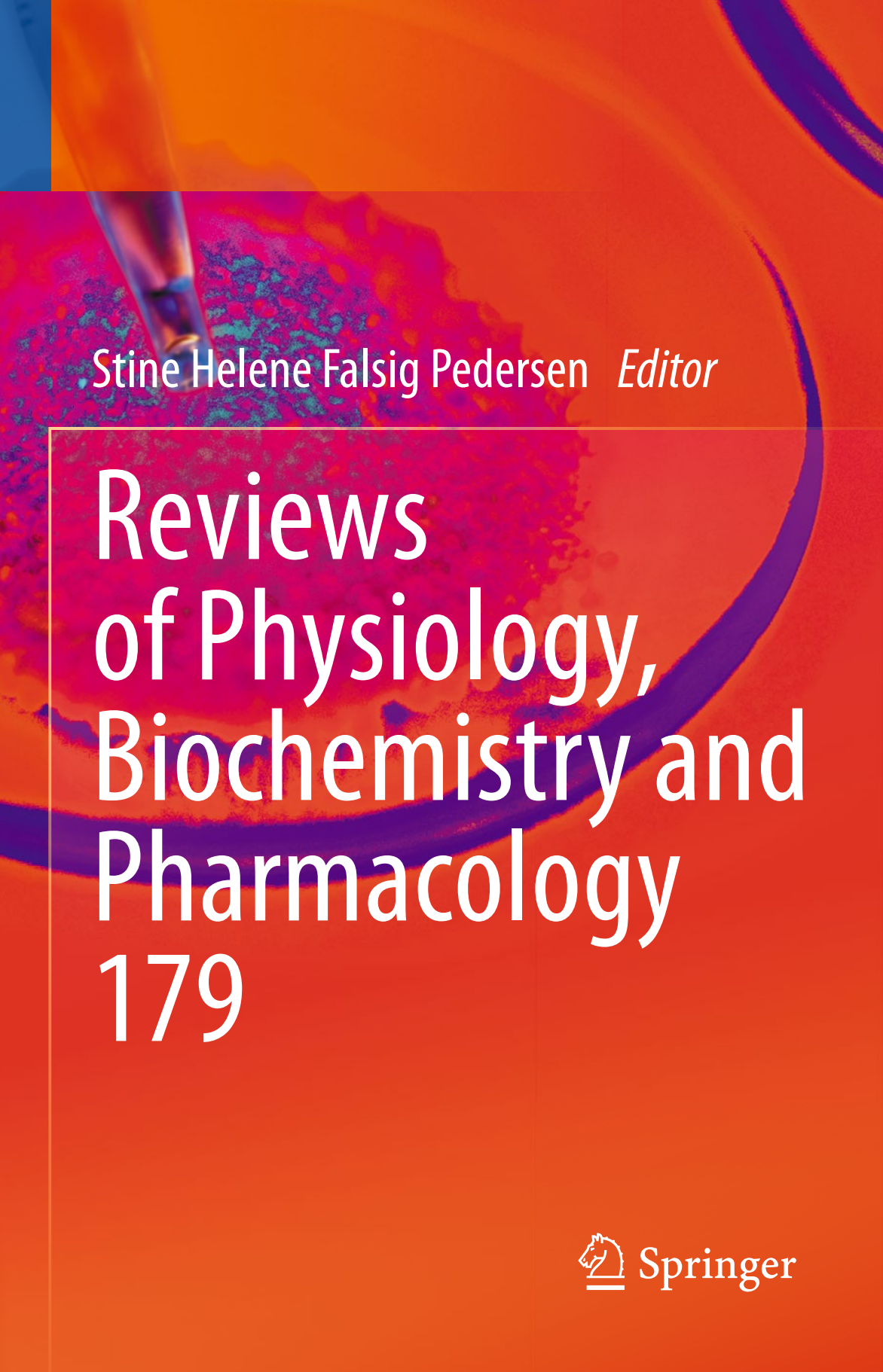
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Immunomodulatory Effects of Curcumin in Rheumatoid Arthritis: Evidence from Molecular Mechanisms to Clinical Outcomes

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Abstract Rheumatoid arthritis (RA) is a chronic immune-mediated inflammatory disorder characterized by the destruction of the joint and bone resorption. The production of pro-inflammatory cytokines and chemokines, dysregulated functions of three important subtypes of T helper (T_H) cells including T_H1, T_H17, and regulator T (Treg) cells are major causes of the initiation and development of RA. Moreover, B cells as a source of the production of several autoantibodies play key roles in the pathogenesis of RA. The last decades have seen increasingly rapid advances in the field of immunopharmacology using natural origin compounds for the management of various inflammatory diseases. Curcumin, a main active polyphenol compound isolated from turmeric, *curcuma longa*, possesses a wide range of pharmacologic properties for the treatment of several diseases. This review comprehensively will assess beneficial immunomodulatory effects of curcumin on the production of pro-inflammatory cytokines and also dysregulated functions of immune cells including T_H1, T_H17, Treg, and B cells in RA. We also seek the clinical efficacy of curcumin for the treatment of RA in several recent clinical trials. In conclusion, curcumin has been found to ameliorate RA complications through modulating inflammatory and autoreactive responses in immune cells and synovial fibroblast cells via inhibiting the expression or function of pro-inflammatory mediators, such as nuclear factor-κB (NF-κB), activated protein-1 (AP-1), and mitogen-activated protein kinases (MAPKs). Of note, curcumin treatment without any adverse effects can attenuate the clinical symptoms of RA patients and, therefore, has therapeutic potential for the treatment of the diseases.

Keywords Curcumin · Inflammation · Rheumatoid arthritis

Abbreviations

AIA	Ag-induced inflammatory arthritis
AIDP	Acute inflammatory demyelinating polyradiculoneuropathies
ALDH1a	Aldehyde dehydrogenase 1a
AN	Autoimmune neuritis
AP-1	Activated protein 1
B10	IL-10-producing B cells
BAFF	B cell-activating factor belonging to the TNF family
BDMC33	2,6-bis[2,5-dimethoxybenzylidene]cyclohexanone
CIA	Collagen-induced arthritis
CNS	Central nervous system

COX-2	Cyclooxygenase-2
CRP	C-reactive protein
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
DAS	Disease activity score
DCs	Dendritic cells
EAE	Experimental allergic encephalomyelitis
EAMG	Experimental autoimmune myasthenia gravis
ER	Endoplasmic reticulum
ESR	Erythrocyte sedimentation rate
FLS	Fibroblast-like synoviocytes
FOXP3	Forkhead box P3
GvHD	Graft versus host disease
HO-1	Heme oxygenase 1
IFN	Interferon
IL	Interleukin
iTreg	Inducible Treg
JAK/STAT	Janus kinase/Signal transducer and activator of transcription
LN	Lupus nephritis
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MMP	Matrix metalloproteinase
MS	Multiple sclerosis
NF-κB	Nuclear factor-κB
NSAIDs	Non-steroidal anti-inflammatory drugs
nTreg	Natural Treg cells
OA	Osteoarthritis
OVA	Ovalbumin
PD1	Programmed cell death 1
PGE2	Prostaglandin E2
PMA	Phorbol 12-myristate 13-acetate
RA	Rheumatoid arthritis
RORγt	RAR-related orphan receptor gamma
sDMARD	Synthetic disease-modifying anti-rheumatic drugs
SLE	Systemic Lupus Erythematosus
TGF-β	Transforming growth factor β
TH	T helper
TNF-α	Tumor necrosis factor-α
Treg	Regulatory T cells
VAS	Visual analog scale

1 Introduction

Rheumatoid arthritis (RA) is one of the most common chronic immune-mediated inflammatory diseases characterized by the uncontrolled inflammatory responses due to the breakdown of self-tolerance (Klareskog et al. 2009). Inflammation of synovium is a dominant feature of RA, which is associated with the joint cartilage destruction and deformity, impaired joint function, and disability (Klareskog et al. 2009).

It is worth mentioning that both innate and adaptive immune responses play critical roles in the initiation and development of RA (Firestein 2005; McInnes and Schett 2011). Local inflammation in the joint is characterized by the infiltration of different types of immune cells such as dendritic cells (DCs), macrophage, neutrophils, T cells, and B lymphocytes as well as other inflammatory cells (Firestein 2005; McInnes and Schett 2011). Subsequently, numerous pro-inflammatory cytokines and chemokines including IL-1β, IL-6, TNF-α, IL-8, IL-12, IFN-γ, and IL-17 are produced in the inflamed joint (Choy and Panayi 2001; Feldmann et al. 1996). In a positive feedback loop, these pro-inflammatory cytokines also recruit more inflammatory cells into inflamed joints, causing tissue damage (Choy and Panayi 2001; Marc Feldmann et al. 1996).

Understanding the underlying mechanisms involved in the immunopathogenesis of RA has led to advances in disease management with several medications including corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), and synthetic disease-modifying anti-rheumatic drugs (sDMARD) (Klareskog et al. 2009; Majithia and Geraci 2007). In addition, new biological agents (e.g. several monoclonal antibodies) targeting specific molecules involved in the immunopathogenesis of RA can be considered as a new treatment approach to prevent tissue damages and progression of the disease (Klareskog et al. 2009; Klinkhoff 2004; Majithia and Geraci 2007). However, there have been some concerns regarding the safety of the prolonged use of these medications for the treatment of RA. Despite their partial clinical success, these therapies have shown several limitations and adverse effects in long-term use, such as infections, liver injury, gastrointestinal damage, and heart failure (Laev and Salakhutdinov 2015; Smolen and Aletaha 2015). Moreover, the use of biologic agents imposes a large economic burden sick. Therefore, there is an urgent need to address alternative treatments for the management of RA.

Recent decades have seen increasingly rapid advances in the field of immunopharmacology using natural origin compounds for the management of several inflammatory diseases (Newman and Cragg 2012). In this regard, curcumin, an active constituent of turmeric, is one of the well-known natural compounds that has shown potential immunomodulatory properties for the treatment of several inflammatory disorders, in which dysregulated functions of immune cells show critical roles in the disease pathogenesis. This review therefore will comprehensively discuss both molecular mechanisms and clinical evidence showing the therapeutic effect of curcumin on RA disease.

2 Curcumin: Molecular Mechanisms of Immunomodulatory Effects

Curcumin is the most active component of the turmeric, derived from *curcuma longa*. It is one of the widely investigated natural compounds and has been extensively used for the treatment of various disorders such as immune-mediated diseases (Abdollahi et al. 2018; Bright 2007; Gupta et al. 2013; Momtazi-Borojeni et al. 2018). The therapeutic potential of curcumin is associated with low cost, low toxicity, pharmacological safety, and wide range pharmacological activities including anti-bacterial, anti-viral, anti-fungal, anti-oxidant, and anti-tumor activities (Bhardwaj et al. 2011; Mirzaei et al. 2016; Momtazi et al. 2016; Sahebkar et al. 2015; Teymouri et al. 2017; Vanichkul et al. 2010). A key aspect of curcumin is its anti-inflammatory and immunomodulatory properties, which have been verified in several animal models and clinical trials (Catanzaro et al. 2018; Ganjali et al. 2014; Gao et al. 2004; Panahi et al. 2015a; Panahi et al. 2015b; Panahi et al. 2012, 2016, 2017; Panahi et al. 2014; Rahimnia et al. 2015). Curcumin effectively interacts with a wide range of immune cells such as macrophages, DCs, as well as B and T cells and, whereby, modulates both innate and acquired immune responses (Shehzad and Lee 2013). Interestingly, it has been shown that curcumin through a pleiotropic interaction with several signaling molecules and transcription factors, including nuclear factor-κB (NF-κB), activated protein 1 (AP-1), Janus kinases/signal transducer and activator of transcriptions (JAKs/STATs), and mitogen-activated protein kinases (MAPKs), can effectively mediate its immunomodulatory roles in the immune system (Abdollahi et al. 2018; Gonzales and Orlando 2008; Han et al. 2002; Momtazi-Borojeni et al. 2018; Pan et al. 2013; Shakibaei et al. 2007; Shehzad and Lee 2013; L. Zhang et al. 2010; Zhao et al. 2016). Concerning this precise evidence, curcumin has been found to be a promising agent for the management of diseases in which uncontrolled inflammatory responses are a major player.

3 Immunopathogenesis of RA

3.1 Role of Pro-Inflammatory Cytokines in the Pathogenesis of RA

One of the main downstream events of both innate and adaptive immune responses in RA is the production of numerous pro-inflammatory cytokines and chemokines including TNF-α, IL-1β, IL-6, IL-12, IL-8, and MCP-1 (E. Y. Kim and Moudgil 2008; Smolen et al. 2005; Vervoordeldonk and Tak 2002). In the synovial (joint) fluid of RA patients, accumulation of these pro-inflammatory cytokines leads to several downstream responses, which may contribute to the development and progression of RA, including (1) more infiltration of leukocytes to the inflamed joints, which those also produce more pro-inflammatory mediators in the positive

feedback loop, (2) activation and proliferation of resident synovial and fibroblast cells, as the main source of proteolytic enzymes such as collagenase, elastase, and matrix metalloproteinases (MMPs), which destroy the cartilage, tendons, and ligaments of the inflamed joints, and (3) activation and differentiation of osteoclasts from osteoclasts precursors, which lead to the bone resorption (E. Y. Kim and Moudgil 2008; Sweeney and Firestein 2004). These pro-inflammatory cytokines also are responsible for systemic symptoms of RA such as fever, cachexia, fatigue, malaise, and elevation of acute phase substances (E. Y. Kim and Moudgil 2008).

Understanding the crucial roles of pro-inflammatory cytokines in the immunopathogenesis of RA has led to the generation of several therapies for the treatment of this disease, in which specific molecules have been targeted. It is noteworthy that several transcription factors such as NF-κB, AP-1, and STATs exert essential roles in the production of pro-inflammatory cytokines and related inflammatory responses in RA and thus can be considered as valuable targets for the treatment of RA (Kyriakis and Avruch 1996; Lawrence 2009; O'shea and Plenge 2012).

3.2 *Role of T_H1 Cells in the Pathogenesis of RA*

For the first time in 1986, Mosmann et al. identified the distinct subsets of T helper (T_H) cells based on the secreted cytokine patterns (Mosmann et al. 1986). T_H1 subset is one of the main subtypes of T helper cells, which is well identified by the production of TNF-α, IL-2, and IFN-γ (also named T_H1 cytokine profile) (Street and Mosmann 1991). Importantly, the presence of IL-12 and IFN-γ is essential for the differentiation of naïve $CD4^+$ T cells into T_H1 subtype (Street and Mosmann 1991). The biological function of T_H1 cells, which is related to their cytokine profile, is mainly defense against intracellular pathogens and tumors via activating function of macrophages (Street and Mosmann 1991). In addition, T_H1 cells influence the proliferation and antibody production of B cells (Coffman et al. 1988; Stevens et al. 1988). Despite the crucial physiological roles of T_H1 immune responses, these cells are also mainly responsible for the Type 4 hypersensitivity, which is strongly associated with several inflammatory and autoimmune diseases like RA (Lochhead et al. 2019; Powrie and Coffman 1993; Scott et al. 1994). Increasing research has found the central role of autoreactive T_H1 cells in initiating and developing autoimmune responses in RA (Harris Jr 1990). In an animal model of RA, T cell culture derived from lymph nodes showed increased rates of IFN-γ production as early as 6 days after immunization (Mauri et al. 1996). Moreover, the assessment of synovial fluid in people with rheumatoid arthritis indicated extremely high levels of IFN-γ (Cañete et al. 2000; Kusaba et al. 1998). IFN-γ as a key cytokine of T_H1 cells by activating and increasing recruitment of macrophages into inflamed joints plays key roles in the immunopathogenesis of the RA (Szekanecz and Koch 2007). As a result, the production of the pro-inflammatory cytokines and several tissue-degrading enzymes by macrophages is increased, causing cartilage and bone resorption

(Szekanecz and Koch 2007). T_H1- secreted IFN-γ is a potent stimulator for the production of various pathogenic antibodies including IgG2a and IgG2b by B cells (Coffman et al. 1988; Stevens et al. 1988). The formation of immune complexes containing autoantibodies and autoantigens also promotes inflammatory responses in inflamed joints, in a positive feedback loop, which causes joint destruction (Mathsson et al. 2006). These immune complexes are also responsible for the systemic complications of the disease, such as vasculitis and lung injury (Song and Kang 2010). Regarding the aforementioned evidence, a promising therapeutic approach for treating RA could be an attempt to modulate the differentiation and function of T_H1 cells.

3.3 Role of T_H17 Cells in the Pathogenesis of RA

T_H17 subset is another subset of $CD4^+$T cells, which is distinct from T_H1 and T_H2 subsets. For the differentiation of naïve $CD4^+$ T cells into T_H17 cells, IL-6, TGF- β, and IL-23 are essential (Mangan et al. 2006; Veldhoen et al. 2006). Indeed, the ability of TGF-β to inhibit the expression of transcription factors T-bet and GATA3, the key regulators for the differentiation of T_H2 cells, prevents differentiation naïve $CD4^+$T cells into T_H1 and T_H2 subsets (Gorelik et al. 2002; Heath et al. 2000). In addition, the lineage-specific transcription factors retinoic acid orphan receptor gamma t (RORγt) and RORα promote differentiation of T_H17 cells (Kurebayashi et al. 2013). Physiologically, T_H17 cell subtype induces cellular-mediated immune responses against extracellular pathogens such as bacteria and fungi by recruiting neutrophils and inducing inflammation (Stockinger and Veldhoen 2007). Following activation, T_H17 cells produce inflammatory cytokines including IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-26, and TNF-α (Kolls and Lindén 2004; Korn et al. 2007; Liang et al. 2006; Ye et al. 2001).

Regarding the wide range of pro-inflammatory cytokines and chemokines produced by T_H17 cells, more attention has been focused on the function of these cells in the immunopathogenesis of several inflammatory autoimmune diseases (Miossec 2009). A growing body of research has shown the critical role of IL-17A and IL-17F in promoting and exacerbating inflammation in RA (van den Berg and Miossec 2009; Yao et al. 1995). An increased level of IL-17 in the RA synovial fluid has highlighted the possible role of IL-17 in RA development (Chabaud et al. 1999). Studies on T_H17-deficient mice (Nakae et al. 2003) or mice treated with IL-17R (Bush et al. 2002) show a significant decline in RA symptoms. IL-17 is one of the main potent stimulators of osteoclastogenesis in the synovium, leading to bone resorption in RA patients (Kotake et al. 1999). Moreover, activation of infiltrated leukocytes, especially neutrophils, by T_H17 cells in inflamed joints promotes the release of numerous tissue-degrading mediators including collagenases, elastase, and MMPs, which contribute to the joint destruction (Chabaud et al. 1999; Miossec 2003). Moreover, IL-17 seems to play an active role in causing degradation of the cartilage matrix by chondrocyte metabolism dysregulation (Benderdour et al. 2002).

Under the influence of IL-17, chondrocytes increase the expression of prostaglandin E2 (PGE2) and nitric oxide (Attur et al. 1997; LeGrand et al. 2001). Together with TNF-α, IL-17A activates synovium fibroblast cells in the synovial fluid to produce IL-6 and IL-8, which contribute to joint inflammation. Interestingly, certain members of the IL-17 family, such as IL-17E or IL-25, may also have direct cartilage breakdown behaviors (Cai et al. 2001). Besides, IL-17 has been found to prevent collagen remodeling (Chabaud et al. 2001).

In summary, the aforementioned findings highlight the key roles of T_H17 cells in the immunopathogenesis of RA and, therefore, suggest that the regulation of T_H17 cells could be used as a promising tool for treating RA.

3.4 Role of Treg Cells in the Pathogenesis of RA

Regulatory T (Treg) cells are a specialized subpopulation of $CD4^+$ T cells and play crucial roles in the maintenance of self-tolerance (or peripheral tolerance) and tissue homeostasis (Sakaguchi et al. 2008; Wing and Sakaguchi 2010). In the processes of T cell development, some of the immature $CD4^+$ T cells, which reorganize self-antigens with high affinity, differentiate into Treg cells [so-called natural Treg cells (nTreg)] (Sakaguchi et al. 2008; Wing and Sakaguchi 2010). On the other hand, in peripheral tissues, recognition of self or exogenous antigens in the absence of inflammation promotes the differentiation of inducible Treg (iTreg) from naïve $CD4^+$ T cells (Sakaguchi et al. 2008). The most specific markers of Treg cells are CD4, $CD25^{high}$ (α chain of IL-2 receptor), Foxp3 (a well-known transcription factor for the generation of Treg), cytotoxic T lymphocyte-associated protein 4 (CTLA4), programmed cell death 1 (PD-1), and $CD127^{low}$ (Sakaguchi et al. 2008). Overall, some of the most important functions of Treg are i) suppressing the activation, maturation, and function of two important cells of innate immunity (i.e. macrophage and DCs) through the production of anti-inflammatory cytokines IL-10 and TGF-β, ii) the disruption of metabolic pathways and also the consumption of IL-2, a key cytokine for the proliferation and differentiation of other T cells, and iii) suppressing the activation, proliferation, differentiation, and function of T and B cells (Sakaguchi et al. 2008; Wing and Sakaguchi 2010). There is a growing body of literature that shows any dysregulations in Treg cell frequency or function may lead to the development of several inflammatory and autoimmune diseases like RA (Behrens et al. 2007; Boissier et al. 2009; Ehrenstein et al. 2004; Kondelkova et al. 2010; Lawson et al. 2006; Morgan et al. 2003; Wing and Sakaguchi 2010; Yudoh et al. 2000). Taken together, this mounting evidence reveals the importance of Treg cells for maintaining immunological tolerance and preventing the generation of immune related-disorders (Sakaguchi et al. 2008). Hence, promoting Treg cell differentiation and function can be a valuable therapeutic approach for the treatment of RA (Esensten et al. 2009; von Boehmer and Daniel 2013).

3.5 Role of B Cells in the Pathogenesis of RA

B cells, besides their protective physiological function, are known to play a critical role in the immunopathogenesis of various autoimmune and inflammatory diseases (Barr et al. 2012; Edwards and Cambridge 2006; Klinman and Steinberg 1987; Martin and Chan 2004). Increased infiltration of B cells, also called pathogenic B cells, has been shown in the synovial of inflamed joints, where activated B cells and plasma cells produce several autoantibodies that lead to joint destruction (Dorner and Burmester 2003; Edwards and Cambridge 2006; Samuels et al. 2005). In contrast to the pathogenic B cells, regulatory B cells (Breg) that suppress inflammatory responses and enhance immune tolerance are recently defined (Mauri and Blair 2010; van de Veen et al. 2016). Two important sub-populations of $FOXP3^+$ Breg cells have been characterized, including IL-10-producing B cells (B10, $CD19^+CD5^+IL10^+$) and TGF-β producing B cells ($CD19^+CD5^+TGF\text{-}\beta^+$) (K. M. Lee et al. 2014; Tedder 2015). Recent in vivo evidence has shown that B10 cells can effectively suppress T cell-mediated inflammatory responses and, therefore, negatively regulate inflammation in various inflammatory diseases such as IBD, RA, and EAE, in which uncontrolled T cell immune responses, especially T_H1 and T_H17, are a major cause of the disease (Daien et al. 2014; Lund and Randall 2010; Matsushita et al. 2008). Regarding this evidence, the expansion of Breg cells can be considered as a promising therapeutic approach in the treatment of RA.

4 Overview of Curcumin's Immunomodulatory Effects

4.1 Modulatory Effects of Curcumin on the Production of Pro-Inflammatory Cytokines

In recent years, a growing body of literature has addressed the potent inhibitory effects of curcumin on the production of several pro-inflammatory mediators, including TNF-α, IL-1β, IL-6, IL-12, MCP-1, and IL-8, in different types of immune cells and animal models (Abe et al. 1999; Hsu et al. 2008; Jain et al. 2009; Kim et al. 2005; Krasovsky et al. 2009; Pan et al. 2012; Rahardjo et al. 2014; Shirley et al. 2008). Of note, there is evidence that curcumin plays a crucial role in regulating inflammation in RA by reducing the production of various pro-inflammatory mediators. Curcumin treatment was found to significantly suppress the expression of IL-6 in IL-1β-stimulated synovial fibroblast cell line and fibroblast-like synoviocytes (FLS) of RA patients (Kloesch et al. 2013). In a study by Joong et al., pre-treatment of RAFLS with curcumin before stimulating with TNF-α markedly reduced the protein expression levels of IL-6, IL-8, MMP-1, and MMP-3 (Ahn et al. 2015). Moreover, treatment of horbol-12-myristate acetate (PMA)-stimulated synovial fibroblast cell line with curcumin analog [2,6-bis[2,5-dimethoxybenzylidene] cyclohexanone (BDMC33)] effectively reduced gene expression of COX-2 and

IL-6 at a dose-dependent manner through targeting NF-κB signaling pathway (K. H. Lee et al. 2015). Under the mentioned findings, another in vitro study also indicated that curcumin dose-dependently via downregulation of the TLR4/NF-κB signaling pathway markedly decreased the protein levels of TNF-α and IL-1β (Li et al. 2018). Also, the anti-arthritic activity and immunomodulatory effects of curcumin were indicated in an in vivo study by Ramadan et al., where it has been found that oral administration of 200 mg/kg turmeric (containing curcumin) for 14 days significantly attenuated the severity and incidence of arthritis by reducing the production of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6 in the animal model of adjuvant-induced arthritis (AIA) (Ramadan et al. 2011). Interestingly, the levels of anti-inflammatory cytokines IL-10 and IL-4 were also increased under treatment with curcumin (Ramadan et al. 2011). These findings demonstrate the immunomodulatory effects of curcumin in RA. A recent study also indicated that topical application of curcumin effectively reduced oxidative stress index and the production of pro-inflammatory mediators including TNF-α, IL-1β, and IL-6 in an animal model of arthritis (Jeengar et al. 2016). Moreover, curcumin in combination with tetramethylpyrazine and resveratrol, by inhibiting the NF-κB signaling pathway, significantly decreased the levels of TNF-α, IL-1β, and IL-6 in collagen-induced arthritis (CIA) rat model (Chen et al. 2017). This evidence is further supported by another in vivo study, in which curcumin treatment, either orally or intrathecal, was able to suppress the mRNA expression levels of pro-inflammatory mediators including TNF-α, IL-1β, MCP-1, and MCP-1α in the glial cells of a rat model of monoarthritis. These findings suggest that continuous treatment with curcumin can moderate hypersensitivity and neuroinflammation in RA (Chen et al. 2015). In another in vivo study, which set out to investigate the clinical efficacy and immunomodulatory effects of Zingier (containing curcumin), it has been found that Zingier with a dose of 50 mg/kg/day not only markedly reduced inflammatory markers, such as IL-1 and IL-6, but also clinically attenuated the severity of the RA disease (Fouda and Berika 2009). An in vivo study on the urate-induced gouty arthritis model showed that curcumin could dose-dependently reduce IL-1β, TNF-α, and elastase expression (Lee et al. 2015). Finally, confirming evidence based on the modulatory effects of curcumin on the production of pro-inflammatory cytokines has been found from more animal models of RA, which indicate curcumin effectively suppresses inflammation mediated by pro-inflammatory cytokines in RA (Dai et al. 2018; Fan et al. 2018; Li et al. 2018; Moon et al. 2010; Wang et al. 2019; Zheng et al. 2015).

4.2 Modulatory Effects of Curcumin on T_H1 Cells Differentiation and Function

It has been reported that curcumin, through suppressing the production of IL-12 (B. Kang et al. 1999) and IL-18 (Yadav et al. 2015) by macrophages, inhibits the

differentiation of naïve $CD4^+$ T cells into T_H1 cells, in vitro (Kang et al. 1999). Moreover, findings of an in vitro study on human DCs show that curcumin inhibits the immunostimulatory function of DCs, which, in turn, results in the decreased differentiation of T_H1 cells (Shirley et al. 2008). Of note, lack of maturation markers, inhibition of cytokine expression, and reduction of migration and endocytosis ability of curcumin-treated DCs were identified as the main reason for the deficiency of T_H1 cell differentiation and production of IFN-γ. In mechanism, curcumin was found to suppress T_H1 cell differentiation and function through inhibiting activation of master transcription factors T-bet, JAK2, and STAT4 that are required for differentiation (Natarajan and Bright 2002). Also, it has been reported that curcumin treatment induces apoptosis of T_H1 cells via distinct mechanisms including activating the stress response of the endoplasmic reticulum (ER), increasing the mitochondrial dysfunctions, and reducing the protein expression of BCL-2, as an anti-apoptotic inducer (Zheng et al. 2013).

The immunomodulatory effects of curcumin on T_H1 cells have been further studied by many researchers using different types of animal models, in which the predominance of T_H1 responses is a major cause of the disease's immunopathogenesis. In an animal model of arthritis, administration of liposome-loaded curcumin, as an NF-κB inhibitor, markedly suppressed Ag-specific responses of T_H1 cells, including both proliferation and production of pro-inflammatory cytokines TNF-α and IFN-γ (Capini et al. 2009).

In vivo studies on an animal model of colitis revealed that curcumin treatment can be a promising therapeutic candidate to ameliorate the severity and progression of the disease by inhibiting differentiation of T_H1 cells and subsequently decreasing the production of T_H1 cytokine profile, especially IFN-γ (Larmonier et al. 2008; Sugimoto et al. 2002; Zhang et al. 2006). These findings are further supported by other in vivo studies on animal models of encephalomyelitis, in which treatment with curcumin attenuated disease symptoms by selectively impairing T_H1 differentiation and function (Bruck et al. 2017; Kanakasabai et al. 2012; Natarajan and Bright 2002). In this regard, the underlying molecular mechanisms of curcumin's immunomodulatory effects have been clearly shown. Following curcumin treatment, the mRNA and protein expression of heme oxygenase 1 (HO-1) was increased in DCs, which in turn, led to the enhanced phosphorylation of STAT3. These events resulted in the generation of anti-inflammatory DCs, with low expression of IL-12, which effectively inhibited T_H1-mediated immune responses (Bruck et al. 2017; Kanakasabai et al. 2012). Moreover, curcumin ameliorated experimental encephalomyelitis through decreasing the production of IL-12, as a key cytokine for the differentiation of T_H1 cells, by macrophage/microglial cells, which resulted in the decreased differentiation of neural Ag-specific T_H1 cells (Natarajan and Bright 2002). In this vein, confirming evidence also emerged from several recent in vivo studies investigating the immunomodulatory effects of curcumin on T_H1 differentiation and function, which finally have shown that curcumin treatment can ameliorate autoimmune neuritis, diabetes, and myasthenia gravis generally through suppressing T_H1 immune responses (Castro et al. 2014; Han et al. 2014; Wang et al. 2016).

4.3 Modulatory Effects of Curcumin on T_H17 Cells Differentiation and Function

Curcumin was reported to inhibit the IL-17 and IL-23 production by spleen cells in vitro and decrease IL-17-producing cells in collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) mice, which was accompanied with reduced clinical symptoms of the disease (Kanakasabai et al. 2012; Okamoto et al. 2011). Another study on a mouse model of psoriasis indicated that curcumin markedly inhibited T_H17 cell proliferation by 50%, and decreased the production of inflammatory cytokines such as IL-17, IL-22, and TNF-α up to 60%. Further investigation revealed a significant reduction in serum levels of inflammatory cytokines such as TNF-α, IFN-γ, IL-22, and IL-23 in curcumin-treated psoriasis mice (Kang et al. 2016). Besides, curcumin has been shown to decrease differentiation of naïve $CD4^+$ T cells into T_H17 cells and thereby prevent T_H17-induced inflammation, resulting in an amelioration of autoimmune-related disorders (Dolati et al. 2018; Han et al. 2014; Handono et al. 2015). Mechanistic studies have indicated that curcumin inhibits both proliferation and differentiation of T_H17 cells through downregulating the expression of IL-6, IL-21, and RORγt (Kanakasabai et al. 2012; Xie et al. 2009).

Immunomodulatory effects of curcumin on T_H17 cells are further supported a study on experimental autoimmune myasthenia gravis (EAMG), in which curcumin improved disease symptoms by increasing the expression of anti-inflammatory cytokines, such as IL-10, and by decreasing the expression level of pro-inflammatory cytokines such as IL-17 and TNF-α (Wang et al. 2016), which was associated with induction of T_H1/T_H17 balance toward T_H2/Treg anti-inflammatory immune responses (Wang et al. 2016). This finding is further explained by investigations that show curcumin could ameliorate disease complications through reducing the population of both T_H17 and T_H1 cells and inhibiting the production of IFN-γ and IL-17 in a murine model of acute graft-versus-host disease (GVHD) characterized by T_H17 and T_H1-mediated destructive immune responses (Park et al. 2013).

It has been also shown that the treatment of $CD4^+$ T cells isolated from SLE patients with curcumin results in a significant reduction in the percentage of T_H17 cells (Handono et al. 2015). Moreover, the expression levels of IL-17 and IL-23 in EAE mice splenocytes were found to be decreased significantly after curcumin treatment (Kanakasabai et al. 2012). The further mechanistic study indicated that treatment of T cells isolated from psoriasis mice with 10 μM curcumin decreased the production of inflammatory cytokines such as IL-17, IL-22, and TNF-α up to 60%. Moreover, the use of 100 μM curcumin markedly decreased T_H17 cell proliferation by 50% (D. Kang et al. 2016).

In summary, curcumin can suppress T_H17 cell differentiation and proliferation by blocking the secretion of IL6, IL21, and IL23 cytokines. It also prevents the production of pro-inflammatory cytokines such as TNF-α and IL-17 in T_H17 cells, thus reduces inflammation and infiltration of inflammatory cells, especially neutrophils, into the site of inflammation like joints. Consequently, the production of more

pro-inflammatory cytokines and also tissue degradation enzymes, such as collagenase and elastase, by neutrophils, as the main cells recruited by T_H17 cells, can be inhibited. Given the role of T_H17 cells and their secreted cytokines in the development of RA, therefore, curcumin can be a potential therapeutic agent for the treatment of RA.

4.4 Modulatory Effects of Curcumin on Treg Cells Differentiation and Function

Curcumin has been found to selectively enhance Treg cell percentage and function, and suppress macrophage and DC-mediated T_H1 and T_H17 cell differentiation. It was found that *s.c.* injection of liposomes containing specific antigens and curcumin (as an NF-κB inhibitor) effectively induced Ag-specific immune tolerance via increasing differentiation of Ag-specific Treg cells in a model of Ag-induced inflammatory arthritis (Capini et al. 2009). Of note, curcumin treatment significantly up-regulated the expression level of FOXP3 that is responsible for the priming of Ag-specific Treg cells (Capini et al. 2009). In downstream of this event, the production of anti-inflammatory cytokine IL-10 was elevated that led to the suppression of T_H1 and T_H17 inflammatory responses (Capini et al. 2009). Rogers et al. studied whether curcumin could induce the generation of Treg cells and have shown that curcumin via induction of the immature state of DCs (or tolerogenic stats) could mediate its immunomodulatory effects. DCs in the immature state, characterized by the low expression of the key co-stimulatory molecules such as CD80, CD86, and CD40 and low production of IL-12 for the T cell priming, not only isn't able to induce the activation and differentiation of naïve T cells but also have a high capacity to induce the generation of Treg cells (Mahnke et al. 2007; Raker et al. 2015). Consequently, T cell responsiveness is significantly decreased due to activation of tolerogenic DC-induced $CD4^+CD25^+Foxp3^+CD127^{low}$ Treg cells (Rogers et al. 2010). Of note, both TGF-β and retinoic acid essentially needed for the generation of intestinal Treg cells (Cong et al. 2009). In this regard, Cong et al. provided new insights into molecular mechanisms of curcumin's effect on Treg cells. It has been shown that curcumin, by inhibiting NF-κB signaling, induced bone marrow-derived DCs to express aldehyde dehydrogenase 1 family (ALDH1a) and TGF-β (Cong et al. 2009). Subsequently, these curcumin-treated DCs showing the features of tolerogenic DCs were found to strongly induce differentiation of naive $CD4^+$ T cells into Treg cells, including both $CD4^+CD25^+$ $Foxp3^+$ Treg and IL-10-producing Tr1 cells (Cong et al. 2009). Clinically, curcumin-DC-induced Treg cells transferred into the mouse model of colitis effectively abrogated the disease in vivo (Cong et al. 2009). Another in vivo study on an animal model of colitis also showed that curcumin nanoparticles, by inducing $CD103^+$ $CD8\alpha^-$ DCs that can promote the generation of Treg cells, increased the number of mucosal $CD4^+FOXP3^+$ Treg cells in the inflamed colon. As a result, the production of several pro-inflammatory

cytokines was decreased following curcumin treatment, which attenuated the development of the disease (Ohno et al. 2017).

The study of curcumin's therapeutic efficacy in experimental autoimmune myasthenia gravis (EAMG; in which dysfunction of Treg cells is one of the possible mechanisms in the disease immunopathogenesis) showed that curcumin can shift the balance of T_H1/T_H17 towards T_H2/Treg (Wang et al. 2016). Consequently, curcumin treatment was found to down-regulate the expression of pro-inflammatory cytokines IFN-γ, TNF-α, and IL-17, and also up-regulate the level of anti-inflammatory cytokine IL-10. Clinically, curcumin treatment for 35 days effectively attenuated symptoms of EAMG (Danikowski et al. 2017; Wang et al. 2016).

it has been also shown that the peroral administration of curcumin in a mouse model of hyper-acute T_H1-type ileitis inhibited ileitis development and acute inflammation in the ileum by increasing the number of $CD4^+FOXP3^+$ Treg cells (Bereswill et al. 2010). In curcumin-treated mice, it was found that an increase in the number of Treg cells and the production of IL-10 strongly suppress T_H1-mediated immune responses.

EAE is an animal model of multiple sclerosis well established by the predominance of T_H1 and T_H17 inflammatory responses, and curcumin was found to ameliorate EAE (Kanakasabai et al. 2012). The inhibition of EAE progression mediated by curcumin was significantly associated with the improved numbers and function of $CD4^+CD25^+FOXP3^+$ Treg cells in the central nervous system (CNS) and peripheral lymphoid organs (Kanakasabai et al. 2012). Following in vivo treatment with curcumin, IL-10 cytokine production was up-regulated, which resulted in shifting the T_H1/T_H17 balance to T_H2/Treg (Kanakasabai et al. 2012).

A study on mice with experimental autoimmune neuritis (EAN) indicated that curcumin treatment (100 mg/kg/day, for 4 weeks) up-regulated the expression level of FOXP3, an essential transcription factor for the differentiation of Treg cells, and altered the differentiation of helper T cells (Han et al. 2014). Another research also revealed that curcumin could significantly up-regulate FOXP3 mRNA expression in a mouse model of lupus nephritis (LN) (H. Lee et al. 2012). This finding further supported by another study that showed curcumin significantly increased FOXP3 expression and Treg cell percentage in the mouse model of autoimmune glomerulonephritis (Eden et al. 2013).

Moreover, an ex vivo study on GVHD mouse model showed that following transplantation of curcumin-treated allogeneic splenocytes, the number of Treg cells was significantly increased. This was accompanied by reduced production of pro-inflammatory cytokines INF-γ and IL-17 as signature cytokines of T_H1 and T_H17 subsets, which directly involved in the pathogenesis of GVHD (Park et al. 2013).

It has been well established that T_H17/Treg imbalance contributes to the breakdown of immunological tolerance in SLE and RA (Handono et al. 2015). Therefore, using a pharmacologic agent to modulating T_H17/Treg balance can be considered as a valuable candidate for the treatment of autoimmune diseases. In this regard, low dose treatment of curcumin (0.1 and 1 μg/ml), without any side effects, could

effectively restore the T_H17/Treg imbalance on $CD4^+$T cells isolated from SLE patients (Handono et al. 2015). After curcumin administration, the number of T_H17 cells was decreased, while Treg cell populations and $CD4^+$ TGF-ß1 producer cells were markedly elevated (Handono et al. 2015). Interestingly, one of the underlying mechanisms by which curcumin mediates modulatory effects on Treg cells was found to be the up-regulation of CD69 (G. Kim et al. 2013). CD69 can inhibit switch from Treg cells into T_H17 effector cells and thus plays as a negative regulator in the generation of several inflammatory diseases (Pilar et al. 2010). Confirming findings have emerged from Kim's comparative study in which treatment with curcumin not only attenuated the declined expression of CD69 but also up-regulated the production of TGF-β1, a key cytokine for the generation of Treg cells, in CD2/CD3/CD28-stimulated T cells (Kim et al. 2013). As a consequence of this event, the percentage of $CD25^+$ $FOXP3^+$ Treg cells was elevated after curcumin treatment (Kim et al. 2013).

Curcumin has also been frequently reported to be effective in ameliorating inflammatory responses in several allergic disorders such as asthma, allergic rhinitis, allergic conjunctivitis, bronchitis, food allergy, and allergic contact dermatitis (Chung et al. 2012; Oh et al. 2011; Thakare et al. 2013). In this regard, the modulatory effects of curcumin on Treg cells' differentiation and function have been indicated in food allergies (Shin et al. 2015). Oral treatment with turmeric extract, *Curcuma longa*, (100 mg/kg) and curcumin (3 mg/kg or 30 mg/kg) for 2 weeks significantly attenuated food allergy symptoms by increasing differentiation of $FOXP3^+$ Treg cells in ovalbumin (OVA)-immunized mice (Shin et al. 2015). In good accordance with the mentioned findings, Ma et al. also found that oral administration of curcumin (50-200 mg/kg) effectively ameliorated allergic airway inflammation in an animal model of asthma by increasing differentiation of Treg cells followed by modulating Treg/TH17 balance (Zingg et al. 2013). Further study revealed that following curcumin treatment, the levels of key anti-inflammatory cytokines including TGF-β, and IL-10 were significantly up-regulated in Treg cells (Shin et al. 2015; Zingg et al. 2013).

In contrast to earlier findings, however, it has been shown that curcumin suppresses the phenotype and function of $CD4^+CD25^+$ Treg cells through the downregulation of Foxp3 and CTLA-4 (Forward et al. 2011; Zhao et al. 2012). Curcumin was found to suppress IL-2 cytokine production by inhibiting NF-κB and JAK/STAT signaling cascades. Following exposer to curcumin, phosphorylation of STAT5 and nuclear translocation of p65 and c-Rel that are essential for the expressions of Foxp3 and CD25 were significantly inhibited in Treg cells (Forward et al. 2011; Zhao et al. 2012).

4.5 *Modulatory Effects of Curcumin on B Cell Differentiation and Function*

Curcumin treatment was found to induce the differentiation of B cells into B_{10} subset, which resulted in the B_{10} cell-mediated immune suppression in an animal model of MG (Wang et al. 2016). In addition, following treatment with curcumin, the level of IgG1 antibody, as a protective or anti-inflammatory mediator, markedly increased, while the level of IgG2b antibody, as a pathogenic mediator, was decreased (Wang et al. 2016). It seems that increased differentiation of B10 cells, which is the favorite for the differentiation of T_H2 cells and subsequent production of protective antibodies, is underling mechanisms of curcumin's immunomodulatory effects in MG (Wang et al. 2016). Further, an in vivo study revealed that the administration of curcumin reduced the levels of pathogenic anti-CII IgG1 and IgG2a in CIA mouse model (Moon et al. 2010). Of note, curcumin (50 mg/kg) was reported to ameliorate the severity and progression of arthritis in CIA model through immunomodulatory effects on B cells (Huang et al. 2013). Curcumin by downregulating the expression of B cell-activating factor belonging to the TNF family (BAFF; a key cytokine for the survival, development, and auto-antibody production of B lymphocytes), significantly suppressed the development and activation of auto-reactive B cells, which produce several autoantibodies in RA (de la Torre et al. 2010; Huang et al. 2013). In line with the mentioned findings, an ex vivo study also showed that in curcumin-treated splenic B cells, isolated from LPS-treated BALB/c mice, the proliferative response and secretion of IgM were efficiently reduced (Decote-Ricardo et al. 2009). In vitro study of curcumin's immunomodulatory effects showed that curcumin can inhibit the activation and proliferation of B cells and suppress the production of several antibodies such as IgG2a by B cells (Sharma et al. 2007). These findings are further supported by another study that showed treatment with curcumin (30 mg/kg) significantly decreased the number of splenic $CD19^{+}CD62L$ B cells and subsequent production of autoantibodies in an animal model of glomerulonephritis (Jacob et al. 2013).

