion but not in platelet migration; on the contrary, MS channels are critically implicated in platelet motility but not in platelets aggregation or secretion (Fig.4.1).

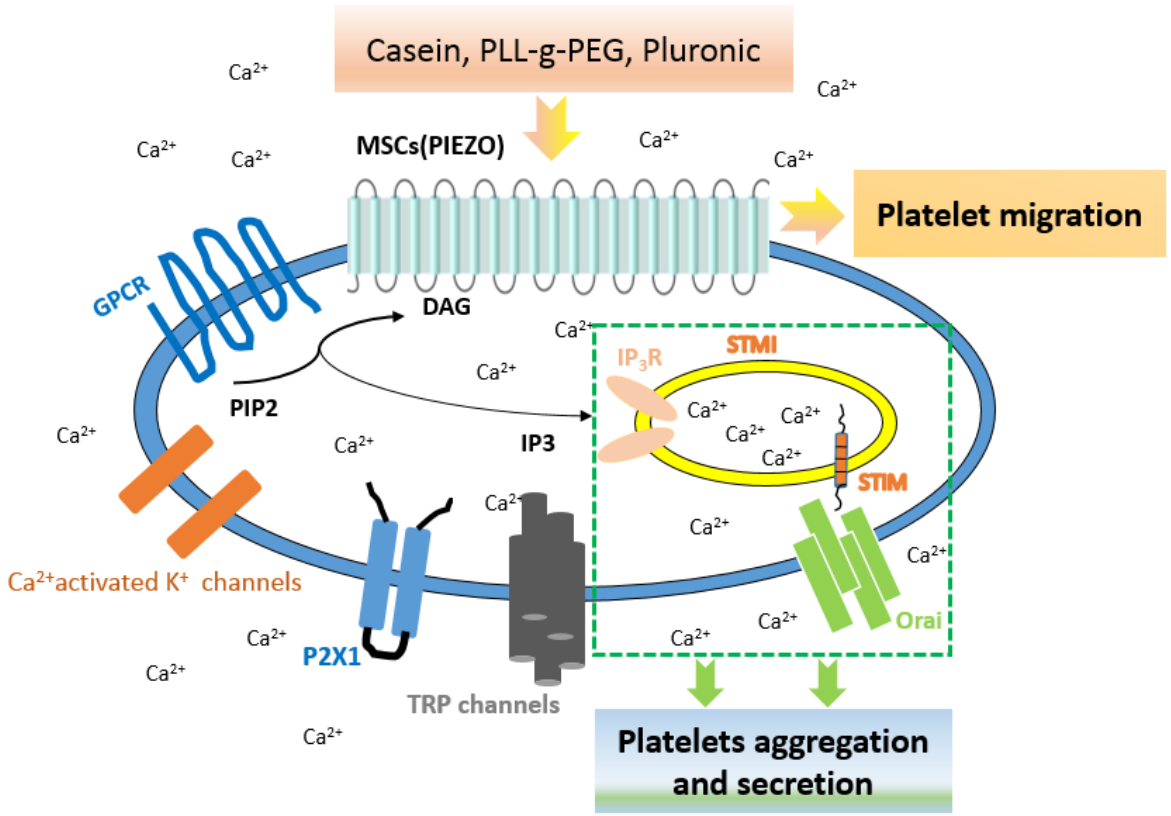


**Fig. 4.1 Schematic of major calcium ion channels involved in platelet aggregation, secretion and migration.** STMI-mediated basal calcium level and calcium influx is crucial for platelets aggregation and secretion, whereas MS channels are critical for platelet migration.

Our previous study suggested that albumin as well as casein proteins facilitated human platelets motility by decreasing the substrate adhesiveness of fibrinogen. However, my results show that casein proteins but not albumin are able to trigger mouse platelet migration. What are disparities between albumin and casein? Albumin, produced by hepatocytes is the most abundant and a heart-shaped tertiary protein in serum and play multiple roles, such as carrying fatty acid, steroids, ions and drugs, maintaining colloidal osmotic pressure and scavenging free radicals (Evans 2002, Quinlan, Martin et al. 2005). Unlike albumin, casein proteins are little in serum, secreted by mammalian cells and constitute the major proteins in milk. Casein is important nutrition for new-borns due to its high content of calcium phosphate and lysine, an essential amino acid for humans (Bhat, Dar et al. 2016). Casein exists in milk as colloidal particles, called casein micelles (Horne 2006, Dalgleish and Corredig 2012,Hristov, Mitkov et al. 2016). One unique feature of casein is that it is naturally unfolded protein, that is to say, casein processes little second or tertiary structure (Redwan, Xue et al. 2015, Thorn, Ecroyd et al. 2015). How these differences in structure between casein and

albumin affect their roles on platelet migration is still a question and required to be further investigated.

By exploring the functional parts of casein proteins, we found that positive charges of casein are likely to explain its effect in triggering platelet migration, since cationic proteins such as poly lysine - albumin conjugate and PLL-g-PEG are also able to induce platelet motility. However, the effect of casein cannot be simply explained by neutralizing negative charges on platelet membrane, because removal of negative charges on platelet membrane does not facilitate platelet migration. Surprisingly, we observe that in the presence of casein some platelets form balloon like blebs prior to death and these blebbing formation is accompanied with the elevation of intracellular calcium. Then our study further reveals that casein proteins reduce the frequency but prolong the duration of calcium oscillations in migrating platelets. More strikingly, the elevated cytosolic calcium sustains for a long time at the initial phase of platelet migration. Calcium oscillation--transient elevated cytoplasmic calcium is a co-ordinated process between calcium release from intracellular stores and calcium influx from outside department and involved in various biological processes, such as cell growth, gene expression, fertilization (Uhlen and Fritz 2010, Parekh 2011). Moreover, calcium oscillations play critical roles in migration of various cell types. For example, transient calcium increase is observed in migrating neutrophils during both chemokinesis and chemotaxis, but the amplitude and duration of calcium oscillations were variable within a given cell as well as different cells (Marks and Maxfield 1990). For another instance, the amplitude and frequency of calcium oscillations were positively correlated with the speed of neuronal cell movement (Komuro and Rakic 1996). In addition, mobile astrocytes (Rondé, Giannone et al. 2000, Hamadi, Giannone et al. 2014) and smooth vascular cells (Scherberich, Campos-Toimil et al. 2000) also exhibit spontaneous calcium transient spikes. In agreement with these studies, our experiments show that migrating platelets display calcium oscillations, moreover casein proteins are involved in regulating these calcium fluctuations.

How does casein proteins affect calcium oscillations? Substrate or extracellular matrix have been demonstrated to modulate calcium oscillations. For example, substrate rigidity affected the calcium oscillations in human mesenchymal stem cells (HMSCs), and lowering the substrate stiffness inhibited both the amplitude and frequency of calcium oscillations (Kim, Seong et al. 2009). Later studies on HMSC showed that matrix protein mediated calcium oscillation regulated the differentiation of

HMSC (Franz, Karagaraj et al. 2014). In addition to HMSCs, calcium oscillations in fibroblast and neurons were regulated by mechanical environment, and increasing the stiffness of substrate enhanced the frequency of calcium oscillations (Godbout, Follonier Castella et al. 2013, Zhang, Zhang et al. 2014, Josephine, Benedikt et al. 2017). Consistent with these studies, our experiments show that platelets exhibit more frequent calcium oscillations in the absence of any proteins on the substrate than in the presence of casein proteins, which raises the possibility that those proteins modulate mechanical properties of the surface.

Although a number of studies show that the substrate stiffness regulates calcium oscillations, the underlying molecular mechanism by which mechanical properties of the environment affect the calcium signal is much unknown. Interestingly, we find that calcium permeant MS channel inhibitor GsMTx4 abolishes platelet motility in the presence of casein, PLL-g-PEG and pluronic, which suggest that these proteins may activate MS channels on platelet membrane and thereby modulate calcium oscillations. How does casein activate MS channels on platelets? Currently, two models regarding the gating of mechanically activated ion channels emerge. One is the lipid bilayer model, and the other is the tethered model (Martinac 2004, Ranade, Syeda et al. 2015). In the lipid bilayer, it is perceived that the membrane deformation is sufficient to activate MS channels. In the tethered model, it is assumed that the extracellular matrix or the cytoskeleton inside the cells are coupled to MS channels, mechanical forces function on the tethers and then gate MS channels. Our results show that mouse platelets are able to migrate when casein proteins and fibrinogen are coated on the substrate, which imply that casein proteins might modulate the extracellular matrix that is connected to MS channels, resulting in the changes of calcium signals in platelets.