5 Clinical Efficacy and Immunomodulatory Effects of Curcumin in Human Studies

During the recent decade, there has been a growing trend towards investigating clinical efficacy and immunomodulatory effects of curcumin in the treatment of various human diseases.

For the first time in a pilot randomized clinical trial in 2012, Chandran et al. showed that treatment with curcumin (500 mg) for the 8 weeks in active RA patients has better outcomes in controlling disease activity as compared with NSAID such as diclofenac (Chandran and Goel 2012). Importantly, without any adverse effects such as dimness of vision, itching and swelling around the eyes, and increasing in the

serum levels of liver function markers detected in diclofenac group, curcumin administration effectively reduced the disease activity score (DAS) as well as tenderness and swelling of joint scores (Chandran and Goel 2012). This finding was the first evidence for the safety and efficacy of curcumin treatment in patients with active RA (Chandran and Goel 2012). Thereafter, the effects of CuroWhite, as a hydrogenated curcuminoids formulation, were investigated by Jobi et al., in which both low and high dose of the CuroWhite for 3 months significantly decreased erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and rheumatoid factor (RF) in RA patients (Jacob et al. 2018). Following administration with CuroWhite, DAS and visual analog scale (VAS) were decreased compared to the placebo receiving group (Jacob et al. 2018). Moreover, the number of tenders and swollen joints was improved in the CuroWhite treated group. These findings clearly show the clinical effectiveness of curcumin in treating RA, without any serious side effects (Jacob et al. 2018). Another randomized clinical trial conducted by Hemmati et al. showed that the administration of Curcumex consisting of ginger and curcumin for the 8 weeks in patients with RA attenuated the severity of the disease by reducing DAS, tenders, and swollen joints (Hemmati et al. 2016) (Fig. 1).

Similarly, a recent randomized, double-blind, controlled trial in patients with RA demonstrated that treatment with curcumin nanomicelle at a dose of 120 mg/day for 12 weeks partially reduced DAS as well as tender and swollen joint counts (Javadi et al. 2019). Importantly, the administration of curcumin nanomicelle had no adverse effects on RA patients (Javadi et al. 2019). Another double-blind, placebo-controlled, clinical study in patients with RA indicated that consumption of turmeric matrix formulation (containing curcuminoids) at doses of 250 and 500 mg for 90 days markedly attenuated clinical symptoms of RA patients (Amalraj et al. 2017). In this study, the levels of CRP, ESR, RF, VAS, and DAS were significantly improved in treated patients as compared to the baseline and also to the placebo group (Amalraj et al. 2017). Moreover, curcumin treatment at both doses, with a greater extent at high dose, indicated considerable clinical efficacy, together with well-tolerability and no side effects in RA patients (Amalraj et al. 2017).

To sum up, it seems that curcumin has therapeutic potential for the treatment of RA, without any serious adverse effects, which may be mainly mediated by its immunomodulatory effects on a wide range of deficient immune cells. Table 1 summarizes the clinical efficacy and immunomodulatory of curcumin treatment in recently completed clinical trials on RA patients.

6 Conclusion

Returning to the aim posed at the beginning of the current review study, it is now possible to suggest that curcumin as an immunomodulatory natural compound can be considered as a promising immunopharmacological agent for the treatment of RA. It can be concluded that the therapeutic effects of curcumin on RA complications arise from its interaction with different types of dis-regulated immune cells,

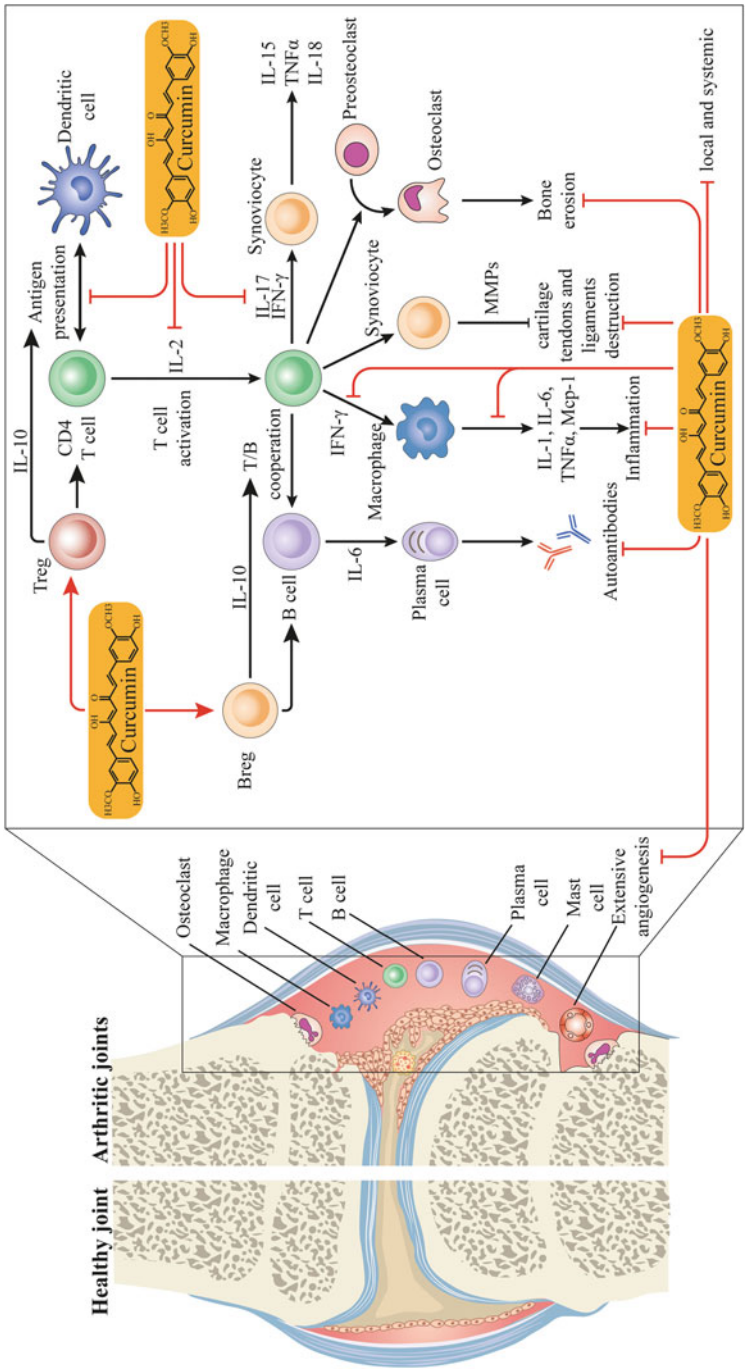

Fig. 1 A schematic view of curcumin's immunomodulatory effects on dis-regulated immune cells in RA. Curcumin inhibits the production of several key pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 by activated macrophages. Curcumin also suppresses activation and cytokine production of auto-reactive T cell, which in downstream leading to the inhibited activation of pathologic synoviocytes and osteoclasts. Curcumin restore the T_H17/Treg balance, which resulted in the increased numbers of Treg cells and subsequently production of anti-inflammatory cytokines IL-10 and TGF-β. Curcumin also suppresses production of autoantibodies by auto-reactive B cells on the one hand and increasing the numbers of Breg cells, which inhibit T cell immune responses, on the other hand. Finally, all of these immunomodulatory effects of curcumin lead to the inhibition of joint and bone destruction in RA

Table 1 An overview on recently completed clinical trials evaluating curcumin's immunomodulatory effects on RA disease

Dose of treatment	Duration of treatment	Population size (N)	Results	Ref.
Curcumin 500 mg/day	8 weeks	45	– Reduction in DAS as well as tenderness and swelling of joint scores	Chandran and Goel (2012)
Curcuminoids 250–500 mg/day	3 months	24	– Reducing the number of tender and swollen joints – Reduction in ESR, CRP, and RF	Jacob et al. (2018)
Curcumin 400 mg/day	8 weeks	60	– Attenuating the severity of the disease by reducing DAS, and also tender and swollen joints	Hemmati et al. (2016)
Curcumin nanomicelle 120 mg/day	12 weeks	32	– Reduction in DAS as well as tender and swollen joint counts	Javadi et al. (2019)
Curcumin 500–1,000 mg/day	90 days	24	– Attenuating clinical symptoms of the disease by reducing DAS, and VAS – Reducing ESR, CRP, and RF	Amalraj et al. (2017)

which have major roles in the immunopathogenesis of RA. Based on evidence emerged from in vitro and experimental studies, curcumin can effectively suppress inflammatory processes, which mediated by both innate and adaptive immune cells in RA, through (i) inhibiting the production of several key pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 by activated macrophages and DCs, (ii) suppressing activation and cytokine production of auto-reactive T cell subsets, especially T_H1 and T_H17, leading to inhibition of type 4 hypersensitivity responses in RA, (iii) restoring the T_H17/Treg balance, resulting in increased numbers of Treg cells in RA and subsequently the production of several anti-inflammatory cytokines by these immune suppressor cells, and (IV) inhibiting the production of autoantibodies by auto-reactive B cells together with increasing the numbers of Breg cells inhibiting T cell immune responses. Interestingly, all of these immunomodulatory effects of curcumin are closely related to its pleiotropic properties through which simultaneously targets various signaling molecules. Importantly, results from clinical-based studies show that the administration of curcumin has satisfying outcomes in treating RA. Concerning this mounting evidence, it is therefore strongly recommended to further evaluate the immunomodulatory effects of curcumin in future clinical trials for the treatment of patients with RA.

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Potential of Renin-Angiotensin-Aldosterone System Modulations in Diabetic Kidney Disease: Old Players to New Hope!

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Abstract Due to a tragic increase in the incidences of diabetes globally, diabetic kidney disease (DKD) has emerged as one of the leading causes of end-stage renal diseases (ESRD). Hyperglycaemia-mediated overactivation of the renin-angiotensin-aldosterone system (RAAS) is key to the development and progression of DKD. Consequently, RAAS inhibition by angiotensin-converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARBs) is the first-line therapy for the clinical management of DKD. However, numerous clinical and preclinical evidences suggested that RAAS inhibition can only halt the progression of the DKD to a certain extent, and they are inadequate to cure DKD completely. Recent studies have improved understanding of the complexity of the RAAS. It consists of two counter-regulatory arms, the deleterious pressor arm (ACE/angiotensin II/AT1 receptor axis) and the beneficial depressor arm (ACE2/angiotensin-(1-7)/Mas receptor axis). These advances have paved the way for the development of new therapies targeting the RAAS for better treatment of DKD. In this review, we aimed to summarise the involvement of the depressor arm of the RAAS in DKD. Moreover, in modern drug discovery and development, an advance approach is the bispecific therapeutics, targeting two independent signalling pathways. Here, we discuss available reports of these bispecific drugs involving the RAAS as well as propose potential treatments based on neurohormonal balance as credible therapeutic strategies for DKD.

Keywords ACE2 activators · AT2R agonist · Diabetic kidney disease · Mas receptor agonist · Neprilysin inhibitors · Renin-angiotensin-aldosterone system

Abbreviations

ACE	Angiotensin-converting enzyme
ACE2	Angiotensin-converting enzyme 2
ACEi	Angiotensin-converting enzyme inhibitors
AKI	Acute kidney injury
Ang I	Angiotensin I
ANP	Atrial natriuretic peptide

ARB	Angiotensin receptor blockers
AT1 receptor	Ang II type 1 receptor
AT2 receptor	Ang II type 2 receptor
BNP	B-type natriuretic peptide
BP	Blood pressure
BUN	Blood urea nitrogen
C21	Compound 21
CKD	Chronic kidney diseases
CNP	C-type natriuretic peptide
CVD	Cardiovascular diseases
DKD	Diabetic kidney disease
DRI	Direct renin inhibitor
ECM	Extracellular matrix
ESRD	End-stage renal disease
GFR	Glomerular filtration rate
HFrEF	Heart failure with reduced ejection fraction
ICAM-1	Intercellular adhesion molecule-1
IL-6	Interleukin-6
LV	Left ventricle
MAPKs	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein-1
NEPi	Neprilysin inhibitor
NEPi	Neprilysin inhibitors
NPS	Natriuretic peptide system
NPS	Natriuretic peptides system
PAI-1	Plasminogen activator inhibitor-1
pGC-A	Particulate guanylyl cyclase A
RAAS	Renin-angiotensin-aldosterone system
rhACE2	Recombinant human ACE2
ROS	Reactive oxygen species
sGC	Soluble guanylyl cyclase
T1DM	Type 1 diabetes mellitus
TGF-β1	Transforming growth factor-β1
TNF-α	Tumour necrosis factor-α
VCAM-1	Vascular cell adhesion molecule-1
VPi	Vasopeptidase inhibitors
ZDF	Zucker diabetic fatty

1 Introduction

The catastrophic escalation in the incidences, along with mortality, morbidity and healthcare expenditure attributable to diabetes, makes it a significant public health threat of the twenty-first century. The International Diabetes Federation estimated the global prevalence of diabetes was 451 million in the adult population in the year 2017, and these figures are anticipated to rise approximately to 693 million by the year 2045 (Cho et al. 2018). Almost 40% of diabetic patients suffered from diabetic kidney diseases (DKD), which is one of the leading causes for the end-stage renal disease (ESRD), globally (Gallagher and Suckling 2016; Goru et al. 2017; Micakovic et al. 2018). Over the past three decades, the increased burden of CKD due to diabetes contributed 50.62% of the increased burden of global CKD (Xie et al. 2018).

Clinically, DKD is diagnosed by progressive albuminuria and impaired renal functions as demonstrated by altered serum creatinine, glomerular hyperfiltration and a swift decline in glomerular filtration rate (GFR), ultimately ESRD (Alicic et al. 2017; Gallagher and Suckling 2016). As elucidated in Fig. 1, the development and progression of DKD govern by altered haemodynamic-mediated glomerular hypertension and pressure overload, perturbed substrate utilisation-driven metabolic remodelling, oxidative stress, inflammation and fibrosis (Dronavalli et al. 2008; Gallagher and Suckling 2016). Metabolic remodelling includes hyperglycaemia-mediated non-enzymatic glycosylation, activation of protein kinase C and the polyol pathway (Arora and Singh 2013; Dronavalli et al. 2008). Activation of these pathological signalling cascades caused adverse structural alterations in the kidney including extracellular matrix (ECM) accumulation, mesangial expansion, glomerular hyperfiltration, thickening glomerular basement membrane and glomerular sclerosis (Alicic et al. 2017; Arora and Singh 2013; Dronavalli et al. 2008; Gallagher and Suckling 2016) (Fig. 1).

Chronic hyperglycaemia-driven incessant renin-angiotensin-aldosterone system (RAAS) activation is key to the development and progression of DKD. Currently, the mainstays of the DKD management are hyperglycaemia and blood pressure (BP) control, along with the RAAS inhibition (Reidy et al. 2014). The RAAS pharmacological interventions like angiotensin receptor blockers (ARB) and angiotensin-converting enzyme inhibitors (ACEi) reduce albuminuria and improve renal functions. However, these drugs may offer imperfect protection, specifically if treatment starts at an advanced stage of the DKD (Anders et al. 2016; Roscioni et al. 2014; Schievink et al. 2016; Stanton 2014). Therefore, there is an urgent need to identify new therapeutic for those diabetic patients who do not respond satisfactorily to RAAS inhibitors.

Researchers are exploring the RAAS from both pathophysiological and therapeutic perspectives for more than a century. These constant systematic research endeavours have led to the breakthrough of a non-conventional RAAS, which has questioned the hypothesis that “the RAAS has an only harmful impact on the cardiovascular and renal systems” (Iwai and Horiuchi 2009; Ocaranza et al. 2019;

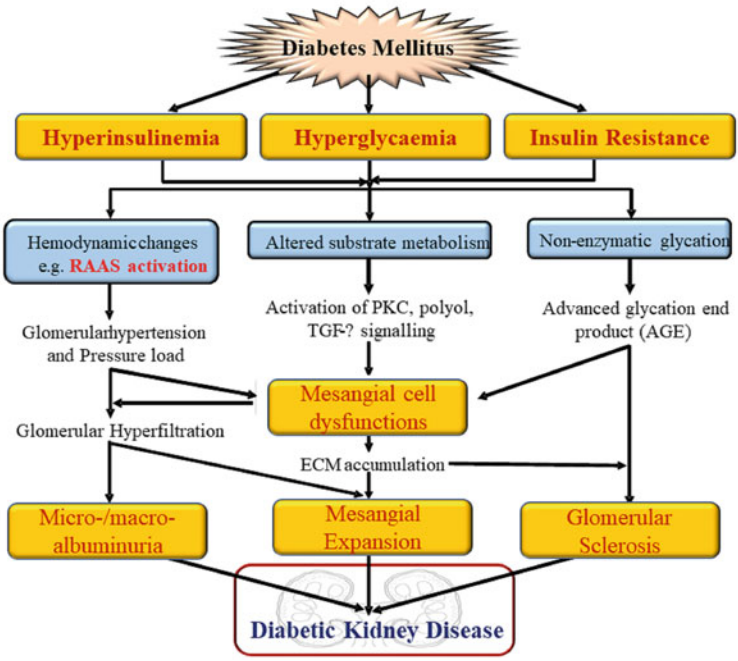

Fig. 1 Diagram recapping the pathophysiology of diabetic nephropathy. Diabetes mellitus comprises hyperinsulinemia, hyperglycaemia and insulin resistance, which leads to haemodynamic alterations, increased TGF-β signalling and advanced glycation end products and results in glomerular hyperfiltration and ECM accumulation. It collectively hastens micro-/macroalbuminuria, mesangial cell expansion and glomerular sclerosis and ultimately precipitates diabetic kidney disease

Pandey and Gaikwad 2017a). This non-conventional RAAS is also known as the depressor arm or the angiotensin-converting enzyme 2 (ACE2)/angiotensin-(1-7) [Ang-(1-7)]/Mas receptor axis. Improved understanding of the RAAS complexity has led to novel tactics aiming upregulation of the depressor RAAS to counteract the detrimental consequences of the conventional RAAS activation (Iwai and Horiuchi 2009; Ocaranza et al. 2019; Pandey and Gaikwad 2017a). Besides, the human body possesses a RAAS opposing natriuretic peptide system (NPS), having BP lowering and renoprotective potential (Malek and Gaikwad 2017).

Hence, in this review, we have discussed how these advances in the understanding of RAAS and neurohormonal imbalance in diabetic condition have paved the way for the development of new therapies for the better treatment of DKD. Besides, we have also focused on novel combination therapies in preclinical development, such as simultaneous Ang II type 1 (AT1) receptor inhibition and Ang II type 2 (AT2) receptor activation, add-on therapy of Ang-(1-7) with RAAS inhibitors, dual AT2 receptor and ACE2 activation, concurrent renin and neprilysin inhibition, combination therapies with Mas receptor agonist and add-on therapy of neprilysin inhibitor (NEPi) with ACE2 activation.

2 The Renin-Angiotensin-Aldosterone System

Under both physiological and pathological conditions, the RAAS remains the central neurohormonal system (Dronavalli et al. 2008). From the physiological viewpoint, RAAS plays an essential part in maintaining haemodynamic stability by regulating the extracellular fluid volume, sodium balance, tissue perfusion and cardiovascular trophic effects. From the pathological perspectives, hyperglycaemia-driven chronic activation of RAAS is the single most significant contributor to the development and progression of diabetes-associated vascular complications including DKD (Burnier and Zanchi 2006; Ruilope and Solini 2011). In DKD, persistent RAAS activation increases intraglomerular pressure, augments the generation of reactive oxygen species (ROS), pledges tissue injury, increases proinflammatory cytokines and stimulates glomerular and tubulointerstitial fibrosis (Carey and Siragy 2003; Mora-Fernández et al. 2014). Hence, in this regard, RAAS inhibition by ARB or ACEi denotes a key pharmacotherapy for DKD (Carey and Siragy 2003; Mora-Fernández et al. 2014).

The RAAS was initially discovered as a paracrine system with major effector, a circulating haemodynamic factor Ang II. However, now it is well established that autonomous RAASs acting in paracrine and autocrine fashion are present in some organs, including the kidney. Though it is contested, several shreds of evidence have suggested the third level of an intracellular functioning RAAS, for example, cells along the whole nephron having their local RAAS (Carey and Siragy 2003; Ellis et al. 2011; Micakovic et al. 2018; Navar et al. 2011). RAAS is comprised of two arms: the pressor arm and the depressor arm. They are the two pans of a balance scale, where the up-/downregulation of one could affect the others' activity (Fig. 2) (Goru et al. 2017; Santos et al. 2017).

3 The Pressor Arm of RAAS

The pressure arm, also known as the conventional RAAS or the deteriorative axis of RAAS, contains angiotensinogen, renin, angiotensin I (Ang I), angiotensin-converting enzyme (ACE), Ang II and AT1 receptor (Fig. 2). Notably, a wide range of the physiological and pathological effects of the RAAS is primarily mediated through the pressor arm (Iwai and Horiuchi 2009).

3.1 Renin

The conventional RAAS activation starts with the synthesis of renin by the juxtaglomerular cells of the renal afferent arteriole. Physiological factors including increased salt content in the distal tubules, low volume states, enhanced renal

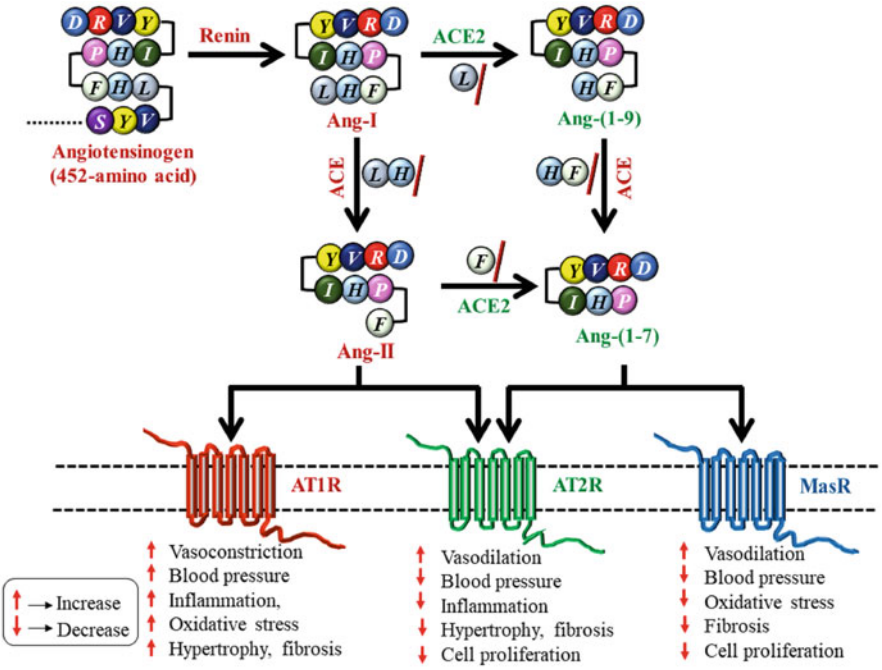

Fig. 2 Schematic representation of the renin-angiotensin-aldosterone system components. In pressor arm, renin converts angiotensinogen to angiotensin I which further breaks into angiotensin II by ACE activity. Ang II exerts its action majorly via angiotensin II type 1 receptor (AT1R). In depressor arm, ACE2 converts angiotensin I and angiotensin II into angiotensin-(1-9) and angiotensin-(1-7) (Ang-(1-7)), respectively, where Ang-(1-7) exerts its renoprotective action via angiotensin II type 2 receptor (AT2R) and Mas receptor

sympathetic tone and abridged renal perfusion are the stimulators for renal renin release (Davis and Freeman 1976). In the systemic circulation, renin acts as an aspartyl protease. It cleaves an α-2-globulin, angiotensinogen, to form a decapeptide, Ang I, having no known biological functions (Fig. 2) (Cat and Touyz 2011; Davis and Freeman 1976). In rats with spontaneous or streptozotocin-induced diabetes, renin protein and mRNA expressions are augmented in both proximal tubule and juxtaglomerular cells, accompanied by an increase in Ang II levels (Zimpelmann et al. 2000). Surprisingly, DKD is a low plasma renin state with an enhanced intrarenal RAAS activity associated with its pathogenesis (Yacoub and Campbell 2015). Urinary renin is increased in type 1 diabetes mellitus (T1DM) patients with DKD and streptozotocin-induced T1DM mice, which attributed to increased glomerular filtration and diminished proximal tubular reabsorption in DKD (Tang et al. 2019).

3.2 Angiotensin-Converting Enzyme

ACE is a monocarboxypeptidase, which cleaves the two amino acid, His-Leu, from the C-terminal portion of Ang I, consequently converting Ang I to Ang II (Fig. 2) (Iwai and Horiuchi 2009). ACE is found predominantly in endothelial cells, especially in pulmonary endothelium. In 1998, Deddish et al. revealed the role of ACE in the degradation of Ang-(1-7). Hence, in the vasculature, ACE plays a central role as a pressor enzyme by increasing the production of a potent vasoconstrictor Ang II and degrading the vasodilator Ang-(1-7) (Fig. 2). It is well established that ACE polymorphism plays a crucial role in the pathogenesis of hypertrophic nephropathy (Mizuiri et al. 1995). Besides, ACE exerted pro-hypertensive action via degradation of endogenous vasodilator, i.e. bradykinin (Cat and Touyz 2011). As per clinical reports, type 2 diabetes mellitus (T2DM) patients with nephropathy had higher serum and renal ACE levels as compared to T2DM without nephropathy (Ustündağ et al. 2000). Additionally, experimental study conducted with OLETF rats demonstrated that elevated ACE is involved in the development of nephropathy and provides evidence that intrarenal ACE rather than circulating ACE might exert a crucial role in T2DM patients with nephropathy (Taniguchi et al. 2002).

3.3 Angiotensin II

Ang II, an octapeptide, is the main effector of the RAAS, which exerts vasopressor action directly by its effects on arteriolar smooth muscles and indirectly by stimulating the production of aldosterone. Efferent arterioles are constricted more than afferent by Ang II in the kidney, thus creating glomerular hypertension (Dikalov and Nazarewicz 2013). Besides, Ang II is an active growth modulator and proinflammatory peptide involved in the pathogenesis of various vascular complications, including DKD (Leehey et al. 2000). The preclinical and clinical reports revealed that the high glucose milieu of diabetes increases Ang II production in the kidney (especially mesangial cells) which stimulates transforming growth factor-β1 (TGF-β1) secretion, resulting in increased synthesis, and decreases degradation of matrix proteins, followed by glomerular sclerosis and fibrosis eventually leading to DKD (Gagliardini et al. 2013; Leehey et al. 2000).

Apart from it, Ang II augments the adrenal production of aldosterone, an endogenous mineralocorticoid, and its primary function is to regulate the sodium absorption and potassium excretion by acting on the late distal tubule and collecting ducts of nephrons in the kidney (Kang and Cha 2009; MacKenzie et al. 2019). It affects the excretion of hydrogen ions by changing the potassium ion concentration in the lumen of the nephron. Additionally, it also affects blood pressure via regulating the sodium concentration by altering the total amount of volume in the extracellular fluid (ECF) (MacKenzie et al. 2019). Basically, aldosterone is synthesised in the zona glomerulosa of the adrenal cortex (Rainey and White 1998). There are

considerable experimental and clinical evidences that aldosterone remains an important driver of inflammation and fibrosis which contributes to the development of nephrosclerosis and renal fibrosis in the diabetic condition (Frimodt-Møller et al. 2020).

3.4 Ang II Type 1 Receptor

The pathophysiological effects of Ang II are primarily mediated via AT1 receptor, which includes aldosterone secretion, vasoconstriction, renal tubular sodium reabsorption, adrenergic facilitation, thirst and vascular remodelling (e.g. inflammation, hypertrophy and fibrosis) (Fig. 2) (Burnier and Zanchi 2006). AT1 receptors are ubiquitously distributed in all the tissues, with higher abundance in the kidney, adrenal glands, lungs and heart (Miyata et al. 1999). The AT1 receptor networks with several heterotrimeric G-proteins and generates second messengers, like diacylglycerol, inositol trisphosphate and ROS (Higuchi et al. 2007). Under clinical and experimental setup, Ang II/AT1 receptor coupling activates the receptor and non-receptor tyrosine kinases, serine/threonine kinases and mitogen-activated protein kinases (MAPKs) and upregulates many proinflammatory and profibrotic gene including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), interleukin-6 (IL-6), TGF-β and plasminogen activator inhibitor-1 (PAI-1) (Higuchi et al. 2007). Moreover, activation of the above-mentioned pathological signalling cascades plays a crucial role in the pathogenesis of CKD (Burnier and Zanchi 2006).

4 The Pressor RAAS Inhibitors in DKD: The Old Players

As discussed above, conventional RAAS activation is one of the main culprits for the development of diabetic vascular complications, including DKD. Hence, since the past few decades, blockage of RAAS inhibition has been "the cornerstone" for the management of CKD associated with diabetes (Barzilay et al. 2016; Ruilope and Solini 2011). Currently, three classes of RAAS inhibitors drugs are available: (1) *direct renin inhibitor (DRI)*, inhibits renin, a rate-limiting enzyme for Ang I synthesis; (2) *ACE inhibitor (ACEi)*, reduced the formation of Ang II; and (3) *AT1 receptor blockers (ARBs)*, block the harmful effects of Ang II at AT1 receptor (Fig. 3).

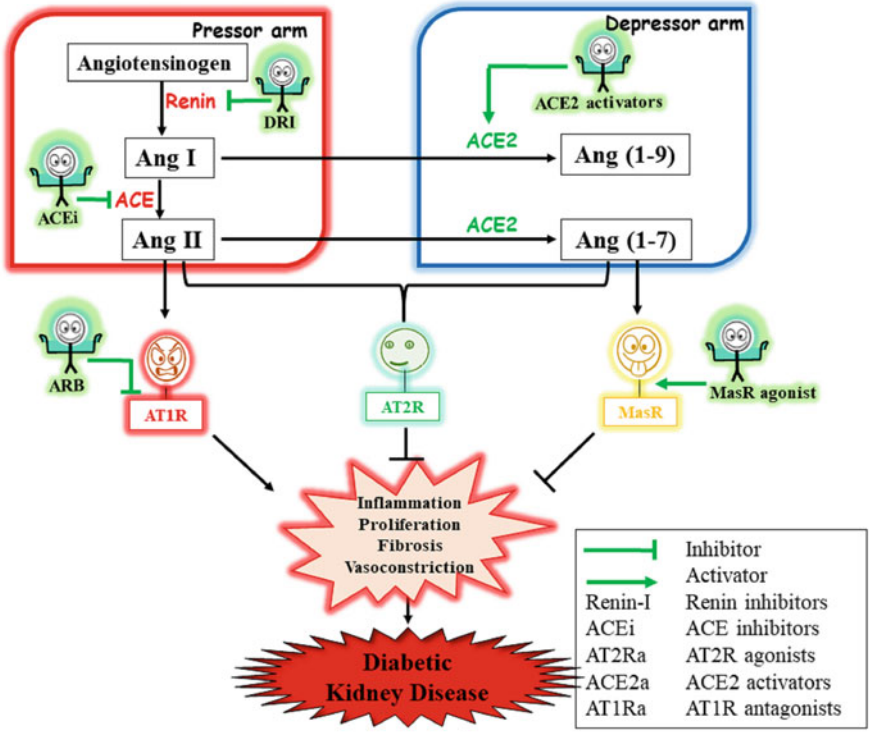

Fig. 3 Explored RAAS targets for the treatment of diabetic kidney disease. Treatment regimen highlighting the effect of the pressor arm (e.g. DRI, ARB, ACEi) and depressor arm (ACE2 activators, Mas receptor agonists) modulators in the prevention of diabetic kidney disease. *DRI* direct renin inhibitors, *ARB* angiotensin receptor blockers, *ACEi* angiotensin-converting enzyme inhibitors

4.1 Direct Renin Inhibitors

Due to renin's role in the upstream of RAAS cascade, its inhibition might prevent the detrimental consequences of both Ang II and aldosterone, resulting in favourable haemodynamic and structural effects on the kidney. Based on this assumption, several direct DRIs have been developed (Roscioni et al. 2014). In 2007, aliskiren became the first clinically approved DRI; it was approved by the United States Food and Drug Administration (US FDA) and the European Medicines Agency (EMA) for the treatment of hypertension (Jensen et al. 2008). In streptozotocin-induced diabetic Ren(2) rats, aliskiren treatment attenuated albuminuria and glomerulosclerosis to a similar level as of perindopril, independent to BP lowering. Moreover, diabetic transgenic (mRen-2)27 rats treated with aliskiren exhibited a more significant reduction in tubulointerstitial fibrosis when compared with the perindopril-treated rats (Kelly et al. 2007). In an initial investigative study, in the DKD patients, aliskiren reduced proteinuria and BP, independent of each other

(Persson et al. 2008). Moreover, In Japanese patients ($n = 31$) with advanced (stage 3–4) DKD, aliskiren treatment reduces SBP, urinary albumin-to-creatinine ratio, oxidative stress, inflammatory chemokine [e.g. monocyte chemoattractant protein-1 (MCP-1)] and inflammatory cytokine (e.g. IL-6) excretions and elevates glomerular filtration rates (Ogawa et al. 2011). Therefore, DRIs have shown renoprotective effect against DKD under clinical as well as preclinical conditions.

4.2 ACE Inhibitors

The ACEi is the first class of drugs targeting the RAAS and has been utilised successfully for the treatment of wide-ranging indications including hypertension, cardiovascular diseases (CVD) and CKD for the past 30 years. In the case of the DKD, numerous preclinical and clinical studies have reported renoprotective effects of ACEi. By limiting the Ang II production, ACEi treatment reduces glomerular hypertension and glomerular permeability to urinary albumin resulting in diminished proteinuria (De et al. 2006). In Zucker diabetic fatty (ZDF) rats, ramipril attenuated proteinuria and renal injury, by limiting upregulation of renal fibroblast growth factor 23 and Klotho expressions (Zanchi et al. 2013). In a breakthrough study, captopril treatment to T1DM patients with DKD (urinary protein excretion $\geq$500 mg/day; serum creatinine $\leq$221 μmol/L, $n = 409$) reduced the risk of a doubling of serum creatinine and DKD-associated death, dialysis or transplantation almost by half (50%) in comparison to the placebo, during a median of 3-year follow-up (Lewis et al. 1993). Renal insufficiency patient receiving ramipril treatment exhibited hazard ratio of 0.80 (95% CI, 0.59–1.09), for the combined risk of myocardial infarction, stroke or cardiovascular death, when compared to placebo treatment (Mann et al. 2001). Two months of lisinopril treatment to DKD patients prevented tubular and glomerular damage and dysfunction, as indicated by reduced urinary liver fatty acid-binding protein (U-LFABP) and albuminuria (Nielsen et al. 2009). Hence, ACEi has effectively attenuated renal injury under DKD condition.

4.3 AT1 Receptor Blockers

An ARB, valsartan attenuated podocyte loss via inhibition of the Notch pathway in the glomeruli of diabetic mice (Gao et al. 2016). Irbesartan prevented metabolic aberrations, podocyte injury and renal dysfunction associated with DKD in the *db/db* mice by conquering the RANKL-RANK-NF-κB pathway (Chen et al. 2016). A meta-analysis of clinical trials revealed that ARB's treatment associated with 23% reductions in the newly diagnosed diabetes cases (Abuissa et al. 2005). In clinical studies, ARB's treatment to diabetic patients resulted in renal and cardiovascular protection; thus, the US FDA approved several ARBs including candesartan, irbesartan, losartan, valsartan and telmisartan. In the reduction of endpoints in

non-insulin-dependent diabetes mellitus with the Ang II antagonist losartan (RENAAL) clinical trial, losartan abridged the occurrence of a doubling of the serum creatinine concentration by 25% ($P = 0.006$) and ESRD by 28% ($P = 0.002$) with no effect on the rate of death (Brenner et al. 2001). In DETAIL® study, telmisartan improved the decline in GFR over 5 years in T2DM patients with early nephropathy which was determined using the iohexol-based direct measurement of GFR (Barnett 2004).

A prospective 1 year trial to compare Micardis® versus losartan in hypertensive T2DM patients with overt nephropathy (AMADEO™) suggests that telmisartan produced a 29.8% reduction in urine albumin-to-creatinine ratio, whereas that with losartan was only 21.4% (Bakris et al. 2008). Ongoing telmisartan alone in combination with ramipril global endpoint trial (ONTARGET) demonstrated that telmisartan controlled albuminuria more efficiently than ACEi, Ramipril and the effect was similar to their combination (Liebson and Amsterdam 2009). The telmisartan versus ramipril in renal endothelium dysfunction (TRENDY®) study showed that 9-week treatment with telmisartan improved the resting renal plasma flow and endothelial function of renal vasculature in better fashion as compared to that of the ACE inhibitor, ramipril (Ritz et al. 2010). Thus, ARBs are proven to be more efficacious in curbing DKD as compared to ACEi.

4.4 Mineralocorticoid Receptor Antagonists

The association between aldosterone levels and declined GFR supports aldosterone as a target for treating DKD patients who are on ACEi or ARB. Spironolactone, a non-selective mineralocorticoid receptor antagonist (MRA), is causing gynecomastia. In 16 studies, spironolactone was tested against T1DM and T2DM patients in combination with ACEi or ARBs demonstrating significant 39% depletion in albuminuria; however, there is a sixfold augmented risk for hyperkalaemia mostly in individuals with impaired renal function (Hou et al. 2015). A 72-week intervention study combining spironolactone with irbesartan has demonstrated 30% antiproteinuric effect in elderly patients with T2D and DKD (Chen et al. 2018).

Eplerenone, a second-generation, more selective but less potent steroidal MRA, has been tested as add-on to ACEi in T2DM patients with nephropathy and demonstrated effective antiproteinuric effects, but persistent hyperkalaemia (Epstein et al. 2002). Further, a meta-analysis survey from observational studies including 693,000 individuals revealed that eplerenone reduced 30% of albuminuria compared to placebo (Heerspink et al. 2019). Till date, not all therapies successfully reducing albuminuria in phase II trials have shown renoprotection in phase III trials (Fried et al. 2013). Besides, finerenone, a novel nonsteroidal MRA, is more selective and potent than spironolactone and eplerenone, and reduced albuminuria in a dose-dependent manner (Bakris et al. 2015). Thus, recent years have progressively developed newer agents to treat hyperkalaemia and novel ideas to inhibit MR with lesser side effects. Till date, the nonsteroidal MRA finerenone is being investigated

in two large phase III studies showing it as a promising additional approach for slowing the progression of DKD.

4.5 ACEi and ARB Combination Therapy

ACEi and ARBs complement each other on Ang II inhibition, which is a backbone of the DKD management. Therefore, the combination of ACEi and ARB is considered as an alternative strategy to attenuate diabetes-related kidney complications as compared to the monotherapies. Moreover, during chronic ACEi therapy, residual Ang II is generated through ACE-independent pathways, which could be neutralised by adding up an ARB to the therapeutic regimen (Roscioni et al. 2014). While in case of long-term ARB treatment, increased compensatory production of Ang II occurs by renin stimulation, which might be prevented by combining an ACEi to the therapy (Forclaz et al. 2003). Based on these facts, several clinical studies have explored the effects of ACEi and ARB combination therapy on surrogate outcomes. A meta-analysis of 21 randomised controlled trials ($n = 654$ patients) has revealed that ACEi and ARB combination therapy resulted in a significant upsurge in serum potassium levels without any changes in the GFR.

Moreover, an ARB addition to ACEi therapy resulted in a further reduction in proteinuria compared to an ACEi monotherapy in diabetic and nondiabetic patients having renal disease (MacKinnon et al. 2006). Likewise, ACEi and ARB combination therapy was found superior in lowering 24-h proteinuria when compared with ACEi alone. This benefit is further coupled with minor impacts on GFR, BP, serum creatinine and potassium (Jennings et al. 2007). However, there should be a careful interpretation of these outcomes as most of the studies were of short-term and the few chronic studies (1 year) have not proven benefits of ACEi and ARB combination therapy.

4.6 ARB or ACEi with Renin Inhibition

In a double-blind, randomised, crossover trial involving 26 patients with T2DM, hypertension and albuminuria, aliskiren and irbesartan combination treatment showed better antiproteinuric effects than monotherapy (Persson et al. 2009). A double-blind, randomised, placebo-controlled trial recruiting 599 patients with T2DM and macroalbuminuria reported that aliskiren and losartan combination produced 20% more reduction in albuminuria as compared with placebo. This therapy was well tolerated but resulted in a considerable increase in the risk of hyperkalaemia (Parving et al. 2008). Moreover, a combination of aliskiren (300 mg) and losartan (100 mg) with optimal antihypertensive regimen produced superior antiproteinuric effects independent of BP in T2DM-associated nephropathic patients

(Persson et al. 2011). Hence, ARB/ACEi along with the renin inhibitor effectively diminished renal damage under DKD condition.

4.7 Hurdles for RAAS Inhibition in DKD

RAAS inhibition is a cornerstone for the treatment of diabetic vascular complications, including DKD. Because of the recognised prophylactic effects, the RAAS inhibitors are commonly prescribed to a diabetic patient without a history of CKD, or other complications associated with diabetes. According to a meta-analysis of the clinical trials, early RAAS interventions are more valuable than late interventions in preventing or delaying the ESRD in T2DM patients (Schievink et al. 2016). However, clinical studies have highlighted that ACEi or ARB therapy does not offer many advantages compared with other antihypertensive medications to diabetic patients (Bangalore et al. 2016; Barzilay et al. 2016; Perez-Gomez et al. 2015). Besides, a phenomenon like "Ang II reactivation" and "aldosterone escape" has been reported during ARB or ACEi treatment, which eventually results into clinical manifestations like increase in water and salt retention and decrease in GFR (Athyros et al. 2007; Schjoedt et al. 2004).

Eagerness about the promising effects of combined RAAS inhibition based on surrogate outcomes of small- and short-term clinical trials has been annealed by the disappointing results of large clinical trials, such as ONTARGET (Mann et al. 2008). One of the clinical features of DKD is albuminuria, which was markedly mitigated by ACEi and ARB combination therapy when compared to respective monotherapies. However, this does not reflect into additional renal benefits, clinically (Pugliese et al. 2019) (Fried et al. 2013; Kunz et al. 2008). Surprisingly, the combination therapy resulted into increased risk of adverse events like hyperkalaemia and acute kidney injury (AKI) in patients with DKD (Fried et al. 2013). In patients with heart failure with reduced ejection fraction (HfrEF) and diabetes, aliskiren and enalapril combination therapy resulted into more adverse events with no improvement in clinical outcomes (Kristensen et al. 2018). Facts highlighted an urgent requirement of a novel therapeutic intervention for the prevention of diabetes-associated CKD. A probable answer to this problem is an auxiliary approach of increasing endogenous BP reducing and RAAS opposing "natriuretic peptide system or the depressor arm of RAAS".

5 The Depressor Arm of RAAS

The depressor arm of RAAS is also named as non-conventional RAAS or the protective axis of RAAS. It consists of ACE2, Ang-(1-7), Ang II type 2 receptors (AT2 receptor) and Mas receptors (Fig. 2) which are discussed below in detail (Goru et al. 2017; Santos et al. 2017).

5.1 Angiotensin-Converting Enzyme 2: Nature's ACEi

In 2000, two identical research groups discovered a new homolog of ACE which was named as ACE2 and captopril insensitive carboxypeptidase, and found to be responsible for Ang-(1-7) synthesis. ACE2 is a monocarboxypeptidase and a type 1 integral membrane protein, having 42% homology with ACE metalloprotease catalytic domains. Nevertheless, ACE has two catalytic domains, whereas ACE2 contains a single catalytic domain (Donoghue et al. 2000; Tipnis et al. 2000). The catalytic mechanism of ACE2 and ACE is similar. As shown in Fig. 2, ACE remove a single amino acid leucine (L) from the C-terminus of Ang I to generate the biologically functional peptide Ang-(1-9) (Donoghue et al. 2000), which further hydrolysed by ACE and neprilysin to produce Ang-(1-7) (Rice et al. 2004). However, the biochemically and physiologically more appropriate way of the generation of Ang-(1-7) is hydrolytic removal of the C-terminal amino acid phenylalanine of Ang II by ACE2 (Fig. 2) (Vickers et al. 2002). Consequently, ACE2 plays an essential role in the body as an endogenous negative regulator of the pressure arm of RAAS, by degrading a vasoconstrictor and proliferative peptide Ang II and producing a vasodilator and antiproliferative peptide Ang-(1-7). ACE2 entirely or partially hydrolysed almost 11 peptides, with highest catalytic efficacy for Ang II (400-fold higher than for Ang I) (Rice et al. 2004). ACE2 is ubiquitously distributed in mammalian tissues, with abundant kidneys, heart, testes and intestines (Santos et al. 2013).

ACE2 is favourably a tissue enzyme, which helps in degrading Ang II in chronic conditions related to Ang II overproduction, including DKD. In the diabetic kidney, ACE2 downregulation caused Ang II accumulation in the glomeruli, eventually increased albuminuria and glomerular damage (Ye et al. 2006). Clinically, diabetic patients showed a significant reduction in ACE2 mRNA expression in the glomeruli and proximal tubules when compared to the controls (Reich et al. 2008). Under DKD, ACE2 over-expression significantly attenuated the Ang II-induced oxidative stress, glomerular mesangial cell proliferation, which in turn reduced the renal ECM accumulation (Liu et al. 2011). Existing literature highlighted the importance of ACE2-mediated renal protection in the development and progression of DKD. Hence, it will be of little surprise that ACE2 activation might halt the pathological development of the DKD, by ACE2/Ang-(1-7)/Mas receptor axis activation.

5.2 Angiotensin-(1-7)

Initially, Ang-(1-7) was viewed as an inactive factor of the RAAS. Later, Ang-(1-7) was found to be the primary product of Ang I, formed via an ACE-independent pathway (Santos et al. 1988). Having the same potency as Ang II to provoke vasopressin release from neurohypophyseal explants (Schiavone et al. 1988) and to lower BP upon microinjection into the nucleus tractus solitarii (Campagnole-

Santos et al. 1989), the importance of Ang-(1-7) as biologically active heptapeptide of the RAAS became gradually noticeable. Ang-(1-7) produces systemic and regional vasodilation, natriuresis and diuresis and exerts antiproliferative in cardiac myocytes and glomerular and proximal tubular cells. Ang-(1-7) mediates its renal protective effects via Mas receptors (Santos et al. 2013). In the development of DKD, streptozotocin-induced diabetic rats exhibited a significant reduction in systemic (plasma), whole kidney and isolated glomerular Ang-(1-7) expressions when compared to nondiabetic rats (Goru et al. 2017). Ang (1-7) is one of the renoprotective peptides of RAAS, and further research is warranted to understand its role in pathogenesis of DKD.

5.3 Angiotensin II Type 2 Receptor

AT2 receptor shares 32% to 34% homology in amino acid sequence with the AT1 receptor (Kambayashi et al. 1993). The AT2 receptor suggested playing a critical role in regulating cellular differentiation and organ development, owing to its high abundance in foetal mesenchymal tissues (Chow and Allen 2016). However, the knowledge regarding the role of AT2 receptor in pathophysiological processes is still embryonic. In adults, the AT2 receptor is inadequately expressed and remains ample only in specific tissues including the kidney, vascular endothelium and brain (Hallberg et al. 2018; Ozono et al. 1997). Under brain and cardiac injuries, expression of AT2 receptor gets significantly upregulated (Li et al. 2005; Sumners et al. 2015). Though the AT2 receptor belongs to the rhodopsin subclass of G-protein-coupled receptor (GPCR) superfamily, it owns a different signalling mechanism from the receptors linked with this family. G-protein GI facilitates the AT2 receptor signal to modulate K^+ channel activity and thereby activates protein-phosphotyrosine phosphatase, consequently leading to reduced MAPK activity and growth inhibition (Nouet and Nahmias 2000). AT2 receptor consists of the SH2 domain which regulates its signal transduction via nitric oxide/cyclic guanosine monophosphate (NO/cGMP) pathway, which is known for its vasodilatory and natriuretic effects (Feng et al. 2002; Siragy et al. 2007).

Wehbi et al. utilised streptozotocin-induced T1DM rats and reported the reduction in AT2 receptor expression in glomeruli and other regions of the kidney, with no change in glomerular mRNA expressions of renin, angiotensinogen and ACE, in its early phase of diabetes. These results indicated that alteration in AT2 receptor expression in the kidney might be an essential determinant of the DKD progression rate (Wehbi et al. 2001). Further, Chang et al. demonstrated that AT2 receptor knockout diabetic mice exhibited significant ECM protein accumulation, renal hypertrophy and tubular apoptosis when compared to wild-type diabetic mice (Chang et al. 2011). Therefore, the AT2 receptor has a renoprotective effect under DKD condition.

5.4 Mas Receptors

The Mas receptor is a proto-oncogene, GPCR. Mas receptors are found in the brain and testis, but also expressed in the heart, kidney and blood vessels (Metzger et al. 1995; Rabelo et al. 2011). Ang-(1-7) acts as a ligand for the Mas receptor, consequently counteracts the action of AT1 receptor through Ang-(1-7)/ACE2/Mas receptor axis, induces vasodilatation, promotes diuresis and natriuresis and reduces BP (Santos et al. 2003). Wistar-Kyoto rats subjected to Ang-(1-7) chronic treatment exhibited increased expression of Mas receptor in the heart, whereas it remains unchanged in the kidney, suggesting a tissue-specific regulation of the Mas receptor (Tan et al. 2011). Mas receptor knockout mice showed significant glomerular hyperfiltration and microalbuminuria and increased glomerular and tubulointerstitial fibrosis accompanied with an upregulation of AT1 receptor and TGF-β mRNA expressions when compared to wild-type animals (Pinheiro et al. 2009). On the other hand, streptozotocin-induced T1DM mice showed downregulation of Mas receptor expression in the kidney, which was significantly normalised by an ARB telmisartan treatment in preventing DKD (Lakshmanan et al. 2011). These studies revealed that upregulation of Mas receptor could protect the kidney damage under DM condition.

6 Modulation of the Depressor Arm of RAAS in DKD: A Better Alternative

As earlier explained, in diabetes along with the RAAS's pressor arm overactivation, the depressor arm suppression is one of the major driving forces for the pathogenesis of DKD. Hence, the depressor arm activation might prevent the development of DKD. In preclinical setup, researchers have attempted to modulate activities of the depressor arm by means of multiple approaches, including pharmacological intervention with ACE2 (e.g. rhACE2), ACE2 activator (e.g. XNT, Dize), ACE2 inhibitor (e.g. DX-600), Mas receptor activator (e.g. AVE 0991), Mas receptor blocker (e.g. A779), AT2 receptor inhibitor (e.g. PD 123319) and AT2 receptor agonist (e.g. compound 21) (Fig. 3) (Hallberg et al. 2018; Pandey and Gaikwad 2017a; Santos et al. 2013).

6.1 ACE2 Activation

Recombinant human ACE2 (*rhACE2*): In cultured glomerular mesangial cells, rhACE2 diminished both Ang II and high glucose-induced oxidative stress and NADPH oxidase activity (Oudit et al. 2010). Diabetic male Akita mice subjected to rhACE2 (2 mg/kg, *i.p.*) treatment for 4 weeks, exhibited increased plasma ACE2

activity, augmented Ang-(1-7) and reduced Ang II levels and NADPH oxidase activity, with a consequent reduction in BP, urinary albumin excretion, glomerular mesangial matrix expansion and collagen III expressions and hence prevented DKD (Oudit et al. 2010).

ACE2 activators: diminazene aceturate (Dize) is a small molecule approved by the US FDA for the treatment of babesiosis, piroplasmosis and trypanosomiasis since 1955 due to its DNA intercalating effect (Kuriakose and Uzonna 2014). Dize belongs to the group of aromatic diamidines and reported to exert an "off-target" ACE2 activating effect, which has nowadays been widely explored (Qi et al. 2013). In genetically diabetic *db/db* mouse, Dize treatment (15 mg/kg/day) activated ACE2, augmented the circulating Ang (1-7) level and alleviated endothelium-dependent relaxation by improving nitric oxide bioavailability and preventing oxidative stress (Zhang et al. 2015b). Dize exposure to lipopolysaccharide-stimulated Kupffer cells repressed NF-kB activity and abridged MCP-1 and IL-6 gene expression (Rajapaksha et al. 2018). Besides, Dize (15 mg/kg/day) treatment reduced glomerular fibrosis and apoptosis through activation of protective Ang-(1-7)/ACE2/AT2 receptor axis and thereby prevented DKD (Goru et al. 2017). Based on the preclinical study results, ACE2 activation has a renoprotective role against DKD. Thus, future research must focus on the development of specific, potent and safe ACE2 activation strategy and its clinical evaluation.

6.2 Ang-(1-7) Therapy

Ang-(1-7) treatment (576 μg/kg/day *i.p.* for 4 weeks) diminished DKD in T1DM rats via reducing proteinuria and renal fibrosis and alleviating endothelial functions without averting tubular damage (Singh et al. 2010). In ZDF rats, administration of Ang-(1-7) (100 ng/kg/min, 2 weeks) reduced systolic BP, oxidative stress and inflammatory markers [e.g. tumour necrosis factor-α (TNF-α), IL-6, neutrophil gelatinase-associated lipocalin and hypoxia-inducible factor-1α] and consequently prevented DKD (Giani et al. 2012). Moreover, Ang-(1-7) administration protected the kidney of *db/db* mice against the development of DKD by attenuating inflammation, oxidative stress and lipotoxicity (Mori et al. 2014). Acute intrarenal infusion of Ang-(1-7) at 400 ng/min dose to T1DM rats improved urinary sodium excretion, reduced proximal tubular reabsorption and normalised GFR, but at the same time augmented kidney oxygen consumption (Persson et al. 2019). Zhang et al. reported that Ang-(1-7) ameliorated streptozotocin-induced DKD better than an ARB valsartan, as indicated by a more significant reduction of oxidative stress, and better attenuation of TGF-β and vascular endothelial growth factor-mediated pathological signalling by Ang-(1-7) treatment when compared to valsartan (Zhang et al. 2015a).

6.3 AT2 Receptor Modulations

For many years, the AT1 receptor is considered as a key drug target for diseases like CKD, whereas the AT2 receptor was only a matter of academic interest. However, this has changed considerably, with the design and synthesis of the first non-peptide orally active AT2 receptor agonist, compound 21 (C21). C21 is increasingly recognised as a budding therapeutic agent owing to its specificity of stimulating the AT2 receptor without influencing the AT1 receptor and the ideal route of administration (Wan et al. 2004). Recently, C21 has received an orphan drug designation by EMA and US FDA for the treatment of idiopathic pulmonary fibrosis (Bruce et al. 2015; Rathinasabapathy et al. 2015). Pandey et al. have extensively reviewed and summarised available reports demonstrating renoprotective effects of AT2 receptor agonists against DKD (Pandey and Gaikwad 2017a). C21 treatment reduced albuminuria, macrophage infiltration, TNF-α expression and glomerular, tubulointerstitial and perivascular fibrosis and thus prevented DKD in ZDF rats (Castoldi et al. 2014). In diabetic ApoE-deficient mice, C21 treatment significantly reduced cystatin C level, albuminuria, mesangial expansion and glomerulosclerosis. Moreover, C21 markedly inhibited oxidative stress and inflammatory and profibrotic signalling, accompanied by decreased ECM production, and thereby prevented the development of DKD (Koulis et al. 2015). A Swedish rare disease company, Vicore Pharma, is already testing C21 clinically for the treatment of idiopathic pulmonary fibrosis, pulmonary fibrosis in systemic sclerosis and COVID-19. Considering promising preclinical data in DKD and ongoing clinical testing in other diseases and conditions, in the future, C21 should be evaluated clinically against DKD.

6.4 Mas Receptor Modulations

AVE 0991, the most explored Mas receptor agonist, is a biaryl derivative that lacks affinity for both AT1 and AT2 receptors (Bosnyak et al. 2011; Santos and Ferreira 2006). AVE 0991 attenuated diabetes-associated atypical vascular responsiveness to Ang II, norepinephrine, carbachol, endothelin-1 and histamine in the isolated carotid and renal arteries (Benter et al. 2007). Diabetic rats treated with AVE 0991 shown alleviation in kidney functions, as demonstrated by reduced blood urea nitrogen (BUN) and proteinuria. AVE 0991 treatment prevented alterations in the proteins related to metabolic and antioxidant enzymes, apoptosis regulators and inflammatory factors in the kidneys of ApoE knockout mice (Suski et al. 2013). AVE 0991, in combination with a low dose of aliskiren synergistically, reduced BP in deoxycorticosterone acetate (DOCA)-salt-induced hypertensive rats (Singh et al. 2013a). Therefore, Mas receptor modulation as monotherapy or as combination therapy with RAAS inhibitors may provide a potential therapeutic treatment for DKD.

7 Neprilysin Inhibition as Add-On to RAAS Blockage

The natriuretic peptides system (NPS) is a peculiar BP lowering system, which opposed the RAAS and arbitrated beneficial actions within the cardiovascular and renal system. Three peptides hormones or endocrine factors – atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) – are the constitutive parts of the NPS. Neprilysin hydrolyses these natriuretic peptides and thereby modulates structural and functional effects on the kidney, heart and other organs (Malek and Gaikwad 2017). As the neprilysin inhibitors (NEPi) increased the level of natriuretic peptides, thus, it has a protective role against kidney dysfunctions, including DKD. However, the stand-alone use of NEPi (both candoxatril and ecadotril) failed in clinical use due to unacceptably low efficacy (McMurray 2015).

7.1 Vasopeptidase Inhibitor (Dual ACEi/NEPi)

Omapatrilat is known to be the most widely explored synthetic vasopeptidase inhibitors (Vpi) against CKD. In uninephrectomised male ZDF rats, omapatrilat reduced systolic and diastolic BP, accompanied by better renal protection when compared to captopril monotherapy (Garcia-Robles et al. 2000). Omapatrilat reduced albuminuria, along with natriuretic peptides, cyclooxygenase and profibrotic connective tissue growth factor expressions, and thus prevented the development of DKD (Cheng et al. 2005). Other Vpi such as S21402, captopril and thiorphan combination, mixanpril, and ilepatril has also been tested in the experimental models of diabetes. Among all, only omapatrilat is the Vpi that underwent clinical testing. However, clinically omapatrilat was unsuccessful in proving its superiority over enalapril in a one-on-one comparison. In OVERTURE trial in which almost 30% of enrolled patients had diabetes, omapatrilat failed to exhibit better effects than enalapril (Solomon et al. 2005). Surprisingly, in the OCTAVE trial, omapatrilat treatment resulted in an augmented risk of angioedema with more severity and prevalence than enalapril treatment (Kostis et al. 2004). Hence, based on the OVERTURE and OCTAVE trial outcomes, the US FDA review panel rejected omapatrilat. Thus its further development was halted (Mangiafico et al. 2013; Pickering 2002).

7.2 Angiotensin Receptor Neprilysin Inhibitors (ARNi)

As mentioned in the previous section, failure of most advanced Vpi, omapatrilats, paved the way for the development of a new class of NEPi, the angiotensin receptor neprilysin inhibitors (ARNi). A hypothesis behind the development is that ARNi

will lower the risk of angioedema by sparing two enzymes, ACE and aminopeptidase P, for bradykinin metabolism, since ARNi will block AT1 receptor instead of ACE, with minimal inhibitory effects on aminopeptidase P (Malek and Gaikwad 2017). The first molecule of ARNi class, LCZ696, is an equimolar combination of an ARB, valsartan and a NEPi, sacubitril (McMurray 2015). Based on the positive outcomes of the PARADIGM-HF trial, LCZ696 has been approved by the US FDA for the treatment of HfrEF patients (Solomon et al. 2015). LCZ696 alleviated serum creatinine and GFR, in HF patients with preserved ejection fraction, among which 36–38% patients were diabetic. Nevertheless, the LCZ696 group exhibited substantially higher urinary albumin-to-creatinine ratio than valsartan group (Voors et al. 2015). Previously, in few review articles, authors have discussed available preclinical and clinical reports regarding the usage of ARNi for the management of diabetic vascular complications, including DKD (Berbari 2020; Esser and Zraika 2019; Idzerda et al. 2019; Malek and Gaikwad 2017).

Uijl et al. has revealed that Sacubitril/Valsartan showed renoprotection in the preclinical model of diabetes and hypertension. The renoprotective effect was independent to antihypertensive efficacy, renal hemodynamics or inflammation, but might be related to the beneficial effects of natriuretic peptides on podocyte integrity (Uijl et al. 2020). In ZDF rats, LCZ696 (68 mg/kg/day, ten weeks) treatment found to be superior in attenuating proteinuria and renal tubular injury as compared to the valsartan or hydralazine alone (Habibi et al. 2019). In an another study, LCZ696 treatment significantly improved the renal functioning which was evidenced by reduced BUN and creatinine levels, delpeted glomerular and tubulointerstitial injury, as compared to the valsartan monotherapy or valsartan and hydralazine combination therapy (Rahman et al. 2020). In our recent study, we have tested another such combination of telmisartan and thiorphan against streptozotocin-induced T1DM (Malek and Gaikwad 2019; Malek et al. 2019b). The combination therapy of telmisartan and thiorphan diminished metabolic perturbations, synthesised RAAS and NPS components, prevented glomerular and tubulointerstitial fibrosis and inhibited profibrotic, inflammatory and apoptotic signalling and consequently improved renal functions in attenuating the development of DKD (Malek et al. 2019b). In a secondary intention-to-treat analysis of PARADIGM-HF clinical trials, involving 3,784 diabetic and 4,615 nondiabetic patients, it has been observed that patient treated with LCZ696 demonstrated a slower rate of decline in estimated GFR than enalapril, and the extent of the benefit was better in patients with diabetes than without diabetes (Packer et al. 2018). Nevertheless, preclinical studies and secondary analysis of clinical trials demonstrated the renoprotective effects of ARNi in diabetes, and a separate clinical trial is to be carried out for the safety and efficacy evaluation of ARNi in the diabetic population.

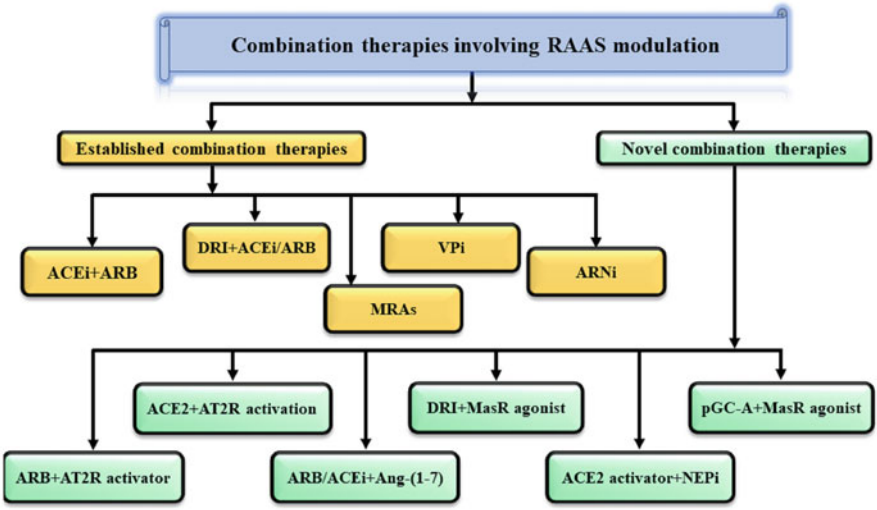

Fig. 4 Combination therapies involving the RAAS modulations. *DRI* direct renin inhibitors, *ARB* angiotensin receptor blockers, *ACEi* angiotensin-converting enzyme inhibitors, *VPi* vasopeptidase inhibitors, *ARNi* angiotensin receptor neprilysin inhibitors, *MasR* Mas receptor, *pGC-A* particulate guanylyl cyclase A, *ACE2* angiotensin-converting enzyme 2, *AT2R* Ang II type 2 receptors

8 Novel Combination Therapies (Fig. 4)

8.1 Simultaneous AT1 Receptor Inhibition and AT2 Receptor Activation

Under the pathogenesis of DKD, AT1 receptor mediates the deteriorative effects whereas AT2 receptor facilitates the reno-protective effects. Thus researchers thought of combining an ARB with AT2 receptor agonist for the treatment of DKD. Castoldi et al. have evaluated the effectiveness of losartan and C21 combination treatment on the development of albuminuria and renal fibrosis in ZDF rats. After 15 weeks of different treatments, rats receiving losartan and C21 combination therapy exhibited significantly alleviated systemic renal functions, reduced urinary albumin-to-creatinine ratio and abridged glomerular, tubulointerstitial and perivascular fibrosis (Castoldi et al. 2014). McKelvey et al. demonstrated that candesartan and C21 combination treatment attenuated albuminuria and glomerulosclerosis and thus provided renoprotection against DKD in streptozotocin-induced T1DM ApoE−/− mice (Mckelvey et al. 2014). Likewise, telmisartan and C21 combination therapy attenuated metabolic and renal anomalies, haemodynamic perturbations and renal morphological and micro-architectural abnormalities in T2DM rats. Besides, C21 complemented telmisartan's anti-apoptotic and anti-inflammatory effects, as revealed by abridged expressions of apoptotic markers (e.g. cPARP and c-Cas-3) and NF-κB downstream inflammatory

molecules (e.g. IL-6, VCAM-1, TNF-α and MCP-1) (Pandey and Gaikwad 2017b) (Fig. 4). Hence, emerging preclinical evidence suggested that combination therapy of an ARB and an AT2 receptor agonist is certainly a silver lining in the dark clouds of DKD and further clinical studies have to be conducted to ascertain this speculated efficacy.

8.2 Add-On Therapy of Ang-(1-7) with RAAS Inhibitors

Considering the potential renoprotective effects of peptide Ang-(1-7), it has been used as add-on therapy to conventional ARB or ACEi treatment. Safari et al. demonstrated that add-on therapy of Ang-(1-7) to an ARB, losartan, attenuates oxidative stress and prevents renal functional decline associated with ischemia-/reperfusion-induced AKI (Safari et al. 2019). The major hurdle in synthesizing the Ang-(1-7) is its small size which quickly gets cleared from the systemic circulation by ACE and other proteases (e Silva and Teixeira 2016). Structurally, a newly synthesized cyclic Ang-(1-7) have a thioether bridge which makes it more ACE and peptidase-resistant than the parent linear peptide (Cassis et al. 2019; Kluskens et al. 2009). Recently, Cassis et al. have tested a combination of cyclic Ang-(1-7) and an ACEi lisinopril against DKD in BTBR ob/ob mice. The add-on therapy of cyclic Ang-(1-7) to lisinopril offered superior renoprotection in mice with DKD when compared to lisinopril monotherapy, as demonstrated by preservation of podocyte proteins and improvement of capillary density (Cassis et al. 2019; Marquez and Batlle 2019). Based on this preclinical evidence, the author claimed that combined therapy of cyclic Ang-(1-7) with ACEi might be advantageous for those diabetic patients who do not respond well to standard RAAS inhibitor therapy.

8.3 Dual AT2 Receptor and ACE2 Activation

Recently, for the first time, in our lab, we targeted two components (AT2 receptor and ACE2) of the RAAS depressor arm simultaneously against hyperglycaemia and AKI-induced renal damage (Sharma et al. 2019). The combination therapy of C21 (an AT2 receptor agonist) and Dize (an ACE2 activator) attenuated renal dysfunction by reducing inflammatory, apoptotic and oxidative stress in acute kidney injury rats under hyperglycaemic condition. Interestingly, the combination therapy augmented the levels of ACE2, Ang 1-7 and AT2R and MasR expressions which suppressed the oxidative stress and inflammatory and apoptosis-induced tubular injury. Therefore, pharmacological activation of the AT2 receptor and ACE2 protects the kidney against ischaemic renal injury under hyperglycaemia (Sharma et al. 2019). Further research is warranted to explore this novel combination for the treatment of diabetes-associated renal dysfunction, which might serve as a breakthrough.

8.4 Mas Receptor Agonist and DRI Combination Therapies

In the existing literature, only one study has explored Mas receptor agonist and DRI combination therapy. In DOCA-induced hypertensive rats, a Mas receptor agonist, AVE 0991, in combination with low doses of aliskiren substantially reduced MAP, as compared with DOCA control rats and respective monotherapy receiving rats (Singh et al. 2013b). Authors have concluded that observed synergistic BP reduction by the proposed combination therapy might be due to downregulation of harmful Ang II/AT1 receptor axis by aliskiren and survival Ang-(1-7)/Mas receptor axis by AVE 0991 (Singh et al. 2013b). Due to added advantage in antihypertensive potential and scarcity of study reporting the renal protection against the DKD, such combination represents an interesting perspective for future investigation.

8.5 Concurrent Renin and Neprilysin Inhibition

Besides the latest addition of Vpi and ARNi in the class of RAAS and NPS dual targeting drugs, a study has reported simultaneous renin and neprilysin inhibition in curbing cardiac and renal dysfunctions. In isoprenaline-induced (5 mg/kg/day, i.p., for 7 days) heat failure rats, a NEPi, sacubitril, in combination with a DRI (aliskiren) or an ACEi (ramipril) effectively reduced systemic levels of cardiac biomarkers, like creatine kinase-MB, matrix metallopeptidase 9 and NT-proBNP (Dizaye and Ali 2019). Moreover, both combination therapies significantly improved renal functions as indicated by increased GFR, urine flow and total urine excretion, along with reduced plasma renin levels. Based on the study results, authors have claimed that complete suppression of the RAAS with aliskiren offers better cardiorenal protection than blocking angiotensin II production with ramipril (Dizaye and Ali 2019). However, further preclinical and clinical studies are necessary to evaluate this combination against the DKD.

8.6 Add-On Therapy of NEPi with ACE2 Activation

The cyclic guanosine monophosphate (cGMP) signalling is critical for upholding normal renal physiological functions. Hence its malfunction or discrepancy is leading to renal anomalies, including DKD (Chen and Burnett 2018; Idzerda et al. 2019; Krishnan et al. 2018). Thus, the therapeutic strategy targeted at improving cGMP balance is well reported for its beneficial effects on the renal system (Buglioni and Burnett Jr. 2016; Idzerda et al. 2019). Interestingly, along with other benefits related to the RAAS and NPS modulation, ACE2 activator and NEPi could increase cGMP levels via two independent mechanisms. ACE2 activator will act on soluble guanylyl cyclase (sGC) via RAAS's depressor arm, whereas NEPi will stimulate

particulate guanylyl cyclase A (pGC-A) by improving natriuretic peptides and bioavailability, both leading to increase in the production and release of cGMP (Buglioni and Burnett Jr. 2016; Chen and Burnett 2018; Krishnan et al. 2018). Hence, add-on therapy of NEPi to ACE2 activator might help in preventing DKD. Recently, we have tested thiorphan (a NEPi) and Dize (an ACE2 activator) combination therapy against the development of DKD in streptozotocin-induced T1DM male Wistar rats (Malek et al. 2019a, b). T1DM rats subjected to thiorphan and Dize combination therapy showed increased cGMP and normalised RAAS component levels and inhibition of key pathological signalling cascades like inflammatory, profibrotic and cell apoptotic and thus prevented DKD (Malek et al. 2019b).

8.7 Dual pGC-A and Mas Receptor Activators

The ultimate target of bispecific drugs is to attain therapeutic synergy, surpassing the effects of single pathway inhibition or activation. Recently, Meems and colleague have exploited advances in peptide engineering to design and synthesise a novel peptide, NPA7, which can activate pGC-A and Mas receptor, simultaneously (Meems et al. 2019). NPA7 was engineered by combining an endogenous Mas receptor agonist, Ang-(1-7), with a 22-amino acid sequence of BNP (a natural ligand of NPR-A/pGC-A axis). In HEK-293 cells overexpressing either human pGC-A or human Mas receptors, NPA7 confirmed its potential as a bispecific ligand of pGC and Mas receptor.

Moreover, in normal male mongrel dogs, NPA7 demonstrated superior diuretic, natriuretic, systemic and renal vasorelaxant effects when compared with the individual endogenous pGC-A or Mas receptor ligands (Meems et al. 2019). However, NPA7 is yet to be tested against the DKD; if successful then it may resolve the unmet need of a new bispecific therapeutic for the treatment of DKD and other CKD. Besides, this initial success of NPA7 has increased the hope for the development of new fusion ligand of other regulatory RAAS peptides, by which we can target more than one receptor at a time to achieve synergistic effects benefiting renal system.

9 Conclusion and Perspective

In diabetes, persistent RAAS activation plays a pivotal role in the pathogenic development of DKD. Thus the RAAS blockade by either ARB or ACEi along with MRAs is currently the mainstay to treat DKD (Table 1). Alternative approaches involve RAAS inhibitor combination therapies. Even if outcomes of small- and short-term clinical trials demonstrated favourable effects of dual RAAS inhibition on BP levels and albuminuria, the disappointing outcomes of large trials, such as ONTARGET and ALTITUDE, highlighted the need for novel therapeutic strategy for the treatment of CKD including DKD.

Table 1 Summary of drugs targeting renin-angiotensin-aldosterone system (RAAS) and used for the treatment of diabetic kidney diseases (DKD)

Class	Drugs	Models	Findings	Outcomes	References
Direct renin inhibitors	Aliskiren	STZ diabetic Ren(2) rats; diabetic transgenic (mRen-2)27 rats; advanced (stage 3–4) DKD	↓ SBP, urinary albumin-to-creatinine ratio, oxidative stress, inflammatory markers like MCP-1, IL-6 ↓ Glomerulosclerosis, tubulointerstitial fibrosis; independent to BP lowering	Elevates GFR and attenuated DKD in rats and human subjects	(Kelly et al. 2007; Ogawa et al. 2011)
ACE inhibitors	Ramipril	ZDF rats	↓ Upregulation of renal fibroblast growth factor 23 and Klotho expressions	Attenuated proteinuria and renal injury	(Zanchi et al. 2013)
	Captopril	T1DM patients with DKD	↓ Serum creatinine and protects renal functions	DKD associated combined end points of death, dialysis, and transplantation was 50% reduced	(Lewis et al. 1993)
	Lisinopril	DKD patients	↓ Urinary liver fatty acid-binding protein (U-LFABP) and albuminuria	DKD patients prevented tubular and glomerular damage and dysfunction as indicated	(Nielsen et al. 2009)
AT1 receptor blockers	Irbesartan	db/db mice	↓ Metabolic aberrations by conquering the RANKL-RANK-NF-κB pathway	Suppressed podocyte injury and renal dysfunction associated with DKD	(Chen et al. 2016)
	Losartan	Hypertensive T2DM patients with overt nephropathy	↓ Serum creatinine concentration by 25%	ESRD by 28% with no effect on the rate of death	(Brenner et al. 2001)
	Telmisartan	T2DM patients	Improved the decline in GFR over 5 years with early nephropathy which was determined using the	Telmisartan significantly improved DKD as compared to enalapril	(Barnett 2004)

			iohexol-based direct measurement of GFR		
		Hypertensive T2DM patients with overt nephropathy	Reduction in urine albumin-to-creatinine ratio to 29.8%	Telmisartan proved as a better therapy than losartan	(Bakris et al. 2008)
Mineralocorticoid receptor antagonists + AT1R/ACEi	Finerenone+ACEi/ARB	T2DM patients with nephropathy	Dose-dependent reduction in urinary albumin excretion rate	Reduced chances of hyperkalaemia episodes	(Bakris et al. 2015)
	Spironolcatone+ irbesartan	Hypertensive T2DM patients with nephropathy	Reduced urinary albumin excretion rate	High-dose irbesartan with spironolactone reduced albuminuria, but caused hyperkalaemia	(Chen et al. 2018)
ACEi and ARB combination therapy	Lisinopril+ losartan; enalapril+ losartan; perindopril+ irbesartan; enalapril+ irbesartan	Diabetic and nondiabetic patients having renal diseases	Chronic proteinuric renal disease is safe, without clinically meaningful changes in serum potassium levels or glomerular filtration rates	Combination therapies remain better than ACEi/or ARB monotherapy	(MacKinnon et al. 2006)
	Enalapril+ losartan; perindopril+ irbesartan; lisinopril+ losartan; candesartan+lisinopril; Enalapril+ irbesartan	DKD patients	This benefit is coupled with minor impacts on GFR, serum creatinine and potassium	ACEi and ARB combination therapy found superior in lowering 24-h proteinuria when compared with ACEi alone	(Jennings et al. 2007)
ARB or ACEi with renin inhibition	Aliskiren and irbesartan combination	DKD patients	Antiproteinuric effects	Aliskiren and irbesartan combination better than monotherapies	(Persson et al. 2009)
	Aliskiren and losartan combination	T2DM patients	Antihypertensive regimen produced superior antiproteinuric effects independent of BP in T2DM-	20% more reduction in albuminuria as compared with placebo	(Persson et al. 2011)

(continued)

Table 1 (continued)

Class	Drugs	Models	Findings	Outcomes	References
			associated nephropathic patients		
ACE2 activator	Recombinant human ACE2 (rhACE2)	Cultured glomerular mesangial cells and diabetic male Akita mice	↑ Plasma ACE2 activity, augmented Ang-(1-7) and reduced Ang II levels and NADPH oxidase activity, with consequent reduction in BP, urinary albumin excretion	Suppressed glomerular mesangial matrix expansion and collagen III expression and hence prevented DKD	(Oudit et al. 2010)
	Diminazene aceturate (Dize)	Diabetic db/db mice	Activated ACE2, ↑circulating Ang-(1-7) level, and alleviated endothelium-dependent relaxation through ↑ nitric oxide bioavailability and inhibiting oxidative stress	Attenuated DKD	(Zhang et al. 2015a)
		T1DM rats with nephropathy	Glomerular fibrosis and apoptosis via activation of protective Ang-(1-7)/ ACE2/AT2 receptor axis	Prevented T1DM-induced renal fibrosis	(Goru et al. 2017)
Ang-(1-7) therapy	Ang-(1-7)	ZDF rats	Reduced systolic BP, oxidative stress and inflammatory markers; TNF- IL-6, neutrophil gelatinase-associated lipocalin and hypoxia-inducible factor-1α	Prevented DKD	(Giani et al. 2012)

		db/db mice	Attenuating inflammation, oxidative stress and lipotoxicity	Prevented DKD	(Mori et al. 2014)
		T1DM rats	Improved urinary sodium excretion, reduced proximal tubular reabsorption and normalised GFR, but at the same time augmented kidney oxygen consumption	Improved renal functions	(Persson et al. 2019)
		T1DM Wistar rats	More significant reduction of oxidative stress and better attenuation of TGF-β and vascular endothelial growth factor-mediated pathological signalling	Ang-(1-7) ameliorated streptozotocin-induced DKD better than an ARB valsartan	(Zhang et al. 2015a)
AT2 receptor modulations	Compound 21	ZDF rats	Reduced albuminuria, macrophage infiltration, TNF-α expression and glomerular, tubulointerstitial and perivascular fibrosis	Prevented DKD	(Castoldi et al. 2014)
		Diabetic ApoE-deficient mice	↓ Cystatin C level, albuminuria, mesangial expansion and glomerulosclerosis, oxidative stress, inflammatory and pro-fibrotic signalling, ↓ ECM production	Prevented the development of DKD	(Koulis et al. 2015)
		T2DM rats with DKD	↓ Oxidative stress, apoptosis, inflammation and renal fibrosis	Renoprotective effects of AT2 receptor agonists against DKD	(Pandey and Gaikwad 2017b)

(continued)

Table 1 (continued)

Class	Drugs	Models	Findings	Outcomes	References
Mas receptor modulations	AVE 0991	Diabetic rats	↓ BUN and proteinuria, diabetes-induced abnormal vascular responsiveness to norepinephrine, endothelin-1, Ang II, carbachol and histamine in the perfused mesenteric bed and isolated renal arteries	Prevented DKD	(Benter et al. 2007)
		ApoE knockout mice	Prevented the alterations in the proteins related to metabolic and antioxidant enzymes, apoptosis regulators and inflammatory factors in the kidneys		(Suski et al. 2013)
Vasopeptidase inhibitor (dual ACEi/NEPi)	Omapatrilat	Uni-nephrectomized male obese Zucker rats	Reduced systolic and diastolic BP	Better renal protection when compared to captopril monotherapy	(Garcia-Robles et al. 2000)
Angiotensin receptor neprilysin inhibitors (ARNi)	LCZ696 (sacubitril/valsartan)	STZ-induced type 1 diabetes in adult male Wistar rats	↓ Inflammatory markers; TNF-α, IL-1β, IL-6, NF-kB and sufficient restoration of antioxidant enzyme levels. Prevention of renal injury was observed with limited necrosis and inflammatory cell infiltration	LCZ696 restricted DKD progression through inhibiting inflammation, oxidative stress and glomerulosclerosis	(Mohany et al. 2020)
	Telmisartan and thiorphan combination	T1DM rats with nephropathy	Alleviated the metabolic alterations, improved renal functions, normalised the RAAS and NPS	Prevented the development of DKD	(Malek et al. 2019b)

			components. Reduced glomerular and tubulointerstitial fibrosis, and suppressed inflammatory, profibrotic as well as apoptotic signalling		
Vasopeptidase inhibitor	AVE7688	ZDF rats	Prevented albuminuria and drastically reduced the incidence and severity of glomerulosclerosis and tubulointerstitial damage	Chronic inhibition of vasopeptidase to prevent DKD	(Schäfer et al. 2003)
Simultaneous AT1 receptor inhibition and AT2 receptor activation	Losartan and compound 21	ZDF rats	Attenuated systemic renal functions, reduced urinary albumin-to-creatinine ratio and abridged glomerular, tubulointerstitial and perivascular fibrosis	Suppressed development of albuminuria and renal fibrosis	(Castoldi et al. 2014)
	Candesartan and compound 21	Streptozotocin-induced T1DM ApoE−/− mice	Attenuated albuminuria and glomerulosclerosis	Renoprotective effect of combination therapy	(Mckelvey et al. 2014)
	Telmisartan and compound 21	T2DM rats	Attenuated metabolic and renal anomalies, haemodynamic perturbations, renal morphological and micro-architectural abnormalities in T2DM rats. Anti-apoptotic and anti-inflammatory effects, as revealed by abridged expressions of apoptotic markers and NF-κB downstream inflammatory	The combination showed a better effect than monotherapies	(Pandey and Gaikwad 2017b)

(continued)

Table 1 (continued)

Class	Drugs	Models	Findings	Outcomes	References
			molecules (e.g. IL-6, VCAM-1, TNF-α and MCP-1)		
Add-on therapy of Ang-(1-7) with RAAS inhibitors	Ang-(1-7) + lisinopril	DKD in BTBR ob/ob mice	Preservation of podocyte proteins and improvement of capillary density	Cyclic Ang-(1-7) to lisinopril offered superior renoprotection in mice with DKD	(Cassis et al. 2019)

Note: *ZDF rats* Zucker diabetic fatty rats; *SBP* systolic blood pressure, *DKD* diabetic kidney disease, *ECM* extracellular matrix, *TNF-α* tumour necrosis factor-α, *VCAM-1* vascular cell adhesion molecule-1, *T1DM* type 1 diabetes mellitus, *T2DM* type 2 diabetes mellitus

In the past decades, our understanding of the RAAS cascade has improved significantly with the discovery of additional components, like ACE2/Ang-(1-7)/Mas receptor axis. Moreover, constant research efforts have revealed the role of new RAAS components in pathophysiology of diabetes and DKD and thus presented us with ample opportunity to develop and study new targets within the RAAS to combat CKD. Now scientists and clinicians believe that pharmacological strategies which block the adverse outcomes and simultaneously promote the beneficial effects of the RAAS, might be represented as better therapeutic options against DKD (Table 1). In the present review, we have discussed such novel combination therapies including AT1 receptor inhibition and AT2 receptor activation, add-on therapy of Ang-(1-7) with RAAS inhibitors, dual AT2 receptor and ACE2 activation, concurrent renin and neprilysin inhibition, combination therapies with Mas receptor agonist, dual pGC-A and Mas receptor activators and add-on therapy of NEPi with ACE2 activation (Table 1). However, most of these strategies are still in the preclinical stage; thus, in the future, vigorous research endeavours are required to take the best therapeutic regimen to clinical use. In summary, our opportunities to improve and extend conventional RAAS blockade are quickly expanding, with the breakthrough of bispecific therapeutics, and sooner or later should result in new treatment modalities with better efficacy, fewer side effects and improved patient compliance.

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Cross-Talk Between the Adenylyl Cyclase/cAMP Pathway and Ca^{2+} Homeostasis

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Contents

Abstract Cyclic AMP and Ca^{2+} are the first second or intracellular messengers identified, unveiling the cellular mechanisms activated by a plethora of extracellular signals, including hormones. Cyclic AMP generation is catalyzed by adenylyl cyclases (ACs), which convert ATP into cAMP and pyrophosphate. By the way, Ca^{2+}, as energy, can neither be created nor be destroyed; Ca^{2+} can only be transported, from one compartment to another, or chelated by a variety of Ca^{2+}-binding molecules. The fine regulation of cytosolic concentrations of cAMP and free Ca^{2+} is crucial in cell function and there is an intimate cross-talk between both messengers to fine-tune the cellular responses. Cancer is a multifactorial disease resulting from a combination of genetic and environmental factors. Frequent cases of cAMP and/or Ca^{2+} homeostasis remodeling have been described in cancer cells. In those tumoral cells, cAMP and Ca^{2+} signaling plays a crucial role in the development

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of hallmarks of cancer, including enhanced proliferation and migration, invasion, apoptosis resistance, or angiogenesis. This review summarizes the cross-talk between the ACs/cAMP and Ca^{2+} intracellular pathways with special attention to the functional and reciprocal regulation between Orai1 and AC8 in normal and cancer cells.