What would be the differences between mouse and human platelets that cause their distinct requirements for migration? Generally, mouse and human platelets function similarly in haemostasis and thrombosis, but mouse platelets are smaller in size, more numerous in count and have more heterogeneous α-granules (Schmitt, Guichard et al. 2001, Jirouskova, Shet et al. 2007). The striking differences between mouse and human platelets are glycoprotein receptors on plasma membrane. First, one well-known dissimilarity is the thrombin receptor- protease activated receptors (PARs) on human and mouse platelets. Human platelets express thrombin receptors PAR1 and PAR4, whereas mouse platelets express PAR3 and PAR4 (Coughlin 2000, Nakanishi-

Matsui, Zheng et al. 2000). However, our results reveal that both human and mouse platelet are capable of migrating in the absence of thrombin, which suggest that PARs are not essential for platelets motility. Therefore, the differences of PARs on human and mouse platelets cannot explain their distinct requirements for migration.

Second, the receptor for the constant Fc region of immunoglobulins (Ig) - Fc receptor (FcR) is one distinct feature between mouse and human platelets. Human platelets express the immunoglobulin G (IgG) Fc receptor- FcγRⅡA that belongs to the class II of FcγRs (Rosenfeld, Looney et al. 1985, Kelton, Smith et al. 1987). On the contrary, mouse platelets are devoid of any FcγRs (McKenzie, Taylor et al. 1999). FcγRⅡA are activated by aggregated IgGs, IgG-opsonized pathogens and implicated in thrombosis and immunity (Arman and Krauel 2015). Recent studies reveal that FcγRⅡA amplifies integrin $\alpha_{IIb}\beta_3$ mediated outside-in signaling in platelets (Boylan, Gao et al. 2008, Zhi, Rauova et al. 2013). Our results show that IgGs are neither required for the migration of human nor mouse platelets, which suggest that the differences of FcRs on human and mouse platelets are not likely to account for their distinct requirements for migration.

Last, but not least, our results suggest that MS channels are crucial in platelet migration. MS channels are conserved proteins expressed both in prokaryotes and eukaryotes (Martinac 2004). So far, the expression or function of MS channels on platelets are not reported. However, one study on embryonic stem cells (ESCs) by inside-out clamp patch recordings shows that mouse ESCs exhibit a higher density of MS channels than human MSCs (70% vs 3% of patches), whereas large conductance $Ca^{2+}$ activated $K^+$ channels (BK) are highly expressed in human ESCs compared with mouse ESCs (50% vs 1% of patches) (Soria, Navas et al. 2013). This report provides a helpful cue that the mechanically gated ion channels on human and mouse platelets could be distinct from each other, which may count for their different responses to mechanical forces. Therefore, in future we will explore the dissimilarities of MS channels on mouse and human platelets.

Calcium, as the second messenger plays crucial roles in platelet functions, like shape change, secretion, and aggregation. The increase of cytoplasmic $Ca^{2+}$ are derived from the internal calcium pools and the extracellular space. Our previous experiments showed that platelets were able to spread in the absence of extracellular calcium (Gärtner, Engelhardt et al. 2015), which was consistent with earlier study that

platelets shape change could occur in calcium free medium (Rink, Smith et al. 1982). Substantial studies supported that platelet secretion was also independent of extracellular calcium. For example, calcium ionophore - A23187 induced platelets ATP release in the absence of exogenous calcium (Feinman and Detwiler 1974). For another instance, thrombin or calcium ionophore - ionomyosin evoked 5-seritonin secretion with little increase of cytosolic free calcium (Rink, Smith et al. 1982). Platelets aggregation is dependent on the increase of intracellular calcium that is partly due to the calcium influx from extracellular department, but to a large extent the release of internal calcium stores in platelets (White, Rao et al. 1974, Feinstein and Fraser 1975). Our results suggest endogenous calcium is insufficient to support platelet migration and platelets motility is strikingly enhanced with the rise of exogenous calcium concentration. This feature differentiates platelet motility from other platelets functions, including shape change, secretion and aggregation. Moreover, our studies that casein proteins, PLL-g-PEG and pluronic that trigger platelet migration do not affect platelet aggregation or secretion provide more evidence that platelet locomotion is distinct from platelets aggregation.

Our results demonstrate that extracellular calcium is indispensable for platelet migration. Besides platelets, exogenous calcium are demonstrated to be crucial for migration of other cell types. For example, extracellular calcium is required for neutrophils chemotaxis, removal of exogenous calcium by EGTA or inhibiting calcium influx by lanthanum chloride reduced neutrophil migration (Boucek and Snyderman 1976, Marks and Maxfield 1990). In fibroblast, the rise of cytosolic calcium from calcium influx instead of calcium release from internal stores is essential for growth factor-induced migration (Yang and Huang 2005). Moreover, nerve growth cone migrate is also dependent on the calcium influx through non-voltage gated calcium channels (Gomez, Snow et al. 1995). Therefore, my results that platelet migration relies on extracellular calcium provides more evidence that exogenous calcium is critical for cell migration.

Exogenous calcium enters into cells via ion channels on plasma membrane, which of these channels are important for platelet migration is an interesting question. SOCE, the major calcium influx way on platelets is mediated by both STIM1, the $Ca^{2+}$ sensor in ER membrane and Orai1, the SOC channel in plasma membrane and contributes significantly to platelets activation (Varga-Szabo, Braun et al. 2011). STIM1 and Orai1 are crucial in breast cancer cell migration by modulating local focal adhesion

turnover. SOCE blocker as well as knocking down of STIM1 or Orai1 inhibit breast tumour metastasis (Yang, Zhang et al. 2009). Besides breast cancer, STIM1 and Orai1 are highly expressed in renal carcinoma and cervical cancer cell and play important roles in cancer cell proliferation and migration (Chen, Chiu et al. 2011, Kim, Lkhagvadorj et al. 2014). However, my results show that platelets in the absence of STIM1 platelets do not display any defects in migration, indicating that STIM1 is not crucial in platelet migration.

STIM2, the isoform of STIM1 is also a single transmembrane proteins located in endoplasmic reticulum (ER) (Liou, Kim et al. 2005). Nevertheless, in contrary to STIM1 that triggers calcium influx in response to calcium depletion of internal pools, STIM2 induces calcium influx upon smaller reduction in ER calcium and functions primarily as the regulator of the basal cytosolic calcium. Consequently, selectively knock down of STIM2 results in lower resting cytosolic calcium concentration (Brandman, Liou et al. 2007, Gruszczynska-Biegala, Pomorski et al. 2011). To further investigate the effect of STIM in platelet migration, platelets in the absence of STIM1/2 were examined in our experiments, but they migrate as well as control platelets, suggesting that neither SOCE activated by STIM1 nor basal calcium contributed by STIM2 are important for platelet migration.

Although the functional defects of STIM1 deficient platelets have been thoroughly investigated, the phenotypes of platelets devoid of both STIM1 and STIM2 have not been reported so far. First, our study reveals that STIM1/2 deficient platelets display both lower resting calcium level and strikingly reduced calcium influx upon activation than wild type control platelets. Then our studies show that different from STIM1 deficient platelets that exhibit normal aggregation to GRCR agonists but impaired aggregation to GPVI agonists (Varga-Szabo, Braun et al. 2008), STIM1/2 knockout platelets display supressed aggregation to GPCR agonists as well as GPVI receptor agonist. Furthermore, ATP secretion upon agonists-evoked activation is markedly diminished in platelets lack of STIM1/2. Since previous study demonstrated that ATP secretion is mediated by intracellular calcium rather than extracellular calcium (Feinman and Detwiler 1974), we could conclude that the impaired ATP release is due to the lower basal calcium level regulated by STIM2 instead of defective calcium influx mediated by STIM1. Taken together, compared with the impaired functions in STIM1 deficient platelets (Varga-Szabo, Braun et al. 2008, Ahmad, Boulaftali et al.

2011), STIM1/2 knockout platelets display more striking defects in secretion and aggregation in vitro. Mice with STIM1 deficient platelets show unstable arterial thrombi (Varga-Szabo, Braun et al. 2008), whether STIM1/2 deficient mice have defects in thrombus formation in vivo needs to be investigated in future.

Mechanosensitive ion channels, particularly stretch activated ion channels play crucial roles in cell migration. Lee et al. demonstrated that the calcium transient in migrating fish keratocytes arised from activation of stretched activated channels, which triggered the calcium influx and subsequently rear retraction (Lee, Ishihara et al. 1999). Fibroblast motility was also regulated by stretch activated channels and inhibited by the heavy metal blocker gadolinium (Munevar, Wang et al. 2004). Furthermore, MS ion channels are expressed in prostate tumour cells, gadolinium or GsMTx4 impaired MS activity and suppressed prostate tumour cell migration (Maroto, Kurosky et al. 2012). My results show the MS channel inhibitor GsMTx4 abolishes platelets motility in the presence of casein, PLL-g-PEG, plurionic and plasma, which strongly suggest that mechanosensitive ion channels mediate platelet migration.

MS channels are non-selective cation channels and consisted of a large quantity of ion channels, we are interested to identify which of these channels are involved in platelet migration. ENaCs are sodium permeable, amiloride-sensitive cation channels that mediate mechanotransduction (Goodman and Schwarz 2003), it is also expressed on platelets and contributes to collagen activation (Cerecedo, Martinez-Vieyra et al. 2014). However, my experiments show that amiloride does not inhibit platelet migration. In addition to eNaC, amiloride is reported to inhibit mechano-gated TREK-1 and TRAAK channels (Maingret, Patel et al. 2000). Therefore, eNaC or $K_{2p}$ do not play significant roles in platelet migration.

Since quite a few TRP channels are mechanically activated, we would like to investigate their roles in platelet migration. TRPC6 is expressed in high levels in platelets (Hassock, Zhu et al. 2002) and can be activated by diacylglycerol as well as membrane stretch (Spassova, Hewavitharana et al. 2006). TRPC1 is also reported to be MS channel that is gated by membrane tension (Maroto, Raso et al. 2005). However, platelets deficient in TRPC1/6 do not show impaired migration, which indicates that TRPC1 or TRPC6 are not critical in platelets motility.

PIEZO1 (Fam38A) is a recently identified mechanically activated ion channel and plays crucial roles in numerous biological processes (Bagriantsev, Gracheva et al. 2014). To name only a few samples regarding the role of PIEZO1 in cell migration.

MS channels in breast cancer cells is formed by PIEZO1 protein and inhibiting PIEZO1 by GsMTx4 results in decreased motility of breast cancer cells. Furthermore, breast cancer patients with high expression of PEIZO1 have reduced overall survival (Li, Rezania et al. 2015). PIEZO1 responds to physical confinement in melanoma cancer cells and GsMTx4 treatment supresses confined migration of melanoma cells (Hung, Yang et al. 2016). It is worth noting that although GsMTx4 is capable of inhibiting PIEZO1 channels (Bae, Sachs et al. 2011), it is regarded as a general blocker of MS channels (Bowman, Gottlieb et al. 2007, Gnanasambandam, Ghatak et al. 2017). For example, GsMTx4 is also able to suppress mechanical activity of TRPC1 and TPPC6 channels (Spassova, Hewavitharana et al. 2006). My results show the GsTMx4 completely inhibits platelets motility, but the contribution of TRPC1/6 can be ruled out by TRPC1/6 deficient platelets that do not display impairment in migration, therefore, PIEZO1 will be a possible candidate of MS channels in platelets that mediates migration. In future, platelets from PIZEO1 knockout mouse will be employed to examine the role of PIEZO1 in platelet motility. Intriguingly, although GsMTx4 abolishes platelets motility, it does not affect platelets aggregation or secretion upon activation. Moreover, GsMTx4 does not calcium influx in the absence of mechanical forces. These results provide further evidence that platelet migration differs from other platelet functions and mechanically activated ion channels are critical in platelet migration but not in aggregation or secretion.

In summary, my studies demonstrate that mouse and human platelets need different requirements for migration, which shed light to disparities between human and mouse platelets. Then my results reveal that mechanosensitive ion channels are critical for platelet migration and STIM1/2 are important for platelets aggregation and secretion. These findings provide a deeper understanding of the cellular mechanisms involved in platelet functions.

# Reference


Ahmad, F., Y. Boulaftali, T. K. Greene, T. D. Ouellette, M. Poncz, S. Feske and W. Bergmeier (2011). "Relative contributions of stromal interaction molecule 1 and CalDAG-GEFI to calcium-dependent platelet activation and thrombosis." J Thromb Haemost **9**(10): 2077-2086.

Aoki, V. W. and D. T. Carrell (2003). "Human protamines and the developing spermatid: their structure, function, expression and relationship with male infertility." Asian J Androl **5**(4): 315-324.

Arman, M. and K. Krauel (2015). "Human platelet IgG Fc receptor FcgammaRIIA in immunity and thrombosis." J Thromb Haemost **13**(6): 893-908.

Bae, C., F. Sachs and P. A. Gottlieb (2011). "The mechanosensitive ion channel Piezo1 is inhibited by the peptide GsMTx4." Biochemistry **50**(29): 6295-6300.

Bagriantsev, S. N., E. O. Gracheva and P. G. Gallagher (2014). "Piezo proteins: regulators of mechanosensation and other cellular processes." J Biol Chem **289**(46): 31673-31681.

Balhorn, R. (2007). "The protamine family of sperm nuclear proteins." Genome Biology **8**(9): 227.

Beigi, R., E. Kobatake, M. Aizawa and G. R. Dubyak (1999). "Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase." Am J Physiol **276**(1 Pt 1): C267-278.

Bergmeier, W., C. L. Piffath, T. Goerge, S. M. Cifuni, Z. M. Ruggeri, J. Ware and D. D. Wagner (2006). "The role of platelet adhesion receptor GPIbalpha far exceeds that of its main ligand, von Willebrand factor, in arterial thrombosis." Proc Natl Acad Sci U S A **103**(45): 16900-16905.

Bergmeier, W. and L. Stefanini (2009). "Novel molecules in calcium signaling in platelets." J Thromb Haemost **7 Suppl 1**: 187-190.

Bergstrand, A., G. Rahmani-Monfared, A. Ostlund, M. Nyden and K. Holmberg (2009). "Comparison of PEI-PEG and PLL-PEG copolymer coatings on the prevention of protein fouling." J Biomed Mater Res A **88**(3): 608-615.

Berridge, M. J., M. D. Bootman and H. L. Roderick (2003). "Calcium signalling: dynamics, homeostasis and remodelling." Nat Rev Mol Cell Biol **4**(7): 517-529.

Bhat, M. Y., T. A. Dar and L. R. Singh (2016). Casein Proteins: Structural and Functional Aspects. Milk Proteins - From Structure to Biological Properties and Health Aspects. I. Gigli. Rijeka, InTech**:** Ch. 01.

Blair, P. and R. Flaumenhaft (2009). "Platelet α–granules: Basic biology and clinical correlates." Blood Rev **23**(4): 177-189.

Blaser, H., M. Reichman-Fried, I. Castanon, K. Dumstrei, F. L. Marlow, K. Kawakami, L. Solnica-Krezel, C. P. Heisenberg and E. Raz (2006). "Migration of zebrafish primordial germ cells: a role for myosin contraction and cytoplasmic flow." Dev Cell **11**(5): 613-627.

Boucek, M. M. and R. Snyderman (1976). "Calcium influx requirement for human neutrophil chemotaxis: inhibition by lanthanum chloride." Science **193**(4256): 905-907.

Bovellan, M., M. Fritzsche, C. Stevens and G. Charras (2010). "Death-associated protein kinase (DAPK) and signal transduction: blebbing in programmed cell death." Febs j **277**(1): 58-65.

Bowman, C. L., P. A. Gottlieb, T. M. Suchyna, Y. K. Murphy and F. Sachs (2007). "Mechanosensitive ion channels and the peptide inhibitor GsMTx-4: history, properties, mechanisms and pharmacology." Toxicon **49**(2): 249-270.

Boylan, B., C. Gao, V. Rathore, J. C. Gill, D. K. Newman and P. J. Newman (2008). "Identification of FcγRIIa as the ITAM-bearing receptor mediating αIIbβ3 outside-in integrin signaling in human platelets." Blood **112**(7): 2780-2786.

Brandman, O., J. Liou, W. S. Park and T. Meyer (2007). "STIM2 is a feedback regulator that stabilizes basal cytosolic and endoplasmic reticulum Ca 2+ levels." Cell **131**(7): 1327-1339.

Braun, A., D. Varga-Szabo, C. Kleinschnitz, I. Pleines, M. Bender, M. Austinat, M. Bosl, G. Stoll and B. Nieswandt (2009). "Orai1 (CRACM1) is the platelet SOC channel and essential for pathological thrombus formation." Blood **113**(9): 2056-2063.

Brohawn, S. G., Z. Su and R. MacKinnon (2014). "Mechanosensitivity is mediated directly by the lipid membrane in TRAAK and TREK1 K+ channels." Proc Natl Acad Sci U S A **111**(9): 3614-3619.

Brownlow, S. L. and S. O. Sage (2005). "Transient receptor potential protein subunit assembly and membrane distribution in human platelets." Thromb Haemost **94**(4): 839-845.

Butterworth, M. B. (2010). "Regulation of the epithelial sodium channel (ENaC) by membrane trafficking." Biochim Biophys Acta **1802**(12): 1166-1177.

Canalle, L. A., D. W. Lowik and J. C. van Hest (2010). "Polypeptide-polymer bioconjugates." Chem Soc Rev **39**(1): 329-353.

Castellino, F. J. and V. A. Ploplis (2005). "Structure and function of the plasminogen/plasmin system." Thromb Haemost **93**(4): 647-654.

Cavallini, L., M. Coassin and A. Alexandre (1995). "Two classes of agonist-sensitive Ca2+ stores in platelets, as identified by their differential sensitivity to 2,5-di-(tert-butyl)-1,4-benzohydroquinone and thapsigargin." Biochem J **310 ( Pt 2)**: 449-452.

Cerecedo, D., I. Martinez-Vieyra, L. Alonso-Rangel, C. Benitez-Cardoza and A. Ortega (2014). "Epithelial sodium channel modulates platelet collagen activation." Eur J Cell Biol **93**(3): 127-136.