Keywords Adenylyl cyclase 8 · Cancer · Inactivation · Orai1α · Store-operated calcium entry

Abbreviations

$[Ca^{2+}]_c$	Cytosolic Ca^{2+} concentration
AC	Adenylyl cyclase
ACBD3	Acyl CoA binding domain protein-3
AKAP	A-kinase anchoring protein
ARC	Arachidonic acid-regulated Ca^{2+}
CAD	CRAC activation domain
CaM	Calmodulin
CaMK	Calmodulin-dependent kinase
CaN	Calcineurin
CAP1	Adenylyl cyclase-associated protein 1
CC	Coiled-coil domain
CDI	Ca^{2+}-dependent inactivation
CRAC	Ca^{2+} release-activated Ca^{2+}
CTPD	C-terminal polybasic domain
DRG	Dorsal root ganglion
Epac	Exchange factor directly activated by cAMP
ER	Endoplasmic reticulum
FCDI	Fast Ca^{2+}-dependent inactivation
FRET	Förster resonance energy transfer
GRK	G-protein coupled receptor kinase
I_{CRAC}	Ca^{2+} released-activated Ca^{2+} current
IP_3	Inositol 1,4,5-trisphosphate
I_{SOC}	Store-operated Ca^{2+} currents
NCX	Na^+-Ca^{2+} exchanger
NFAT	Nuclear factor of activated T-cells
OASF	ORAI1-activating small fragment
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PLC	Phospholipase C
PM	Plasma membrane
PMCA	Plasma membrane Ca^{2+} ATPase
PTH	Parathyroid hormone
RGS	Regulator of G protein signaling

SAM	Sterile alpha motif
SCDI	Slow Ca^{2+}-dependent inactivation
SERCA	Sarco/endoplasmic reticulum Ca^{2+} ATPase
SOAR	STIM1 Orai-activating region
SOC	Store-operated channels
SOCE	Store-operated Ca^{2+} entry
STIM1	Stromal interaction molecule 1
TRP	Transient receptor potential channels
TRPC	Transient receptor potential-canonical

1 Adenylyl Cyclases: Overview

The adenylyl cyclase (AC) family comprises a group of enzymes involved in the generation of the second messenger cAMP from ATP. In eukaryotic cells, cAMP is involved in the activation of a plethora of intracellular signaling pathways required for many cellular functions, hence AC/cAMP signaling pathways are highly conserved in the evolution of eukaryotes and prokaryotes. The increase of cAMP leads to the activation of the cAMP-dependent protein kinase A (PKA) and the subsequent modulation by protein phosphorylation of many enzymes, secondary kinases, transcription factors, receptors, and channels (Taylor et al. 1990; Dessauer et al. 2017). Later, it was demonstrated that cAMP also modulates cyclic nucleotide-gated channels and phosphodiesterases by direct activation of small molecular weight G proteins. The mammalian ACs belong to the Class III of ACs, the only subtype of the six unrelated AC subtypes (I–VI) that is expressed in eukaryotic cells, since the expression of the other five classes (I, II, IV, V, VI) is limited to prokaryotic cells. Likewise, AC Class III groups four subclasses (IIIa–d). Since class III ACs are also expressed in bacteria, and not in archaea, it has been suggested that eukaryotic ACs were originated by the spread of bacterial proteins into the eukaryotic cells during the endosymbiotic process that led to the origin of mitochondria and chloroplasts (Dessauer et al. 2017; Bassler et al. 2018).

The *ADCY1–10* genes encode ten different AC mammalian isoforms (AC1–10): the membrane-delimited ACs (AC1–9) belong to the IIIa subclass, and the soluble AC (AC10) belongs to the IIIb subclass (Linder and Schultz 2003). Membrane-delimited ACs present differences between their sequence and length (1080–1353 amino acids) (about 60% of homology) and were numbered following the order in which they were cloned and not according to the homology between them. All membrane-associated ACs share a common protein structure that consists of cytoplasmic N- and C-terminal domains, with a variable length, separated by two transmembrane regions (TM1 and TM2), each consisting of six transmembrane α-helices (Dessauer et al. 2017). The large intracellular regions include two cytosolic domains (C1 and C2), the ATP-binding site, subdivided into catalytic (C1a, C2a), and regulatory (C1b and C2b) subunits (Dessauer et al. 2017). The soluble AC that

shares an amino acid sequence and protein structure closely related to bacterial ACs (Buck et al. 1999) does not have the two transmembrane regions and, thus, it is not located in the plasma membrane (PM) (Steegborn 2014).

Some membrane-associated ACs present structural similarities and can be organized in four groups. AC1, 3, and 8 present a high grade of homology, the same happens between the transmembrane domains of ACs 2, 4, and 7, and in a similar way between AC5 and AC6. Finally, AC9 membrane domain does not resemble any of the other ACs (Bassler et al. 2018). Another difference between the membrane-delimited ACs is their presence or not in the lipid rafts domains, PM cholesterol-rich domains that are enriched in certain signaling proteins. While AC1, AC3, AC5, AC6, and AC8 are embedded in lipid rafts (Pagano et al. 2009; Averaimo et al. 2016; Thangavel et al. 2009; Crossthwaite et al. 2005; Cooper and Tabbasum 2014), the other isoforms are excluded from this region (Crossthwaite et al. 2005; Cooper and Tabbasum 2014; Foster et al. 2003; Smith et al. 2002; Ostrom et al. 2003).

However, the most used ACs classification is based on their regulatory properties. All membrane-delimited ACs are activated by the α-subunit of stimulatory G-proteins ($G_{\alpha s}$) and by forskolin, a diterpene isolated from the root of the plant *Coleus forskohlii* (Dessauer et al. 2017; Patel et al. 2001; Hanoune and Defer 2001; Smit and Iyengar 1998; Sadana and Dessauer 2009), although this molecule only induces a weak activation of the isoform AC9 (Hacker et al. 1998). Nevertheless, AC isoforms are differently regulated by other agonists and antagonists. The group I, Ca^{2+}/calmodulin (CaM)-activated and $G_{\beta\gamma}$-inhibited ACs, consists of AC1, AC3, and AC8 isoforms which are activated by rises in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in the submicromolar range via CaM, although it has been demonstrated that AC1 and AC3 are inhibited by supramicromolar $[Ca^{2+}]_c$ via Ca^{2+}/CaM-dependent kinase IV (CaMKIV) (Wayman et al. 1996) and CaMKII (Wei et al. 1998), respectively. The βγ-subunit of heterotrimeric G-proteins ($G_{\beta\gamma}$) can also act as an activator or inhibitor depending on the AC isoform. $G_{\beta\gamma}$ subunit boosts the activation of AC2, AC4, and AC7 induced by $G_{\alpha s}$ and forskolin (Tang and Gilman 1991; Gao and Gilman 1991), but it has an inhibitory role in the activation of Ca^{2+}/CaM-activated ACs (Steiner et al. 2006; Diel et al. 2006). AC2, AC4, and AC7 form Group II ($G_{\beta\gamma}$-stimulated and Ca^{2+}-insensitive ACs). $G_{\alpha s}$- or forskolin-induced AC5 and AC6 activity are inhibited by both the α-subunit of inhibitory G-proteins ($G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, and $G_{\alpha z}$) (Chen-Goodspeed et al. 2005; Taussig et al. 1994; Dessauer et al. 1998) and physiological concentrations of Ca^{2+} (Yoshimura and Cooper 1992; Katsushika et al. 1992; Guillou et al. 1999). These inhibitory G-proteins also inhibit Ca^{2+}/CaM-induced AC1 activity (Taussig et al. 1994). AC5 and AC6 isoforms have been found to be also inhibited by the overexpression of $G_{\beta\gamma}$ in in vitro conditions (Bayewitch et al. 1998) and both ACs form group III ($G_{i\alpha}$/Ca^{2+}-inhibited ACs). The isoform AC9 is the only member of Group IV (forskolin-, Ca^{2+}-, and $G_{\beta\gamma}$ -insensitive AC). The regulatory properties of soluble AC are completely different from its membrane-delimited counterparts since this isoform is activated by bicarbonate and Ca^{2+}, but not by $G_{\alpha s}$ proteins (Chen et al. 2000; Litvin et al. 2003). The bicarbonate ion promotes structural changes of the active site similar to those

induced by $G_{\alpha s}$ in membrane-delimited ACs (Steegborn et al. 2005; Kleinboelting et al. 2014).

In addition to the regulatory mechanisms described above, both the AC activity and the downstream signaling cascades via PKA activation are finely regulated by the A-kinase anchoring proteins (AKAPs). AKAPs constitute a group of proteins with a high grade of divergency, but all of them can interact with PKA and act as spatial and temporal regulators by localizing PKA with other proteins into discrete signaling complexes, including those involved in the cAMP signal pathway. However, AKAPs proteins also regulate by direct binding the activity of different key proteins involved in the cAMP signal pathway, such as protein kinase C (PKC), G protein-coupled receptors, calcineurin (CaN), or the own ACs (Zhang et al. 2019; Baldwin and Dessauer 2018; Dessauer 2009). AKAP79/150 (AKAP5), Yotiao (AKAP 9), and mAKAP (AKAP6) are the members of the family whose isoform-specific interaction with ACs has been demonstrated and all of them have an inhibitory role. AKAP79/150 binds to different AC isoforms, including AC2, AC3, AC5, AC6, AC8, and AC9 (Bauman et al. 2006; Willoughby et al. 2010; Efendiev et al. 2010). Yotiao inhibits AC2 and AC3 activity, although this AKAP protein can also bind to AC1 and AC9 without affecting their activity (Piggott et al. 2008), while mAKAP inhibits AC2 and AC5 activity (Kapiloff et al. 2009). Finally, the activity of certain AC isoforms is also modulated by PKA and PKC-mediated phosphorylation. PKA inhibits AC activity, while PKC induces the opposite effects in the activation of AC isoforms depending on the PKC and AC isoforms. Other regulatory proteins of AC activity include regulators of G-protein signaling proteins, which also induce opposite effects depending on the AC isoform and P-site inhibitors, which inhibit all ACs with the exception of AC9 (Sadana and Dessauer 2009).

Altogether, the AC isoform-selective mechanisms of regulation and localization explain that each AC isoform is involved in different cellular functions. Most mammalian cells express multiple AC isoforms, depending on the type of tissue, and AC activity results essential for the proper function of the tissue. The use of transgenic models and pharmacological approaches has allowed establishing specific role of each ACs. For example, it has been demonstrated that AC1 and AC8 have overlapping roles in cognitive functions such as learning and memory (Liauw et al. 2005; Zhang et al. 2011; Wieczorek et al. 2012). In the heart, AC5 and AC6 are the most important isoforms and regulate heart rate and contractility (Vatner et al. 2013). Recently it has been demonstrated that AC5 is involved in the vascular reactivity in response to acute hyperglycemia and diabetes (Syed et al. 2019). AC5 is also the major isoform expressed in medium spiny neurons, and its activity is required for the integration of both stimulatory and inhibitory midbrain signals activated by the dopaminergic system (Doyle et al. 2019; Kheirbek et al. 2009). Renal epithelial cells express multiple isoforms, including AC3, AC4, AC6 and AC9, even though only the role of AC6 has been demonstrated in the control of renal electrolyte and water transport (Rieg and Kohan 2014). Similar observations were found in the olfactory cilia, where AC2, AC3, and AC4 are expressed (Wong et al. 2000), but only AC3 is implicated in olfactory responses (Wang et al. 2006). AC2, AC4, and AC6 are widely expressed in airway smooth muscle cells, and the first is involved in

the generation of interlukin-6 and in the regulation of muscle tone in response to prostaglandins (Dessauer et al. 2017; Bogard et al. 2012, 2014). AC7 is highly expressed in brain and in B and T lymphocytes and an increased AC7 activity is related to neuropsychiatric disorders, including depression and alcoholism (Price and Brust 2019), while the loss of AC7 activity is associated with a high mortality due to the impairment of antigen-specific antibodies and prolonged inflammation during bacterial infections (Duan et al. 2010). The AC9 isoform is widely expressed in brain and heart (Palvolgyi et al. 2018), where it regulates the response of motoneurons (Defer et al. 2000) and some important heart functions, including repolarization and stress responses (Marsden and Dessauer 2019). Mammalian soluble AC is widely expressed in the cardiovascular system (Chen et al. 2012) and is involved in the activation of intrinsic apoptotic pathways (Kumar et al. 2009, 2014), as it happens during the development of cardiac hypertrophy in cardiomyocytes (Schirmer et al. 2018). Soluble AC has been also related to the glucose-induced insulin release and to the pathogenesis of several types of cancer, such as prostate cancer and melanoma (Steegborn 2014). Finally, it is important to highlight that genetic deletion of AC3 (Tong et al. 2016), AC7 (Duan et al. 2010), and AC9 (Li et al. 2017) is embryonically lethal in mice, while the deletion of the other isoforms causes dysfunction in the tissues in which they are expressed (Cosson et al. 2019; Holz et al. 2014; Dunn et al. 2009).

1.1 Adenylyl Cyclases and Cancer

AC/cAMP signaling has been shown to regulate positively and negatively different hallmarks of cancer, depending on the tissue type and AC isoform (Table 1), by the activation of MAPK/ERK signaling pathways via exchange factor directly activated by cAMP (Epac)-mediated small GTPase Rap stimulation or its inactivation via PKA-dependent Raf1 and B-raf inhibition (Ramos-Espiritu et al. 2016). It has been described several ways that alter AC activity in cancer. *ADCY* gene expression has been shown to be controlled by promoter CpG methylation and its dysregulation is related to the altered expression of AC isoforms observed in different types of cancer. Hence, AC3 overexpression is associated with a decreased DNA methylation levels in the *ADCY3* gene in gastric cancer cells (Hong et al. 2013), while *ADCY1* promoter hypermethylation reduces the expression of AC1 in glioblastoma and liver cancer cells (Fan et al. 2019; Ma et al. 2015). Hypermethylation of the promoter of *ADCY3*, *ADCY4*, *ADCY7* and *ADCY8* also leads to downregulation of AC3, AC4, AC7 and AC8 expression observed in breast cancer cells, lung cancer cells, colorectal cancer cells, and cervical cancer cells, respectively (Fan et al. 2019). A remodeling of AC isoform expression has been observed in pancreatic cancer cells compared to healthy pancreatic cells. AC6 is the main isoform involved in the increase of the level of cAMP and in the physiological response of pancreatic cells to secretagogues or forskolin via cAMP/PKA pathway activation; however, in tumoral cells AC3 is overexpressed while AC2 expression is diminished in these

Table 1 Changes in AC expression in cancer cells

AC isoform	Increased expression	Reduced expression	Interplay between Ca^{2+}/AC signal in cancer cells
AC1	Non-small cell lung cancer cells (He et al. 2017) Melanoma cancer cells (Chen et al. 2019)	Glioblastoma (Ma et al. 2015) Liver cancer cells (Fan et al. 2019; Ma et al. 2015) Metastatic rectal adenocarcinoma cells (Hua et al. 2017)	*Increments in the level of cytosolic cAMP:* activation of cyclic nucleotide-gated channels-, EPACs-, and PKAs-dependent intracellular signaling pathways (Zou et al. 2019)
AC2	Small intestinal neuroendocrine tumors (Duerr et al. 2008)	Pancreatic cancer cells (Quinn et al. 2017)	N.D.
AC3	Gastric cancer cells (Hong et al. 2013) Pancreatic cancer cells (Quinn et al. 2017)	Breast cancer cells (Fan et al. 2019)	N.D.
AC4	N.D.	Lung cancer cells (Fan et al. 2019)	N.D.
AC5	N.D.	N.D.	N.D.
AC6	Non-small cell lung cancer cells (He et al. 2017)	N.D.	N.D.
AC7	Acute myeloid leukemia cancer cells (Li et al. 2015)	Colorectal cancer cells (Fan et al. 2019)	N.D.
AC8	N.D.	Cervical cancer cells (Fan et al. 2019)	*SOCE regulation and NFAT activation* (Sanchez-Collado et al. 2019; Zhang et al. 2019)
AC9	Colon cancer cells z.	N.D.	
AC10	Human prostate cancer cells (Flacke et al. 2013) Breast cancer cells (Onodera et al. 2014)	N.D.	*Increments in the level of cytosolic cAMP:* activation of EPAC/Rap1/B-Raf signaling pathway (Ramos-Espiritu et al. 2016)

cells. These AC isoforms are differentially regulated by Ca^{2+} as described in Sect. 2, while AC6 is inhibited by Ca^{2+} and AC2 is insensitive to this ion, AC3 is activated by increments in $[Ca^{2+}]_c$. Since this increment in $[Ca^{2+}]_c$ has been observed in pancreatic cancer cells, AC3 is the responsible isoform for a chronic increase in cAMP levels in these cells (Quinn et al. 2017). Finally, another explanation for an altered AC activity in cancer cells could be that the same AC isoform is localized in distinct microdomains in cancer cells with respect to their counterpart non-tumoral cell, as it occurs with the soluble AC isoform (Zippin et al. 2010; Magro et al. 2012).

Alteration in AC1 expression has been observed in different cancer cells with a differential effect on the prognosis and overall survival depending on the cancer type. Hence AC1 is overexpressed in non-small cell lung cancer cells and melanoma

cancer cells, and its expression is positively correlated with a better prognosis in non-small cell lung cancer, while this correlation is negative in melanoma (He et al. 2017; Chen et al. 2019) Conversely, AC1 is downregulated in metastatic rectal adenocarcinoma cells (Hua et al. 2017) and glioblastoma cells, and in the latter cancer type, its expression is directly correlated with a poor prognosis in patients (Ma et al. 2015). AC2 expression is enhanced in small intestinal neuroendocrine tumors (Duerr et al. 2008), while its expression is diminished in pancreatic tumors (Quinn et al. 2017). Regarding AC3, this isoform has shown to be overexpressed in gastric cancer cells isolated from human patients and in different gastric cancer cell lines, such as SNU-216, SNU-638, SNU-718, AGS, and MKN28 cells. AC3 overexpression promotes, via cAMP/PKA/CREB pathway activation, the development of different hallmarks of cancer, which includes cell migration, cell invasion, and cell proliferation in tumoral cells. In addition, the silencing of AC3 expression in SNU-216 cells reduced all these hallmarks of cancer (Hong et al. 2013). Conversely, AC3 is overexpressed in pancreatic cancer cells and forms a complex together with adenylyl cyclase-associated protein 1 (CAP1), which reduces cell migration and cell invasion by forskolin-induced increase in cAMP levels and CREB phosphorylation (Quinn et al. 2017). CAP1 is an actin-binding protein that plays an essential role in cytoskeleton organization and the Ras/cAMP pathway. In yeast, CAP is involved in AC activation and expression, although this function remains unclear in mammalian cells (Kakurina et al. 2018; Zhang et al. 2013). However, it has been demonstrated that its expression and activity are increased in other types of cancer, including non-small cell lung cancer (Kolegova et al. 2019) and breast cancer (Hasan and Zhou 2019). AC4 expression is diminished in breast cancer cells and its expression is positively correlated with a better prognosis in patients (Fan et al. 2019). AC6 is overexpressed in non-small cell lung cancer cells (He et al. 2017), and in other tumor cells, hypoxia via hypoxic-induced factor (HF-1) generates an increment in the expression of AC6 and AC7. This event is involved in the enhanced hypoxia-induced cAMP synthesis that promotes cell migration and pH regulation through PKA activation in HeLa and C33a cervical cancer cells, RKO colorectal cancer cells, and MCF7 breast cancer cells (Simko et al. 2017). In addition, diminished AC7 activity is related with growth inhibition and increased apoptosis in primary human acute myeloid leukemia cancer cells and its expression inversely correlates with the overall survival of patients (Li et al. 2015). AC9 is overexpressed in colon cancer cells and its expression is directly correlated with a poor prognosis in patients (Yi et al. 2018). Soluble AC has been shown to underlie cancer genesis and tumor progression in several types of tumoral cells and this isoform has been considered as tumor suppressor protein, since its expression is reduced in a wide variety of types of human cancers. It has been proposed that reduced soluble AC activity impairs the control of this AC isoform on MAPK/ERK pathway regulation, giving a selective advantage to tumoral cells and allowing them to proliferate (Ramos-Espiritu et al. 2016). Conversely, several studies have demonstrated that the overexpression of soluble AC or an increase in its activity is involved in the enhanced proliferation required for metastasis and invasion in human prostate cancer cells and breast cancer cells via Epac/Rap1/B-raf pathway (Flacke et al. 2013; Onodera et al. 2014).

2 Regulation of ACs by Ca^{2+}

Ca^{2+} signaling is critical for a wide array of physiological functions, encompassing from embryonic development to cell death. In non-excitable cells, Ca^{2+} signaling typically results from the activation of G protein-coupled receptors or tyrosine kinase receptors, which causes phospholipase C (PLC) stimulation. Active PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into two second messengers: (inositol 1,4,5-trisphosphate) IP_3 and diacylglycerol. While diacylglycerol activates PKC and also regulates other signaling mechanisms independently from PKC, IP_3 induces Ca^{2+} release from the ER through activation of its receptor (Streb et al. 1983). Upon ER depletion, a mechanism for Ca^{2+} influx from the extracellular medium, named store-operated Ca^{2+} entry (SOCE), favors the signaling of biological functions and the replenishment of intracellular Ca^{2+} stores (Putney 1986; Kwan et al. 1990). As described in the previous section, increments in $[Ca^{2+}]_c$ has differential effects in AC activity depending on each of the enzyme isoforms by direct Ca^{2+}-AC interaction or by Ca^{2+}-activated signaling pathways (Table 2). AC8 is, together with AC1 and soluble AC10, the unique enzyme that couples activity-dependent Ca^{2+} influx to the activation of cAMP signaling pathways. Hence, AC1, AC8, and in a lesser extent AC3 are activated by Ca^{2+}/CaM at submicromolar $[Ca^{2+}]_c$, but only AC1 and AC8 are activated by SOCE (Fagan et al. 1996). CaM is a Ca^{2+}-binding protein with two N- and C-terminal lobes and each contains two Ca^{2+} binding EF-hands domains (Kawasaki and Kretsinger 2017). AC1 possesses one CaM-binding domain located in the C1b region (Vorherr et al. 1993), while AC8 presents two CaM-binding domains, one in N-terminal domain (an amphipathic helix) and other in C2b region (an IQ-like motif) (Gu and Cooper 1999). In resting conditions, both AC1 and AC8 are in autoinhibited state due to steric hindrance caused mainly by the C-terminal domain that prevents ATP binding and cAMP synthesis (Simpson et al. 2006; Masada et al. 2012). The mechanism of Ca^{2+}/CaM-dependent activation of AC1 is not well known, although it is supposed that Ca^{2+}/CaM binding to the CaM-binding domain induces a conformational change leading to AC1 activation by relieving the autoinhibition (Masada et al. 2012). However, the mechanism of Ca^{2+}-dependent activation of AC8 was well established by Simpson and coworkers (Fig. 1). This study demonstrated that, in resting conditions, AC8 is in autoinhibited state due to steric hindrance caused mainly by the C-terminal domain that prevents ATP binding and cAMP synthesis. In this condition, CaM is tethered to the N-terminal domain of AC8 by the C-terminal lobe with this lobe loaded with Ca^{2+} while the N-terminal lobe remains Ca^{2+}-free. After local rises in $[Ca^{2+}]_c$, Ca^{2+} binds to the EF-hand motifs at the N-terminal lobe of CaM leading to the interaction of both lobes with the C-terminal CaM-binding domain to mediate a conformational change that leads to AC8 activation (Simpson et al. 2006). Later, it was demonstrated that partially loaded CaM lobes can promote AC8 activation by a cooperative mechanism, while AC1 is activated only when both CaM lobes are fully loaded (Masada et al. 2009). In regard to the soluble isoform, AC10 has been shown to be activated directly by Ca^{2+} at submicromolar and supramicromolar $[Ca^{2+}]_c$ after endoplasmic

Table 2 Regulation of AC isoforms by Ca^{2+} signaling

AC isoform	Activation	Inhibition
AC1	*Ca^{2+}/CaM:* Via SOCE (Fagan et al. 1996; Masada et al. 2012) Via ER-Ca^{2+}depletion (Masada et al. 2009) *Ca^{2+}-dependent enzymes:* Via PKC (Jacobowitz et al. 1993)	*Direct AC-Ca^{3+} binding:* At supramicromolar $[Ca^{2+}]_c$ (Mou et al. 2009) *Ca^{2+}-dependent enzymes:* Via CaMKIV (Wayman et al. 1996)
AC2	*Ca^{2+}-dependent enzymes:* Via PKC (Bol et al. 1997)	*Direct AC-Ca^{3+} binding:* At supramicromolar $[Ca^{2+}]_c$ (Mou et al. 2009)
AC3	*Ca^{2+}/CaM* (Fagan et al. 1996) *Ca^{2+}-dependent enzymes:* Via PKC (Jacobowitz et al. 1993)	*Direct AC-Ca^{3+} binding:* At supramicromolar $[Ca^{2+}]_c$ (Mou et al. 2009) *Ca^{2+}-dependent enzymes:* Via CaMKII (Wei et al.1996, 1998)
AC4	N.D.	*Direct AC-Ca^{3+} binding:* At supramicromolar $[Ca^{2+}]_c$ (Mou et al. 2009) *Ca^{2+}-dependent enzymes:* Via PKC (Zimmermann and Taussig 1996)
AC5	*Direct AC-STIM1 binding:* Via ER-Ca^{2+} depletion (Lefkimmiatis et al. 2009; Spirli et al. 2017) *Ca^{2+}-dependent enzymes:* Via PKC (Kawabe et al. 1994)	*Direct AC-Ca^{3+} binding:* At submicromolar $[Ca^{2+}]_c$ (Tesmer et al. 1999) At supramicromolar $[Ca^{2+}]_c$ (Mou et al. 2009)
AC6	*Direct AC-STIM1 binding:* Via ER-Ca^{2+} depletion (Motiani et al. 2018)	*Direct AC-Ca^{3+} binding:* At submicromolar $[Ca^{2+}]_c$ (Tesmer et al. 1999) At supramicromolar $[Ca^{2+}]_c$ (Mou et al. 2009) *Ca^{2+}-dependent enzymes:* Via PKC (Lai et al. 1997)
AC7	*Ca^{2+}-dependent enzymes:* Via PKC (Haslauer et al. 1998)	*Direct AC-Ca^{3+} binding:* At supramicromolar $[Ca^{2+}]_c$ (Mou et al. 2009)
AC8	*Ca^{2+}/CaM:* Via SOCE (Fagan et al. 1996; Simpson et al. 2006; Masada et al. 2009)	*Direct AC-Ca^{3+} binding:* At supramicromolar $[Ca^{2+}]_c$ (Mou et al. 2009)
AC9	*Ca^{2+}-dependent enzymes:* Via PKC (Liu et al. 2014) Via CaMKII (Cumbay and Watts 2005)	*Direct AC-Ca^{3+} binding:* At supramicromolar $[Ca^{2+}]_c$ (Mou et al. 2009) *Ca^{2+}-dependent enzymes:* Via CaN (Antoni et al. 1998)
AC10	*Direct AC-Ca^{3+} binding:* Via SOCE (Parker et al. 2019) Via voltage-gated Ca^{2+} channels (Ramos et al. 2008) Via ER-Ca^{2+} depletion (Jaiswal and Conti 2003)	*Direct AC-Ca^{3+} binding:* At supramicromolar $[Ca^{2+}]_c$ (Mou et al. 2009)

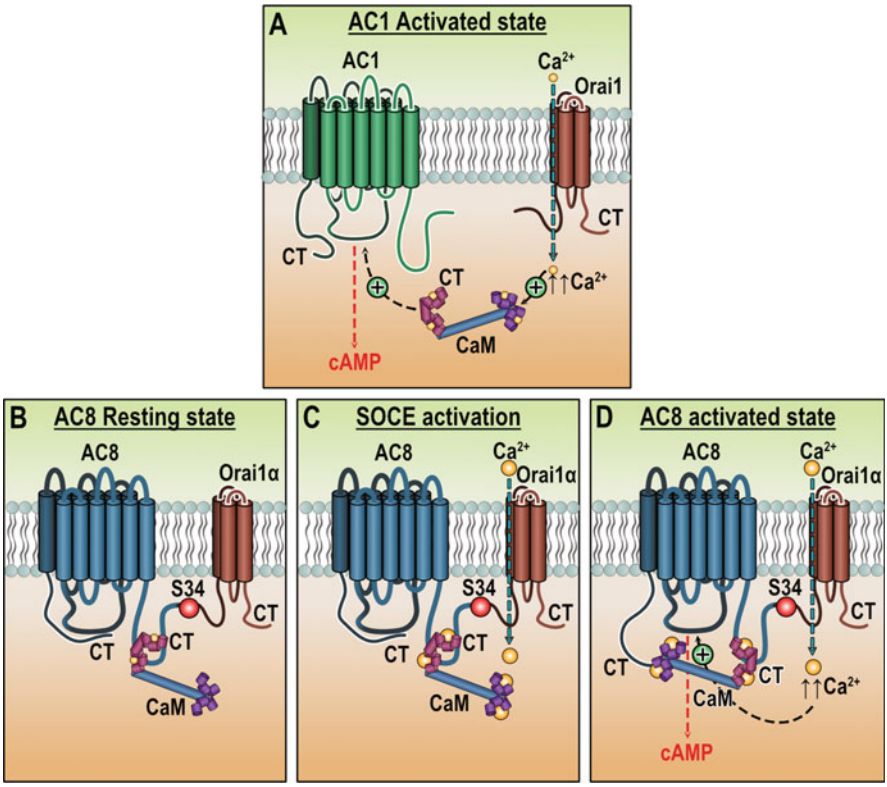

Fig. 1 Cartoon summarizing the activation of AC1 and AC8 mediated by Ca^{2+}/CaM. (**a**) Ca^{2+}/CaM binding to the CaM-binding domain of AC1 promotes a conformational change that relieves the autoinhibition state of AC1 and leads to cAMP synthesis. In the case of AC8 activation mechanism, (**b**) AC8 stays in an autoinhibition state that avoids cAMP synthesis in which CaM is binding to the N-terminal domain of AC8 by its Ca^{2+} loaded C-terminal lobe, while the N-terminal lobe remains free of Ca^{2+}. (**c**) After SOCE activation and local increases in $[Ca^{2+}]_c$, Ca^{2+} binds to the EF-hands domains at the N-terminal lobe of CaM that, in turn, (**d**) leads to the interaction of both N- and C-terminal CaM lobes with AC8, promoting a conformational change in AC8 and its activation

reticulum (ER)-Ca^{2+} depletion (Jaiswal and Conti 2003) or Ca^{2+} entry through store-operated Ca^{2+} channels (Parker et al. 2019) or voltage-dependent Ca2+ channels (Ramos et al. 2008).

AC1 and AC3 are, respectively, inhibited by CaMKIV (Wayman et al. 1996) and CaN (Wei et al. 1998) at the supramicromolar $[Ca^{2+}]_c$, while AC5 and AC6 are inhibited by both sub- and supramicromolar $[Ca^{2+}]_c$; in fact, all AC isoforms are inhibited by rises in $[Ca^{2+}]_c$, at non-physiological supramicromolar range since ACs require Mg^{2+} to catalyze the conversion of ATP to cAMP, and Ca^{2+} acts as a competitive inhibitor at the active site (Cooper et al. 1995; Willoughby and Cooper 2007; Choi et al. 1992; Cali et al. 1994; Krupinski et al. 1989). Conversely, AC2,

AC4, AC7, and AC9 activity are not modified at submicromolar $[Ca^{2+}]_c$ (Dessauer et al. 2017; Halls and Cooper 2011). Finally, AC activity can be differentially regulated by PKC (Dessauer et al. 2017; Halls and Cooper 2011).

2.1 Store-Operated Calcium Entry-Dependent AC Activation

2.1.1 Store-Operated Calcium Entry

The biophysical characterization of SOCE in mast cells led to the identification of the calcium released-activated current (I_{CRAC}), a highly Ca^{2+} selective, inward rectified current with positive reversal potential at around 60 mV (Hoth and Penner 1992). The first attempts to identify the molecular components of SOCE proposed the transient receptor potential-canonical (TRPC) channel subfamily, especially TRPC1, as candidates to mediate SOCE (Venkatachalam et al. 2003). However, the characterization of TRPC currents revealed biophysical and pharmacological properties distinct from I_{CRAC}. The involvement of TRPC channels in SOCE is still under debate, although there are several studies that highlight the role of TRPC family members in an alternative store-operated current, known as I_{SOC} (Jardin et al. 2008a; Desai et al. 2015; Ambudkar et al. 2017; Cheng et al. 2011; Ong et al. 2016; Worley et al. 2007; Lopez et al. 2020). In 2005 and 2006, after an onerous search, stromal interaction molecule 1 (STIM1) and Orai1 proteins were identified as the major constituents of the Ca^{2+} release-activated Ca^{2+} (CRAC) channel, and, thus, SOCE. Since then, it has been extensively demonstrated that STIM1 operates as a reticular Ca^{2+} transducer which communicates the filling state of the intracellular Ca^{2+} stores to the PM pore-forming Orai1, in order to trigger I_{CRAC} currents (Zhang et al. 2005; Roos et al. 2005; Feske et al. 2006).

STIM1 is a single pass transmembrane protein located mainly in the ER, which is able to sense the reticular Ca^{2+} concentrations, thanks to its intraluminal EF-hand domain. STIM1 luminal segment also contains a hidden EF-hand and a sterile alpha motif (SAM) (Gudlur et al. 2018). On its cytosolic side, three coiled-coil domains (CC1–3), a Ser/Pro-rich domain, and a C-terminal polybasic domain 7(CTPD) complete STIM1 sequence. Once Ca^{2+} efflux from the ER decreases the availability of free Ca^{2+} inside the store, STIM1 EF-hand dissociates from Ca^{2+}. This event triggers a conformational change in STIM1, its oligomerization with other STIM molecules and the translocation to the PM-ER junctions in order to trigger SOCE (Zhang et al. 2005; Luik et al. 2008). Four independent groups identified the STIM1 cytosolic fragment that contacts and activates Orai proteins (Muik et al. 2009; Kawasaki et al. 2009; Park et al. 2009; Yuan et al. 2009). All these fragments (termed OASF (ORAI1-activating small fragment), CAD (CRAC activation domain), SOAR (STIM1 Orai-activating region) and CCb9) have a common sequence located in the second and third CC domains and 19 additional amino acids. In resting cells, when ER Ca^{2+} stores are full, STIM1 adopts a compact structure where the cytosolic CC1 sequesters the SOAR domain. This folded

conformation facilitates STIM1 Brownian movement along the ER, but upon activation, the conformational change of its cytosolic region allows the SOAR domain and the CTPD to interact with Orai1 and PM phospholipids, respectively. It has been demonstrated that these interactions are crucial for STIM1 migration and clustering at the PM-ER junctions, via a diffusion trap mechanism (Park et al. 2009; Korzeniowski et al. 2009; Wu et al. 2014; Zheng et al. 2018).

Orai1 is a PM protein containing four transmembrane domains, with both N- and C-termini facing the cytosol. The first efforts to describe Orai1 channel structure proposed a tetrameric approach, but crystal structure of Orai from *Drosophila melanogaster* (dOrai1) revealed a hexameric pore structure. According to this model, the pore would be formed by the TM1 of each subunit, and the remaining TMs would describe concentric rings around the conducting pore. Structural analysis of dOrai1 has also revealed that a negatively charged glutamate (Glu106), placed near the extracellular site of the pore, creates a filter that ensures Ca^{2+} selectivity (Hou et al. 2012). Immediately beneath this filter, two hydrophobic residues (Val102 and Phe99) form a free energy barrier, which is strong enough to prevent ion conductance at the resting state (Yamashita et al. 2017). The final segment of the pore contains a basic ring, formed by arginine residues (Arg91), which creates an electrostatic gate by projecting their side chains into the center of the pore. Patients carrying the Orai1-Arg91Trp mutation suffer from severe combined immunodeficiency because tryptophan side chains completely block the channel and abrogate SOCE in T cells and fibroblasts (Feske et al. 2006). The C-terminal domain of Orai1 is a helical extension of the fourth transmembrane domain which contains a CC segment. Structural analysis of dOrai1 has revealed an antiparallel arrangement between the C-terminal CC of two adjoining Orai1 subunits. The binding of STIM1 to these CCs in order to activate the channel has been extensively documented and involves electrostatic and hydrophobic interactions between STIM1 CC2 and Orai1 C termini (Calloway et al. 2009, 2010; Stathopulos et al. 2013). However, STIM1-mediated Orai channel activation involves the conformational change of Orai proteins. Initially it was suggested that this change was mediated by the interaction between STIM1 SOAR domain and Orai1 N-terminal region (Gudlur et al. 2014), but more recent studies challenge this hypothesis. Crystallographic and cryo-EM studies of dOrai1 and constitutively open dOrai1 mutants have proposed that the conformational change and activation of Orai1 channels only depend on the interaction between the SOAR domain and Orai1 C-terminal region. This association triggers an allosteric signal that is transmitted from the channel periphery to the central pore and allows Ca^{2+} conductance by modifying the pore structure. Nevertheless, because of the multiple dOrai1 mutants studied and the limitations of each technique there is not a unique open configuration (Yamashita et al. 2017), (Liu et al. 2019; Hou et al. 2018).

In order to prevent Ca^{2+} overload, Orai1-dependent currents display two spatiotemporally distinct inhibitory mechanisms. The faster, termed fast Ca^{2+}-dependent inactivation (FCDI), develops over milliseconds and results by the action of Ca^{2+} in the proximity of the pore (Zweifach and Lewis 1995a; Fierro and Parekh 1999). It has been proposed that the intracellular loop and the selectivity filter of Orai1 play a

critical role in FCDI. Orai1 intracellular loop was described as a possible Ca^{2+} binding region and as the inactivating particle which blocks the pore (Srikanth et al. 2010). Additionally, Mullins and Lewis have reported that STIM1 mediates FCDI, suggesting that full-strength Ca^{2+} dependent inactivation demands an allosteric interaction between three acidic STIM1 residues (Asp476, Asp478, and Asp479) and Orai1 Trp76 (Mullins and Lewis 2016). Tenths of a second after Orai1 channel activation the global increases in cytosolic Ca^{2+} concentration evoke the second inhibitory mechanism, which is called slow Ca^{2+}-dependent inactivation (SCDI) (Zweifach and Lewis 1995b; Parekh 1998). SARAF and CaM have been proposed to mediate SCDI, although there are strong evidences to suggest that they trigger slightly different Orai1 inhibitory mechanisms. SARAF is a single pass transmembrane protein, which binds STIM1 SOAR domain to prevent its spontaneous activation. Upon store depletion, SARAF transiently dissociates from STIM1, allowing STIM1–Orai1 interaction and the opening of the channel. Later, the cytosolic Ca^{2+} concentration rise and the refilling of the internal Ca^{2+} stores induce the inhibition of Orai1 current by SARAF, which interacts again with STIM1 molecules and promotes their disaggregation (Palty et al. 2012; Albarran et al. 2016a). CaM is a ubiquitous protein involved in the transduction of Ca^{2+} signaling into physiological events. To accomplish this task, CaM monitors cytosolic Ca^{2+} concentrations and subsequently binds and regulates several proteins and enzymes, including ion channels. Recently, two hydrophobic regions of STIM1 (Leu374, Val375 and Leu390, Phe391) have been proposed as CaM-binding domains and therefore as the region responsible to facilitate CaM mediated SCDI (Bhardwaj et al. 2020).

The continuous research about the molecules involved in SOCE has led to the description of multiple proteins related to STIM1 and Orai1. STIM1L is a longer variant of STIM1 generated after a process of mRNA alternative splicing which constitutively colocalizes with Orai1 establishing permanent clusters. This advanced position enables STIM1L to trigger SOCE faster than the canonical STIM1 (Darbellay et al. 2011). STIM2 is a STIM1 homolog which contains an EF-hand with lower affinity for Ca^{2+}, this feature allows STIM2 to detect smaller reticular Ca^{2+} depletions and reverse homeostatic disruptions by triggering small and prolonged store-operated currents (Brandman et al. 2007; Zhou et al. 2009). Due to a process of alternative splicing three STIM2 mRNA isoforms are produced, these messengers give rise to the already described variant (STIM2.2; also STIM2α), to a shorter protein containing an alternative exon 13 (STIM2.3), and to a third variant which includes an additional exon 9 (STIM2.1; also STIM2β). STIM2.1 contains an additional sequence within its SOAR domain that results in defective dimer formation and impaired Orai1 interaction, thus STIM2.1 antagonizes the function of other STIM family members and inhibits SOCE (Miederer et al. 2015). Since the identification of the three mammalian Orai homologs by Feske et al. (2006), Orai1 has been the focal point for a great number of scientific efforts, relegating Orai2 and Orai3 to a secondary role. Although the three Orai isoforms are widely expressed and present a highly conserved structure, the involvement of Orai2 and Orai3 in SOCE is not fully understood and their participation in other signaling mechanism

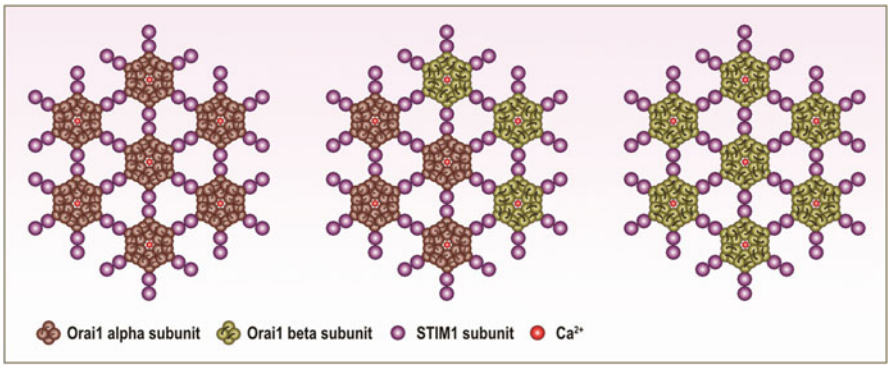

Fig. 2 This Cartoon depicts the clustering of Orai1 channels by STIM1. Orai1 clusters might include Orai1α or Orai1β, although a combination of both variants is not discarded

has been suggested. After overexpression with STIM1, Orai2 and Orai3 mediate store-operated I_{CRAC}-like currents showing less efficacy than Orai1; additionally, Orai2 and Orai3 currents present more pronounced FCDI, especially in the case of Orai3, and weaker SCDI (Mercer et al. 2006; Lis et al. 2007; DeHaven et al. 2007). The hypothesis of heteromultimeric channels, formed by the association of different Orai homologs, has been extensively discussed. The first paper confirming this association led to the description of the arachidonate-regulated Ca^{2+} channel, which is formed by Orai1 and Orai3 (Mignen et al. 2008). In 2017, Vaeth et al. reported that Orai1 and Orai2 form heteromeric capacitative channels in mice T cells, suggesting that Orai2 attenuates the magnitude of SOCE, due to its stronger FCDI, and controls the I_{CRAC}-mediated immune response (Vaeth et al. 2017). Additionally, a recent study reinforces these results showing that native CRAC channel is formed by heteromultimerization of Orai1 with Orai2 and/or Orai3 (Yoast et al. 2020). In 2012, Fukushima et al. reported that mammalian Orai1 proteins are expressed as two variants generated by alternative translation initiation of the Orai1 mRNA. Orai1β is the shorter form, which exists in all vertebrates, while Orai1α is a mammalian-specific variant that includes a longer N-terminal region (63 additional amino acids) (Fukushima et al. 2012). Although these isoforms do not co-assemble to form heteromeric channels (Fig. 2), both are able to interact with STIM1 and mediate I_{CRAC} y I_{SOC} currents, nevertheless, Orai1β conducts a greater current as Orai1α exhibits a more pronounced Ca^{2+}-dependent inactivation (Desai et al. 2015; Fukushima et al. 2012). The additional N-terminal region of Orai1α contains several novel domains that confer some singular features to this isoform in comparison with Orai1β. Within this fragment, three regions intended for the association with caveolin, PIP_2, and AC8 have been described (topic that will be extensively discussed later). Overlapping the AC8 binding region, two PKC phosphorylation sites (Ser27 and Ser30) have been proposed to mediate Orai1α channel inhibition (Yu et al. 2010; Kawasaki et al. 2010). STIM and Orai proteins participate in other non-capacitative calcium currents, such as the arachidonic acid-regulated

Ca^{2+} current (I_{arc}). Initially, Mignen et al. proposed that Orai1, Orai3, and STIM1 were the molecular components of the I_{arc} channel, but the characterization of Orai1 isoforms highlighted that only Orai1α is involved in this signaling mechanism (Desai et al. 2015; Mignen et al. 2008, 2007).