Chalfie, M. (2009). "Neurosensory mechanotransduction." Nat Rev Mol Cell Biol **10**(1): 44-52.

Charras, G. T. (2008). "A short history of blebbing." J Microsc **231**(3): 466-478.

Chaudhary, J., M. Walia, J. Matharu, E. Escher and A. K. Grover (2001). "Caloxin: a novel plasma membrane Ca2+ pump inhibitor." Am J Physiol Cell Physiol **280**(4): C1027-1030.

Chen, Y. F., W. T. Chiu, Y. T. Chen, P. Y. Lin, H. J. Huang, C. Y. Chou, H. C. Chang, M. J. Tang and M. R. Shen (2011). "Calcium store sensor stromal-interaction molecule 1-dependent signaling plays an important role in cervical cancer growth, migration, and angiogenesis." Proc Natl Acad Sci U S A **108**(37): 15225-15230.

Christensen, A. P. and D. P. Corey (2007). "TRP channels in mechanosensation: direct or indirect activation?" Nat Rev Neurosci **8**(7): 510-521.

Christensen, T., M. Wienrich, H. A. Ensinger and N. H. Diemer (2005). "The broad-spectrum cation channel blocker pinokalant (LOE 908 MS) reduces brain infarct volume in rats: a temperature-controlled histological study." Basic Clin Pharmacol Toxicol **96**(4): 316-324.

Clapham, D. E. (2003). "TRP channels as cellular sensors." Nature **426**(6966): 517-524.

Clapham, D. E. (2007). "Calcium signaling." Cell **131**(6): 1047-1058.

Clapham, D. E., L. W. Runnels and C. Strubing (2001). "The TRP ion channel family." Nat Rev Neurosci **2**(6): 387-396.

Clark, K., M. Langeslag, B. van Leeuwen, L. Ran, A. G. Ryazanov, C. G. Figdor, W. H. Moolenaar, K. Jalink and F. N. van Leeuwen (2006). "TRPM7, a novel regulator of actomyosin contractility and cell adhesion." Embo j **25**(2): 290-301.

Corey, D. (2003). "Sensory transduction in the ear." J Cell Sci **116**(Pt 1): 1-3.

Corey, D. P. (2003). "New TRP channels in hearing and mechanosensation." Neuron **39**(4): 585-588.

Coste, B., J. Mathur, M. Schmidt, T. J. Earley, S. Ranade, M. J. Petrus, A. E. Dubin and A. Patapoutian (2010). "Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels." Science **330**(6000): 55-60.

Coste, B., B. Xiao, J. S. Santos, R. Syeda, J. Grandl, K. S. Spencer, S. E. Kim, M. Schmidt, J. Mathur, A. E. Dubin, M. Montal and A. Patapoutian (2012). "Piezo proteins are pore-forming subunits of mechanically activated channels." Nature **483**(7388): 176-181.

Coughlin, S. R. (2000). "Thrombin signalling and protease-activated receptors." Nature **407**(6801): 258-264.

Czapiga, M., J. L. Gao, A. Kirk and J. Lekstrom-Himes (2005). "Human platelets exhibit chemotaxis using functional N-formyl peptide receptors." Exp Hematol **33**(1): 73-84.

Dalgleish, D. G. and M. Corredig (2012). "The structure of the casein micelle of milk and its changes during processing." Annu Rev Food Sci Technol **3**: 449-467.

Daniel, J. L., C. Dangelmaier, J. Jin, B. Ashby, J. B. Smith and S. P. Kunapuli (1998). "Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets." J Biol Chem **273**(4): 2024-2029.

Dean, W. L., D. Chen, P. C. Brandt and T. C. Vanaman (1997). "Regulation of platelet plasma membrane Ca2+-ATPase by cAMP-dependent and tyrosine phosphorylation." J Biol Chem **272**(24): 15113-15119.

Dedman, A., R. Sharif-Naeini, J. H. Folgering, F. Duprat, A. Patel and E. Honore (2009). "The mechano-gated K(2P) channel TREK-1." Eur Biophys J **38**(3): 293-303.

Duquesnoy, R. J., D. F. Lorentsen and R. H. Aster (1975). "Platelet migration inhibition: a new method for detection of platelet antibodies." Blood **45**(6): 741-747.

Eigel, W. N., J. E. Butler, C. A. Ernstrom, H. M. Farrell, V. R. Harwalkar, R. Jenness and R. M. Whitney (1984). "Nomenclature of Proteins of Cow's Milk: Fifth Revision1." Journal of Dairy Science **67**(8): 1599-1631.

Endo, Y. and M. Nakamura (1992). "The effect of lipopolysaccharide, interleukin-1 and tumour necrosis factor on the hepatic accumulation of 5-hydroxytryptamine and platelets in the mouse." Br J Pharmacol **105**(3): 613-619.

Enouf, J., R. Bredoux, B. Papp, I. Djaffar, A. M. Lompre, N. Kieffer, O. Gayet, K. Clemetson, F. Wuytack and J. P. Rosa (1992). "Human platelets express the SERCA2-b isoform of Ca(2+)-transport ATPase." Biochem J **286 ( Pt 1)**: 135-140.

Erhardt, J. A., J. R. Toomey, S. A. Douglas and D. G. Johns (2006). "P2X1 stimulation promotes thrombin receptor-mediated platelet aggregation." J Thromb Haemost **4**(4): 882-890.

Evans, T. W. (2002). "Review article: albumin as a drug--biological effects of albumin unrelated to oncotic pressure." Aliment Pharmacol Ther **16 Suppl 5**: 6-11.

Eylar, E. H., M. A. Madoff, O. V. Brody and J. L. Oncley (1962). "The contribution of sialic acid to the surface charge of the erythrocyte." J Biol Chem **237**: 1992-2000.

Feinman, R. D. and T. C. Detwiler (1974). "Platelet secretion induced by divalent cation ionophores." Nature **249**(453): 172-173.

Feinstein, M. B. and C. Fraser (1975). "Human platelet secretion and aggregation induced by calcium ionophores. Inhibition by PGE1 and dibutyryl cyclic AMP." The Journal of General Physiology **66**(5): 561-581.

Feng, D., J. A. Nagy, K. Pyne, H. F. Dvorak and A. M. Dvorak (1998). "Platelets exit venules by a transcellular pathway at sites of F-met peptide-induced acute inflammation in guinea pigs." Int Arch Allergy Immunol **116**(3): 188-195.

Fine, B. P., K. A. Hansen, J. R. Salcedo and A. Aviv (1989). "Calcium-Activated Potassium Channels in Human Platelets." Proceedings of the Society for Experimental Biology and Medicine **192**(2): 109-113.

Fink, M., F. Duprat, F. Lesage, R. Reyes, G. Romey, C. Heurteaux and M. Lazdunski (1996). "Cloning, functional expression and brain localization of a novel unconventional outward rectifier K+ channel." Embo j **15**(24): 6854-6862.

Fink, M., F. Lesage, F. Duprat, C. Heurteaux, R. Reyes, M. Fosset and M. Lazdunski (1998). "A neuronal two P domain K+ channel stimulated by arachidonic acid and polyunsaturated fatty acids." Embo j **17**(12): 3297-3308.

Franz, D., J. Karagaraj and S. Sun (2014). Matrix-mediated regulation of Ca2+ oscillations in osteodifferentiation of human mesenchymal stem cells. Physics and Chemistry of Stem Cells. **1**.

Gachet, C. (2001). "Identification, characterization, and inhibition of the platelet ADP receptors." Int J Hematol **74**(4): 375-381.

Gachet, C. (2008). "P2 receptors, platelet function and pharmacological implications." Thromb Haemost **99**(3): 466-472.

Gao, H., X. Chen, X. Du, B. Guan, Y. Liu and H. Zhang (2011). "EGF enhances the migration of cancer cells by up-regulation of TRPM7." Cell Calcium **50**(6): 559-568.

Gärtner, F. R., S. Engelhardt, A. Görlach and D. Busch (2015). Autonomous Migration of Anucleated Platelets Facilitates Thrombus Consolidation.

Gee, J. M., N. A. Smith, F. R. Fernandez, M. N. Economo, D. Brunert, M. Rothermel, S. C. Morris, A. Talbot, S. Palumbos, J. M. Ichida, J. D. Shepherd, P. J. West, M. Wachowiak, M. R. Capecchi, K. S. Wilcox, J. A. White and P. Tvrdik (2014). "Imaging activity in neurons and glia with a Polr2a-based and cre-dependent GCaMP5G-IRES-tdTomato reporter mouse." Neuron **83**(5): 1058-1072.

Ghoshal, K. and M. Bhattacharyya (2014). "Overview of Platelet Physiology: Its Hemostatic and Nonhemostatic Role in Disease Pathogenesis." The Scientific World Journal **2014**: 16.

Gillespie, P. G. and R. G. Walker (2001). "Molecular basis of mechanosensory transduction." Nature **413**(6852): 194-202.

Gnanasambandam, R., C. Ghatak, A. Yasmann, K. Nishizawa, F. Sachs, A. S. Ladokhin, S. I. Sukharev and T. M. Suchyna (2017). "GsMTx4: Mechanism of Inhibiting Mechanosensitive Ion Channels." Biophys J **112**(1): 31-45.

Godbout, C., L. Follonier Castella, E. A. Smith, N. Talele, M. L. Chow, A. Garonna and B. Hinz (2013). "The mechanical environment modulates intracellular calcium oscillation activities of myofibroblasts." PLoS One **8**(5): e64560.

Gomez, T. M., D. M. Snow and P. C. Letourneau (1995). "Characterization of spontaneous calcium transients in nerve growth cones and their effect on growth cone migration." Neuron **14**(6): 1233-1246.

Goodman, M. B. and E. M. Schwarz (2003). "Transducing touch in Caenorhabditis elegans." Annu Rev Physiol **65**: 429-452.

Griffin, J. D. and L. Ellman (1978). "Epsilon-aminocaproic acid (EACA)." Semin Thromb Hemost **5**(1): 27-40.

Grimm, C., R. Kraft, S. Sauerbruch, G. Schultz and C. Harteneck (2003). "Molecular and functional characterization of the melastatin-related cation channel TRPM3." J Biol Chem **278**(24): 21493-21501.

Grosse, J., A. Braun, D. Varga-Szabo, N. Beyersdorf, B. Schneider, L. Zeitlmann, P. Hanke, P. Schropp, S. Muhlstedt, C. Zorn, M. Huber, C. Schmittwolf, W. Jagla, P. Yu, T. Kerkau, H. Schulze, M. Nehls and B. Nieswandt (2007). "An EF hand mutation in Stim1 causes premature platelet activation and bleeding in mice." J Clin Invest **117**(11): 3540-3550.

Gruszczynska-Biegala, J., P. Pomorski, M. B. Wisniewska and J. Kuznicki (2011). "Differential roles for STIM1 and STIM2 in store-operated calcium entry in rat neurons." PLoS One **6**(4): e19285.

Hamad, M. I., M. Krause and P. Wahle (2015). "Improving AM ester calcium dye loading efficiency." J Neurosci Methods **240**: 48-60.

Hamadi, A., G. Giannone, K. Takeda and P. Rondé (2014). "Glutamate involvement in calcium–dependent migration of astrocytoma cells." Cancer Cell Int **14**: 42.

Hamill, O. P. and B. Martinac (2001). "Molecular basis of mechanotransduction in living cells." Physiol Rev **81**(2): 685-740.

Hamley, I. W. (2014). "PEG-peptide conjugates." Biomacromolecules **15**(5): 1543-1559.

Harper, M. T. and A. W. Poole (2011). "Store-operated calcium entry and non-capacitative calcium entry have distinct roles in thrombin-induced calcium signalling in human platelets." Cell Calcium **50**(4): 351-358.

Hassock, S. R., M. X. Zhu, C. Trost, V. Flockerzi and K. S. Authi (2002). "Expression and role of TRPC proteins in human platelets: evidence that TRPC6 forms the store-independent calcium entry channel." Blood **100**(8): 2801-2811.

Hechler, B., N. Lenain, P. Marchese, C. Vial, V. Heim, M. Freund, J. P. Cazenave, M. Cattaneo, Z. M. Ruggeri, R. Evans and C. Gachet (2003). "A role of the fast ATP-gated

P2X1 cation channel in thrombosis of small arteries in vivo." J Exp Med **198**(4): 661-667.

Heemskerk, J. W., M. A. Feijge, L. Henneman, J. Rosing and H. C. Hemker (1997). "The Ca2+-mobilizing potency of alpha-thrombin and thrombin-receptor-activating peptide on human platelets -- concentration and time effects of thrombin-induced Ca2+ signaling." Eur J Biochem **249**(2): 547-555.

Hermanson, G. T. (2013). Chapter 10 - Fluorescent Probes. Bioconjugate Techniques (Third edition). Boston, Academic Press**:** 395-463.

Honke, K. and N. Taniguchi (2002). "Sulfotransferases and sulfated oligosaccharides." Med Res Rev **22**(6): 637-654.

Horne, D. S. (2006). "Casein micelle structure: Models and muddles." Current Opinion in Colloid & Interface Science **11**(2): 148-153.

Hou, Y., N. Carrim, Y. Wang, R. C. Gallant, A. Marshall and H. Ni (2015). "Platelets in hemostasis and thrombosis: Novel mechanisms of fibrinogen-independent platelet aggregation and fibronectin-mediated protein wave of hemostasis." J Biomed Res **29**.

Hristov, P., I. Mitkov, D. Sirakova, I. Mehandgiiski and G. Radoslavov (2016). Measurement of Casein Micelle Size in Raw Dairy Cattle Milk by Dynamic Light Scattering. Milk Proteins - From Structure to Biological Properties and Health Aspects. I. Gigli. Rijeka, InTech**:** Ch. 02.

Hung, W. C., J. R. Yang, C. L. Yankaskas, B. S. Wong, P. H. Wu, C. Pardo-Pastor, S. A. Serra, M. J. Chiang, Z. Gu, D. Wirtz, M. A. Valverde, J. T. Yang, J. Zhang and K. Konstantopoulos (2016). "Confinement Sensing and Signal Optimization via Piezo1/PKA and Myosin II Pathways." Cell Rep **15**(7): 1430-1441.

Ingerman-Wojenski, C. M. and M. J. Silver (1984). "A quick method for screening platelet dysfunctions using the whole blood lumi-aggregometer." Thromb Haemost **51**(2): 154-156.

Ishikawa, J., K. Ohga, T. Yoshino, R. Takezawa, A. Ichikawa, H. Kubota and T. Yamada (2003). "A Pyrazole Derivative, YM-58483, Potently Inhibits Store-Operated

Sustained Ca2+ Influx and IL-2 Production in T Lymphocytes." The Journal of Immunology **170**(9): 4441-4449.

Jackson, S. P. (2007). "The growing complexity of platelet aggregation." Blood **109**(12): 5087-5095.

Jirouskova, M., A. S. Shet and G. J. Johnson (2007). "A guide to murine platelet structure, function, assays, and genetic alterations." J Thromb Haemost **5**(4): 661-669.

Josephine, L., S. Benedikt and A. S. Howard (2017). "Calcium oscillations in wounded fibroblast monolayers are spatially regulated through substrate mechanics." Physical Biology **14**(4): 045006.

Jurk, K. and B. E. Kehrel (2005). "Platelets: physiology and biochemistry." Semin Thromb Hemost **31**(4): 381-392.

Kabanov, A. V., P. Lemieux, S. Vinogradov and V. Alakhov (2002). "Pluronic block copolymers: novel functional molecules for gene therapy." Adv Drug Deliv Rev **54**(2): 223-233.

Kahner, B. N., H. Shankar, S. Murugappan, G. L. Prasad and S. P. Kunapuli (2006). "Nucleotide receptor signaling in platelets." J Thromb Haemost **4**(11): 2317-2326.

Kakoma, I., C. A. Carson, M. Ristic, E. M. Stephenson, P. K. Hildebrandt and D. L. Huxsoll (1978). "Platelet migration inhibtion as an indicator of immunologically mediated target cell injury in canine ehrlichiosis." Infect Immun **20**(1): 242-247.

Kaplan, J. E. and T. M. Saba (1978). "Platelet removal from the circulation by the liver and spleen." Am J Physiol **235**(3): H314-320.

Kardash, E., J. Bandemer and E. Raz (2011). "Imaging protein activity in live embryos using fluorescence resonance energy transfer biosensors." Nat Protoc **6**(12): 1835-1846.

Kellenberger, S. and L. Schild (2002). "Epithelial sodium channel/degenerin family of ion channels: a variety of functions for a shared structure." Physiol Rev **82**(3): 735-767.

Kelton, J. G., J. W. Smith, A. V. Santos, W. G. Murphy and P. Horsewood (1987). "Platelet IgG Fc receptor." Am J Hematol **25**(3): 299-310.

Kim, J. H., S. Lkhagvadorj, M. R. Lee, K. H. Hwang, H. C. Chung, J. H. Jung, S. K. Cha and M. Eom (2014). "Orai1 and STIM1 are critical for cell migration and proliferation of clear cell renal cell carcinoma." Biochem Biophys Res Commun **448**(1): 76-82.

Kim, T. J., J. Seong, M. Ouyang, J. Sun, S. Lu, J. P. Hong, N. Wang and Y. Wang (2009). "Substrate rigidity regulates Ca2+ oscillation via RhoA pathway in stem cells." J Cell Physiol **218**(2): 285-293.

Kloda, A. and B. Martinac (2001). "Mechanosensitive channel of Thermoplasma, the cell wall-less archaea: cloning and molecular characterization." Cell Biochem Biophys **34**(3): 321-347.

Koedam, J. A., E. M. Cramer, E. Briend, B. Furie, B. C. Furie and D. D. Wagner (1992). "P-selectin, a granule membrane protein of platelets and endothelial cells, follows the regulated secretory pathway in AtT-20 cells." J Cell Biol **116**(3): 617-625.