2.1.2 Regulation of Adenylyl Cyclases by Store-Operated Calcium Entry

It has been demonstrated that AC1 and AC8 are also differentially regulated by SOCE. Both AC1 and AC8 isoforms are activated by SOCE, but not by Ca^{2+} influx induced by ionophores (Fagan et al. 1996). Accordingly, these isoforms have shown to be located in the lipid rafts (Smith et al. 2002; Fagan et al. 1996; Masada et al. 2009), but only AC8 can interact with SOC channels (Willoughby et al. 2012) as discussed in more detail below. However, it has been demonstrated that AC1 has a higher affinity for Ca^{2+} that explains the AC1 activation, but not AC8, by agonist-induced Ca^{2+} release from intracellular stores and that AC1 could be located more distant from store-operated Ca^{2+} channels than AC8 (Masada et al. 2009). All these differences in the mechanisms of activation of both ACs could explain why AC8 is activated before AC1 by extracellular Ca^{2+} influx, despite that AC1 exhibits a greater affinity for Ca^{2+}, their different cAMP generation pattern and, therefore, their involvement in different cell responses (Masada et al. 2009, 2012).

The first evidence that AC8 is activated by Ca^{2+} entry, and not by Ca^{2+} released from intracellular stores, was reported by Fagan et al. at the end of the last century. This study suggested that AC8 and SOC channels colocalize in the same PM domains and that Ca^{2+} entry through these channels is required for AC8 activation in transfected HEK-293 cells (Fagan et al. 1996). Later, the relevance of SOCE in the activation of AC8-mediated cAMP synthesis was demonstrated in native non-excitable cells (Watson et al. 1998) and this activation takes place into lipid raft transmembrane domains (Smith et al. 2002). This functional coupling between Ca^{2+} influx and AC8 activation is specific to SOCE, since non-capacitative Ca^{2+} currents mediated by the arachidonic acid-regulated Ca^{2+} (ARC)-selective channel (Shuttleworth and Thompson 1999) and diacylglycerol-activated TRPC channels (Martin and Cooper 2006) failed to induce AC8-mediated cAMP synthesis. Nevertheless, AC8 has also been found to be activated by Ca^{2+} currents via L-type ($Ca_v1.2$) Ca^{2+} channels in excitable cells (Fagan et al. 2000; Everett and Cooper 2013).

It was not until a few years after the identification of Orai1 as the pore subunit of the CRAC channel in the PM in 2006 (Yeromin et al. 2006; Vig et al. 2006; Prakriya et al. 2006), when it was demonstrated that AC8 activity is regulated by Orai1 in the lipid raft domains of the PM (Martin et al. 2009), and that the disruption of these domains impairs SOCE and AC8 activation (Smith et al. 2002; Dionisio et al. 2011). Later, Willoughby et al. confirmed the direct interaction between the cytoplasmic N-terminal region of Orai1 and AC8 using Förster resonance energy transfer (FRET), glutathione S-transferase pulldown and coimmunoprecipitation assays

with different constructs of AC8 and Orai1 (Willoughby et al. 2012). Although the revelance of the N-terminal domain of AC8 in Ca^{2+}-dependent activation, and therefore with any type of interaction with SOCE components, was previously demonstrated (Smith et al. 2002; Cooper 2015), this seminal study showed that residues 26–34 (Gly26, Ser27, Arg28, Arg29, Ser30, Arg31, Arg32, Arg33, and Ser34) of the Arg-rich N-terminal domain of Orai1 are involved in the interaction with AC8. The first five amino acids form the Orai1-AC8 binding site while the residues of arginine are essential for Orai1–AC8 interaction, since the double alanine substitutions, but not the single one, impair the interaction between both proteins (Willoughby et al. 2012). This AC8-binding region is absent in the short variant of Orai1, Orai1β, since unlike the long Orai1 variant, Orai1α, this isoform lacks the first 63 amino acids of the N-terminal region, and as a consequence, AC8 cannot interact with Orai1β. According to that, two recent studies have demonstrated by FRET, glutathione S-transferase pulldown and coimmunoprecipitation assays the interaction of AC8 with Orai1α, but not Orai1β, after the activation of SOCE (Zhang et al. 2019; Sanchez-Collado et al. 2019). Furthermore, the AC8-binding site is also absent in the other Orai isoforms, Orai2 and Orai3, suggesting that AC8 specifically interacts with Orai1 (Willoughby et al. 2012). Accordingly, SOC currents (I_{SOC}) mediated by TRPC1 can also be involved in the Ca^{2+}-dependent activation of AC8 when it is located in the same nanodomain as Orai1 and STIM1, and this effect seems to depend on the development of functional Orai1–TRPC1 interaction since direct TRPC1-AC8 coupling has not been demonstrated (Willoughby et al. 2014).

As described in the previous section, the activity of AC8 is attenuated by its interaction with AKAP79/150 (Willoughby et al. 2010). It is interesting that AKAP79/150 also interacts with Orai1 (Zhang et al. 2019; Kar et al. 2014) and $Ca_v1.2$ channels (Everett and Cooper 2013; Oliveria et al. 2007; Murphy et al. 2014), regulating their functions and leading to the activation of the nuclear factor of activated T-cells (NFAT) signaling pathway coupled to Ca^{2+} entry through both types of channels. AKAP79 is also involved in PKA-mediated STIM1 phosphorylation at Tyr389, which results to be involved in the activation of ARC channels, but not SOC channels (Thompson and Shuttleworth 2015).

Recently it has been demonstrated that soluble AC is also activated by SOCE in human coronary arterial smooth muscle cells and is involved in the activation of cAMP/PKA/CREB signal pathway responsible for the change of a quiescent to a proliferative phenotype in these cells (Parker et al. 2019). The direct binding of Ca^{2+} to a saturable site, with a high affinity for Ca^{2+} (in the nanomolar range) and very low or no affinity for Mg^{2+}, located in the catalytic core or in its vicinity results in an increase in V*max* of the enzyme without affecting the K_m for substrates (Jaiswal and Conti 2003). In contrast to transmembrane delimited ACs, Ca^{2+}-binding to the Mg^{2+} site B also increases sAC activity at supramicromolar and submicromolar $[Ca^{2+}]_c$, suggesting that physiological activation of this isoform occurs with Mg^{2+} binding to site A and Ca^{2+} to site B (Steegborn 2014).

2.2 STIM1-Dependent AC Activation

In addition to the SOCE-mediated AC activation, it has been demonstrated another mechanism that associates the Ca^{2+} stores to cAMP/AC signaling activation, a process that occurs independently of increases in $[Ca^{2+}]_c$, but is strongly dependent on STIM1 translocation to the PM after ER Ca^{2+}-store depletion (Lefkimmiatis et al. 2009; Spirli et al. 2017; Motiani et al. 2018). Cyclic AMP signaling activated by this mechanism has been proposed to be involved in ER stress responses activated by prolonged Ca^{2+} depletion. However, it is important to remark that this mechanism has been demonstrated with different intensity of activation in some cell types, including HEK-293 cells as well as the human colon cancer HT-29 and normal human colon mucosal epithelial NCM460 cell lines, but not in other cell types as HeLa cells. The proposed mechanism of activation suggests a direct AC5–STIM1 interaction as responsible for cAMP/AC signaling activation, but this interaction was not confirmed by immunoprecipitation (Lefkimmiatis et al. 2009). However, AC5–STIM1 interaction was later confirmed by Spirli et al. in mouse cholangiocytes using PLA assay. This study shows a remodeling in the Ca^{2+}-dependent regulation of AC5 that is associated with the pathogenesis of polycystic liver disease associated with autosomal dominant kidney disease. Polycistin-2 defective cholangiocytes are characterized not only by a decreased Ca^{2+} accumulation in the ER Ca^{2+} stores and reduced SOCE, but also by enhanced oligomerization and membrane translocation of STIM1 that leads to STIM1–AC5, but not STIM1–AC6 interaction, impairing Orai1 activation. As a consequence of the decreased $[Ca^{2+}]_c$ levels, AC5-mediated cAMP/PKA signaling is increased and promotes ERK1/2 phosphorylation and vascular endothelial growth factor production, which are responsible for liver cysts growth (Spirli et al. 2017). A similar AC activation mechanism that involves AC6 isoform but not AC4, AC5, AC7, and AC9 has been widely described during melanogenesis. Melanocyte stimulation with α-melanocyte-stimulating hormones induces ER Ca^{2+} store depletion in order to increase, by a positive feedback loop, the AC activity required for melanogenesis. Hence, stimulatory G_s protein coupled to melanocortin-1 receptor activates AC/cAMP signaling, promoting ER depletion via PKA/PLC/IP_3 or Epac/PLC/IP_3 signal pathway activation. As a result, STIM1 is translocated to the PM and promotes SOCE activation by interaction with Orai1, leading to cell proliferation. However, FRET studies have demonstrated that a population of STIM1 recruited at PM can also interact directly with AC6, through its Ser/Pro-rich domain, in order to enhance cAMP signaling (Motiani et al. 2018).

2.3 Ca^{2+}-Dependent AC Inactivation

To understand the regulation of AC5 and AC6 activity by Ca^{2+} is necessary to know the catalytic and kinetic mechanism that catalyzes, using Mg^{2+} as cofactor, the conversion of ATP to cAMP (for a more detailed explanation, see Mou et al.

(2009)). Briefly, the catalytic core of AC contains two Mg^{2+} sites, named as site A and site B that must be occupied to facilitate the nucleophilic attack of the 3-′-hydroxyl group on the oxygen of the α-phosphate of ATP and the formation of pyrophosphate (PPi) (Tesmer et al. 1999). The Mg^{2+} site A has less affinity for Ca^{2+} than the site B, hence, AC inhibition at submicromolar $[Ca^{2+}]_c$ is a consequence of the binding of Ca^{2+} to the Mg^{2+} site A in a non-competitive manner that stabilizes an ATP-Ca^{2+}-bound open conformation, avoiding the nucleophilic attack of the 3-′-hydroxyl group on the oxygen of the α-phosphate of ATP. The insensitivity of the rest of AC isoforms to their inhibition at submicromolar $[Ca^{2+}]_c$ has been related to specific features, attributed to non-conserved amino acids, in the catalytic core of each AC isoform (Tesmer et al. 1999). Kinetic studies have demonstrated that cAMP is released before PPi from the catalytic core, leading to a PPi-bound stable open conformation that inactivates the enzyme by the impairment of ATP binding (Mou et al. 2009; Dessauer and Gilman 1997). At supramicromolar concentration, Ca^{2+} binds to both A and B sites and with the latter in a competitive manner, stabilizing a Ca^{2+}-PPi-bound conformation and promoting the inhibition of all ACs (Mou et al. 2009).

2.4 *AC Regulation by Ca^{2+}-Dependent Enzymes: PKCs, CMKs, and CaN*

PKC differentially stimulates AC isoforms, while AC1 (Jacobowitz et al. 1993), AC2 (Bol et al. 1997), AC3 (Jacobowitz et al. 1993), AC5 (Kawabe et al. 1994), AC7 (Haslauer et al. 1998), and AC9 (Liu et al. 2014) have been shown to be activated by PKC-mediated phosphorylation, AC6 activity is reduced (Lai et al. 1997), AC4 activity is reduced or unaffected depending on the activation pathway (Zimmermann and Taussig 1996) and AC8 activity is unaffected by PKC (Dessauer et al. 2017; Halls and Cooper 2011). There are three known family of PKC: conventional (α, βI, βII, and γ), novel (δ, ε, θ, and μ) and atypical (ζ, ι, and λ). Only conventional PKCs require Ca^{2+} for their activation (Webb et al. 2000), and AC activity is also differentially regulated by the members of this PKC family. $G_{\alpha s}$ and $G_{\beta\gamma}$- mediated AC2 activity is enhanced by PKCβII- (Baldwin et al. 2019) and PKCα-mediated phosphorylation (Zimmermann and Taussig 1996). Ser490, Ser543, and Thr1073 have been identified as PKC phosphorylation sites involved in the activation of AC2 (Bol et al. 1997; Shen et al. 2012). AC5 is activated by PKCα, although it is activated more powerfully by the atypical isoform PKCζ (Kawabe et al. 1996). AC9 is activated by PKCβII in human neutrophils (Liu et al. 2014), although a recent study has demonstrated that PKCβII did not affect $G_{\beta\gamma}$- AC9 activity in human fibroblast-like COS-7 cells (Baldwin et al. 2019). Conversely, several studies reported a PKC-mediated inhibition of AC9 by novel PKC isoforms (Cumbay and Watts 2004, 2005). It has been described that PKCα-activation has no effect in forskolin-stimulated or βγ-stimulated AC4 activities, but decreases both the

$G_{\alpha s}$-stimulated and $G_{\alpha s}/G_{\beta\gamma}$ $G_{s\alpha}$-stimulated activities of this isoform (Zimmermann and Taussig 1996). Regarding the known regulation by the other PKC family, AC6 activity is inhibited by novel PKCδ and ε-mediated phosphorylation at residues Ser10, Ser568, Ser674, and Thr931 (Lai et al. 1997; Lin et al. 2002), while ethanol-induced AC7 phosphorylation is mediated by atypical PKCδ (Nelson et al. 2003).

CaMKs also differentially regulate AC activity, but it only affects few AC isoforms. CaMKIV-mediated phosphorylation at residues Ser545 and Ser553 induces AC1, but not AC8, inhibition in transfected HEK-293 cells (Wayman et al. 1996), while CaMKII-mediated phosphorylation at residue Ser-1,076 induces AC3 inhibition in neurons and transfected HEK-293 cells (Wei et al. 1996, 1998). Conversely, $G_{\alpha q}$-mediated AC9 activity is enhanced by CaMKII (Cumbay and Watts 2005), while AC9 activity is inhibited by the protein phosphatase CaN (Antoni et al. 1998).

3 Regulation of Ca^{2+} Homeostasis by cAMP Signaling

The interplay between the signaling pathways mediated by cAMP and Ca^{2+} has long been investigated. Both cAMP and Ca^{2+} are ubiquitous intracellular messengers that cooperate positively or negatively to precisely regulate a large number of cellular functions. There is a body of evidence demonstrating that cAMP- and cytosolic Ca^{2+}-dependent pathways participate jointly and cooperatively in the modulation of cell functions, such as the inotropic role of ATP in cardiomyocytes or sperm acrosome reaction upon treatment with progesterone (Gramajo-Buhler et al. 2016; Cerny et al. 2017). In addition, as discussed above, cAMP and cytosolic Ca^{2+} cascades might interact modulating each other's signaling mechanisms. Ca^{2+} either directly or indirectly might regulate the synthesis of cAMP as mentioned in the previous section, meanwhile cAMP modulates intracellular Ca^{2+} signals, through the activation of PKA, Epac, or cyclic nucleotide-activated cation channels.

Most physiological agonists mobilize intracellular Ca^{2+} by occupation of G-protein coupled receptors or tyrosine kinase receptors, which, in turn, leads to the activation of different PLC isoforms and results in the IP_3 generation. IP_3, as well as other second messengers, including NAADP and Ca^{2+} itself, is responsible for the release of Ca^{2+} from intracellular agonist-sensitive Ca^{2+} stores (Berridge et al. 2000). Since Ca^{2+} efflux from finite intracellular Ca^{2+} stores is often insufficient to fully activate and maintain cellular processes, agonists also evoke Ca^{2+} influx through PM channels, including receptor-operated, second messenger-operated, and store-operated Ca^{2+}-permeable channels (Albarran et al. 2016b).

The modulation of PLC activity by cAMP has been found to be isoform-specific. Cyclic AMP generation has been reported to stimulate PLCε activation and Ca^{2+} mobilization in N1E-115 neuroblastoma cells. The activation of PLCε was reported to be independent of PKA but mediated by the small GTPase Rap2B, which, in turn, is activated by an Epac (Schmidt et al. 2001). By contrast, activation of Epac by cAMP blocks the oxidative burst in neutrophils via inhibition of the PLCβ activity

(Cerny et al. 2017). Another regulatory mechanism of PLC function by cAMP is based on the modulation of its expression. For instance, cAMP downregulates the expression of PLCδ3 in the WI38, U373, and H1299 human cell lines (Lin et al. 2001). A role for PKA in the regulation of PLC has also been demonstrated. In polarized opossum kidney cells, occupation of dopamine receptors leads to the activation of the AC/PKA pathway, which in turn results in the activation of PLCβ and subsequent PKC-mediated attenuation of the Na^+-K^+-ATPase function (Gomes and Soares-da-Silva 2002). By contrast, PKA inhibits Gβγi-dependent PLC-β3 activity by direct phosphorylation of PLC-β3 in rabbit gastric smooth muscle cells (Nalli et al. 2014). The regulation of PLC by PKA might also be mediated by upstream mechanisms as reported in rabbit gastric smooth muscle cells, where PKA activation results in phosphorylation of the proteins GRK2 (G-protein coupled receptor kinase-2), RGS4 (regulator of G protein signaling-4), and RGS2 leading to rapid inactivation of Gαq and, subsequently, PIP_2 hydrolysis by PLC (Nalli et al. 2014; Huang et al. 2007). Altogether, these findings suggest that the effect of PKA on PLC activity seems to be isoform- or cell type-specific.

Furthermore, the IP_3 receptor function has been demonstrated to be regulated by PKA in different ways. Activation of the cAMP/PKA pathway has long been associated with phosphorylation of the IP_3 receptors and potentiation of the IP_3-evoked Ca^{2+} release from the intracellular stores (Burgess et al. 1991). While phosphorylation of types I and II IP_3 receptors increases the open probability of the channels in the presence of IP_3, the functional role of PKA-dependent phosphorylation of type III IP_3 receptor remains unclear (Soulsby and Wojcikiewicz 2007; Betzenhauser and Yule 2010; Yu et al. 2017; Soulsby et al. 2004). In prostate cancer cells, PKA induces IP_3 receptor hyperphosphorylation at Ser1716, which has been associated with Ca^{2+} leakage from the ER and resistance to androgen deprivation-induced apoptosis (Boutin et al. 2015). In addition to the regulation of IP_3 receptors by PKA, these receptors have been found to be modulated by cAMP by a mechanism that is independent of the known cAMP effectors. This non-canonical effect of cAMP has been suggested to be mediated by allosteric interaction with a low-affinity binding site in the IP_3 receptors or an associated regulatory protein that increases the apparent effectiveness of IP_3 and Ca^{2+} to evoke channel opening (Tovey et al. 2010; Taylor 2017). Konieczny et al. demonstrated a further mechanism for the regulation of IP_3 receptors by cAMP. By stimulating stably expressing type 1 human parathyroid hormone (PTH) receptor HEK293 cells with carbachol and PTH to induce IP_3 and cAMP generation, respectively, it was found that PTH, which per se was unable to mobilize intracellular Ca^{2+}, potentiates the response to carbachol. These findings indicate that cAMP recruits intracellular Ca^{2+} compartments by unmasking high-affinity IP_3 receptors (Konieczny et al. 2017). Furthermore, Epac has been reported to play an important role in Ca^{2+} signaling through the regulation of IP_3, but also ryanodine receptors, which are phosphorylated by a PKA-independent pathway that involves activation of PLC and Ca^{2+}/CaMKII (Ruiz-Hurtado et al. 2013). As described for IP_3 receptors, ryanodine receptors are targeted by cAMP by PKA-dependent phosphorylation. A role of cAMP in the phosphorylation and activation of type 2 ryanodine receptors has been reported in

pancreatic beta cells (Islam et al. 1998). Phosphorylation of ryanodine receptor 2 at Ser2030 has been reported to play an important role in the modulation of its activity during β-adrenergic stimulation (Potenza et al. 2019). Therefore, activation of the cAMP pathway plays an important role in the modulation of Ca^{2+} release from the intracellular stores.

A role for cAMP in the modulation of SERCA (sarco/endoplasmic reticulum Ca^{2+} ATPase) function has been suggested. SERCA is regulated by phospholamban, a phosphoprotein that reduces the Ca^{2+} affinity of SERCA and PKA-dependent phosphorylation of phospholamban enhances Ca^{2+} re-uptake into the ER (Akin et al. 2013). Attenuation of PKA activity leads to impaired SERCA activity in rat ventricular myocytes, which is involved in the pathogenesis of glucose-induced cardiomyopathy (Dutta et al. 2002). In addition, urotensin II has been shown to induce neonatal cardiomyocyte hypertrophy through the activation of PKA, which, in turn, results in phosphorylation of phospholamban at Ser16 and the activation of Ca^{2+} re-uptake into the ER by SERCA (Xu et al. 2017). Consistent with the effect of PKA on SERCA function in cardiomyocytes, ischemic post-conditioning attenuates Ca^{2+} uptake by SERCA at the onset of reperfusion by delaying phospholamban phosphorylation via activation of PKG and inhibition of PKA (Inserte et al. 2014).

The cAMP signaling cascade also modulates the activity of Ca^{2+} transport mechanisms across the PM, including Ca^{2+}-ATPases, exchangers, and Ca^{2+} permeable channels. It has been reported that PKA activates Ca^{2+} efflux through the Na^{+}-Ca^{2+} exchanger (NCX) in t-tubules of rat ventricular myocytes (Chase and Orchard 2011). A more recent study has revealed that while Ca^{2+} extrusion induced by NCX3 operating in the forward mode is unaffected by PKA, Ca^{2+} uptake by reverse exchange activity of NCX3 is enhanced by PKA-dependent phosphorylation of the residue Ser524 (Michel et al. 2017). Concerning the mechanism of Na^{+}-Ca^{2+} exchange, it is also worth to mention that PKA-dependent phosphorylation of the mitochondrial Na^{+}/Ca^{2+} exchanger has also been reported to enhance Ca^{2+} efflux toward the cytosol modulating mitochondrial Ca^{2+} homeostasis (Kostic et al. 2015). The PM Ca^{2+}-ATPase (PMCA) can also be regulated by phosphorylation at Ser/Thr and Tyr residues. PKA, as well as PKC, is among the protein kinases involved in the regulation of PMCA function. In mouse parotid acinar cells, Bruce et al. have reported that cAMP modulates Ca^{2+} signaling by potentiating the PMCA activity in a Ca^{2+}-dependent manner (Bruce et al. 2002). In mouse parotid acinar cells and rat parotid gland Par-C10 cells, PKA has been reported to potentiate $[Ca^{2+}]_c$ clearance by apical PMCA, which, together with the modulation of Ca^{2+} release, provides a mechanism to tightly regulate the temporal and spatial properties of the Ca^{2+} signals underneath the apical membrane and thus secretion (Baggaley et al. 2007). The regulation of PMCA by PKA has been described in different cell types, for instance, in renal proximal tubule cells the inhibitory effect of angiotensin II on Ca^{2+} extrusion through the basolateral membrane by PMCA has been reported to be impaired by angiotensin-(3,4), an angiotensin-II derived dipeptide, by a mechanism that involves activation of PKA (Axelband et al. 2012).

Probably, a major mechanism to control Ca^{2+} signals by cAMP involves the regulation of PM channels. From a physiological point of view, Ca^{2+} might enter

mammalian cell through different channels gated by electrical, chemical, and mechanical stimuli. Mechanosensitive or stretch-activated Ca^{2+} channels respond to mechanical forces along the plane of the cell membrane. Among the most relevant Ca^{2+}-permeable mechanosensitive channels in mammalian cells are the Piezo channels and several members of the polymodal transient receptor potential (TRP) family (Geng et al. 2017; Xiao and Xu 2010). Cyclic AMP has been reported to sensitize TRPV1 channels by a mechanism involving PKA-dependent phosphorylation (Rathee et al. 2002), furthermore, there is a body of evidence supporting that cAMP, via activation of Epac1, potentiates Piezo2 function (Eijkelkamp et al. 2013; Singhmar et al. 2016).

Voltage-gated Ca^{2+} channels respond to depolarized membrane potentials and are expressed in a variety of electrically excitable cells, including muscle cells, glial cells, and neurons (Hofmann et al. 2014), as well as in certain non-excitable cells, such as lymphocytes and mast cells (Stokes et al. 2004b; Yoshimaru et al. 2009) where these channels are inhibited by a mechanism involving STIM1 (Dionisio et al. 2015; Wang et al. 2010). The regulation of voltage-gated channels by the cAMP pathway has long been described. The cardiac $Ca_v1.2$ channel is a well-known target of PKA. Stimulation of β-adrenergic receptors results in enhanced whole cell current in cardiac cells (Sculptoreanu et al. 1993) by a mechanism that involves phosphorylation of the α_1 subunit (Bunemann et al. 1999). In fact, PKA-dependent phosphorylation has been reported to protect voltage-dependent Ca^{2+} channels from the inhibitory role exerted by PIP_2 (Michailidis et al. 2007). In addition to PKA, the cAMP pathway has been reported to modulate $Ca_v1.2$ channels by an Epac-dependent mechanism. In pancreatic α-cells, adrenaline-evoked enhanced Ca^{2+} influx through $Ca_v1.2$ channels and glucagon exocytosis is reduced in Epac2-deficient islets (De Marinis et al. 2010). Other Ca_v channels have been found to be regulated by the cAMP/PKA signaling cascade. Studies in *Xenopus* oocytes have demonstrated that PKA enhances Ca^{2+} influx through Cav1.3 channels by protein phosphorylation (Kim et al. 2006). By contrast, it has been reported that the incretin hormone glucagon-like peptide-1 attenuates the release of glucagon by pancreatic α-cells by elevating cAMP levels, thus leading to PKA-dependent inhibition of $Ca_v2.1$ (P/Q-type) and Cav2.2 (N-type) Ca^{2+} channels (De Marinis et al. 2010; Ramracheya et al. 2018). The findings mentioned above are consistent with studies performed in mouse presynaptic taste cells, where elevation of cAMP results in PKA-dependent Ca^{2+} influx that was found to be blocked by nifedipine, a $Ca_v1.2$ inhibitor, but insensitive to omega-agatoxin IVA and omega-conotoxin GVIA ($Ca_v2.1$ and $Ca_v2.2$ channel inhibitors, respectively) (Roberts et al. 2009).

Calcium permeable channels gated by chemical stimuli can be grouped into receptor-operated channels, second messenger-operated channels, and store-operated channels. Receptor-operated channels are ionotropic hormone receptors such as the nicotinic cholinergic, glutamate or purinergic P2X receptors. Nicotinic α4β2 and α7 receptors have been reported to be phosphorylated by different protein kinases including PKA, and this mechanism has been shown to underlie the inhibitory role of dopamine receptors on nicotinic neurotransmission in prefrontal cortical interneurons (Wecker and Rogers 2003; Komal et al. 2015). The GluR6 glutamate

receptor is phosphorylated by PKA, which results in potentiation of the glutamate response (Raymond et al. 1993). Concerning purinergic receptors, PKA is involved in the glucocorticoid-induced inhibition of P2X receptor current in mouse HT4 neuroblastoma cells (Han et al. 2005). The PKA catalytic subunit is sufficient to induce a reduction in the amplitude of $P2X_2$ receptor current in HEK293 cells stably transfected with rat $P2X_2$ receptor (Chow and Wang 1998). PKA has also been reported to modulate ATP-mediated $P2X_4$ receptor currents by a mechanism independent of protein phosphorylation but involving the trafficking of the receptor to the PM (Brown and Yule 2010). In addition to PKA, cAMP-activated Epac induces sensitization of native $P2X_3$ receptors in rat DRG neurons (Wang et al. 2007).

Finally, the cAMP signaling pathway plays a relevant role in the modulation of second-messenger and store-operated Ca^{2+} influx mechanisms. Among the most relevant second messengers-activated Ca^{2+} channels are some members of the TRP family. TRP channels serve as polymodal-regulated sensors expressed in several cell types and tissues. A number of physical and chemical stimuli, including mechanical stress (as described above in this section), temperature, voltage, pH, regulatory proteins, and chemical ligands, such as second messengers, activate these channels. Diacylglycerol has been demonstrated to activate TRPC3, TRPC6, and TRPC7 (Hofmann et al. 1999; Albarran et al. 2014; Bouron et al. 2016) and a number of TRP channels are modulated by PIP_2 (Jardin et al. 2008b; Gamper and Rohacs 2012). TRPC channels, such as TRPC1, have also been found to play a relevant role in store-operated Ca^{2+} entry (Huang et al. 2006; Jardin et al. 2008a; Desai et al. 2015; Ambudkar et al. 2017), which will be discussed in the next section. TRPC channels have been reported to be regulated by the cAMP signaling pathway. This event has been demonstrated in TRPM7, whose activity is enhanced by elevations of cytosolic cAMP levels by a mechanism that involves PKA activity and its endogenous kinase (Takezawa et al. 2004). These findings have been supported by a more recent study reporting that prostaglandin E increases TRPM7 currents in HEK293 and human glioblastoma A172 cells by a mechanism dependent on the phosphorylation of Ser1269 by PKA (Tian et al. 2018). However, these studies have been challenged by a more recent study showing that TRPM7-mediated sustained Ca^{2+} influx is abrogated upon elevation of cAMP levels through PKA-dependent phosphorylation of Ser1269 (Broertjes et al. 2019). The reasons of these discrepancies might underlie in the methodological approaches but are still unsolved. Furthermore, as mentioned above, PKA may also phosphorylate TRPV1 on Ser502, leading to sensitization of its response to a variety of stimuli; in addition, phosphorylation of Thr-370 and Ser116 by PKA in rat TRPV1 appears to reduce channel desensitization (Yao et al. 2005). TRPV2 is another substrate of PKA, which interacts with the channel through ACBD3 (Acyl CoA binding domain protein-3), an A kinase adapter protein, and enhances TRPV2-conducted Ca^{2+} entry in response to heat (Stokes et al. 2004a). Moreover, TRPP2 phosphorylation on Ser829 by PKA enhances cation transport in reconstituted apical membranes of term human syncytiotrophoblast (Cantero Mdel et al. 2015). These findings suggest that the AC/cAMP signaling pathway, via PKA-dependent phosphorylation, is involved in the regulation of a variety of TRP channels in mammalian cells.

SOCE is a major mechanism for Ca^{2+} influx in non-electrically-excitable, but also relevant in electrically-excitable cells. The cross-talk between SOCE and the cAMP signaling pathway has long been reported. Ca^{2+} influx by SOCE modulates the function of Ca^{2+}-dependent ACs, as described in Sect. 2. Reciprocally, cAMP has been found to regulate SOCE in different cell types. In human and rat platelets, cAMP-elevating agents attenuate SOCE by mechanisms involving the activation of protein tyrosine phosphatases (Rosado et al. 2001) and indirect inhibition of store depletion by PKA (Heemskerk et al. 1994), respectively. More recent studies in rat aortic smooth muscle cells have revealed that cAMP is able to reduce SOCE by activation of PKA and Epac, thus reducing the contraction of rat aorta (Cuinas et al. 2016). Recently, a cross-talk between SOCE and AC8 has been described to have a significant relevance in cell function. This mechanism and its remodeling in cancer cells will be reviewed in the next section.

4 Regulation of Store-Operated Calcium Entry by Adenylyl Cyclases

4.1 Regulation of Orai1 Function by AC8

As mentioned above, AC8 is the only AC isoform able to interact with Orai1 in the lipid rafts membrane domains so far, and this interaction is required for SOCE-induced AC8 activation (see Sect. 2). However, recent studies have demonstrated a reciprocal cross-talk between cAMP and Ca^{2+} signaling since Orai1 function is also regulated by AC8 activity (Zhang et al. 2019; Sanchez-Collado et al. 2019). The N-terminal domain of Orai1 contains three phosphorylation sites at Ser27, Ser30, and Ser34 that reduce Orai1 channel activity. The phosphorylation of Ser27 or Ser30 by PKC reduces Orai1-mediated Ca^{2+} influx (Kawasaki et al. 2010), while the phosphorylation of Ser34 by PKA has been recently associated with Orai1 Ca^{2+}-dependent inactivation (CDI) (Zhang et al. 2019). CDI, together with cytosolic Ca^{2+} clearance mainly mediated by SERCA and PMCA, is required for the regulation of $[Ca^{2+}]_c$, avoiding Ca^{2+} overload and its deleterious effects (Parekh 2017; Jardin et al. 2018a). However, it has recently been reported that CDI could also act as modulator of Ca^{2+} signaling, fine-tuning the Ca^{2+}-dependent activation of certain transcription factors under physiological conditions (Zhang et al. 2019). As it is described previously, two spatial and temporally separated mechanisms for CDI have been described; FCDI mechanism that occurs within milliseconds after Orai1 activation and requires the interaction of Ca^{2+} with residues in the Orai1 pore, and SCDI mechanism that is triggered over ten seconds after Orai1 activation and requires a global increase in $[Ca^{2+}]_c$ (Parekh 2017; Jardin et al. 2018a). Zhang et al. have revealed that AC8-mediated signaling is involved in Orai1 channel activity regulating its FCDI by a signaling pathway that also involves the participation of PKA and AKAP79. The proposed CDI model suggests that Orai1 and AC8 interact under

resting conditions and Orai1-dependent AC8 activation evokes an increase in local [cAMP] leading to PKA activation, which, in turn, promotes Orai1 phosphorylation at Ser34 in an AKAP79-dependent manner (Fig. 3) (Zhang et al. 2019). Both the Arg-rich AC8-binding domain and the three phosphorylation sites are located in Orai1α exclusive N-terminal region, which explains why Orai1α activity, but not Orai1β, is strongly reduced by CDI activation, as previously demonstrated (Desai et al. 2015; Fukushima et al. 2012). This differential regulation of both Orai1 variants by CDI has been associated with the differential activation of transcription factors. Hence, NFAT1 activation and nuclear translocation that requires a high increase in $[Ca^{2+}]_c$ is mediated by both Orai1α and Orai1β, while NFAT4 activation that requires small rises in $[Ca^{2+}]_c$ is more efficiently activated by Orai1β (Zhang et al. 2019).

The pivotal role of AC8 in Orai1-CDI activation is based on different findings: (1) AC8 constitutively interacts with Orai1α, but not with Orai1β, by using pulldown and FRET microscopy assays. (2) Orai1α-, but not Orai1β-, CDI is empowered by increments in cAMP synthesis secondary to AC8 overexpression or forskolin-induced AC activation, and by direct introduction of cAMP into the cytosol. (3) AC8 expression silencing reduces Orai1-CDI triggering. (4) The co-overexpression of an AC8 construct that lacks N-terminal Orai1-binding domain together with eYFP-STIM1 and ORAI1-CFP constructs failed to activate Orai1-CDI, but not Orai1β-CDI. (5) The co-expression of an Orai1 construct with double alanine substitutions in Arg31 and Arg33, originating a defective AC8-binding domain, with a YFP-STIM1 in Orai1-KO HEK-293 cells reduces Orai1-CDI compared with cells that overexpress wild-type Orai1 and YFP-STIM1. However, this reduction in Ora1-CDI is less than that observed in Orai1β overexpressing cells, suggesting that other mechanism, besides Orai1-AC8 binding, is involved in Orai1-CDI activation. This study also analyzed the role of other domains present in the first 63 N-terminal residues of Orai1, including a PIP_2-binding domain (Arg28, Arg29, Ser30, Arg31, Arg32, Arg33) (Calloway et al. 2011), a caveolin-binding domain (Tyr52, Pro53, Asp54, Trp55, Ile56, Gly57, Gln58, Ser59, Tyr60) (Yu et al. 2010) and the two above-mentioned PKC-phosphorylatable serine residues (Ser27 and Ser30) that negatively regulates Orai1 function (Kawasaki et al. 2010). Hence, the co-transfection of Orai1-KO HEK-293 cells with an Orai1 construct with double alanine substitutions in Tyr52 and Trp55, that generates a non-functional caveolin-binding domain impairing the localization of Orai1 in lipid rafts, also reduces Orai1-CDI, supporting that AC8-mediated Orai1-CDI activation occurs in lipid rafts domains. However, neither the PKC phosphorylation sites nor the PIP_2-binding domain is involved in Orai1-CDI activation as demonstrated by the use of an Orai1 construct with double alanine substitutions in Ser27 and Ser30 and the water-soluble PIP_2 analogue diC8-PIP_2, respectively (Zhang et al. 2019).

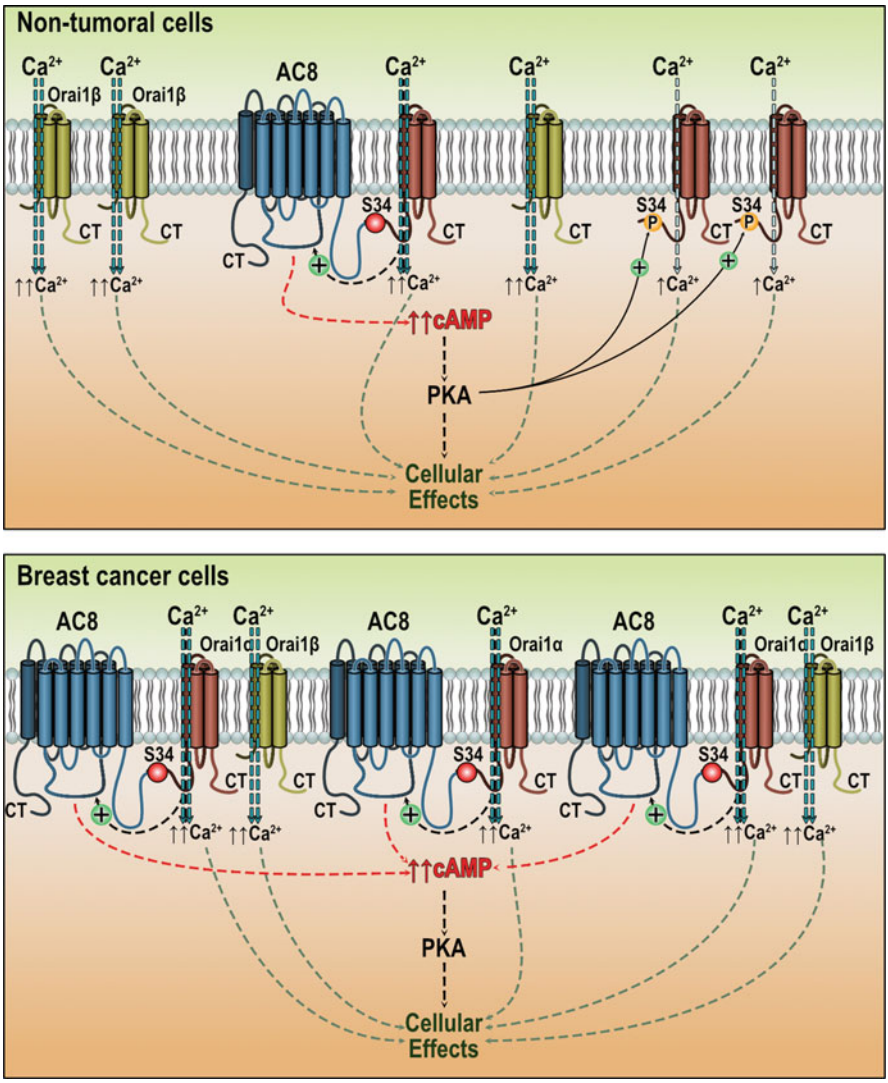

Fig. 3 Cartoon summarizing the inactivation of Orai1α mediated by PKA-dependent phosphorylation of Orai1 at Ser34 and how this mechanism is bypassed in breast cancer cells. In non-tumoral cells, AC8 interacts with Orai1α through the AC8-binding site (amino acids 26–34) located in the N-terminal region. Ca^{2+} influx via Orai1α leads to AC8 activation and the generation of cAMP, which, in turn, modulates Orai1α inactivation, via phosphorylation at Ser34, by a mechanism involving PKA. As the AC8-binding site overlaps with the PKA-phosphorylatable Ser residue, the Orai1α pool associated with AC8 is not susceptible to inactivation by this mechanism. In breast cancer cells, overexpression of AC8 enhances the pool of Orai1α associated with AC8, which impairs phosphorylation of Orai1α at Ser34 and thus Orai1α inactivation, leading to enhanced Ca^{2+} entry

4.2 *Remodeling of the Regulation of Orai1 by AC8 in Cancer Cells*

As mentioned above, cancer cells have been reported to remodel Ca^{2+} signals in order to shift the balance toward enhanced proliferation and migration while suppressing mechanism leading to apoptosis and cell death. SOCE is among the Ca^{2+} signaling events remodeled in cancer cells. Studies in patient samples and cancer cell lines have revealed a plethora of mechanisms that alter the expression and function of SOCE constituents. For instance, STIM1 expression has been reported to be overexpressed in a variety of cancer cells including glioblastoma multiforme, cervical cancer siHa and CaSki cells, colorectal carcinoma, hepatocellular carcinoma, pancreatic adenocarcinoma, or melanoma among others (Yang et al. 2013; Umemura et al. 2014; Kim et al. 2014; Liu et al. 2015; Holzmann et al. 2013; Li et al. 2013; Chen et al. 2011). In addition, Orai1 is overexpressed in a number of cancer cells including those derived from colorectal carcinoma, pancreatic adenocarcinoma, melanoma, and breast cancer (Kim et al. 2014; Holzmann et al. 2013; Motiani et al. 2010; Jardin et al. 2018b). In addition to the altered expression of STIM1 and Orai1 in cancer cells, the function of the SOCE components has been found to be remodeled by a variety of mechanisms. In some cells the predominant role of STIM1 and Orai1 in SOCE is shifted toward other isoforms, this is the case of Orai3 in luminal breast cancer cells (Motiani et al. 2010; Jardin et al. 2018c) or shared with other components that gain protagonism, such as TRPC1 in colorectal carcinoma cells (Sobradillo et al. 2014), Orai2 in leukemia cells (Diez-Bello et al. 2017), or STIM2 in luminal breast cancer cells (Jardin et al. 2018c). Furthermore, in prostate cancer cells, Orai3 overexpression favors heteromerization with Orai1 to form ARC channels in detriment of SOCE, thus leading to enhanced proliferation and apoptosis resistance (Dubois et al. 2014). In addition, in breast cancer cells, TRPC6 modulates the PM expression of Orai channels, which, in turn, is involved in the maintenance of different hallmarks of cancer (Jardin et al. 2018b). In triple negative breast cancer cells, we have recently reported a mechanism that remodels the regulation of Orai1 by AC8. As described above, in normal cells AC8 induces Orai1 inactivation in order to shape Ca^{2+} signals and fine-tune NFAT activation (Zhang et al. 2019). Luminal and triple negative breast cancer cells exhibit a high expression of AC8 and both Orai1 variants, Orai1α and Orai1β, as compared to non-tumoral breast epithelial cells. As for non-tumoral cells, the interaction with AC8 in breast cancer cells is restricted to Orai1α. In these cells we have found that while phosphorylation of Orai1α at Ser27 and Ser30 does not alter AC8-Orai1α coupling, the interaction of AC8 with the N-terminal Orai1α AC8-binding site impairs Orai1α phosphorylation at Ser27, Ser30, and Ser34 (Fig. 3) (Sanchez-Collado et al. 2019). Therefore, considering that Orai1α phosphorylation at serines-27, 30, and 34 is associated with channel inactivation (Zhang et al. 2019; Kawasaki et al. 2010) and that breast cancer cells overexpress AC8, these observations reveal that luminal and triple negative breast tumoral cells impair Orai1α inactivation by overexpressing AC8, which provides an explanation to the enhanced

Ca^{2+} influx observed in these cells as compared to non-tumoral breast epithelial cells. As a result, AC8 expression silencing attenuates TG-induced SOCE as well as Ca^{2+} entry induced by co-expression of Orai1α and the ORAI1-activating small fragment (OASF, amino acids 233–450/474) of STIM1, and, reciprocally, AC8 overexpression enhances Ca^{2+} entry induced by TG or co-expression of Orai1α and OASF (Sanchez-Collado et al. 2019). In triple negative breast cancer cells, AC8 was found to play a relevant role in the maintenance of a highly proliferative phenotype and the ability to migrate, the latter is likely mediated by a significant effect on the phosphorylation of the focal adhesion kinase, a protein with a crucial role in the formation of focal adhesions and, thus, cell migration and invasion (Wagner et al. 2008). The functional role of AC8 in breast cancer cells, which was found to be dependent on both PKA activation and enhancement of Ca^{2+} influx (Sanchez-Collado et al. 2019), points at AC8 as a potential target for therapeutic intervention in breast cancer.