Komuro, H. and P. Rakic (1996). "Intracellular Ca2+ fluctuations modulate the rate of neuronal migration." Neuron **17**(2): 275-285.

Kraemer, B. F., O. Borst, E. M. Gehring, T. Schoenberger, B. Urban, E. Ninci, P. Seizer, C. Schmidt, B. Bigalke, M. Koch, I. Martinovic, K. Daub, T. Merz, L. Schwanitz, K. Stellos, F. Fiesel, M. Schaller, F. Lang, M. Gawaz and S. Lindemann (2010). "PI3 kinase-dependent stimulation of platelet migration by stromal cell-derived factor 1 (SDF-1)." J Mol Med (Berl) **88**(12): 1277-1288.

Krishnamurti, C., S. J. Vukelja and B. M. Alving (1994). "Inhibitory effects of lysine analogues on t-PA induced whole blood clot lysis." Thromb Res **73**(6): 419-430.

Lee, J., A. Ishihara, G. Oxford, B. Johnson and K. Jacobson (1999). "Regulation of cell movement is mediated by stretch-activated calcium channels." Nature **400**(6742): 382-386.

Lee, S. and N. D. Spencer (2008). "Adsorption properties of poly(l-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) at a hydrophobic interface: influence of tribological stress, pH, salt concentration, and polymer molecular weight." Langmuir **24**(17): 9479-9488.

Lentz, B. R. (2003). "Exposure of platelet membrane phosphatidylserine regulates blood coagulation." Prog Lipid Res **42**(5): 423-438.

Lentz, B. R. (2003). "Exposure of platelet membrane phosphatidylserine regulates blood coagulation." Progress in Lipid Research **42**(5): 423-438.

Lesage, F., E. Guillemare, M. Fink, F. Duprat, M. Lazdunski, G. Romey and J. Barhanin (1996). "TWIK-1, a ubiquitous human weakly inward rectifying K+ channel with a novel structure." Embo j **15**(5): 1004-1011.

Lesage, F. and M. Lazdunski (2000). "Molecular and functional properties of two-pore-domain potassium channels." Am J Physiol Renal Physiol **279**(5): F793-801.

Lesage, F., C. Terrenoire, G. Romey and M. Lazdunski (2000). "Human TREK2, a 2P domain mechano-sensitive K+ channel with multiple regulations by polyunsaturated fatty acids, lysophospholipids, and Gs, Gi, and Gq protein-coupled receptors." J Biol Chem **275**(37): 28398-28405.

Li, C., S. Rezania, S. Kammerer, A. Sokolowski, T. Devaney, A. Gorischek, S. Jahn, H. Hackl, K. Groschner, C. Windpassinger, E. Malle, T. Bauernhofer and W. Schreibmayer (2015). "Piezo1 forms mechanosensitive ion channels in the human MCF-7 breast cancer cell line." Scientific Reports **5**: 8364.

Li, J., D. E. van der Wal, G. Zhu, M. Xu, I. Yougbare, L. Ma, B. Vadasz, N. Carrim, R. Grozovsky, M. Ruan, L. Zhu, Q. Zeng, L. Tao, Z. M. Zhai, J. Peng, M. Hou, V. Leytin, J. Freedman, K. M. Hoffmeister and H. Ni (2015). "Desialylation is a mechanism of Fc-independent platelet clearance and a therapeutic target in immune thrombocytopenia." Nat Commun **6**: 7737.

Liou, J., M. L. Kim, W. D. Heo, J. T. Jones, J. W. Myers, J. E. Ferrell, Jr. and T. Meyer (2005). "STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx." Curr Biol **15**(13): 1235-1241.

Lowenhaupt, R. W., H. I. Glueck, M. A. Miller and D. L. Kline (1977). "Factors which influence blood platelet migration." J Lab Clin Med **90**(1): 37-45.

Lowenhaupt, R. W., M. A. Miller and H. I. Glueck (1973). "Platelet migration and chemotaxis demonstrated in vitro." Thrombosis Research **3**(5): 477-487.

Lytton, J., M. Westlin and M. R. Hanley (1991). "Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps." J Biol Chem **266**(26): 17067-17071.

Mahaut-Smith, M. P. (1995). "Calcium-activated potassium channels in human platelets." J Physiol **484**(Pt 1): 15-24.

Mahaut-Smith, M. P. (2012). "The unique contribution of ion channels to platelet and megakaryocyte function." J Thromb Haemost **10**(9): 1722-1732.

Mahaut-Smith, M. P., S. Jones and R. J. Evans (2011). "The P2X1 receptor and platelet function." Purinergic Signal **7**(3): 341-356.

Maingret, F., A. J. Patel, F. Lesage, M. Lazdunski and E. Honore (2000). "Lysophospholipids open the two-pore domain mechano-gated K(+) channels TREK-1 and TRAAK." J Biol Chem **275**(14): 10128-10133.

Marks, P. W. and F. R. Maxfield (1990). "Transient increases in cytosolic free calcium appear to be required for the migration of adherent human neutrophils." J Cell Biol **110**(1): 43-52.

Maroto, R., A. Kurosky and O. P. Hamill (2012). "Mechanosensitive Ca(2+) permeant cation channels in human prostate tumor cells." Channels (Austin) **6**(4): 290-307.

Maroto, R., A. Raso, T. G. Wood, A. Kurosky, B. Martinac and O. P. Hamill (2005). "TRPC1 forms the stretch-activated cation channel in vertebrate cells." Nat Cell Biol **7**(2): 179-185.

Martinac, B. (2004). "Mechanosensitive ion channels: molecules of mechanotransduction." Journal of Cell Science **117**(12): 2449-2460.

Martinac, B., J. Adler and C. Kung (1990). "Mechanosensitive ion channels of E. coli activated by amphipaths." Nature **348**(6298): 261-263.

McKenzie, S. E., S. M. Taylor, P. Malladi, H. Yuhan, D. L. Cassel, P. Chien, E. Schwartz, A. D. Schreiber, S. Surrey and M. P. Reilly (1999). "The Role of the Human Fc Receptor FcγRIIA in the Immune Clearance of Platelets: A Transgenic Mouse Model." The Journal of Immunology **162**(7): 4311-4318.

McManus, O. B. (1991). "Calcium-activated potassium channels: regulation by calcium." J Bioenerg Biomembr **23**(4): 537-560.

McNicol, A. and S. J. Israels (1999). "Platelet dense granules: structure, function and implications for haemostasis." Thromb Res **95**(1): 1-18.

Miyamoto, T., T. Mochizuki, H. Nakagomi, S. Kira, M. Watanabe, Y. Takayama, Y. Suzuki, S. Koizumi, M. Takeda and M. Tominaga (2014). "Functional role for Piezo1 in stretch-evoked Ca(2)(+) influx and ATP release in urothelial cell cultures." J Biol Chem **289**(23): 16565-16575.

Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa and V. E. Papaioannou (1992). "RAG-1-deficient mice have no mature B and T lymphocytes." Cell **68**(5): 869-877.

Munevar, S., Y. L. Wang and M. Dembo (2004). "Regulation of mechanical interactions between fibroblasts and the substratum by stretch-activated Ca2+ entry." J Cell Sci **117**(Pt 1): 85-92.

Nakamura, M., M. Shibazaki, Y. Nitta and Y. Endo (1998). "Translocation of platelets into Disse spaces and their entry into hepatocytes in response to lipopolysaccharides, interleukin-1 and tumour necrosis factor: the role of Kupffer cells." J Hepatol **28**(6): 991-999.

Nakanishi-Matsui, M., Y. W. Zheng, D. J. Sulciner, E. J. Weiss, M. J. Ludeman and S. R. Coughlin (2000). "PAR3 is a cofactor for PAR4 activation by thrombin." Nature **404**(6778): 609-613.

Nevo, A., A. De Vries and A. Katchalsky (1955). "Interaction of basic polyamino acids with the red blood cell. I. Combination of polylysine with single cells." Biochim Biophys Acta **17**(4): 536-547.

Ni, H., C. V. Denis, S. Subbarao, J. L. Degen, T. N. Sato, R. O. Hynes and D. D. Wagner (2000). "Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen." J Clin Invest **106**(3): 385-392.

Numata, T., T. Shimizu and Y. Okada (2007). "Direct mechano-stress sensitivity of TRPM7 channel." Cell Physiol Biochem **19**(1-4): 1-8.

Oury, C., M. J. Kuijpers, E. Toth-Zsamboki, A. Bonnefoy, S. Danloy, I. Vreys, M. A. Feijge, R. De Vos, J. Vermylen, J. W. Heemskerk and M. F. Hoylaerts (2003). "Overexpression of the platelet P2X1 ion channel in transgenic mice generates a novel prothrombotic phenotype." Blood **101**(10): 3969-3976.

Oury, C., C. Lecut, A. Hego, O. Wera and C. Delierneux (2015). "Purinergic control of inflammation and thrombosis: Role of P2X1 receptors." Comput Struct Biotechnol J **13**: 106-110.

Packham, M. A. (1994). "Role of platelets in thrombosis and hemostasis." Can J Physiol Pharmacol **72**(3): 278-284.

Parekh, A. B. (2011). "Decoding cytosolic Ca2+ oscillations." Trends Biochem Sci **36**(2): 78-87.

Parekh, A. B. and J. W. Putney, Jr. (2005). "Store-operated calcium channels." Physiol Rev **85**(2): 757-810.

Paszty, K., T. Kovacs, C. Lacabaratz-Porret, B. Papp, J. Enouf, A. G. Filoteo, J. T. Penniston and A. Enyedi (1998). "Expression of hPMCA4b, the major form of the plasma membrane calcium pump in megakaryoblastoid cells is greatly reduced in mature human platelets." Cell Calcium **24**(2): 129-135.

Patel, A. J., E. Honore, F. Maingret, F. Lesage, M. Fink, F. Duprat and M. Lazdunski (1998). "A mammalian two pore domain mechano-gated S-like K+ channel." Embo j **17**(15): 4283-4290.

Patel, S. R., J. H. Hartwig and J. E. Italiano, Jr. (2005). "The biogenesis of platelets from megakaryocyte proplatelets." J Clin Invest **115**(12): 3348-3354.

Patterson, R. L., D. B. van Rossum and D. L. Gill (1999). "Store-Operated Ca2+ Entry: Evidence for a Secretion-like Coupling Model." Cell **98**(4): 487-499.

Paul, B. Z., J. Jin and S. P. Kunapuli (1999). "Molecular mechanism of thromboxane A(2)-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors." J Biol Chem **274**(41): 29108-29114.

Pegg, A. E. (2009). "Mammalian polyamine metabolism and function." IUBMB Life **61**(9): 880-894.

Pegg, A. E. (2014). "The function of spermine." IUBMB Life **66**(1): 8-18.

Perozo, E., A. Kloda, D. M. Cortes and B. Martinac (2002). "Physical principles underlying the transduction of bilayer deformation forces during mechanosensitive channel gating." Nat Struct Biol **9**(9): 696-703.

Perozo, E. and D. C. Rees (2003). "Structure and mechanism in prokaryotic mechanosensitive channels." Curr Opin Struct Biol **13**(4): 432-442.

Pickles, J. O., S. D. Comis and M. P. Osborne (1984). "Cross-links between stereocilia in the guinea pig organ of Corti, and their possible relation to sensory transduction." Hear Res **15**(2): 103-112.

Pingle, S. C., J. A. Matta and G. P. Ahern (2007). "Capsaicin receptor: TRPV1 a promiscuous TRP channel." Handb Exp Pharmacol(179): 155-171.

Pitchford, S. C., S. Momi, S. Baglioni, L. Casali, S. Giannini, R. Rossi, C. P. Page and P. Gresele (2008). "Allergen induces the migration of platelets to lung tissue in allergic asthma." Am J Respir Crit Care Med **177**(6): 604-612.

Prakriya, M., S. Feske, Y. Gwack, S. Srikanth, A. Rao and P. G. Hogan (2006). "Orai1 is an essential pore subunit of the CRAC channel." Nature **443**(7108): 230-233.

Qi, Z., S. Chi, X. Su, K. Naruse and M. Sokabe (2005). "Activation of a mechanosensitive BK channel by membrane stress created with amphipaths." Mol Membr Biol **22**(6): 519-527.

Quinlan, G. J., G. S. Martin and T. W. Evans (2005). "Albumin: biochemical properties and therapeutic potential." Hepatology **41**(6): 1211-1219.

Ramanathan, G., S. Gupta, I. Thielmann, I. Pleines, D. Varga-Szabo, F. May, C. Mannhalter, A. Dietrich, B. Nieswandt and A. Braun (2012). "Defective diacylglycerol-induced Ca2+ entry but normal agonist-induced activation responses in TRPC6-deficient mouse platelets." J Thromb Haemost **10**(3): 419-429.

Ranade, S. S., Z. Qiu, S. H. Woo, S. S. Hur, S. E. Murthy, S. M. Cahalan, J. Xu, J. Mathur, M. Bandell, B. Coste, Y. S. Li, S. Chien and A. Patapoutian (2014). "Piezo1, a mechanically activated ion channel, is required for vascular development in mice." Proc Natl Acad Sci U S A **111**(28): 10347-10352.

Ranade, S. S., R. Syeda and A. Patapoutian (2015). "Mechanically Activated Ion Channels." Neuron **87**(6): 1162-1179.

Ranade, S. S., S.-H. Woo, A. E. Dubin, R. A. Moshourab, C. Wetzel, M. Petrus, J. Mathur, V. Begay, B. Coste, J. Mainquist, A. J. Wilson, A. G. Francisco, K. Reddy, Z. Qiu, J. N. Wood, G. R. Lewin and A. Patapoutian (2014). "Piezo2 is the major transducer of mechanical forces for touch sensation in mice." Nature **516**(7529): 121-125.

Redwan, E. M., B. Xue, H. A. Almehdar and V. N. Uversky (2015). "Disorder in milk proteins: caseins, intrinsically disordered colloids." Curr Protein Pept Sci **16**(3): 228-242.

Reed, P. W. and H. A. Lardy (1972). "A23187: a divalent cation ionophore." J Biol Chem **247**(21): 6970-6977.

Rendu, F. and B. Brohard-Bohn (2001). "The platelet release reaction: granules' constituents, secretion and functions." Platelets **12**(5): 261-273.

Retailleau, K., F. Duprat, M. Arhatte, S. S. Ranade, R. Peyronnet, J. R. Martins, M. Jodar, C. Moro, S. Offermanns, Y. Feng, S. Demolombe, A. Patel and E. Honore (2015). "Piezo1 in Smooth Muscle Cells Is Involved in Hypertension-Dependent Arterial Remodeling." Cell Rep **13**(6): 1161-1171.

Rink, T. J. and S. O. Sage (1990). "Calcium signaling in human platelets." Annu Rev Physiol **52**: 431-449.

Rink, T. J., S. W. Smith and R. Y. Tsien (1982). "Cytoplasmic free Ca2+ in human platelets: Ca2+ thresholds and Ca-independent activation for shape-change and secretion." FEBS Lett **148**(1): 21-26.

Roberts, D. E., A. McNicol and R. Bose (2004). "Mechanism of collagen activation in human platelets." J Biol Chem **279**(19): 19421-19430.

Rondé, P., G. Giannone, I. Gerasymova, H. Stoeckel, K. Takeda and J. Haiech (2000). "Mechanism of calcium oscillations in migrating human astrocytoma cells." Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1498**(2): 273-280.

Roopenian, D. C., B. E. Low, G. J. Christianson, G. Proetzel, T. J. Sproule and M. V. Wiles (2015). "Albumin-deficient mouse models for studying metabolism of human albumin and pharmacokinetics of albumin-based drugs." MAbs **7**(2): 344-351.

Rosado, J. A., S. L. Brownlow and S. O. Sage (2002). "Endogenously expressed Trp1 is involved in store-mediated Ca2+ entry by conformational coupling in human platelets." J Biol Chem **277**(44): 42157-42163.

Rosado, J. A. and S. O. Sage (2000). "Coupling between inositol 1,4,5-trisphosphate receptors and human transient receptor potential channel 1 when intracellular Ca2+ stores are depleted." Biochem J **350 Pt 3**: 631-635.

Rosado, J. A. and S. O. Sage (2001). "Activation of store-mediated calcium entry by secretion-like coupling between the inositol 1,4,5-trisphosphate receptor type II and human transient receptor potential (hTrp1) channels in human platelets." Biochem J **356**(Pt 1): 191-198.

Rosenfeld, S. I., R. J. Looney, J. P. Leddy, D. C. Phipps, G. N. Abraham and C. L. Anderson (1985). "Human platelet Fc receptor for immunoglobulin G. Identification as a 40,000-molecular-weight membrane protein shared by monocytes." J Clin Invest **76**(6): 2317-2322.

Scherberich, A., M. Campos-Toimil, P. Ronde, K. Takeda and A. Beretz (2000). "Migration of human vascular smooth muscle cells involves serum-dependent repeated cytosolic calcium transients." J Cell Sci **113 ( Pt 4)**: 653-662.

Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J. Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak and A. Cardona (2012). "Fiji: an open-source platform for biological-image analysis." Nat Methods **9**(7): 676-682.

Schlessinger, J. and A. Ullrich (1992). "Growth factor signaling by receptor tyrosine kinases." Neuron **9**(3): 383-391.

Schmidt, E. M., P. Munzer, O. Borst, B. F. Kraemer, E. Schmid, B. Urban, S. Lindemann, P. Ruth, M. Gawaz and F. Lang (2011). "Ion channels in the regulation of platelet migration." Biochem Biophys Res Commun **415**(1): 54-60.

Schmitt, A., J. Guichard, J. M. Masse, N. Debili and E. M. Cramer (2001). "Of mice and men: comparison of the ultrastructure of megakaryocytes and platelets." Exp Hematol **29**(11): 1295-1302.