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Developmental Changes in Phosphate Homeostasis

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Contents

Abstract Phosphate is a multivalent ion critical for a variety of physiological functions including bone formation, which occurs rapidly in the developing infant. In order to ensure maximal bone mineralization, young animals must maintain a positive phosphate balance. To accomplish this, intestinal absorption and renal phosphate reabsorption are greater in suckling and young animals relative to adults. This review discusses the known intestinal and renal adaptations that occur in young animals in order to achieve a positive phosphate balance. Additionally, we discuss

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the ontogenic changes in phosphotropic endocrine signalling as it pertains to intestinal and renal phosphate handling, including several endocrine factors not always considered in the traditional dogma of phosphotropic endocrine signalling, such as growth hormone, triiodothyronine, and glucocorticoids. Finally, a proposed model of how these factors may contribute to achieving a positive phosphate balance during development is proposed.

Keywords Development · Phosphate · Phosphorus · Postnatal

Abbreviations

AA	Arachidonic acid
ALP	Alkaline phosphatase
BBMV	Brush-border membrane vesicles
CVD	Cardiovascular disease
FGF23	Fibroblast growth factor 23
GH	Growth hormone
NaPiIIa	Sodium-phosphate cotransporter isoform IIa
NaPiIIb	Sodium-phosphate cotransporter isoform IIb
NaPiIIc	Sodium-phosphate cotransporter isoform IIc
NHE3	Sodium hydrogen exchanger isoform III
Pi	Phosphate
PLA_2	Phospholipase A2
PTH	Parathyroid hormone
T_3	Triiodothyronine

1 Introduction

Phosphate (Pi) is critical for a myriad of physiological functions including cell signalling, cell metabolism, as a constituent of the phospholipid bilayer as well as nucleic acids and in the maintenance of bone integrity (Penido and Alon 2012; Peacock 2020). In bone, Pi and calcium form a hydroxyapatite salt ($(Ca)_{10}(PO_4)_6(OH)_2$) which is interspersed in the extracellular matrix of bone tissue (Bhadada and Rao 2020). In blood, the concentration of phosphate is maintained within a narrow range which varies with age, and in adults is approximately 0.9–1.5 mM. The highest serum Pi concentration is observed in infants and it decreases with age to typical adult values (Greenberg et al. 1960). Females tend to display higher serum Pi than males (Zhang et al. 2014). This difference is particularly pronounced approaching and following menopause, which may be in part due to the decrease in oestrogen which has a phosphaturic effect (Zhang et al. 2014; Faroqui et al. 2008). The concentration of intracellular Pi greatly exceeds that of serum by approximately 100-fold (Penido and Alon 2012). However, this does not reflect total intracellular inorganic Pi content as inorganic Pi is found in intracellular

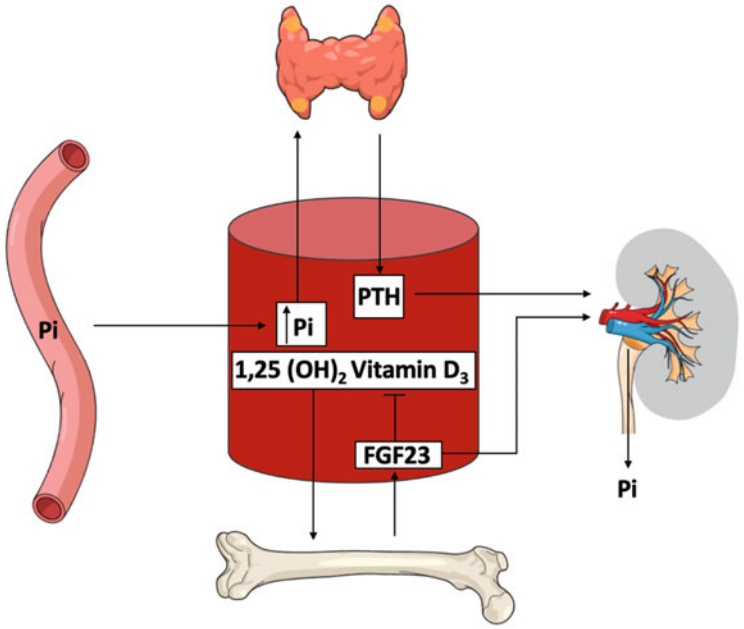

Fig. 1 Endocrine modulation of phosphate homeostasis. Phosphate is absorbed from the small intestine into the systemic circulation. An increase in serum Pi elicits secretion of PTH from the parathyroid gland. PTH increases the synthesis of 1,25 $(OH)_2$ Vitamin D_3 in the kidney. Increased serum 1,25 $(OH)_2$ Vitamin D_3 stimulates secretion of FGF23 above baseline levels from osteoblasts and osteocytes in the bone. An elevation in serum 1,25 $(OH)_2$ Vitamin D_3 also elicits increased Pi absorption from the intestine. The aforementioned increases in serum [PTH] and [FGF23] decreases renal Pi reabsorption; increasing urinary Pi excretion. Consequently, serum [Pi] decreases towards the normal physiological range

molecules such as nucleoside triphosphates and cyclic nucleoside monophosphates. Phosphate-dependent bone formation is particularly important early in life, as neonates and infants experience rapid bone formation with growth (Sanders et al. 2017). Phosphate homeostasis is maintained by a delicate balance between intestinal absorption, deposition to and resorption from bone, renal reabsorption and urinary excretion. Perturbations in one or more of these sites of regulation can result in disease including hypophosphatemic rickets, osteoporosis and osteomalacia (Bitzan and Goodyer 2019; Francis and Selby 1997; Lee and Cho 2015; Imel 2020; Marcucci and Brandi 2020; Ito and Fukumoto 2020).

Phosphate homeostasis is subject to hormonal control at all of the aforementioned regulatory points (Fig. 1). For instance, in response to an increase in serum Pi, parathyroid hormone (PTH) is released from the parathyroid gland into the blood (Almaden et al. 1996; Almaden et al. 1998; Centeno et al. 2019; Kritmetapak and Kumar 2019). Circulating PTH increases bone resorption by liberating Pi from hydroxyapatite. It also has a direct phosphaturic effect via inhibiting Pi reabsorption from the renal proximal tubule (Lee et al. 2017). In addition, PTH indirectly

enhances intestinal Pi absorption by increasing the expression of 1α-hydroxylase, an enzyme that catalyses the production of 1,25 $(OH)_2$ Vitamin D_3 (also known as calcitriol). 1,25 $(OH)_2$ Vitamin D_3 increases the expression and activity of the predominant intestinal Na^+-dependent Pi cotransporter and consequently intestinal Pi absorption (Xu et al. 2002; Yagci et al. 1992; Katai et al. 1999). Despite enhanced intestinal Pi absorption, the net effect of PTH secretion is a slight decrease in serum Pi levels secondary to renal loss of Pi. Parathyroid hormone also has an indirect but noteworthy phosphaturic effect, both directly and indirectly. PTH stimulates the production of 1,25 $(OH)_2$ Vitamin D_3 which is a stimulus for secretion of FGF23 from osteocytes and osteoblasts (Kaneko et al. 2015). Fibroblast growth factor 23 (FGF23) has a pronounced phosphaturic effect by reducing Pi reabsorption from the proximal tubule (Gattineni et al. 2009). FGF23 acts by binding to the FGF receptor and its coreceptor alpha-klotho which enhances its binding (Urakawa et al. 2006). Interestingly, a cleaved soluble form of klotho also induces phosphaturia independent of FGF23 (Hu et al. 2010). Importantly FGF23 increases in response to prolonged (days) high Pi consumption but does not respond to transient increases in serum Pi (Ferrari et al. 2005; Layunta et al. 2019).

1.1 Transcellular Intestinal Phosphate Absorption/Renal Phosphate Reabsorption

Dietary free Pi, hereafter referred to simply as Pi, is a common food additive and a common constituent of the Western diet (Calvo et al. 2014). Pi, as it is not covalently linked to amino acids in protein or another organic molecule, does not require enzymatic cleavage to be liberated and absorbed. Inorganic Pi can thus be freely absorbed from the small intestine. In contrast, phosphorus bound to carbon-containing molecules such as polypeptides, lipids, and nucleic acids, henceforth referred to as P, must be enzymatically cleaved prior to absorption. This is typically accomplished by alkaline phosphatase (ALP), a hydrolase that catalyses the nonspecific cleavage of phosphomonoester bonds. Once liberated from an organic molecule, Pi flux across intestinal and renal epithelia can occur via either the transcellular or paracellular pathway (Fig. 2) (Saurette and Alexander 2019). The transcellular pathway is characterized by entry of Pi into the epithelial cell via movement across the apical membrane, followed by exit across the opposing basolateral membrane (N.B. there is no evidence of transcellular phosphate secretion, i.e. basolateral to apical movement). Given that transcellular Pi flux in both the small intestine and the kidney depends on the activity of secondary active transporters, the transcellular pathway is saturable. In the small intestine, transcellular Pi absorption is mediated at the apical membrane by Na^+-dependent secondary active transport (Fig. 3). Specifically, the type-II Na^+-Pi cotransporter NaPiIIb (gene name human/mouse – *SLC34A2*/*Slc34a2*) transports Na^+ and Pi in a 3:1 stoichiometric ratio and is recognized as the principal mediator contributing apical Pi uptake in the

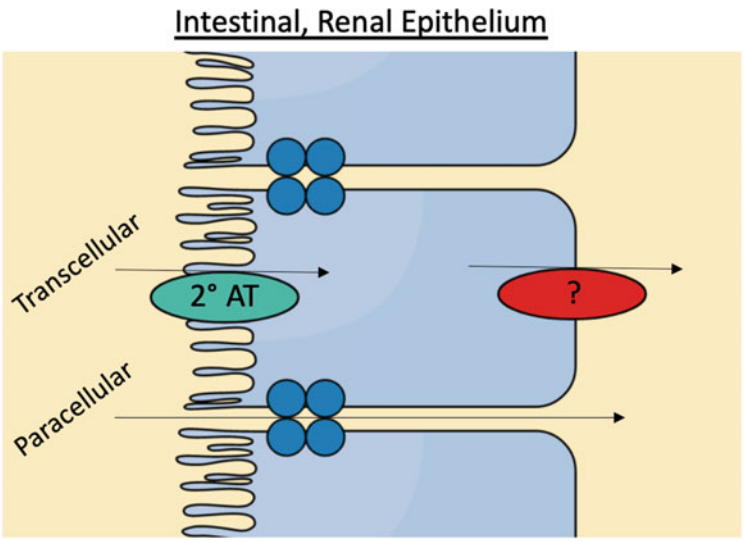

Fig. 2 The transcellular and paracellular routes of epithelial ion flux. In contrast to the inherently saturable nature of transcellular ion movement, paracellular flux is governed by the transepithelial electrochemical gradient. Paracellular ion flux is largely regulated by claudins (indicated by blue circles) expressed in the epithelial tight junction. With respect to transcellular Pi absorption and reabsorption, both processes begin with apical secondary active transport (2° AT), coupled to sodium influx. The identity of the basolateral mechanism(s) of Pi extrusion in the intestine and kidney have not been clearly delineated (indicated by red question mark on basolateral membrane)

small intestine (Hilfiker et al. 1998; Sabbagh et al. 2011). However, the overall contribution of NaPiIIb to the maintenance of phosphate homeostasis in humans is questionable, as patients with biallelic mutations in this gene have pulmonary alveolar microlithiasis and do not display a phosphate phenotype (Corut et al. 2006; Huqun et al. 2007). Moreover, clinical trials employing a NaPiIIb inhibitor were unable to lower plasma Pi in humans (Larsson et al. 2018). Also expressed in the small intestine are the type III Na^+-Pi cotransporters Pit1 (gene name human/mouse – *SLC20A1*/*Slc20a1*) and Pit2 (gene name human/mouse – *SLC20A2*/*Slc20a2*) that transport Na^+ and Pi in a 2:1 stoichiometric ratio. However, the contribution of Pit1/2 to apical Pi uptake in the intestine is likely minimal given negligible measurable sodium coupled Pi uptake in the NaPiIIb intestinal knockout mice (Sabbagh et al. 2011; Pastor-Arroyo et al. 2020). Further, Pit2 is certainly dispensable to maintaining global Pi homeostasis under normal dietary Pi conditions as Pit2 null mice have unaltered Pit homeostasis on this diet (Pastor-Arroyo et al. 2020). Pit1 and 2 have also been proposed to act as extracellular Pi sensors (Bon et al. 2018). The basolateral mechanism of Pi extrusion in the intestine has not been identified at the molecular level (Cross et al. 1990).

In the kidney, the vast majority, if not all of the Pi reabsorption occurs in the proximal tubule (Fig. 4) (Pastoriza-Muñoz et al. 1978; Biber et al. 2013). Chiefly responsible for Pi reabsorption in this nephron segment are the apically expressed

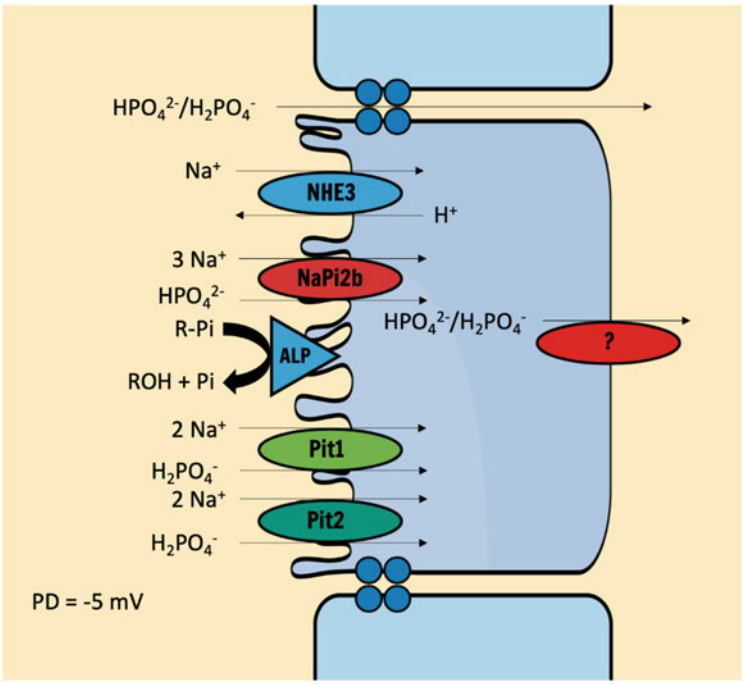

Fig. 3 Intestinal phosphate absorption. Alkaline phosphatase (ALP) liberates P from organic molecules as Pi. This is accomplished by the hydrolysis reaction depicted above. Phosphate attached to a generic organic 'R' group is cleaved off by the hydrolase ALP, resulting in R-OH and H-Pi. The free Pi can then be absorbed via the Na^+-dependent secondary active transporters NaPiIIb, Pit1 or Pit2. NaPiIIb preferentially transports the divalent Pi species (HPO_4^{2-}), whereas Pit1/2 preferentially transport the monovalent Pi species ($H_2PO_4^-$); conferring versatility of intestinal Pi transport with respect to luminal pH. NHE3 plays a role in paracellular Pi absorption. Little is known regarding the basolateral mechanism of Pi extrusion. PD = potential difference, which is approximately −5 mV in the small intestine

type-II Na^+-Pi cotransporters NaPiIIa (*SLC34A1*/*Slc34a1*) and NaPiIIc (*SLC34A3*/*Slc34a3*). NaPiIIc is not electrogenic, co-transporting 2 Na^+:1 Pi (Segawa et al. 2002). Individuals with mutations in SLC34A3 present with hereditary hypophosphatemic rickets with hypercalciuria during childhood (Lorenz-Depiereux et al. 2006), which appears to be a lifelong disease suggesting an important role for NaPiIIc across all developmental stages. In contrast NaPiIIa takes up Na^+ and Pi in a 3:1 ratio and is therefore electrogenic (Forster et al. 1999). Both isoforms contribute significantly to Pi homeostasis in humans. Consistent with this, loss of function mutations in both transporters can cause hypophosphatemia due to renal Pi wasting (Bergwitz et al. 2006; Schlingmann et al. 2016; Schönauer et al. 2019). Patients with SLC34A1 mutations, however, can display a range of phenotypes including a renal Pi wasting syndrome similar to SLC34A3 mutations, infantile neonatal hypercalcemia or simply kidney stones (Schlingmann et al. 2016; Rajagopal et al. 2014; Braun et al. 2016). However, the clinical manifestations tend to improve with age

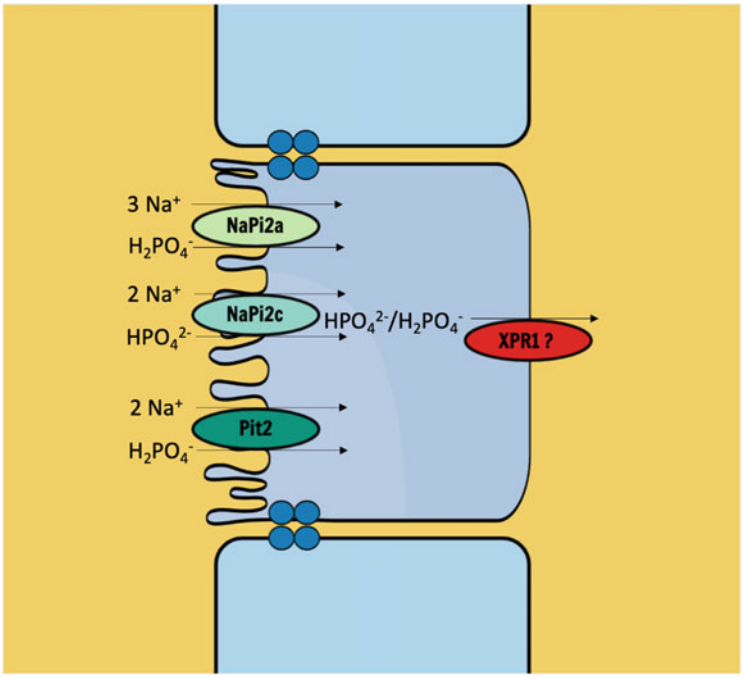

Fig. 4 Renal phosphate reabsorption. Apical NaPiIIa, NaPiIIc preferentially takes up inorganic Pi in the divalent form, whereas Pit2 shows preference for the monovalent form. The mechanism of basolateral Pi extrusion is not understood. There is no data supporting paracellular flux across the renal tubule

highlighting the importance of NaPiIIa early in life. Also expressed in the proximal tubule is Pit2 which, akin to its role in the small intestine, contributes less to apical Pi uptake, in adults at least (Villa-Bellosta et al. 2009; Bon et al. 2018). Xenotropic and polytropic retrovirus receptor 1 (XPR1) is expressed in the renal proximal tubule, where recent findings are consistent with a role in basolateral Pi efflux (Ansermet et al. 2017). This finding, however, should be interpreted tentatively as XPR1 has not been localized to the basolateral membrane in the proximal tubule, nor have heterozygous expression studies demonstrated the ability for XPR1 to mediate Pi flux. Recent efforts identified NaPiIIb in the thick ascending limb, but its role in renal Pi homeostasis is unclear at present (Motta et al. 2020). For a recent review of phosphate transporters please see (Levi et al. 2019).

1.2 Paracellular Phosphate Flux

Paracellular flux is the movement of an ion across an epithelium between epithelial cells (Fig. 1). The paracellular pathway is unsaturable and is driven by the

transepithelial electrochemical gradient (King et al. 2018; Knöpfel et al. 2019). Thus, depending on the electrochemical gradient Pi could either be absorbed (i.e. apical to basolateral) or secreted (i.e. basolateral to apical) via the paracellular pathway. The concentration of Pi in the intestinal lumen of rodents has been reported to be in the millimolar range (Ikuta et al. 2018; Marks et al. 2015). Assuming similar luminal Pi concentrations exist in humans, a chemical gradient would exist between the lumen and the blood favouring apical-to-basolateral paracellular Pi movement. Further, the mammalian small intestine displays a negative transepithelial potential difference (lumen negative), which would also favour apical-to-basolateral flux of negatively charged ions such as Pi, especially at an alkaline pH that favours the divalent species (Gustke et al. 1981). Taken together, the electrochemical gradient for Pi across the intestine strongly favours apical-to-basolateral absorption of inorganic Pi via the paracellular pathway, which may be more pronounced when consuming a western diet high in inorganic Pi. In contrast, there does not appear to be significant paracellular flux contributing to renal Pi reabsorption (Kaufman and Hamburger 1987; Edwards and Bonny 2018).

The paracellular movement of an ion requires a permeable pore. Claudins are a family of four pass transmembrane proteins expressed in the tight junctions of epithelia including the small intestine and the renal epithelium (Günzel and Yu 2013). As hetero- or homo-tetramers, claudins form pores which confer ion selectivity to epithelia (Piontek et al. 2020). The sodium-hydrogen-exchanger isoform III (NHE3) is apically expressed in the small bowel (Fig. 3) and appears to regulate intestinal Pi absorption as its pharmacological inhibition reduces paracellular Pi permeability and intestinal Pi absorption (King et al. 2018). While the roles of the Na^+-Pi cotransporters in Pi homeostasis have been largely established (Biber et al. 2013), the contribution of paracellular Pi movement to whole-body Pi status remains largely unexplored, as does the contribution of paracellular Pi absorption to maintaining a positive Pi balance throughout development. This article therefore focuses largely on transcellular mechanisms by which Pi homeostasis is maintained. Specifically, we cover the current body of literature characterizing the changes in intestinal and renal Pi handling throughout mammalian postnatal development. Included in the discussion is a review of the developmental hormonal changes influencing Pi homeostasis. Data from a number of animal models are included in this review. The reader should consider that physiological differences exist between humans and the models discussed, especially rodents which are multiparous. Thus, findings from nonhuman mammalian models should be interpreted with these considerations. We have chosen to include results from nonhuman models as these studies are often not feasible to conduct on humans for technical and/or ethical reasons and we believe they contribute insight that is likely translatable.

2 Ontogeny of Intestinal Phosphate Absorption

Given the need to rapidly mineralize bone as a neonate, it is not surprising that functional adaptations exist to optimize Pi absorption from the small intestinal lumen. These adaptive mechanisms diminish as the neonate matures into adulthood and peak bone mineralization is achieved. Recent work revealed greater ^{33}P absorption from the jejunum, ex vivo, of 10-week-old (i.e. not fully grown) Sprague-Dawley rats relative to 20- or 30-week-old (fully grown) animals (Vorland et al. 2018). Corroborating this, *Slc34a2* mRNA abundance was significantly greater in the duodenum and jejunum at 10 weeks of age relative to 20 weeks of age (Vorland et al. 2018). Further, Borowitz and Granrud found that in New Zealand White Rabbits, duodenal brush-border membrane vesicles (BBMV) from 2- to 4-week-old rabbits (preweaning age) displayed higher Na^+-dependent Pi uptake than 6- or 12-week-old animals, consistent with greater transcellular Pi uptake in vivo at a younger age (Borowitz and Granrud 1992). This study also demonstrated that at any given Pi concentration, Na^+-dependent Pi uptake was higher in duodenal BBMV vesicles derived from 2-week old rather than older animals (Borowitz and Granrud 1992). Finally, they observed the highest ALP activity in small intestine BBMV of young rabbits, which decreased with age. The in situ and ex vivo Pi flux data is consistent with enhanced transcellular intestinal Pi uptake in the neonate, which decreases with age. The high intestinal ALP activity in the infant described by Borowitz and Granrud also supports this. Increased ALP enzyme activity would enable the liberation and subsequent absorption of Pi found in organic sources, such as casein in milk. It is noteworthy that enterocyte turnover in the small intestine is much lower in young animals in comparison to adults (Smith and Jarvis 1978). Thus, increased intestinal ALP activity observed in younger animals may reflect longer enterocyte presence in the small intestine accommodating increased ALP expression and subsequent activity. Taken together the data is consistent with young animals having an increased capacity for transcellular Pi absorption from the small intestine relative to adults.

The relative importance of paracellular versus transcellular intestinal Pi absorption, particularly in the young, is unclear. Pi permeability in the jejunum and ileum is significantly greater at pH = 6 than pH = 8.4 (Knöpfel et al. 2019). The human duodenal pH is approximately 6.6, which increases to 7.5 at the terminal ileum, consistent with Pi permeability being highest in the most acidic segment, i.e. the duodenum (Evans et al. 1988). However, the brief sojourn time of dietary Pi in the duodenum compounded with the rate-limiting step of ALP liberation of dietary P from organic sources likely limits the contribution of the duodenum to overall Pi absorption (Duflos et al. 1995). This phenomenon may be particularly relevant in the infant, given that most, if not all of the Pi in breastmilk is organic; i.e. found in whey, casein and phospholipids (Gridneva et al. 2018; Suzuki et al. 1991; Lönnerdal et al. 2017). In the case of breastmilk, the protein-bound P component would need to be enzymatically liberated prior to absorption. By the time this occurs the previously bound Pi may be in a more distal segment given the brief sojourn time of the

duodenum. The conceivably low luminal [Pi] contributed by enzymatic cleavage may necessitate secondary active transport and thus may be absorbed in a transcellular fashion. Unfortunately, the precise percentage breakdown between free Pi and bound P in mammalian breastmilk has not been established. By the stated rationale, however, if the majority of phosphorus content in breastmilk exists as bound P, the duodenum may not be a site of high Pi absorption during suckling. Duodenal Pi absorption may become more important after weaning from breastmilk to a diet containing free ionized Pi, which is freely absorbed. Furthermore, a post-weaning diet higher in free Pi likely contributes a large electrochemical gradient for Pi across the duodenum, which combined with an acidic luminal pH, would favour paracellular Pi absorption from the proximal small bowel. As the pH progressively increases in the distal small intestine and as the luminal sojourn time increases, Pi absorption in the jejunum and ileum may become a balance between transcellular and paracellular flux.

3 Ontogeny of Renal Phosphate Handling

Renal tubular Pi reabsorption is greater early in life compared to older animals (Bistarakis et al. 1986; Kaskel et al. 1988; Neiberger et al. 1989). A 1988 in situ study demonstrated functional differences between juvenile and adult guinea pigs with respect to renal Pi handling (Kaskel et al. 1988). Proximal tubular micropuncture experiments found that Pi reabsorption (normalized to GFR) and fractional Pi reabsorption (i.e. the percentage of filtered load) significantly decreases from 1 week (preweaning) to 7 weeks (post-weaning) of age. Similarly, in vitro studies employing renal cortical BBMVs from juvenile (3–14 days old, i.e. suckling) guinea pigs had a significantly higher rate of Na^+-dependent Pi uptake than BBMVs derived from older (>57 days old, i.e. post-weaning) animals (Neiberger et al. 1989). This work also revealed that the V_{max} of Na^+-Pi uptake in juvenile-derived kidney BBMV was significantly greater than that of adults. These findings are consistent with increased reabsorption of Pi in the neonatal proximal tubule relative to the adult. Of note, the V_{max} of Na^+-Pi cotransport in BBMVs isolated from adult kidneys is inversely correlated to dietary Pi content, whereas juvenile animals did not show the same capacity to regulate Na^+-Pi cotransport in response to changes in dietary Pi (Neiberger et al. 1989). Consistent with this, serum Pi significantly increases in young animals when given an oral gavage of inorganic Pi (Neiberger et al. 1989). A failure to attenuate tubular Pi reabsorption in response to increased dietary Pi in juvenile animals likely also contributes to maintaining a positive Pi balance while suckling. Evidence supporting enhanced tubular Pi reabsorption in neonates has also been found in humans. The urinary fractional excretion of Pi increases between 3 and 6 months of age (Bistarakis et al. 1986). However, the mechanism conferring increased renal Pi reabsorption in suckling mammals is unclear (Segawa et al. 2002). Western Blot analysis of kidney BBMV from suckling, weaning and adult rats found the lowest expression of NaPiIIc in animals that were suckling, with significantly

higher NaPiIIc expression in weaning rats relative to the adult. Increased renal Pi reabsorption in suckling animals, despite lower NaPiIIc expression, may be secondary to greater abundance or apical membrane expression of NaPiIIa or because of increased Pit1 or 2 activity. Regardless of the mechanism, the data strongly supports increased proximal tubule Pi reabsorption in young mammals compared to adults. This increased renal Pi reabsorption likely contributes to a positive Pi balance necessary for optimal growth.

4 Ontogeny of Hormonal Factors Directing Intestinal and Renal Phosphate Handling

PTH Developmental alterations in hormonal signalling likely contribute to the observed differences with respect to renal and intestinal Pi handling in younger animals. PTH is a central hormone fundamental to Pi homeostasis. The ability for PTH to inhibit proximal tubular Pi reabsorption in the kidney changes with mammalian development (Johnson and Spitzer 1986). In contrast to the well-recognized effect of PTH to enhance renal Ca^{2+}-reabsorption, which is present in the neonate, PTH-mediated phosphaturia is not present in the newborn and appears to develop in older animals (Johnson and Spitzer 1986). A potential mechanism for this phenomenon is reduced phospholipase A_2 (PLA_2) activity in the neonatal proximal tubule. Of note, PLA_2 is a downstream signalling molecule after activation of the PTH receptor, which produces arachidonic acid (AA) via hydrolysis of phospholipid substrates (Friedlander and Amiel 1994). Sheu et al. (1997) found that ^{14}C-AA production by liposomes purified from the adult rabbit kidney significantly exceeded that of the juvenile (Sheu et al. 1997). The authors interpreted increased ^{14}C-AA production as a hallmark of elevated PLA_2 activity in adult proximal tubule. Inhibition of Na^+/Pi transport by AA metabolites has been demonstrated in opossum kidney (OK) cells, a common proximal tubular cell model (Silverstein et al. 1999). The absence of PTH-induced phosphaturia in the neonate could therefore be secondary to reduced PLA_2 activity in the neonatal proximal tubule, resulting in lower intracellular [AA], and thus reduced or absent inhibition of apical Na^+/Pi cotransport, thereby limiting phosphaturia. Parathyroid hormone-mediated Ca^{2+} reabsorption combined with the absence of PTH-induced phosphaturia in the neonate likely enhances the retention of Ca^{2+} and Pi so as to optimize skeletal mineralization during early development (Linarelli 1972).

FGF23 Another hormone central to Pi homeostasis is the phosphatonin, FGF23. This hormone also inhibits proximal tubule phosphate reabsorption but in contrast to PTH, inhibits rather than stimulates calcitriol synthesis. FGF23 levels nearly double between 5 days and 3 months of age. This rapid increase is followed by a gradual decline to levels at 1 year of age which are comparable to adult values (Braithwaite et al. 2016; Schoppet et al. 2012). Low serum FGF23 levels in the immediate

postnatal period likely also contribute to an overall positive Pi balance early in life by minimizing phosphaturia.

Calcitriol The production and handling of 1,25 $(OH)_2$ vitamin D_3/calcitriol changes throughout mammalian development. Serum calcitriol increases rapidly following birth, followed by a marked decline into adulthood (Ross and Dorsey 1991). Consistent with this, the production rate of calcitriol declines with age. We are only aware that the ontogeny of 1,25 $(OH)_2$ Vitamin D_3 production has been studied in sheep, however, it is plausible that heightened neonatal calcitriol production occurs ubiquitously in mammals. Elevated calcitriol levels in the neonate may contribute to the increased intestinal Pi absorption observed. In addition to altered 1,25 $(OH)_2$ Vitamin D_3 levels across development, calcitriol signalling in the small intestine undergoes developmental change. The administration of 1,25 $(OH)_2$ Vitamin D_3 induces similar changes in magnitude of Na^+-dependent Pi uptake in small intestine BBMV in both juvenile and adult rats. However, the fold-increase in Slc34a2 (gene encoding NaPiIIb) mRNA abundance in young animals is significantly greater than that of adults, which may reflect greater NaPiIIb abundance if protein expression similarly increases (Xu et al. 2002). Post-transcriptional regulation of NaPiIIb by calcitriol is possible given its regulation of other target genes at this level (Moor et al. 2018). While functional response to 1,25 $(OH)_2$ Vitamin D_3 administration with respect to Na^+-dependent Pi absorption is similar between juvenile and adult mammals, the mechanisms underpinning this response differ between age groups. Since the BBMV technique examines predominately the transcellular pathway, the results of the Xu et al. suggest that 1,25 $(OH)_2$ Vitamin D_3 may increase Slc20a1 and/or Slc20a2 expression in the small intestine in adults (Keasey et al. 2016). However, since vitamin D is a transcriptional regulator, it is also possible that calcitriol regulates the transcription of claudins mediating paracellular Pi flux in addition to regulating NaPiIIb. Consistent with this possibility, claudin-2 and -12, two calcium permeable claudins are upregulated in the intestine in response to vitamin D (Fujita et al. 2008; Zhang et al. 2015). Thus, the functional distinction in Pi absorption between young and adult animals could be due to a combination of increased capacity for paracellular Pi absorption and increased NaPiIIb expression in the neonate.

GH In addition to the roles of PTH and calcitriol in contributing to a positive Pi balance in young animals, growth hormone (GH) also displays phosphotropic properties that may contribute to a positive Pi balance early in life. The administration of an antagonist to GH-releasing factor in juvenile (aged 4–5 weeks) rats significantly increased renal fractional Pi excretion (Mulroney et al. 1989). As GH levels in rodents peak in the first week of life, high serum GH in the neonate may contribute to the elevated Pi reabsorption observed in the kidneys of neonates (Toriz et al. 2019).

Glucocorticoids May also contribute to renal mediated regulation of Pi homeostasis. The incubation of renal BBMVs with dexamethasone is associated with a significant decrease in the V_{max} of Na^+-Pi uptake (Levi et al. 1995). Additionally,

dexamethasone also decreases mRNA and protein abundance of a NaPiII transporter (N.B. this work was performed prior to the differentiation between the two renal NaPiII isoforms; NaPiIIa and c). Corticosterone, the predominant rodent glucocorticoid, has low serum levels until postnatal day 12 in rats, when it increases dramatically to a maximum at 24 hrs of life (Henning 1978). Given data supporting a role for glucocorticoids mediating phosphaturia, low glucocorticoid levels early in life likely also limit renal phosphate excretion thereby contributing to a positive Pi balance.

Triiodothyronine (T_3) Triiodothyronine (T_3) is another phosphotropic factor that is not commonly thought of in this regard. Exogenous administration of T_3 to suckling Sprague-Dawley rats was associated with several physiological alterations consistent with enhanced renal Pi reabsorption (Euzet et al. 1995). The T_3-treated animals displayed a significant decrease in fractional renal excretion of Pi compared to controls. Animals receiving T_3 also had significantly higher NaPiII (NB – this work was performed pre-distinction between renal NaPiII isoforms) protein abundance and elevated Pi uptake into renal cortical BBMVs relative to vehicle-administered rats. Given the marked postnatal increase in T_3 observed in Sprague-Dawley rats (Chanoine et al. 1993), elevated serum T_3 levels may contribute to a positive Pi balance in young animals. Of note, the increase in renal BBMV Pi uptake as well as the increase in NaPiII expression in response to exogenous T_3 are blunted in old (24 month) Wistar rats compared to young (3 month) rats (Alcalde et al. 1999). While a 3-month old rat is certainly not neonatal, this finding nevertheless supports the possibility that T_3 may contribute to greater Pi-retention in younger animals.

5 A Proposed General Model: Intestinal and Renal Ontogeny of Phosphate Handling

Given the abundance of evidence, it is reasonable to conclude that increased intestinal NaPiIIb expression in the neonate likely contributes to a positive Pi balance. Increased neonatal 1,25 $(OH)_2$ Vitamin D_3 production in the neonate, which would result in enhanced intestinal Pi absorption, also likely contributes to a positive Pi balance. In the young mammal, the mechanism for 1,25 $(OH)_2$ Vitamin D_3-mediated increase in Pi absorption appears to be secondary to an increase in intestinal NaPiIIb expression whereas the mechanism in the adult is less clear. While certainly increased NaPiIIb expression contributes, the possibility that 1,25 $(OH)_2$ Vitamin D_3 also increases Pit1 and/or Pit2 expression in the adult which in turn would confer increased Na^+-Pi transport cannot be dismissed. The route of intestinal Pi absorption may change from pre- to post-weaning as the dietary Pi source changes from largely bound P to a combination of P and Pi. The rate-limiting step of intestinal ALP liberating Pi from organic molecules may generate a low luminal concentration of free Pi, necessitating the use of secondary active transport (i.e. NaPiIIb or Pit1,2) to absorb Pi derived from organic sources. The consumption

of free ionized Pi post-weaning, however, will generate a high luminal Pi concentration resulting in an electrochemical gradient sufficient to drive paracellular Pi absorption. With the exception of the role of ALP, this mechanism has been previously proposed (Knöpfel et al. 2019). Thus, transcellular absorption may predominate in the preweaning period, whereas paracellular absorption may take on a larger role post-weaning for persons consuming a western diet. Furthermore, the relative importance of different intestinal segments to Pi absorption may change across development. As children begin to consume ionized dietary Pi, the duodenum may take on a more significant role in intestinal Pi absorption; whereas the net contribution of the jejunum and ileum to Pi absorption may decrease.

In addition to the intestine, the neonatal kidney also contributes to a positive Pi balance through functional adaptations resulting in enhanced Pi reabsorption. Renal Pi excretion is lowest in the neonate. Given that NaPiIIc expression is at a lifetime low in suckling animals, increased abundance of other Na^+-Pi cotransporters in the kidney may contribute the high Pi reabsorption observed in the neonate. Increased basolateral XPR1 expression in the proximal tubule may play an additional role in conferring elevated Pi reabsorption in the neonate (Ansermet et al. 2017). Finally, the absence of PTH-directed phosphaturia in the neonate likely also contributes to an overall positive Pi balance.

6 Inorganic Phosphate: Its High Consumption in the Western World and Potential Ramifications for the Infant

Phosphate is a common constituent of the Western diet and it is much more completely absorbed from the intestinal lumen than its organic counterpart (Noori et al. 2010). The high bioavailability of free Pi presents a potential public health issue, as elevated serum Pi is associated with renal and vascular damage as well as with increased all-cause mortality (Hong et al. 2015; Tonelli et al. 2005). It has been noted that this latter phenomenon occurs even within the normal physiological serum Pi range (Tonelli et al. 2005). The exact mechanism leading to cardiovascular complications in persons with elevated serum Pi is unclear. However, one possibility is increased FGF23 secretion in order to lower serum Pi by inducing phosphaturia. FGF23 induces Pi-dependent vascular calcification in vitro and is independently associated with aortic calcification in humans (Jimbo et al. 2014; Schoppet et al. 2012). This phenomenon is of concern when consuming a 'Western Diet' given the high consumption of preservative-containing foods of which free Pi is a component. It is thus not unreasonable to postulate that the consumption of ionized Pi is associated with chronically elevated FGF23, which in turn increases the risk of developing cardiovascular disease (CVD). If this association, i.e. increased CVD risk in persons with higher plasma Pi, can be extrapolated to infants it suggests formulae is a risk for CVD, since infants consuming baby formula display elevated

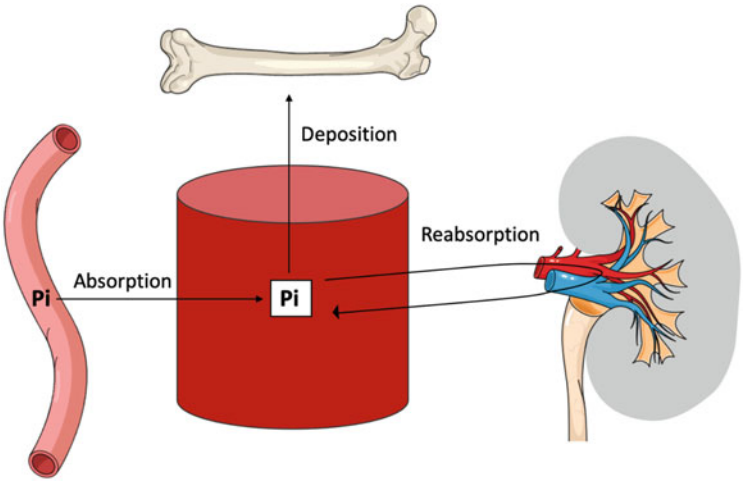

Fig. 5 Proposed general adaptations of the juvenile mammal to establish positive Pi balance. The juvenile mammal possesses increased absorption and reabsorption of Pi in the small bowel and kidney, respectively. This establishes a sufficiently positive Pi balance such that rapid bone deposition during growth may occur

serum Pi compared to breast fed infants (Greer 1989). For a pictorial representation of the general adaptations of the juvenile mammal to establish a high Pi balance, the reader is referred to Fig. 5.

7 Future Research

Studies Examining the Expression and Function of Mediators of Pi Transport Across Development Much remains to study with respect to the physiological mechanisms underlying the maintenance of a positive Pi balance in young animals. The detailed characterization of the normal physiological state will enable the identification of pathophysiological mechanisms and inform their treatment. To this end, the ontogeny of Pit1/2 expression across the lifespan remains unknown and should be delineated. In light of the postulated Pi-sensing role of Pit1/2 heterodimers (Bon et al. 2018), this information may suggest the extent to which this mechanism is active in the small bowel throughout the life span. Additionally, investigation is warranted into whether Pi sensing by Pit1/2 has a downstream effect on NaPiIIb or other NaPi's analogous to the regulation of the Trpv6 calcium channel by the calcium-sensing receptor (Lee et al. 2019). Further, the effect of 1,25 $(OH)_2$ Vitamin D_3 on Pit1/2 expression in the adult should be determined. Recently, it was demonstrated in Caco-2 cells that calcitriol upregulates the mRNA abundance of several intestinal ALP splice variants (Noda et al. 2017). Given the role of ALP in intestinal Pi absorption, future studies are warranted to examine the change in ALP

expression in response to 1,25 $(OH)_2$ Vitamin D_3 administration in animal models at different ages. This information would be informative regarding if and how calcitriol contributes Pi liberation from macromolecules in milk in the suckling infant. Since studying transcript abundance is not sufficient to analyse calcitriol regulation of NaPIIb at different ages, changes in NaPiIIb protein abundance in response to calcitriol in juvenile and adult animals should be studied.

Studies Aimed at Understanding the Mechanisms Mediating Increased Renal Tubular Pi Absorption in Young Animals As the ontogeny of NaPiIIa expression in the kidney has not been examined at all, future studies should delineate the potential role of NaPiIIa in maintaining a positive Pi balance in young animals. Other experiments aimed at determining the possibility of another transporter such as Pit2 contributing a compensatory role in Pi reabsorption in the suckling animal should also be considered. To enhance our understanding of the parathyroid-kidney interaction in development of renal Pi handling, the ontogeny of PTH receptor expression in the proximal tubule should be elucidated. Given the absence of PTH-induced phosphaturia in the newborn, low PTH receptor abundance in the proximal tubule early in life might explain this phenomenon. Additionally, studies examining the developmental changes in expression of downstream factors in PTH-mediated phosphaturia such as the Sodium-Hydrogen Exchanger Regulating Factor 1 should also be conducted (Lee et al. 2017).

Studies Aimed to Understand the Mechanisms Leading to Developmental Changes in Phosphotropic Hormone Levels In light of the multifaceted role of 1,25 $(OH)_2$ Vitamin D_3 in intestinal Pi handling, future research should seek to determine how the synthesis and degradation of this endocrine factor is altered across mammalian development. To this end, the assessment of transcript levels for, and overall abundance of, 25 hydroxylase (CYP2R1) and 1α hydroxylase (CYP27B1) in the liver and kidney, respectively, should be ascertained. Studies examining the ontogeny of calcitriol deactivation by measuring 24 hydroxylase (CYP24A1) abundance in target tissues would also be informative. Future studies examining intestinal vitamin D absorption at different ages will further our understanding of the developmental changes in the absorption of dietary cholecalciferol prerequisite to the synthesis and action of active calcitriol. Further the abundance of VDR and RXR should be examined across ages and consideration made to the effects of calcitriol on post-transcriptional modification of phosphate transporters such as NaPi2b examined.