Schoenwaelder, S. M., Y. Yuan, E. C. Josefsson, M. J. White, Y. Yao, K. D. Mason, L. A. O'Reilly, K. J. Henley, A. Ono, S. Hsiao, A. Willcox, A. W. Roberts, D. C. Huang, H. H. Salem, B. T. Kile and S. P. Jackson (2009). "Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function." Blood **114**(3): 663-666.

Smyth, J. T., S.-Y. Hwang, T. Tomita, W. I. DeHaven, J. C. Mercer and J. W. Putney (2010). "Activation and regulation of store-operated calcium entry." Journal of Cellular and Molecular Medicine **14**(10): 2337-2349.

Solum, N. O., I. Hagen, C. Filion-Myklebust and T. Stabaek (1980). "Platelet glycocalicin. Its membrane association and solubilization in aqueous media." Biochim Biophys Acta **597**(2): 235-246.

Soria, B., S. Navas, A. Hmadcha and O. P. Hamill (2013). "Single mechanosensitive and Ca(2)(+)-sensitive channel currents recorded from mouse and human embryonic stem cells." J Membr Biol **246**(3): 215-230.

Spassova, M. A., T. Hewavitharana, W. Xu, J. Soboloff and D. L. Gill (2006). "A common mechanism underlies stretch activation and receptor activation of TRPC6 channels." Proc Natl Acad Sci U S A **103**(44): 16586-16591.

Sullivan, R., S. K. Koliwad and D. L. Kunze (1998). "Analysis of a Ca<sup>2+</sup>-activated K<sup>+</sup> channel that mediates hyperpolarization via the thrombin receptor pathway." American Journal of Physiology - Cell Physiology **275**(5): C1342-C1348.

Sun, J., P. Bird and H. H. Salem (1993). "Interaction of annexin V and platelets: effects on platelet function and protein S binding." Thromb Res **69**(3): 289-296.

Szewczyk, M. M., J. Pande and A. K. Grover (2008). "Caloxins: a novel class of selective plasma membrane Ca2+ pump inhibitors obtained using biotechnology." Pflugers Arch **456**(2): 255-266.

Tan, C. W., C. L. Poh, I. C. Sam and Y. F. Chan (2013). "Enterovirus 71 uses cell surface heparan sulfate glycosaminoglycan as an attachment receptor." J Virol **87**(1): 611-620.

Thastrup, O., P. J. Cullen, B. K. Drobak, M. R. Hanley and A. P. Dawson (1990). "Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase." Proc Natl Acad Sci U S A **87**(7): 2466-2470.

Thiagarajan, P. and J. F. Tait (1990). "Binding of annexin V/placental anticoagulant protein I to platelets. Evidence for phosphatidylserine exposure in the procoagulant response of activated platelets." J Biol Chem **265**(29): 17420-17423.

Thorn, D. C., H. Ecroyd, J. A. Carver and C. Holt (2015). "Casein structures in the context of unfolded proteins." International Dairy Journal **46**(Supplement C): 2-11.

Tiedt, R., T. Schomber, H. Hao-Shen and R. C. Skoda (2007). "Pf4-Cre transgenic mice allow the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo." Blood **109**(4): 1503-1506.

Traving, C. and R. Schauer (1998). "Structure, function and metabolism of sialic acids." Cell Mol Life Sci **54**(12): 1330-1349.

Uhlen, P. and N. Fritz (2010). "Biochemistry of calcium oscillations." Biochem Biophys Res Commun **396**(1): 28-32.

Valone, F. H., K. F. Austen and E. J. Goetzl (1974). "Modulation of the random migration of human platelets." J Clin Invest **54**(5): 1100-1106.

Varga-Szabo, D., K. S. Authi, A. Braun, M. Bender, A. Ambily, S. R. Hassock, T. Gudermann, A. Dietrich and B. Nieswandt (2008). "Store-operated Ca(2+) entry in platelets occurs independently of transient receptor potential (TRP) C1." Pflugers Arch **457**(2): 377-387.

Varga-Szabo, D., A. Braun, C. Kleinschnitz, M. Bender, I. Pleines, M. Pham, T. Renne, G. Stoll and B. Nieswandt (2008). "The calcium sensor STIM1 is an essential mediator of arterial thrombosis and ischemic brain infarction." J Exp Med **205**(7): 1583-1591.

Varga-Szabo, D., A. Braun and B. Nieswandt (2009). "Calcium signaling in platelets." J Thromb Haemost **7**(7): 1057-1066.

Varga-Szabo, D., A. Braun and B. Nieswandt (2011). "STIM and Orai in platelet function." Cell Calcium **50**(3): 270-278.

Vergara, C., R. Latorre, N. V. Marrion and J. P. Adelman (1998). "Calcium-activated potassium channels." Current Opinion in Neurobiology **8**(3): 321-329.

Vig, M., W. I. DeHaven, G. S. Bird, J. M. Billingsley, H. Wang, P. E. Rao, A. B. Hutchings, M. H. Jouvin, J. W. Putney and J. P. Kinet (2008). "Defective mast cell effector functions in mice lacking the CRACM1 pore subunit of store-operated calcium release-activated calcium channels." Nat Immunol **9**(1): 89-96.

Vig, M., C. Peinelt, A. Beck, D. L. Koomoa, D. Rabah, M. Koblan-Huberson, S. Kraft, H. Turner, A. Fleig, R. Penner and J. P. Kinet (2006). "CRACM1 is a plasma

membrane protein essential for store-operated Ca2+ entry." Science **312**(5777): 1220-1223.

Volkers, L., Y. Mechioukhi and B. Coste (2015). "Piezo channels: from structure to function." Pflugers Arch **467**(1): 95-99.

Wang, L., O. Ostberg, A. K. Wihlborg, H. Brogren, S. Jern and D. Erlinge (2003). "Quantification of ADP and ATP receptor expression in human platelets." J Thromb Haemost **1**(2): 330-336.

White, J. G., G. H. Rao and J. M. Gerrard (1974). "Effects of the Ionophore A23187 on blood platelets I. Influence on aggregation and secretion." Am J Pathol **77**(2): 135-149.

Wolfs, J. L., S. J. Wielders, P. Comfurius, T. Lindhout, J. C. Giddings, R. F. Zwaal and E. M. Bevers (2006). "Reversible inhibition of the platelet procoagulant response through manipulation of the Gardos channel." Blood **108**(7): 2223-2228.

Woo, S.-H., S. Ranade, A. D. Weyer, A. E. Dubin, Y. Baba, Z. Qiu, M. Petrus, T. Miyamoto, K. Reddy, E. A. Lumpkin, C. L. Stucky and A. Patapoutian (2014). "Piezo2 is required for Merkel-cell mechanotransduction." Nature **509**(7502): 622-626.

Wright, J. R., S. Amisten, A. H. Goodall and M. P. Mahaut-Smith (2016). "Transcriptomic analysis of the ion channelome of human platelets and megakaryocytic cell lines." Thromb Haemost **116**(2): 272-284.

Yang, S. and X. Y. Huang (2005). "Ca2+ influx through L-type Ca2+ channels controls the trailing tail contraction in growth factor-induced fibroblast cell migration." J Biol Chem **280**(29): 27130-27137.

Yang, S., J. J. Zhang and X. Y. Huang (2009). "Orai1 and STIM1 are critical for breast tumor cell migration and metastasis." Cancer Cell **15**(2): 124-134.

Yavin, E. and Z. Yavin (1974). "Attachment and culture of dissociated cells from rat embryo cerebral hemispheres on polylysine-coated surface." J Cell Biol **62**(2): 540-546.

Zhang, Q. Y., Y. Y. Zhang, J. Xie, C. X. Li, W. Y. Chen, B. L. Liu, X. A. Wu, S. N. Li, B. Huo, L. H. Jiang and H. C. Zhao (2014). "Stiff substrates enhance cultured neuronal network activity." Sci Rep **4**: 6215.

Zhang, Y., F. Gao, V. L. Popov, J. W. Wen and O. P. Hamill (2000). "Mechanically gated channel activity in cytoskeleton-deficient plasma membrane blebs and vesicles from Xenopus oocytes." J Physiol **523**(Pt 1): 117-130.

Zheng, J. (2013). "Molecular mechanism of TRP channels." Compr Physiol **3**(1): 221-242.

Zhi, H., L. Rauova, V. Hayes, C. Gao, B. Boylan, D. K. Newman, S. E. McKenzie, B. C. Cooley, M. Poncz and P. J. Newman (2013). "Cooperative integrin/ITAM signaling in platelets enhances thrombus formation in vitro and in vivo." Blood **121**(10): 1858-1867.

Zitt, C., B. Strauss, E. C. Schwarz, N. Spaeth, G. Rast, A. Hatzelmann and M. Hoth (2004). "Potent inhibition of Ca2+ release-activated Ca2+ channels and T-lymphocyte activation by the pyrazole derivative BTP2." J Biol Chem **279**(13): 12427-12437.

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