It is surprising that there is a paucity of literature surrounding how the production/secretion of FGF23 and PTH change across development given their instrumental role in Pi homeostasis. Serum [FGF23] changes across development have been documented, the same should be done for PTH to better understand its endocrine role during growth. Researchers may additionally examine the ontogeny of PTH and FGF23 transcript abundance in the parathyroid gland and osteocyte/osteoblast, respectively. This information may prove useful in understanding developmental changes in a mammal's capacity to generate a robust endocrine response to an increase in serum Pi.

Examination of the Effects of Inorganic Pi on Infants and Children The evidence linking increased serum Pi levels to all-cause mortality and CVD, even when in the normal range in adults begs further inquiry in children. Does the increased plasma Pi level observed in bottle fed infants translate into increased CVD or overall mortality later in life? The effect of breast vs bottle feeding on Pi homeostasis, FGF23 and PTH levels should also be considered. This information would be valuable to families choosing between formula and breast milk.

8 Conclusions

Given the fundamental role of Pi in skeletal development we know surprisingly little about Pi absorption in young animals. It is clear that there is increased intestinal and renal Pi absorption/reabsorption in suckling animals. While it is likely that increased NaPiIIb mediated intestinal absorption accounts for the increased Pi absorption from the small bowel, the mechanisms mediating increased renal Pi reabsorptions remains unknown and is an area in need of further study.

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Ligands and Signaling of Mas-Related G Protein-Coupled Receptor-X2 in Mast Cell Activation

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Contents

Abstract Mas-related G protein-coupled receptor-X2 (MRGPRX2) is known as a novel receptor to activate mast cells (MCs). MRGPRX2 plays a dual role in promoting MC-dependent host defense and immunomodulation and contributing to the pathogenesis of pseudo-allergic drug reactions, pain, itching, and

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inflammatory diseases. In this article, we discuss the possible signaling pathways of MCs activation mediated by MRGPRX2 and summarize and classify agonists and inhibitors of MRGPRX2 in MCs activation. MRGPRX2 is a low-affinity and low-selectivity receptor, which allows it to interact with a diverse group of ligands. Diverse MRGPRX2 ligands utilize conserved residues in its transmembrane (TM) domains and carboxyl-terminus Ser/Thr residues to undergo ligand binding and G protein coupling. The coupling likely initiates phosphorylation cascades, induces Ca^{2+} mobilization, and causes degranulation and generation of cytokines and chemokines via MAPK and NF-κB pathways, resulting in MCs activation. Agonists of MRGPRX2 on MCs are divided into peptides (including antimicrobial peptides, neuropeptides, MC degranulating peptides, peptide hormones) and nonpeptides (including FDA-approved drugs). Inhibitors of MRGPRX2 include non-selective GPCR inhibitors, herbal extracts, small-molecule MRGPRX2 antagonists, and DNA aptamer drugs. Screening and classifying MRGPRX2 ligands and summarizing their signaling pathways would improve our understanding of MRGPRX2-mediated physiological and pathological effects on MCs.

Keywords Agonist · Antagonist · Degranulation · Generation of cytokines and chemokines · Host defense · Inflammatory diseases · Inhibitors · Mas-related G protein-coupled receptor-X2 · Mast cells · Pseudo-allergic drug reactions

1 Introduction

Mast cells (MCs) are tissue-resident granulocytes of hematopoietic origin and play a pivotal role in the physiological and pathological inflammatory and immune processes including immunoglobulin E (IgE)-dependent anaphylaxis (Helm et al. 1988), IgE-independent pseudo-allergic reactions (McNeil et al. 2015; Yu et al. 2016), immune surveillance (St John et al. 2011), and modulation of immune responses to microorganisms such as bacteria, viruses, and parasites (Abraham and St John 2010). The classical pathway of MCs activation mediates IgE-dependent anaphylaxis by cross-linking the high affinity IgE receptor FcεRI. In addition to FcεRI, MCs express a large number of G protein-coupled receptors (GPCRs) for diverse ligands, including lipids, chemokines, adenosine, anaphylatoxins C3a and C5a, and peptides (Ahamed et al. 2004; Jiang et al. 2007; McNeil et al. 2015; Okayama et al. 2008; Wojta et al. 2002). Mas-related G protein-coupled receptor-X2 (MRGPRX2) has been known as a novel receptor that activates MCs.

Mas-related G protein-coupled receptors (MRGPRs) are a class of seven-transmembrane GPCRs comprised of approximately 50 members in mice, rats, humans, and monkeys (Bader et al. 2014). MRGPRs are divided into several subfamilies: MrgprA, B, C, D, E, F, G, H, and a primate-specific MRGPRX subfamily (Fujisawa et al. 2014; Subramanian et al. 2016; Zylka et al. 2003). The MRGPRs family was discovered by its expression in specific dorsal root and trigeminal ganglia

neurons, MCs, tumors, and cardiovascular organs (Bader et al. 2014; Dong et al. 2001; Tatemoto et al. 2006). MRGPRX2 is most abundant in specific dorsal root and trigeminal ganglia neurons and MCs and has a little expression in both the peripheral and central nervous system (Robas et al. 2003). In human MCs, hMRGPRX2 is the primary MRGRPRs, with small amounts of hMRGPRX1 also being expressed, but not hMRGPRX3 or hMRGPRX4 (Subramanian et al. 2011b; Tatemoto et al. 2006). MRGPRX2 is exclusively expressed in MC_{TC} (which express tryptase and chymase) or connective tissue MCs, such as human skin MCs (HSMC), human cultured MCs which are connective tissue type ($HCMC_{CT}$), cord blood-derived MCs, $CD34^+$ cell-derived MCs, and human LAD2 MCs (McNeil et al. 2015; Tatemoto et al. 2006). Conversely, MC_T and immature MC lines lack or express little of the receptor, such as human cultured MCs which are cultured in serum-containing medium ($HCMC_M$), human mast cell line-1 (HMC-1), and bone marrow-derived MCs (BMMC) (Moon et al. 2003; Wu et al. 2015). In addition to humans, *MrgprX2* is also expressed in other primates, including chimpanzees, orangutans, gibbons, macaques, and marmosets (Choi and Lahn 2003).

MRGPRX2 is a non-canonical GPCR that is expressed on human MCs and has physiological and pathological effects on MCs. The characteristics of low affinity and low selectivity allow it to interact with a diverse group of ligands, such as antimicrobial peptides (AMPs), neuropeptides (NPs), mast cell degranulating peptides (MCDPs), and FDA-approved drugs (Bader et al. 2014; Subramanian et al. 2016). Recent studies have demonstrated that MRGPRX2 plays a critical role in promoting MCs-mediated host defense and its activation by AMPs, such as defensins and LL-37, enhances microbial clearance, and promotes wound healing in MCs (Chen et al. 2007; Subramanian et al. 2011a, 2013). The MRGPRX2-mediated activation of MCs in close proximity to nerve endings induced by some NPs, such as substance P (SP), increases degranulation and contributes to neurogenic inflammation, pain, and itch (Barrocas et al. 1999; Hagermark et al. 1978; McNeil et al. 2015; Tatemoto et al. 2006). In addition, MRGPRX2 ligands such as compound 48/80 (C48/80), SP, and many FDA-approved drugs are known to induce degranulation of MCs via MRGPRX2, releasing storage granules containing a lot of proinflammatory mediators such as histamine, and causing pseudo-allergic diseases (Che et al. 2018; Lansu et al. 2017; Liu et al. 2017; McNeil et al. 2015; Wang et al. 2016; Yaksh et al. 2019). Activation of MRGPRX2 by the ligands contributes to host defense, immunomodulation, inflammatory diseases, and pseudo-allergic drug reactions (McNeil et al. 2015; Solinski et al. 2014; Subramanian et al. 2016).

In this article, we discuss the signaling pathways of MRGPRX2-mediated activation of MCs and illuminate its mechanism and review the reported agonists and antagonists of MRGPRs in MCs-dependent antimicrobial activity, immunomodulation, inflammatory diseases, and pseudo-allergic reactions.

2 mMrgprB2 and rMrgprB3 as Orthologs of Human MRGPRX2

mMrgprB2 and rMrgprB3 are the mouse and rat orthologs of human MRGPRX2. In mice, the expression of mMrgprB2 is restrictedly detected in connective tissue MCs in skin, gut, and trachea (McNeil et al. 2015; Tatemoto et al. 2006). The peritoneum of the mouse is a major source of connective tissue-type MCs. Mouse peritoneal MCs (MPMC) expresses mMrgprB2. hMRGPRX2 and mMrgprB2 have certain unique characteristics as these receptors are expressed in connective tissue MCs and are activated by some ligands such as SP and C48/80 (McNeil et al. 2015). However, hMRGPRX2 and mMrgprB2 show a considerable difference in the ability of ligands to activate or inhibit these receptors. The EC50 values of most ligands for MrgprB2 are significantly higher than those for MRGPRX2 (McNeil et al. 2015). A few compounds even display a selectively inhibiting effect. Neurokinin 1 receptor (NK-1R) antagonist QWF could inhibit SP-induced activation of MrgprB2, and MRGPRX2 as well as MrgprA1 in HeLa cells. However, NK-1R antagonists L733060 and aprepitant are dual antagonists of NK-1R and mouse MrgprB2 but not human MRGPRX2 (Azimi et al. 2016) because NK-1R does not mediate SP-induced MCs degranulation and because these NK-1R antagonists can interact with MrgprB2 but not with human MRGPRX2 (McNeil et al. 2015; Azimi et al. 2016). The differential effect of NK-1R antagonists on mouse versus human MRGPRs can explain the inconsistencies with respect to their efficacy in mouse models as compared with a range of human conditions (Azimi et al. 2016). The extracellular (EC) domains and transmembrane extracellular regions (TM-EC) of GPCR contribute to ligand binding, whereas intracellular (IC) domains are involved in G protein coupling (Katritch et al. 2012). The differences in the amino acid sequences of hMRGPRX2 and mMrgprB2, which is only ~53% overall sequence, 34% N-terminal 60 amino acids sequence, and 47% C-terminal 80 amino acids sequence similarity between these receptors, may contribute to differences in the ability of ligands to act on these receptors (Subramanian et al. 2016; Tatemoto et al. 2006). In rats, rMrgprB3 and B8 are expressed in rat peritoneal MCs (RPMC), alongside low levels of rMrgprB1, B2, B6, and B9, but exhibiting no rMrgprA, rMrgprX1, or any other rMrgprB expression (Tatemoto et al. 2006). Rat basophilic leukemia-2H3 (RBL-2H3) cells also do not express MRGPRX2 (Subramanian et al. 2013).

3 Physiological and Pathological Functions of MRGPRX2 in Human MCs

MRGPRX2 is a non-canonical GPCR expressed on human MCs and plays an important role in host defense, immunomodulation, inflammatory diseases, and pseudo-allergic drug reactions. AMPs such as defensins, LL-37, catestatin, and

small-molecule nonpeptide host-defense peptides (smHDPs) have the effect of the clearance of microbial pathogens and play a critical role in host defense. These AMPs have direct antimicrobial activities via interacting with the negatively charged phospholipid moieties and disrupting the membrane of microbial pathogens (Hazlett and Wu 2011). In addition to direct antimicrobial activities, AMPs also activate MCs via MRGPRX2 and cause the release of proinflammatory mediators and the generation of cytokines and chemokines that play an important role in host defense, immunomodulation, and wound healing by causing increased vascular permeability and by recruiting or activating other inflammatory cells, such as macrophages, T cells, and basophilic cells (Chen et al. 2007; Subramanian et al. 2011a, 2013). The positive effects of AMPs-induced MCs activation via MRGPRX2 likely outweigh the risks of developing adverse reactions.

In addition to host defense, MRGPRX2 has been identified as a MC specific receptor which is responsible for pseudo-allergic drug reactions (McNeil et al. 2015). MrgprB2, the mouse ortholog of human MRGPRX2, serves as a model to develop potential therapeutic targets for drug-induced pseudo-allergic drug reactions in mice. MRGPRX2 ligands such as C48/80, SP, and many FDA-approved drugs including neuromuscular blocking agents (NMBAs), fluoroquinolone antibiotics, opioid drugs, and some herbal extracts are known to induce degranulation of MCs, releasing storage granules containing large amounts of proinflammatory mediators such as histamine and cause pseudo-allergic diseases (Che et al. 2018; Lansu et al. 2017; Liu et al. 2017; McNeil et al. 2015; Wang et al. 2016; Yaksh et al. 2019). Therefore, MRGPRX2 is also considered to be a therapeutic target for pseudo-allergic diseases. Some non-selective GPCR inhibitors such as pertussis toxin (PTx) and QWF, cytokines, herbal extracts (including saikosaponin A, resveratrol, quercetin, osthole, genistein, shikonin, piperine, and paeoniflorin), small-molecule MRGPRX2 antagonists, and single-stranded DNA (ssDNA) aptamer drugs were effective in inhibiting or blocking MRGPRX2-mediated signaling and displayed the effect of anti-pseudo-allergic reactions (Callahan et al. 2020; Chen et al. 2007; Ogasawara et al. 2019; Suzuki et al. 2020; Wang et al. 2020b). Discovering and identifying inhibitors or antagonists of MRGPRX2 has become a research focus for anti-pseudo-allergic reactions.

The MRGPRX2-mediated MCs activation induced by NPs, such as SP, plays an important role in the modulation of neurogenic inflammation, pain, and itch (Barrocas et al. 1999; Hagermark et al. 1978; McNeil et al. 2015; Tatemoto et al. 2006). MCs are found in close proximity to nerve endings that can release peptides such as SP. Moreover, tryptase released from MCs activates primary afferent neurons resulting in the release of pre-stored SP via proteinase activated receptor-2 (PAR-2) (Kempkes et al. 2014). SP released from neurons and MCs can provide a positive feedback for further MCs activation via MRGPRX2 that increases MCs degranulation and contributes to neurogenic inflammation, pain, and itch (McNeil et al. 2015; Tatemoto et al. 2006). In addition to neurogenic inflammation, MRGPRX2-mediated MCs activation also contributes to the pathogenesis of chronic inflammatory diseases. The expression of MRGPRX2 in chronic urticaria patients' MCs is higher than that in the healthy subjects (Fujisawa et al. 2014). SP-induced

MCs degranulation and generation of cytokines, such as prostaglandin D (PGD) 2, contribute to the pathogenesis of chronic urticaria (Fujisawa et al. 2014).

4 Signaling Pathways and Regulation of MRGPRX2 in Activation of MCs

4.1 MRGPRX2-Mediated MCs Degranulation and Generation of Cytokines/Chemokines

Agonists of MRGPRs including AMPs, NPs, MCDPs, and FDA-approved drugs induce degranulation of MCs, releasing storage granules containing quantities of proinflammatory mediators such as histamine. Histamine binds to histamine receptors (HR) including H1R, H2R, H3R, and H4R. The activation of H1R which is expressed on endothelial cells and bronchial smooth muscle cells is involved in pseudo-allergic drug reactions (Borriello et al. 2017; Seifert et al. 2013). The activation of H2R and H4R modulates the migration and activation of immune cells (e.g., MCs, basophils, eosinophils, monocytes, dendritic cells, NK, T cells, Treg, and Th2 cells) and plays a major role in pseudo- or allergic reactions and immune-mediated disorders (Borriello et al. 2017). H3R regulates behavior and body temperature at central nervous (Borriello et al. 2017). In addition to histamine release, AMPs such as human β-defensins (hBDs), LL-37, and angiogenic peptide-30/5C (AG-30/5C) induce production of chemokines (such as interleukin 8 (IL-8), monocyte chemoattractant proteins (MCPs) s, and macrophage inflammatory protein (MIPs)) and cytokines (tumor necrosis factor-α (TNFα), prostaglandins (PGs), and cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF)) (Chen et al. 2007; Kanazawa et al. 2016; Niyonsaba et al. 2019; Subramanian et al. 2011a). These chemokines and cytokines contribute to host defense, immunomodulation, and wound healing by recruiting or activating inflammatory cells, such as MCs, lymphocytes, macrophages, memory T cells, and basophilic cells (Griffith et al. 2014; Mukai et al. 2018; Ridiandries et al. 2018). MRGPR-mediated MCs activation by NPs leads to the release of proinflammatory cytokines and chemokines and contributes to neurogenic inflammation, pain, and itch (Green et al. 2019). Moreover, the release of tryptase from MCs activates primary afferent neurons via proteinase activated receptor-2 (PAR-2) resulting in the release of pre-stored NPs (Steinhoff et al. 2000). MRGPRX2-mediated MCs activation contributes to host defense, immunomodulation, pain, itching, inflammatory disease, and pseudo-allergic drug reactions by degranulation which releases storage granules containing a wide array of proinflammatory mediators and induces the generation of cytokines and chemokines.

4.2 Binding Sites of Ligands on MRGPRX2

The gene for MRGPRX2 is a two-exon gene located on chromosome 11p15 in humans (Bader et al. 2014; Choi and Lahn 2003). The sequence of MRGPRX2 is 2036 bp in length, encoding a protein with 330 amino acids (Wu et al. 2015). The structure of MRGPRX2 is composed of the extracellular (EC) domain including the N-terminus (N-term), seven-transmembrane (TM) bundles connected by three extracellular loops (ECL1, ECL2, and ECL3) and three intracellular loops (ICL1, ICL2, and ICL3), and the intracellular (IC) part including helix VIII and a C-terminal sequence. The EC and their closest TM regions with the greatest structural diversity are responsible for ligand binding, while the IC and its closest TM regions are involved in G protein coupling and downstream signaling (Yan and Hancock 2001). Missense variants G165E (rs141744602) and D184H (rs372988289) in MRGPRX2's ECL and TM cause a loss of MCs activation function by smHDPMs (Alkanfari et al. 2019). E164Q or D184N substitution in the 4th and 5th TM domains of MRGPRX2 retains the steric property of the wild-type residues but without negative charges resulting in the loss of receptor activity for cationic ligands (Lansu et al. 2017). In addition to E164Q or D184N substitutions, naturally occurring rare MRGPRX2 variants in its 4th, 5th, 6th, or 7th TM (G165E, D184H, W243R, or H259Y) fail to respond to SP, hemokinin-1(HK-1), hBD3, and icatibant to induce MCs degranulation (Alkanfari et al. 2018). However, naturally occurring rare MRGPRX2 variants in its N-term, 1st, or 2nd TM variants (N16H, L31V, V43I, and F78L) have no influence on SP-induced Ca^{2+} mobilization and degranulation (Alkanfari et al. 2018). In addition, highly conserved residues in TM6 (I225) and TM7 (Y279) of MRGPRX2 are essential for SP-induced Ca^{2+} mobilization and degranulation in MRGPRX2-RBL-2H3 cells (Chompunud Na Ayudhya et al. 2019). MRGPRX2 variants in conserved residues (V123F and V282M) and intracellular loops (R138C and R141C) failed to respond to SP, suggesting that these regions contribute to MRGPRX2 activation and G protein coupling (Chompunud Na Ayudhya et al. 2019). Thus, it is likely that TM4 (E164 and G165), TM5 (D184), TM6 (W243), and TM7 (W243 and Y279) of MRGPRX2 are responsible for ligands binding, and highly conserved residues in TM3 (V123), TM6 (I225), TM7 (Y282 and Y279), and intracellular loops (R138C and R141C) are involved in G protein coupling and downstream signaling (Fig. 1). In addition, naturally occurring rare MRGPRX2 variants in its N-term (N16H), TM1 (L31V and V43I), and TM2 (F78L) are negative (Fig. 1).

4.3 Activation of MRGPRX2 in MCs Via the Gαi and Gαq Families

The activation of MRGPRX2 induced by agonists, such as NPs and AMPs, causes MCs chemotaxis, degranulation, and cytokine generation, most likely involving the

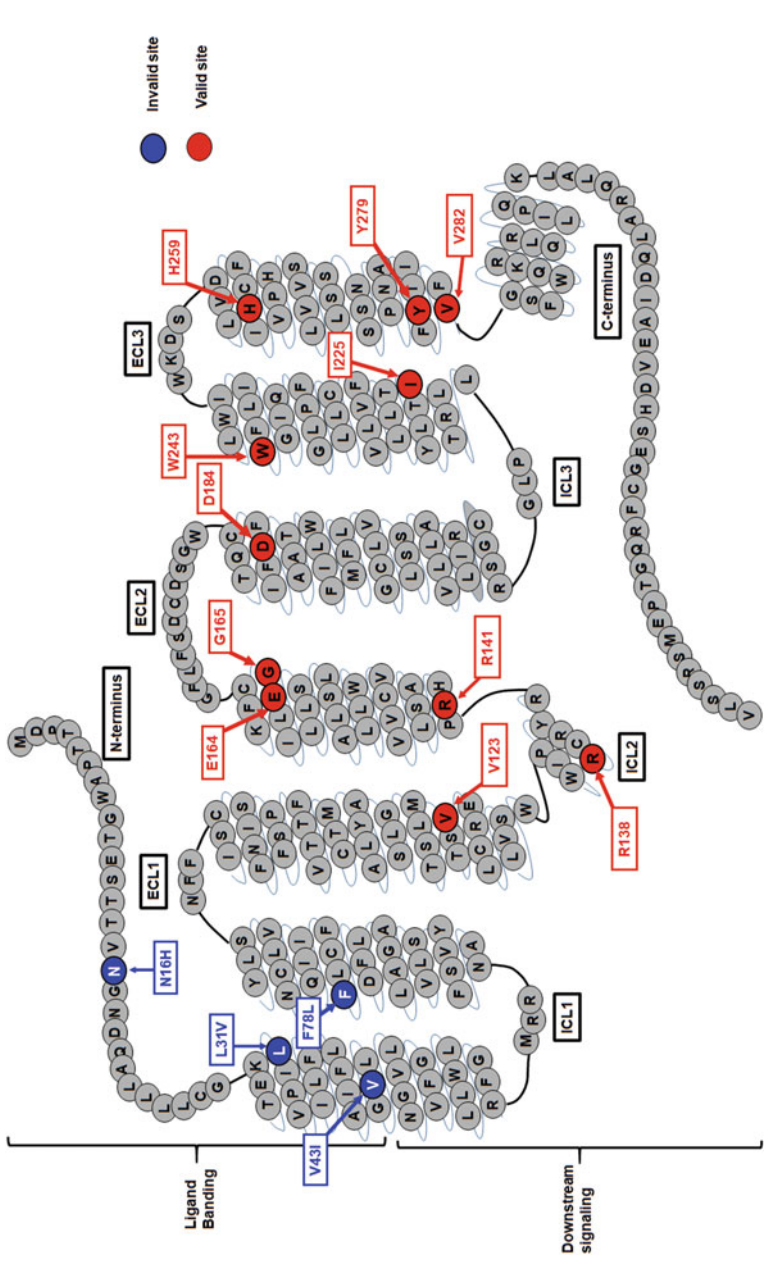

Fig. 1 Snake diagram of secondary structure of MRGPRX2 showing potential binding and activation sites. Each circle with one letter code represents a particular amino acid residue. Extracellular (ECL), intracellular (ILC), and transmembrane (TM) domains are shown. Solid background indicates the naturally occurring missense variants in the structure of MRGPRX2. Red circles represent valid ligands response sites including TM3 (V123), ICL2 (R138C), TM4 (R141, E164, and G165), TM5 (D184), TM6 (W243 and I225), and TM7 (V282, W243, and Y279). Blue circles represent invalid ligands response sites including N-term (N16H), TM1 (L31V and V431), and TM2 (F78L)

activation of Gαi and Gαq. PTx, an inhibitor of Gαi proteins, restrains Ca^{2+} mobilization or degranulation induced by MRGPRX2 agonists such as C48/80 (Mori et al. 2000b), SP (Barrocas et al. 1999), Icatibant (Roy et al. 2019a), pituitary adenylate cyclase-activating peptide (PACAP) (6–27) (Tatemoto et al. 2006), and hBDs (Chen et al. 2007). Cortistatin increases intracellular Ca^{2+} but has no effect on cyclic adenosine monophosphate (cAMP) levels in MRGPRX2-HEK293 cells, suggesting that this receptor is not Gas-coupled (Robas et al. 2003). Mastoparan specifically activates MRGPRX2-HEK293 cells coexpressing NFAT-RE but not SRE-RE or CRE, indicating that mastoparan specifically activates Gαq (Arifuzzaman et al. 2019). A Gαi-specific inhibitor, PTx, and a Gαq-specific inhibitor, YM-254890, abolished SP-induced degranulation of MCs (Chompunud Na Ayudhya et al. 2019). These findings suggest that both Gαi and Gαq are involved in MRGPRX2 activation (Fig. 2).

4.4 Signaling of Ca^{2+} in MRGPRX2-Mediated Activation of MCs

Calcium signaling is essential for both FcεRI-mediated and MRGPRX2-mediated MCs activation. While FcεRI-mediated degranulation is attributed to a slow and sustained Ca^{2+} response, MRGPRX2-mediated degranulation is induced by a rapid and transient Ca^{2+} mobilization (Ali 2017). Induction of FcεRI aggregation by antigens initiates phosphorylation cascades that involve Src tyrosine kinases Lyn, Fyn and Syk Src tyrosine kinases recruit additional signaling molecules by these adaptors and ultimately product phospholipase C (PLC) (Gilfillan et al. 2011; Schneider et al. 1992). Gαi-coupling PLC signaling involves a Ca^{2+} mobilization in MRGPRX2-mediated MCs activation. Early work thought that regulation of PLCγ isoforms through receptor and non-receptor tyrosine kinases distinguished this family from the regulation of PLCβ isoforms by Gα and Gβγ subunits of heterotrimeric G proteins (Bunney and Katan 2011). However, MRGPRX2-mediated Ca^{2+} mobilization seems to favor coupling PLCγ. Phosphorylation of PLCγ and inositol 1,4,5-trisphosphate receptor (IP_3R) increases in sinomenine-, C48/80-, and mivacurium-induced MCs activation via MRGPRX2 (Liu et al. 2017). U-73122, an inhibitor of PLCγ, suppressed AMP-IBP5-induced MCs activation (Niyonsaba et al. 2019). Src tyrosine kinases, such as pLyn, which can activate PLCγ, are involved in sinomenine- and C48/80-induced MCs activation via MRGPRX2 (Wang et al. 2016). In addition, evidence indicates that it is possible to interplay between different PLC families (Bunney and Katan 2006). Synergistic activation between PLCγ and PLCβ leads to enhanced store operated Ca^{2+} entry that permits reinforcement of signals for degranulation in MCs (Kuehn et al. 2008). La^{3+} and 2-APB, the inhibitor of Orai 1 and Orai 2, restrain hBD-induced Ca^{2+} mobilization and degranulation in LAD2 cells, suggesting that Orai is involved in MRGPRX2-mediated Ca^{2+} mobilization (Subramanian et al. 2013). Unlike FcεRI-

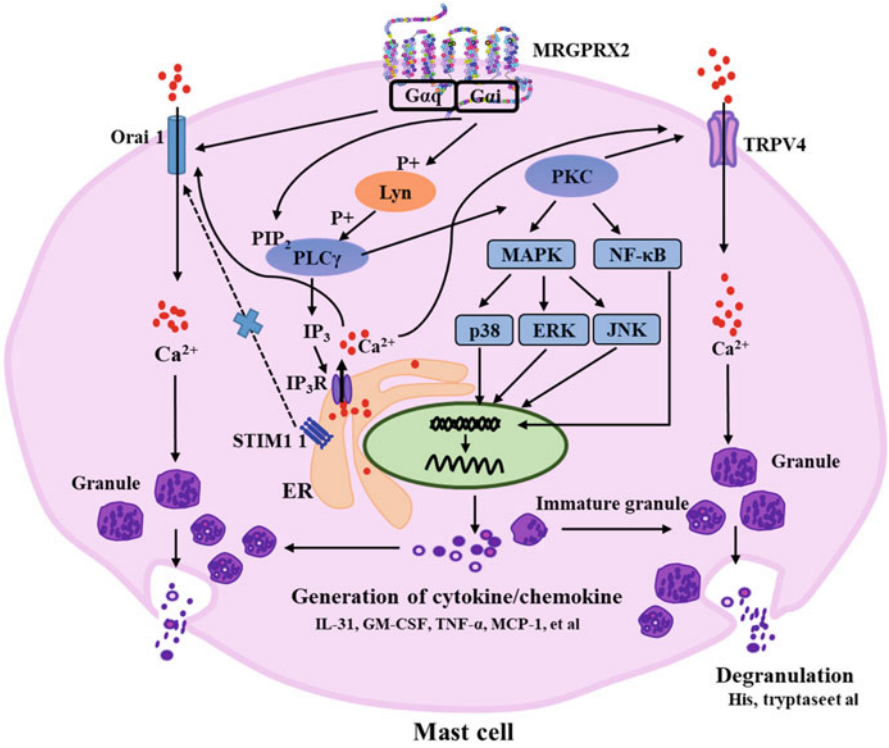

Fig. 2 Signaling pathways of MCs activation via MRGPRX2. The activation of MRGPRX2 is both Gαq- and Gαi- coupled, and the coupling likely initiates phosphorylation cascades that involve Src tyrosine kinase Lyn, phospholipase C (PLC)γ, and inositol 1,4,5-trisphosphate receptor (IP_3R), subsequently triggering rapid and transient Ca^{2+} mobilization via the Ca^{2+} release-activated Ca^{2+} channel protein Orai or the transient receptor potential cation channel subfamily V4 (TRPV4), and the mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway activation results in degranulation and generation of cytokines/chemokines

mediated SOCE, STIM1 is not involved in C48/80- and sinomenine-induced Ca^{2+} mobilization (Liu et al. 2017). Transient receptor potential channels of the vanilloid subfamily (TRPV) 4 are also likely involved in Ca^{2+} influx. LL-37 can directly increase TRPV4 expression in human MCs and the upregulation of TRPV4 is dependent on MRGPRX2 activity (Mascarenhas et al. 2017). Local stimulation by diacylglycerol and protein kinase C (PKC) signaling results in TRPV4-mediated Ca^{2+} influx (Mercado et al. 2014). In addition, IP_3R can directly interact with TRPV4 to activate or sensitize these channels (Alessandri-Haber et al. 2009). Therefore, MRGPRX2-mediated rapid and transient Ca^{2+} mobilization initiates phosphorylation cascades that involve Lyn, PLCγ, and IP_3R, subsequently triggering influx of extracellular Ca^{2+} via Orai or elicits influx of Ca^{2+} via TRPV4 (Fig. 2).

4.5 MAPK and NF-κB Pathways in MRGPRX2-Mediated MCs Activation

The activation of MCs via MRGPRX2 results in not only degranulation, releasing stored mediators but also the generation of new inflammatory mediators (Chen et al. 2007). hBDs and LL-37 enhance the production of IL-31, PGE2, and leukotriene C4 via the phosphorylation of p38, ERK, and JNK in MCs (Chen et al. 2006; Niyonsaba et al. 2010). Furthermore, PTx and inhibitors of p38, ERK, and JNK suppress the hBD/LL-37-induced production of IL-31 (Niyonsaba et al. 2010). SB203580 (a p38 inhibitor), JNK inhibitor II, and NF-κB activation inhibitor II inhibit the AMP-IBP5-induced generation of cytokines and chemokines, including GM-CSF, TNF-α, MCP-1, and MIP-1α (Niyonsaba et al. 2019). Sinomenine hydrochloride and C48/80 induce the phosphorylation of PKC and P38 in LAD2 cells (Liu et al. 2017). Therefore, it is likely that both the MAPK pathway including p38, ERK, and JNK and the NFeκB pathway are involved in the MRGPRX2 ligand-induced generation of inflammatory mediators (Fig. 2).

5 Agonists

Agonists of MRGPRs on MCs are mainly divided into two major categories: peptides and nonpeptides. Peptide agonists of MRGPRs on MCs primarily include AMPs, NPs, MCDPs, peptide hormones, and other endogenous protein fragments (Table 1). In addition to C48/80, a majority of nonpeptide agonists of MRGPRs on MCs are FDA-approved drugs, including NMBAs, fluoroquinolone antibiotics, opioids, polymyxins, contrast media, herbal extracts, and others (Table 2). Also, some compounds have been identified as MRGPRX2-selective ligands by compound libraries.

5.1 Peptides

5.1.1 Antimicrobial Peptides

β-Defensins and Analogs

hBDs are small cationic AMPs that are mainly produced by epithelial cells and platelets and play an important role in antimicrobial and immunomodulatory properties (Weinberg et al. 2012). hBD-3 and hBD-4 induce degranulation, PGD2 production, intracellular Ca^{2+} mobilization, and chemotaxis in RPMC or LAD2 cells and increased vascular permeability in wild-type (WT) rats, but not in MC-deficient rats (Chen et al. 2007). hBD2 and hBD3 induce degranulation or

Table 1 Peptide agonists of Mas-related G protein-coupled receptors in mast cell activation

Classification	Agonist	Receptor	EC50	Activated cell types	Effect on MCs	References
AMPs	hBDs	MRGPRX2	EC: hBD2, 1 μM; hBD3, 1 μM	MRGPRX2-HEK293(+), MRGPRX2-RBL-2H3(+), HA-MRGPRX2-BMMCs (+), RBL-2H3(−), MPMC (−), BMMC(−)	Ca^{2+}mobilization; degranulation	Chen et al. (2007), Subramanian et al. (2013)
	θ-Defensins analogs	MRGPRX2	EC: RC-100, RC-101, Protegrin-1, 1–5 μg/mL	LAD2(+), MRGPRX2-RBL-2H3(+)	Ca^{2+} mobilization; degranulation	Gupta et al. (2015)
	LL-37	MRGPRX2	EC 1–10 μM	LAD2(+), RPMC(+), MRGPRX2-RBL-2H3(+), MRGPRX2-HMC-1 (+), MRGPRX1-RBL-2H3(−), shMRGPRX2-LAD2(−), HMC-1(−)	Ca^{2+} mobilization; degranulation; generation of IL-8 and CCL4	Gupta et al. (2016), Niyonsaba et al. (2002), Subramanian et al. (2011a), Yu et al. (2017)
	AG-30/5C	MRGPRX2; MrgprX4	EC 0.1–1.5 μM	LAD2(+), MRGPRX2-RBL-2H3(+), MRGPRX4-HTLA(+), RBL-2H3(−)	Degranulation; production of cytokines/chemokines	Kanazawa et al. (2016), Roy et al. (2019a)
	AMP-IBP5	MRGPRX2	EC 1.3–10 uM	LAD2(+), siMrgprX2-LAD2(−), siMrgprX1-LAD2(+), siMrgprX3-LAD2(+), siMrgprX4-LAD2(+)	Degranulation and migration; Ca^{2+} mobilization; production of cytokines/chemokines	Niyonsaba et al. (2019)
	Magainin 2	MRGPRX2	28.1 ± 5.9 μM	RPMC(+), MRGPRX2-HEK293(+), MRGPRX1-HEK293(−)	Ca^{2+} mobilization; degranulation	Hook et al. (1990), Tatemoto et al. (2006)
	Indolicidin	MRGPRX2; MRGPRX1	MRGPRX2 2.9 ± 0.9 μM; MRGPRX1 7.6 ± 6.9 μM	$HCMC_{CT}$(+), MRGPRX2-HEK293(+), MRGPRX1-HEK293(+)	Ca^{2+} mobilization; degranulation	Tatemoto et al. (2006)

	Catestatin	MRGPRX2 MRGPRX1	MRGPRX2 28.3 ± 13.2 μM; MRGPRX1 4.6 ± 1.4 μM	MRGPRX2-HEK293(+), MRGPRX1-HEK293(−)	Ca^{2+} mobilization	Tatemoto et al. (2006)
	smHDPs	MRGPRX2	Cpd 4, 0.4 μM; Cpd 5, 0.4 μM; Cpd 1, 0.8 μM; Cpd 2, 3 μM; Cpd 3, 1.8 μM	LAD2(+), BMMCs (+), MRGPRX2-RBL-2H3(+), siMrgprX2-LAD2(−), $MrgprB2^{MUT}$ MPMC (−), RBL-2H3(−)	Degranulation	Alkanfari et al. (2019)
	Polymyxins	MRGPRX2 MrgprB2	MRGPRX2 Polymyxin B, 8.9 μg/mL; Polymyxin E, 12.2 μg/mL	LAD2(+), MRGPRX2-HEK293(+), MrgprB2-HEK293(+), siMrgprX2-LAD2(−)	Ca^{2+} mobilization; degranulation; secretion of TNFα, PGD2	Zhan et al. (2019)
NPs	SP	MRGPRX2 MrgprB2	MRGPRX2 8.0 ± 4.5 μM; MrgprB2 54.3 ± 1.9 μM; MRGPRX1 >100 μM	$HCMC_{CT}$(+), HSMC(+), MPMC(+), RPMC(+), LAD2(+), MRGPRX2-HEK293(+), MRGPRX2-RBL-2H3(+), MrgprB2-HEK293(+), MRGPRX1-HEK293(−), $MrgprB2^{MUT}$ MPMC (−)	Ca^{2+} mobilization; degranulation	Azimi et al. (2016), Barrocas et al. (1999), McNeil et al. (2015), Tatemoto et al. (2006)
	PACAP-38	MrgprB3	--	Dura MCs(+), MrgprB3-Xenopus laevis oocytes(+), RPMC(+)	Degranulation	Pedersen et al. (2019)
	PACAP(6–27)	MRGPRX2 MrgprB3	MRGPRX2 1.6 ± 0.6 μM	$HCMC_{CT}$(+), MRGPRX2-HEK293(+), MrgprB3-HEK293(+), MRGPRX1-HEK293(−)	Ca^{2+} mobilization; degranulation	Tatemoto et al. (2006)
	Angiopeptin	MRGPRX2	4.4 ± 2.3 μM		Ca^{2+} mobilization; degranulation	Tatemoto et al. (2006)

(continued)

Table 1 (continued)

Classification	Agonist	Receptor	EC50	Activated cell types	Effect on MCs	References
				$HCMC_{CT}$(+), MRGPRX2-HEK293(+), MRGPRX1-HEK293(−)		
	Somatostatin	MRGPRX2	MRGPRX2 5.7 ± 2.1 μM	$HCMC_{CT}$(+), RPMC(+), MRGPRX2-HEK293(+), MRGPRX1-HEK293(−)	Ca^{2+} mobilization; degranulation; decrease of cAMP	Tatemoto et al. (2006), Waalkens et al. (1981)
	Cortistatin-14	MRGPRX2 MrgprB2	MRGPRX2 106.7 ± 39.3 nM; MrgprB2 21.3 ± 0.9 μM	LAD2(+), MRGPRX2-CHO(+), MRGPRX2-HEK293(+), MrgprB2-HEK293(+), MRGPRX1-CHO(−), siMrgprX2-LAD2(−)	Ca^{2+} mobilization; degranulation; increase of cAMP	Kamohara et al. (2005), McNeil et al. (2015), Subramanian et al. (2013)
Peptide hormone	PAMP-12	MRGPRX2 MrgprB2	MRGPRX2 166.0 ± 35.7 nM; MrgprB2 12.4 ± 1.6 μM	MPMC(+), LAD2(+), MRGPRX2-CHO(+), MRGPRX1-CHO(−), MRGPRX2-HEK293(+), MRGPRX1-HEK293(+), $MrgprB2^{MUT}$ MPMC (−), siMrgprX2-LAD2(−)	Ca^{2+} mobilization; degranulation	Lu et al. (2018), McNeil et al. (2015), Meixiong et al. (2019)
	BAM(8–22)	MRGPRX1	MRGPRX1 0.04 ± 0.02 μM	$HCMC_{CT}$(−), MRGPRX2-HEK293(−), MRGPRX1-HEK293(+)	Ca^{2+} mobilization	Tatemoto et al. (2006)
	VIP	MRGPRX2 MRGPRX1	MRGPRX2 2.6 ± 2.8 μM; MRGPRX1 17.7 ± 17.6 μM	$HCMC_{CT}$(+), MRGPRX2-HEK293(+), MRGPRX1-HEK293(+)	Ca^{2+} mobilization; degranulation	Tatemoto et al. (2006)
	GnRHR antagonist and agonist	MRGPRX2 MrgprB2	MRGPRX2: sermorelin, octreotide, leuprolide,	MPMC (+), MRGPRX2-HEK293(+), MrgprB2-	Ca^{2+} mobilization	McNeil et al. (2015)

			4–10 μg/ml cetrorelix, 223 ± 63 ng/ml MrgprB2: octreotide, sermorelin, cetrorelix, 10–30 μg/ml; leuprolide, 152 ± 7.1 μg/ml	HEK293(+), MrgprB2^{MUT} MPMC (−)		
	Kallidin	MrgprB2	–	MPMC (+), MrgprB2^{MUT} MPMC (−)	Ca^{2+} mobilization	McNeil et al. (2015)
MCDPs	MCDP	MRGPRX2 MrgprB3	MRGPRX2 1.3 ± 0.7 μM;	HCMC$_{CT}$(+), MRGPRX2-HEK293(+), MrgprB3-HEK293(+), MRGPRX1-HEK293(−)	Ca^{2+} mobilization; degranulation	Tatemoto et al. (2006)
	Granuliberin R	MRGPRX2 MRGPRX1	MRGPRX2 24.3 ± 2.2 μM; MRGPRX1 38.3 ± 11.6 μM;	RPMC(+), MRGPRX2-HEK293(+), MRGPRX1-HEK293(+)	Ca^{2+} mobilization; degranulation	Tatemoto et al. (2006)
	Mastoparan	MRGPRX2 MrgprB2	MRGPRX2 3.9 ± 0.7 nM; MrgprB2 24.0 ± 3.6 μM	LAD2(+), RPMC(+), ROSA(+), MC/9(+), MPMC (+), MRGPRX2-HEK293(+), MrgprB2-HEK293(+), MRGPRX2-HEK293-NFAT-RE(+), MRGPRX2-HEK293-RE-RE(−), MRGPRX2-HEK293-CRE (−), RBL-2H3(−), MrgprB2^{MUT} MPMC (−)	Ca^{2+} mobilization; degranulation	Arifuzzaman et al. (2019), McNeil et al. (2015), Mousli et al. (1989), Nakao et al. (2011)

(continued)

Table 1 (continued)

Classification	Agonist	Receptor	EC50	Activated cell types	Effect on MCs	References
Endogenous protein fragments	CNP 1–17	MRGPRX2	8 μM	$HCMC_{CT}$(+), MRGPRX2-HEK293(+)	Ca^{2+} mobilization; degranulation	Tatemoto et al. (2018)
	Chaperonin-10	MRGPRX2	50 μM	$HCMC_{CT}$(+), MRGPRX2-HEK293(+)	Ca^{2+} mobilization; degranulation	Tatemoto et al. (2018)
	Tropomyosin 1–12	MRGPRX2	50 μM	$HCMC_{CT}$(+), MRGPRX2-HEK293(+)	Ca^{2+} mobilization; degranulation	Tatemoto et al. (2018)
	HSAs	MRGPRX2	408–423 < 409–423 < 407–423	LAD2(+), MRGPRX2-HEK293(+), MRGPRX1-HEK293(−)	Ca^{2+} mobilization; degranulation	Karhu et al. (2017)
	PF4	MRGPRX2	9.0 ± 2.6 μM	RPMC(+), MRGPRX2-HEK293(+), MRGPRX1-HEK293(−),	Ca^{2+} mobilization; degranulation	Suzuki et al. (2002), Tatemoto et al. (2006)
	HK-1	MRGPRX2	EC 3 μM	LAD2(+), MRGPRX2-RBL-2H3(+)	Degranulation	Manorak et al. (2018)

Table 2 Nonpeptide agonists of Mas-related G protein-coupled receptors in mast cell activation

Classification	Agonist	Receptor	EC50	Activated cell types	Effect on MCs	References
C48/80	C48/80	MRGPRX2 MrgprB2 MRGPRX1	MRGPRX2 3.6 ± 1.6 μg/ml; MrgprB2 3.7 ± 0.5 μg/ml; MRGPRX1 70.0 ± 23.2 μg/ml	$HCMC_{CT}$(+), MPMC(+), RPMC(+), LAD2(+), $HCMC_{M}$(−), MRGPRX2-HEK293(+), MRGPRX2-RBL-2H3 (+), MRGPRX1-HEK293 (+), MrgprB2-HEK293 (+), MrgprB1-HEK293(-), $MrgprB2^{MUT}$ MPMC (−), siMrgprX2-LAD2 (−)	Ca^{2+} mobilization; degranulation; production of TNF, PGD2	Kashem et al. (2011), Lumry et al. (2011), McNeil et al. (2015), Moon et al. (2003), Nazarov and Pronina (2012), Tatemoto et al. (2006)
NMBAs	Atracurium	MRGPRX2 MrgprB2	MRGPRX2 28.6 ± 2.4 μg/ml; MrgprB2 44.8 ± 1.4 μg/ml	HSMC(+), HLMC(+), MPMC (+), MRGPRX2-HEK293(+), MrgprB2-HEK293(+), $MrgprB2^{MUT}$ MPMC (−)	Ca^{2+} mobilization; degranulation	Marone et al. (1993), McNeil et al. (2015)
	Rocuronium	MRGPRX2 MrgprB2	MRGPRX2 261.3 ± 14.4 μg/ml; MrgprB2 22.2 ± 3.3 μg/ml	MPMC (+), MRGPRX2-HEK293(+), MrgprB2-HEK293(+), $MrgprB2^{MUT}$ MPMC (−)	Ca^{2+} mobilization; degranulation	McNeil et al. (2015)
	Mivacurium	MRGPRX2 MrgprB2	EC 100 μg/ml	LAD2(+), MRGPRX2-HEK293(+), siMrgprX2-LAD2(−), $MrgprB2^{MUT}$ mice(−)	Ca^{2+} mobilization; degranulation	Che et al. (2018)
Fluoroquinolone antibiotics	Fluoroquinolone antibiotics	MRGPRX2 MrgprB2	MRGPRX2: ciprofloxacin <moxifloxacin < levofloxacin < ofloxacin	MRGPRX2-HEK293(+), MrgprB2-HEK293(+)	Ca^{2+} mobilization	McNeil et al. (2015)

(continued)

Table 2 (continued)

Classification	Agonist	Receptor	EC50	Activated cell types	Effect on MCs	References
			MrgprB2: moxifloxacin < ofloxacin < levofloxacin			
Herbal extracts	Sinomenine	MRGPRX2 MrgprB2	MRGPRX2 2.8 ± 0.4 μM; MrgprB2 2,318 ± 314 μM	P815(+), LAD2(+), MPMC(+), MRGPRX2-HEK293(+), MrgprB2-HEK293(+), siMrgprX2-LAD2(−), $MrgprB2^{MUT}$ MPMC (−)	Ca^{2+} mobilization; degranulation; secretion of TNFα, MCP-1, IL-8, and MIP-1α/β	Liu et al. (2017), Wang et al. (2016)
	Complanadine A	MRGPRX2	5.5 μM	–	Activity for MRGPRX2(+)	Johnson and Siegel (2014)
Opioid drugs	Opioid drugs	MRGPRX2	LogEC50: (+)-TAN67 < dynorphin A (1–13) < dynorphin A (1–17) <dextromethorphan <dextrorphan <alpha-neoendorphin <RTI-4612 < dynorphin B (1–13) <(−)-morphine < (−)-codeine < (+)-morphine < (−)-metazocine < (+)-metazocine	LAD2(+)	Ca^{2+} mobilization; degranulation; PRESTO-tango actives for MRGPRX2(+)	Lansu et al. (2017), Yaksh et al. (2019)

			<(+)-Cis-N-normetazocine < (+)-codeine < (+)-alpha-propoxyphene <6-acetyl-codeine <6-acetyl-morphine <dynorphin A (1–9) <levorphanol thebaine			
BKRB2 antagonist	Icatibant	MRGPRX2 MrgprB2	MRGPRX2 15.8 ± 2.7 μg/mL; MrgprB2 32.5 ± 2.0 μg/mL	MPMC(+), LAD2(+), MRGPRX2-HEK293(+), MRGPRX2-RBL-2H3 (+), siMrgprX2-LAD2 (−), $MrgprB2^{MUT}$ MPMC (−), RBL-2H3 (−)	Ca^{2+} mobilization; degranulation; secretion of TNF, PGD2	McNeil et al. (2015), Roy et al. (2019a)
Contrast media	Iopamidol	MRGPRX2 MrgprB2	–	LAD2(+), MPMC (+), MRGPRX2-HEK293(+), siMrgprX2-LAD2(−), $MrgprB2^{MUT}$ MPMC(−)	Ca^{2+} mobilization; degranulation	Jiang et al. (2019)
MRGPRX2-selective ligands from compound library	ZINC	MRGPRX2	ZINC-9232,10 μM; (R)ZINC-3573, 760 nM	LAD2(+)	Ca^{2+} mobilization; degranulation; PRESTO-tango actives for MRGPRX2(+)	Lansu et al. (2017)

Ca^{2+} mobilization in LAD2 cells, $CD34^{+}$ cell-derived MCs, MRGPRX2 overexpressing HEK293 cells (MRGPRX2-HEK293 cells), MRGPRX2 overexpressing RBL-2H3 cells (MRGPRX2-RBL-2H3 cells), as well as HA-MRGPRX2-BMMC, but not in BMMC or RBL-2H3 cells (Subramanian et al. 2013). In addition, knockdown of MRGPRX2 inhibits hBD2 and hBD3 induced MCs activation (Subramanian et al. 2013). CHRG01 is a C-terminal 14 amino acid hBD3 peptide with all Cys residues replaced with Ser that displays antimicrobial activity similar to hBD3. CHRG01 induces degranulation and Ca^{2+} mobilization in LAD2 cells. Moreover, CHRG01 induces Ca^{2+} mobilization in MRGPRX2-RBL-2H3 cells (Gupta et al. 2016). β-defensins as endogenous ligands are likely to regulate innate immunity via MRGPRX2-mediated activation of human MCs (Subramanian et al. 2013).

θ-Defensin Analogs

θ-defensins, isolated from the leukocytes of rhesus macaques and baboons, are the only cyclic peptides that possess antimicrobial activity (Lehrer et al. 2012). Retrocyclin-1 (RC-100) and its analog RC-101 are synthetic cyclic octadecapeptide humanized θ-defensins with antimicrobial and anti-HIV effects and show neither hemolytic nor cytotoxic effects (Cole et al. 2002, 2007). Protegrin-1 has structural similarities with θ-defensin and was originally isolated from porcine leukocytes and has been shown to have immense therapeutic potential against infectious diseases (Kokryakov et al. 1993). RC-100, RC-101, and protegrin-1 induce degranulation and Ca^{2+} mobilization in LAD2 cells and MRGPRX2-RBL-2H3 cells (Gupta et al. 2015). Harnessing this novel feature and low cost production of θ-defensin analogs to activate MC-mediated host defense and wound-healing properties via MRGPRX2 could expand their clinical potential overcoming major hurdles in current production systems (Gupta et al. 2015).

LL-37 and LL-37-Derived Peptides

LL-37 is a C-terminal peptide of human cathelicidin antimicrobial peptide (CAMP, hCAP18) that displays direct antibacterial effects against gram-positive and gram-negative bacteria and promotes inflammation, wound healing, angiogenesis, and tumor metastasis (Burton and Steel 2009; Gupta et al. 2016; Niyonsaba et al. 2002; Subramanian et al. 2011a; Yu et al. 2017). LL-37 induces Ca^{2+} mobilization and degranulation in LAD2, $CD34^{+}$ cell-derived MCs, and RPMC (Niyonsaba et al. 2002; Subramanian et al. 2011a; Yu et al. 2017). LL-37 induces Ca^{2+} mobilization, chemotaxis, and chemokine generation in HMC-1 cells stably expressing MRGPRX2 (MRGPRX2-HMC-1 cells), but not in HMC-1 cells (Subramanian et al. 2011a). Moreover, LL-37 induces degranulation in MRGPRX2-RBL-2H3 cells but not in MRGPRX1-RBL-2H3 cells. In addition, knockdown of MRGPRX2 inhibits LL-37-induced Ca^{2+} mobilization and degranulation in LAD2 cells

(Subramanian et al. 2011a). LL-37 activates MCs via MRGPRX2 as an endogenous ligand.

FK-13 is an LL-37 peptide consisting of residues 17–29 that displays antimicrobial activity but lacks immunomodulatory properties. FK-13 induces Ca^{2+} mobilization in MRGPRX2-RBL-2H3 cells but not in untransfected RBL-2H3 cells (Gupta et al. 2016). FK-13 serves as an agonist for MRGPRX2 MCs-dependent antimicrobial activity, but not for MRGPRX1. Lipopolysaccharide causes substantial inhibition of hBD3- and LL-37-induced MCs activation via MRGPRX2, but has no effect on FK-13-induced MCs responses (Gupta et al. 2016, 2017). These findings suggest that AMPs derivatives that kill microbes, harness host defense and wound-healing properties of MCs via the activation of MRGPRX2 but are resistant to inhibition by lipopolysaccharide, could be utilized for the treatment of antibiotic-resistant microbial infections (Gupta et al. 2016).

AG-30/5C

Angiogenic peptide-30/5C (AG-30/5C), a modified version of AG-30, is generated by replacing several of its neutral amino acids with cationic amino acids. It displays greater antimicrobial activity and the ability to promote angiogenesis and wound healing (Kanazawa et al. 2016; Nakagami et al. 2012). AG-30/5C induces chemotaxis and cytokine and chemokine production including leukotriene C4 (LT-C4), PGD2, PGE2, GM-CSF, TNFα, IL-8, MCP-1, MCP-3, MIP-1α, and MIP-1β in LAD2 cells and Ca^{2+} mobilization in MRGPRX2-RBL-2H3 cells (Kanazawa et al. 2016; Roy et al. 2019a). However, AG-30/5C does not induce a robust β-arrestin activation as determined by transcriptional activation following arrestin translocation (Tango). AG-30/5C serves as a G protein-biased agonist for MRGPRX2 in MCs. An important clinical significance of these findings is that lack of functional desensitization of MRGPRX2 by AG-30/5C likely enhances its potential therapeutic efficacy as an immunomodulator in host defense (Roy et al. 2019a).

AMPs-Derived from Insulin-Like Growth Factor-Binding Protein 5

AMPs derived from insulin-like growth factor-binding protein 5 (AMP-IBP5) were originally isolated from rat brains and intestines by the cleavage of insulin-like growth factor-binding protein 5 (IGFBP-5) by serine proteases (Osaki et al. 2011). In addition to antimicrobial properties, AMP-IBP5 modulates immune responses and contributes to wound healing by recruiting and activating MCs at inflammation sites and wounds (Niyonsaba et al. 2019). AMP-IBP5 evokes MCs activation resulting in the degranulation of LAD2 cells, releasing β-hexosaminidase, cysteinyl ILs (CysLTs: LTC4, LTD4, and LTE4), PGD2, and PGE2 and producing cytokines/chemokines, including GM-CSF, TNF-α, IL-8, MCP-1, MCP-3, MIP-1α, and MIP-1β (Niyonsaba et al. 2019). Of course, AMP-IBP5-induced LAD2 cell activation depends on intracellular Ca^{2+} mobilization. MRGPRX2 siRNA suppresses the

AMP-IBP5-mediated intracellular Ca^{2+} mobilization, but not MRGPRX1, MRGPRX3, or MRGPRX4 siRNAs (Niyonsaba et al. 2019). AMP-IBP5 is an agonist for MRGPRX2, but not for MRGPRX1, MRGPRX3, or MRGPRX4. AMP-IBP5 displays immunomodulatory activity through its ability to recruit and activate MCs via MRGPRX2 at wound sites, similar to those of hBDs and LL-37 (Niyonsaba et al. 2019).

Magainin-2

Magainin-2 is a pore-forming antimicrobial peptide on the lipid matrix of bacterial membranes with broad-spectrum antimicrobial activity that was discovered in the skin of *Xenopus laevis* (Lee and Lee 2014). Magainin-2 induces histamine release in RPMC (Hook et al. 1990). Magainin-2 elicits Ca^{2+} mobilization in MRGPRX2-HEK293 cells, but not in MRGPRX1-HEK293 cells (Tatemoto et al. 2006). Magainin-2 acts as an agonist for MRGPRX2, but not for MRGPRX1.

Indolicidin

Indolicidin is a short peptide with broad-spectrum antimicrobial and cytolytic activity derived from bovine neutrophils. Indolicidin induces degranulation in $HCMC_{CT}$, and Ca^{2+} mobilization not only in MRGPRX2-HEK293 cells, but also in MRGPRX1-HEK293 cells (Tatemoto et al. 2006). Indolicidin acts as an agonist for both MRGPRX2 and MRGPRX1. It is likely that MRGPRX participates in the process of indolicidin-induced host defenses. Unsatisfactorily, severe cytotoxicity and aggregation propensity associated with indolicidin limit its probable therapeutic application even though indolicidin displays rapid and efficient antimicrobial effect (Dwivedi et al. 2019).

Catestatin

Catestatin is derived from chromogranin A and involved in multiple processes such as innate immunity, inflammatory and autoimmune reactions, and antimicrobial action (Deng and Xu 2017). Catestatin also induces degranulation or Ca^{2+} mobilization in MRGPRX2-HEK293 cells and MRGPRX1-HEK293 cells (Tatemoto et al. 2006). Catestatin acts as an agonist for MRGPRX2 and MRGPRX1. Catestatin might be a major agent of the innate defense and a potential therapeutic agent to treat multiple diseases like skin infection (Deng and Xu 2017).

Small-Molecule Nonpeptide Host-Defense Peptides

Host-defense peptides (HDPs) display antifungal and antibacterial activity, contribute to host defense, and also regulate angiogenesis and promote wound healing (Diamond et al. 2009; Kanazawa et al. 2016; Subramanian et al. 2011a). However, synthetic HDPs are prohibitively expensive, have low antimicrobial activity, are metabolically unstable, and display cytotoxicity (Gupta et al. 2015). Therefore, a series of smHDPMs which are relatively inexpensive and have distinct advantages over HDPs in terms of stability, bioavailability, and low toxicity have been developed (Alkanfari et al. 2019; Scott and Tew 2017). Ibrahim Alkanfari synthesized five smHDPs, including compound (Cpd)1, Cpd 2, Cpd 3, Cpd 4, and Cpd 5 and tested their effects on antimicrobial activity and MCs degranulation (Alkanfari et al. 2019). Five smHDPMs with low cytotoxicity displayed differences in their ability to kill bacteria and fungi and induced MCs degranulation via MRGPRX2 or MrgprB2 (Alkanfari et al. 2019). It is possible that these novel synthetic peptide mimetic MRGPRX2/MrgprB2 agonists could form the basis of developing novel therapeutic agents for the treatment of drug-resistant fungal and bacterial infection via harnessing immunomodulatory properties of MCs.

Polymyxins

Polymyxins are a group of polycationic lipopeptide antibiotics including polymyxin A, B, C, D, and E that are used for the treatment of gram-negative infection-caused meningitis, pneumonia, sepsis, and urinary tract infections (Hoeprich 1970). Polymyxins induced MCs activation primarily elicits pseudo-allergic reactions with symptoms such as mild itching, episodes of rash, and contact dermatitis (eczema and erythematous eruption) (Henao and Ghaffari 2016; Knowles and Shear 1995; Zhan et al. 2019). Polymyxin B and polymyxin E evoke the release of histamine and β-hexosaminidase and increased the secretion of TNFα and PGD2 in LAD2 cells, but not in siMrgprX2-LAD2 cells (Zhan et al. 2019). Moreover, polymyxin B and polymyxin E increase Ca^{2+} influx both in MRGPRX2-HEK293 cells and MrgprB2-HEK293 cells (Zhan et al. 2019). Polymyxin B and polymyxin E cause capillary permeability and paw swelling in WT mice, but not in $MrgprB2^{MUT}$ mice (Zhan et al. 2019). The MRGPRX2- or MrgprB2-mediated MCs activation by polymyxin B and polymyxin E may mainly contribute to pseudo-allergic reactions.

5.1.2 Neuropeptides

Substance P

SP is secreted by neurons and participates in many biological processes, including nociception and inflammation (Mashaghi et al. 2016). In 1978, Östen Hägermark

found that synthetic SP could induce flare, wheal, and itching mediated by histamine released from dermal MCs (Hagermark et al. 1978). SP induces degranulation and Ca^{2+} mobilization in a variety of MCs, including HSMC, LAD2 cells, RPMC, MPMC, and $HCMC_{CT}$, but not $HCMC_{M}$ (Barrocas et al. 1999; Hagermark et al. 1978; McNeil et al. 2015; Tatemoto et al. 2006). SP induced MCs activation by exciting two subtypes of MRGPRs as endogenous ligands: MRGPRX2 and MrgprB2, but not MRGPRX1, MRGPRX3, MRGPRX4, MrgprB1, MrgprB10, or MrgprB11 (Azimi et al. 2016; McNeil et al. 2015; Tatemoto et al. 2006). [d-$Trp^{7,9,10}$]-substance P and [d-Pro^{4}, d-$Trp^{7,9,10}$]-substance P also induce Ca^{2+} mobilization in MRGPRX2-HEK293 cells, but not in MRGPRX1-HEK293 cells (Tatemoto et al. 2006). MRGPRX2 and MrgprB2 mediate neurogenic inflammatory mechanical and thermal hyperalgesia induced by SP and are required for recruitment of innate immune cells at the injury site, suggesting that MRGPRX2/MrgprB2 acts as an important neuroimmune modulator and a potential target for treating inflammatory pain (Green et al. 2019).

Pituitary Adenylate Cyclase-Activating Peptides

Pituitary adenylate cyclase-activating peptides (PACAPs) are NPs of the vasoactive intestinal polypeptide/secretin/glucagon family (Kinhult et al. 2000; Reglodi et al. 2018). PACAP-38 is an endogenous 38-amino acid neuropeptide located in both sensory and parasympathetic perivascular nerve fibers (Moller et al. 1993). PACAP-induced MCs activation presents as pseudo-allergic reactions. Infusion of PACAP-38 causes not only migraine attacks but also heat sensation and long-lasting flushing (Schytz et al. 2009). PACAP-38 induces degranulation of RPMC and meningeal MCs via the orphan MrgprB3 receptor (Pedersen et al. 2019). PACAP (6–27) is a long-term administration of PACAP receptor antagonists. PACAP (6–27) could induce the degranulation of $HCMC_{CT}$ (Tatemoto et al. 2006). PACAP (6–27) induced Ca^{2+} mobilization in MRGPRX2-HEK293 cells and MrgprB3-HEK293 cells, but not in MRGPRX1-HEK293 cells (Tatemoto et al. 2006). PACAPs act as agonists for MRGPRX2 and MrgprB3. PACAPs may modulate the immune system via MRGPRX2/MrgprB3 mediated MCs activation during the pathogenesis development of inflammatory diseases like atopic dermatitis, psoriasis, and rosacea (Choi and Di Nardo 2018; Pedersen et al. 2019).

Somatostatin and Its Analogues

Somatostatin regulates the endocrine system and affects neurotransmission and cell proliferation via its interaction with GPCR and inhibits the release of growth hormones from the pituitary. Angiopeptin is a cyclic octapeptide analogue of somatostatin-14 and displays immunosuppressive effects via modulation of leukocyte–endothelial interactions (Leszczynski et al. 1995). Somatostatin elicits histamine release and Ca^{2+} mobilization and lowered intracellular cAMP levels in

RPMC (Waalkens et al. 1981). In addition, somatostatin and angiopeptin induce the degranulation of $HCMC_{CT}$ (Tatemoto et al. 2006). Angiopeptin induces Ca^{2+} mobilization in MRGPRX2-HEK293 cells, but not in MRGPRX1-HEK293 cells (Tatemoto et al. 2006).

Cortistatin-14 is a neuropeptide that is structurally and functionally related to somatostatin-14 and binds the somatostatin receptor which is a GPCR (de Lecea et al. 1996; Tostivint et al. 1996). Cortistatin-14 also activates the MRGPRX2 receptor. Cortistatin-14 increases intracellular cAMP and Ca^{2+} mobilization in Chinese hamster ovary (CHO) cells stably expressing MRGPRX2 (MRGPRX2-CHO cells), but not in CHO cells expressing MRGPRX1, MRGPRX3, and MRGPRX4 (Kamohara et al. 2005). Cortistatin-14 induces an increase of intracellular Ca^{2+} concentration in MRGPRX2-HEK293 cells and MrgprB2-HEK293 cells (McNeil et al. 2015). Knockdown of MRGPRX2 suppresses cortistatin-mediated intracellular Ca^{2+} mobilization in LAD2 cells (Subramanian et al. 2013). Somatostatin and its analogues act as agonists for MRGPRX2, but not for MRGPRX1. MRGPRX2-mediated MCs activation is likely to contribute to inflammation and allergic diseases induced by somatostatin and its analogues.

5.1.3 Peptide Hormones

Pro-adrenomedullin Peptides

Pro-adrenomedullin peptides (PAMPs) are potent hypotensive peptides that can be isolated from human pheochromocytoma tissue (Kuwasako et al. 1997; Mahata et al. 1998). PAMPs give rise to hypotension through inhibition of catecholamine secretion from sympathetic nerve endings and adrenal chromaffin cells (Niina et al. 1995; Shimosawa et al. 1995). The ion-complementary self-assembling peptide PAMP-12, also known as PAMP9–20, is modified by self-assembling peptide RADA4 which is composed of alternating hydrophobic and hydrophilic amino acids (Lu et al. 2018). PAMP-12 and RADA4-GG-PAMP-12 induce degranulation of LAD2 cells (Lu et al. 2018). Both PAMP-12 and PAMP-20 increase intracellular cAMP or calcium mobilization in MPMC, Chinese hamster ovary (CHO) cells, or HEK293 expressing MRGPRX2, but not in cells expressing MRGPRX1, MRGPRX3, MRGPRX4, MrgprB1, MrgprB10, MrgprB11, as well as $MrgprB2^{MUT}$ MPMC (Bairamashvili et al. 1989; Kamohara et al. 2005; McNeil et al. 2015). The rank order of appropriate ligand activities was "PAMP-12 > PAMP-20." Subcutaneous injection of PAMP-12 elicited itching in wild-type mice, while the itching was reduced in MrgprB2-deficient mice (Meixiong et al. 2019). PAMPs mainly excite two MRGPRs subtypes: MRGPRX2 and MrgprB2.

Bovine adrenal medulla (BAM) 8–22, an endogenous itch-inducing peptide, was first isolated from bovine adrenal medulla. BAM(8–22) is involved in cholestatic pruritus through MRGPRX1 in dorsal root ganglia (Sanjel et al. 2019). BAM(8–22) has little effect on the degranulation of $HCMC_{CT}$. BAM(8–22) increases the intracellular Ca^{2+} concentration in MRGPRX1-HEK293 cells but not in

MRGPRX2-HEK293 cells(Tatemoto et al. 2006). However, BAM(8–22) could induce the release of chemokine ligand 2 (CCL2) in LAD2 cells, suggesting that MRGPRX1 also exerts its action in cells of the immune system (Solinski et al. 2013). BAM(8–22) acts as a specific agonist for MRGPRX1.

Vasoactive Intestinal Peptide

Vasoactive intestinal peptide (VIP) was initially isolated from porcine duodenum and has multiple physiological effects through regulation of gut motility, immune responses, ion secretion, nutrient absorption, and circadian rhythms and has therapeutic potential in inflammatory disorders (Iwasaki et al. 2019). VIP not only activates MCs, but can also release from MCs. VIP has been detected in MPMC, BMMC, cultured PT-18, and C1.MC/C57.1 lines of MCs, as well as in RBL-2H3 cells (Wershil et al. 1993). VIP induces the degranulation of $HCMC_{CT}$ (Tatemoto et al. 2006). VIP induces Ca^{2+} mobilization not only in MRGPRX2-HEK293 cells, but also in MRGPRX1-HEK293 cells (Tatemoto et al. 2006). VIP acts as an endogenous agonist for both MRGPRX2 and MRGPRX1.

Cetrorelix

Cetrorelix is a gonadotropin-releasing hormone antagonist that competitively blocks binding with gonadotropin-releasing hormone (GnRH) receptors on pituitary cell to inhibit secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Tur-Kaspa and Ezcurra 2009). Cetrorelix increases Ca^{2+} mobilization in MrgprB2-HEK293 cells, MRGPRX2-HEK293 cells, and MPMC cells from WT mice (WT MPMC), but not in MPMC cells from $MrgprB2^{MUT}$ mice ($MrgprB2^{MUT}$ MPMC) (McNeil et al. 2015). Cetrorelix is an agonist for both MRGPRX2 and MrgprB2.

Leuprolide

Leuprolide is a nonapeptide agonist of GnRH that induces secretion of LH and FSH (Swayzer and Gerriets 2019). Leuprolide is similar to cetrorelix in that it activates MrgprB2-HEK293 cells, MRGPRX2-HEK293 cells, as well as WT MPMC, but not in $MrgprB2^{MUT}$ MPMC (McNeil et al. 2015). Leuprolide is an agonist for both MRGPRX2 and MrgprB2.

Sermorelin

Sermorelin is the shortest synthetic analogue of human growth hormone-releasing hormone (GHRH) known to stimulate growth hormone secretion from the anterior

pituitary (Prakash and Goa 1999). Sermorelin increased Ca^{2+} mobilization in MrgprB2-HEK293 cells, MRGPRX2-HEK293 cells, and WT MPMC, but not in MrgprB2^{MUT} MPMC (McNeil et al. 2015).

Octreotide

Octreotide is a long-acting synthetic somatostatin analog that plays an inhibitory role in pituitary, pancreatic, and gastrointestinal hormone secretion (Katz and Erstad 1989). Octreotide increases Ca^{2+} mobilization in MrgprB2-HEK293 cells, MRGPRX2-HEK293 cells, and MPMC but not in MrgprB2^{MUT} MPMC (McNeil et al. 2015). Octreotide acts as an agonist for both MRGPRX2 and MrgprB2.

Kallidin

Kallidin, a decapeptide kinin, is released from kininogens mainly by the action of kallikrein (Werle and Trautschold 1963). Kallidin induces Ca^{2+} mobilization in WT MPMC but not in MrgprB2^{MUT} MPMC (McNeil et al. 2015). Kallidin acts as an agonist for MrgprB2. These peptide hormones, such as PAMPs, VIP, some agonists and antagonists of GnRH, and kallidin, mainly induce pseudo-allergic diseases via MRGPRX2-mediated activation of MCs.

5.1.4 Mast Cell Degranulating Peptides

Mast Cell Degranulating Peptide

Mast cell degranulating peptide (MCDP) is a 22-amino acid residue component of bee venom with allergy and inflammation effects (Buku 1999). The peptide is a strong mediator of MCs degranulation and histamine release at low concentrations and was the first named as MCDP (Jasani et al. 1979). MCDP induced the degranulation of $HCMC_{CT}$ (Tatemoto et al. 2006). MCDP induces Ca^{2+} mobilization in MRGPRX2-HEK293 cells and MrgprB3-HEK293 cells, but not in MRGPRX1-HEK293 cells (Tatemoto et al. 2006). MCDP acts as an agonist for MRGPRX2 and MrgprB3.

Granuliberin R

Granuliberin R is a MCDP that is found in the skin of the frog. Granuliberin R induced K^+ efflux in bacteria, erythrocytes, and MCs and histamine release in RPMC (Nakao et al. 2011). Granuliberin R induced Ca^{2+} mobilization not only in MRGPRX2-HEK293 cells, but also in MRGPRX1-HEK293 cells (Tatemoto et al. 2006). Granuliberin R is an agonist for both MRGPRX2 and MRGPRX1.

Mastoparan

Mastoparan is a membrane-active amphipathic component of wasp and displays a wide variety of biological activities, including antimicrobial activity, the release of histamine from MCs, hemolytic activity, and lytic activity (Cabrera et al. 2011; Mukai et al. 2008; Yibin et al. 2005). Mastoparan induces K^+ efflux and the release of histamine and increases Ca^{2+} mobilization in RPMC (Mousli et al. 1989; Nakao et al. 2011). Mastoparan evokes degranulation in a human MCs line ROSA, a murine CTMC line (MC/9), as well as mouse skin MCs (MSMC), but not in RBL-2H3 cells (Arifuzzaman et al. 2019). Mastoparan increases the release of TNF, GM-CSF, IL-8, chemokine (C-C motif) ligands (CCL) 2, and CCL3 in LAD2 cells (Arifuzzaman et al. 2019). Mastoparan activates MrgprB2-HEK293 cells, MRGPRX2-HEK293 cells, and MPMC cells, but not $MrgprB2^{MUT}$ MPMC (McNeil et al. 2015). Mastoparan is a stimulant of MRGPRX2 and MrgprB2. MRGPRX2 contributes to MCDPs-induced pseudo-allergic diseases and inflammatory diseases.

5.1.5 Other Endogenous Protein Fragments

There are other endogenous protein fragments which can activate MCs via MRGPRX2, such as chaperonin-10, 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNP) (1–17), tropomyosin (1–12), endogenous protein fragments from human serum albumin (HSA), platelet factor-4 (PF-4), and HK-1. MRGPRX2 may recognize various endogenous ligands and plays an important role in regulating inflammatory responses to endogenous harmful stimuli (Tatemoto et al. 2018).

CNP (1–17) and Tropomyosin (1–12)

Chaperonin-10 (1–20), 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNP) (1–17), and tropomyosin (1–12) are isolated from porcine brain and intestinal extracts (Tatemoto et al. 2018). These peptides cause the release of β-hexosaminidase on $HCMC_{CT}$ (Tatemoto et al. 2018). They cause increases in Ca^{2+} influx and activation of G proteins in MRGPRX2-HEK293 cells (Tatemoto et al. 2018). Chaperonin-10 (1–20), CNP(1–17), and tropomyosin(1–12) act as agonists for MRGPRX2.

Human Serum Albumin Sequences

Karhu et al. isolated endogenous protein fragments from HSA407–423, 408–423, and 409–423 (Karhu et al. 2017). HSA sequences 407–423, 408–423, and 409–423 increased Ca^{2+} mobilization or degranulation in LAD2 cells and MRGPRX2-HEK293 cells, but not in MRGPRX1-HEK293 cells (Karhu et al. 2017). Surprisingly, HSA (175–182) induces MCs activation via MRGPRX2 as well as MRGPRX1 (Karhu et al. 2017).

Platelet Factor-4

PF-4 is released from the alpha-granules of activated platelets that binds with high affinity to heparin to promote blood coagulation and plays a role in wound repair and inflammation. A PF-4 biologically active carboxyl-terminal fragment stimulates histamine release in RPMC (Suzuki et al. 2002). PF-4 induces Ca^{2+} mobilization in MRGPRX2-HEK293 cells, but not in MRGPRX1-HEK293 cells (Tatemoto et al. 2006). PF-4 acts as an agonist for MRGPRX2, but not MRGPRX1.

Hemokinin-1

HK-1 is produced by human bronchial cells and lung macrophages and can cause the contraction of bronchi (Grassin-Delyle et al. 2010). As a NK-1R antagonist, HK-1 activates NK-1R on MCs as an adjuvant for IgE-mediated anaphylaxis and lung inflammation in a MC-dependent chronic asthma model (Sumpter et al. 2015). In addition to NK-1R, HK-1 also activates MRGPRX2 on MCs. HK-1 induces degranulation in LAD2 cells and in MRGPRX2-RBL-2H3 cells (Manorak et al. 2018). The antagonist of NK-1R, CP96345, causes substantial inhibition of HK-induced degranulation in NK-1R-RBL-2H3, but not in LAD2 or MRGPRX2-RBL-2H3 cells. Moreover, knockdown of MRGPRX2 reduces the degranulation in LAD2 cells (Manorak et al. 2018). HK-1 acts as an agonist for both MRGPRX2 and NK-1R.

5.2 Nonpeptides

5.2.1 Compound 48/80

C48/80 is regarded as a histamine liberator or a MC activator that promotes MCs degranulation and is a condensation product of N-methyl-p-methoxyphenethylamine with formaldehyde (Paton 1951). C48/80 displays a strong ability to activate MCs, including RMMC, MPMC, LAD2 cells, and $HCMC_{CT}$, but not $HCMC_{M}$ (McNeil et al. 2015; Rothschild 1970; Tatemoto et al. 2006). C48/80 induces degranulation or Ca^{2+} mobilization in MRGPRX2-HEK293 cells and MRGPRX1-HEK293 cells, as well as MRGPRX2-RBL-2H3 cells, but not in RBL-2H3 cells, HMC-1 cells, MRGPRX1-HEK293 cells, MrgprB1-HEK293 cells, MrgprB10-HEK293 cells, MrgprB11-HEK293 cells, or $MrgprB2^{MUT}$ MPMC (Kashem et al. 2011; McNeil et al. 2015; Roy et al. 2019a; Tatemoto et al. 2006). Moreover, C48/80 pretreatment causes reduction in cell surface MRGPRX2 expression in MRGPRX2-RBL-2H3 cells (Roy et al. 2019a). The activation is significantly reduced in LAD2 cells transfected with MRGPRX2 siRNA (siMrgprX2-LAD2 cells) or in $MrgprB2^{MUT}$ MPMC (Lumry et al. 2011; McNeil et al. 2015). C48/80-triggered tracheal contraction and hindpaw inflammation are almost completely absent in $MrgprB2^{MUT}$ mice

(McNeil et al. 2015). Therefore, MRGPRX2, MRGPRX1, and MrgprB2 are mainly involved in C48/80-induced pseudo-allergic reactions.

5.2.2 Neuromuscular Blocking Agents

NMBAs are widely used to provide muscle relaxation during anesthesia (Martinez 2002). The activation of MCs induced by NMBAs mainly displays as adverse reaction anaphylaxis or pseudo-allergic drug reactions. The incidence rate of hypersensitivity has been estimated at about 1/5,000 for NMBAs (de Pater et al. 2017). Atracurium, more than vecuronium, induces histamine release from HSMC and human lung parenchyma MCs (HLMC) (Marone et al. 1993). The perspective that NMBAs mainly cause anaphylaxis via an IgE-mediated mechanism has been challenged (Spoerl et al. 2017). NMBA-induced hypersensitivity is mediated by MRGPRX2. Mivacurium induces Ca^{2+} mobilization and degranulation of LAD2 cells resulting in the release of β-hexosaminidase, histamine, and TNFα, but has no effect in siMrgprX2-LAD2 cells (Che et al. 2018). Atracurium, rocuronium, and mivacurium increase Ca^{2+} mobilization in MrgprB2-HEK293 cells, MRGPRX2-HEK293 cells, and WT MPMC, but not in $MrgprB2^{MUT}$ MPMC (Che et al. 2018; McNeil et al. 2015). Moreover, mivacurium induces pseudo-allergic reactions in WT mice but not in $MrgprB2^{MUT}$ mice (Che et al. 2018). NMBAs act as agonists for MRGPRX2 and MrgprB2.

5.2.3 Fluoroquinolone Antibiotics

Fluoroquinolone antibiotics are bactericidal agents that inhibit DNA synthesis in bacteria. Quinolones are the second most common antibiotic class to be associated with drug-induced hypersensitivity (McGee et al. 2019). Based on estimates of exposure, the corresponding hypersensitivity rates are 3.3, 0.6, 0.2, and 0.2 per one million for moxifloxacin, levofloxacin, ciprofloxacin, and ofloxacin, respectively (Sachs et al. 2006). Ciprofloxacin, levofloxacin, and moxifloxacin induced MCs activation and cause hypersensitivity. Ciprofloxacin induces histamines from RSMC, RPMC, and canine skin MCs (CSMC) (Mori et al. 2000a; Roy et al. 2019b). The sensitivity level of MCs is CSMC>RPMC>RSMC (Mori et al. 2000a). Ciprofloxacin increases Ca^{2+} mobilization in WT MPMC, but not in $MrgprB2^{MUT}$ MPMC (McNeil et al. 2015). Knockdown of MRGPRX2 inhibits Ca^{2+} mobilization induced by ciprofloxacin in LAD2 cells (Han et al. 2017). Ciprofloxacin, levofloxacin, moxifloxacin, and ofloxacin increase Ca^{2+} mobilization in MrgprB2-HEK293 cells and MRGPRX2-HEK293 cells (McNeil et al. 2015). The rank order of ligand activities for MRGPRX2 and MrgprB2 is "ciprofloxacin> moxifloxacin > levofloxacin > ofloxacin" and "moxifloxacin > ciprofloxacin > levofloxacin > ofloxacin," respectively.

5.2.4 Opioid Drugs

Opiates activate MRGPRX2, which degranulates MCs, activates fibroblasts, and results in intrathecal mass formation (Yaksh et al. 2019). Morphine, but not PZM21 (biased μ agonist) or 2′,6′-dimethyl tyrosine-(Tyr-D-Arg-Phe-Lys-NH2) (DMT-DALDA), stimulates degranulation of LAD2 cells (Yaksh et al. 2019). MRGPRX activation was produced by morphine, DMT-DALDA, and TAN67 (∂− -opioid agonist), but not by PZM21 or TRV130 (μ biased ligand) (Yaksh et al. 2019). MRGPRX2 is activated by many dextro-enantiomers and N-methyl scaffolds of opioids (Lansu et al. 2017). The rank order of morphinan ligand activities for MRGPRX2 is dextrorphan > dextrabenzylorpan > levorphanol, while levallorphan is completely inactive (Lansu et al. 2017). MRGPRX2 prefers dextrorotary morphinan sinomenine and dextrorotary benzomorphan compounds including (+)-cis-normetazocine. Moreover, (+)- and (−)-morphine enantiomers, (+)- and (−)-codeine, and thebaine have similar activities for MRGPRX2 (Lansu et al. 2017). MRGPRX2 is also activated by endogenous opioid ligands. Prodynorphin-derived peptides including dynorphin A(1–17), dynorphin A(1–13), and dynorphin A(1–9) activate MRGPRX2, while both dynorphin A(1–7), A(1–8), dynorphin A(1–6) truncated C-terminal amino acid, and dynorphin A(13–17) truncated N-terminal YGGF motif are completely inactive (Lansu et al. 2017). Dynorphin A(1–13), dynorphin B(1–13), and α- and β-neoendorphin also activate MRGPRX2 (Lansu et al. 2017).

5.2.5 Herbal Extracts

Sinomenine

Sinomenine is a natural alkaloid and mainly extracted from the root of the traditional Chinese medicinal plant *Caulis sinomenii*. It possesses immunomodulatory, anti-inflammatory, anti-hypertensive, and anti-arrhythmic activity (Qian et al. 2007; Zhao et al. 2012). However, sinomenine hydrochloride induces anaphylactoid reactions (Huang et al. 2017). Sinomenine hydrochloride induces histamine release and Ca^{2+} mobilization in RBL-2H3 cells, P815 cells, LAD2 cells, MPMC, MrgprB2-HEK293 cells, and MRGPRX2-HEK293 cells, but not in siMrgprX2-LAD2 cells or $MrgprB2^{MUT}$ MPMC (Liu et al. 2017; Wang et al. 2016). In addition to degranulation, sinomenine increases chemokine secretion such as MCP-1, IL-8, and MIP-1α via P38 pathway. Sinomenine hydrochloride evokes extensive extravasation and swelling in WT mice, but not in $Kit^{W\text{-}sh/W\text{-}sh}$ mice and $MrgprB2^{MUT}$ mice (Liu et al. 2017).

Complanadine A

Complanadine A is an alkaloid with a lycodine-type skeleton that can be isolated from the club moss *Lycopodium complanatum* (Kobayashi et al. 2000). Complanadine A was found to modulate MRGPRX2 as an agonist with the ability to mobilize Ca^{2+} (Johnson and Siegel 2014).

5.2.6 Other Drugs

Icatibant

Icatibant, a bradykinin B2 receptor (BKRB2) antagonist, is used for the treatment of hereditary angioedema. The most common adverse events at icatibant injection sites are pseudo-allergic drug reactions and angioedema, which may involve MCs activation (Lumry et al. 2011). The activation of LAD2 cells in response to icatibant is characterized by the release of β-hexosaminidase, TNF, PGD2, and histamine (McNeil et al. 2015). Icatibant induces degranulation or Ca^{2+} mobilization in MRGPRX2-HEK293 cells, MRGPRX2-RBL-2H3 cells, MrgprB2-HEK293 cells, and MPMC, but not in siMrgprX2-LAD2 cells, or MrgprB2^{MUT} MPMC (McNeil et al. 2015; Roy et al. 2019a). Icatibant acts as an agonist for MRGPRX2 and MrgprB2.

Iopamidol

Iopamidol, a radiographic contrast medium, causes a very high incidence (13.9%) of hypersensitivity (Cutroneo et al. 2007). Iopamidol induces hindpaw swelling and extravasation in WT mice, but not in MrgprB2^{MUT} mice (Jiang et al. 2019). Iopamidol increases intracellular Ca^{2+} and the release of β-hexosaminidase, histamine, and TNFα in MRGPRX2-HEK293 cells, MPMC, and LAD2 cells, but not in MrgprB2^{MUT} MPMC or siMrgprX2-LAD2 cells (Jiang et al. 2019). These FDA-approved drugs, such as NMBAs, fluoroquinolone antibiotics, opioid drugs, some herbal extracts, and iopamidol, induce degranulation of MCs via MRGPRX2 and cause pseudo-allergic diseases.

5.2.7 MRGPRX2-Selective Ligands from a Compound Library

ZINC is a free public resource database for ligand discovery and can be searched by structure, biological activity, physical property, vendor, and other parameters (Irwin et al. 2012). Katherine Lansu et al. (2017) assayed 20 compounds from the top-ranked compounds for MRGPRX2 activity in ZINC. Of these compounds, ZINC-9232 (EC50 of <10 μM) and ZINC-3573 (EC50 of <1 μM) activate MRGPRX2 both in Tango and Ca^{2+} mobilization assays (Lansu et al. 2017).

ZINC-9232 and ZINC-3573 also induce intracellular calcium release and degranulation in LAD2 cells and MRGPRX2-HEK293 cells, but not in siMrgprX2-LAD2 cells (Lansu et al. 2017).

6 Inhibitors

Some non-selective GPCR inhibitors, cytokines, herbal extracts, and small-molecule MRGPRX2 antagonists, as well as DNA aptamer drugs can non-competitively or competitively bind with MRGPRX2, or inhibit MRGPRX2-mediated signaling, display the effect of anti-pseudo-allergic reactions or anti-inflammation. Inhibitors of MRGPRX2-mediated MCs activation are shown in Table 3. The inhibition of MCs degranulation by these inhibitors has important clinical implication for modulating pseudo-allergic drug reactions and inflammatory diseases.

6.1 GPCR Inhibitors

6.1.1 Pertussis Toxin

PTx is a multisubunit protein toxin secreted by *Bordetella pertussis* that displays an important effect in bacterial colonization and in immunomodulation to evade innate or adaptive immunity (Carbonetti 2015). PTx non-selectively inhibits GPCR signaling through Gαi proteins (Asano et al. 1984; Katada 2012). PTx inhibits Ca^{2+} mobilization or degranulation induced by agonists for Mrgprs, such as hBDs (Chen et al. 2007), LL-37 (Niyonsaba et al. 2002), C48/80 (Mori et al. 2000b), SP (Barrocas et al. 1999), icatibant (Roy et al. 2019a), PACAP(6–27) (Tatemoto et al. 2006), MCDP (Tatemoto et al. 2006), [Lys^{10}, Leu^{13}] mastoparan (Mukai et al. 2007), and AG-30/5C (Roy et al. 2019a).

6.1.2 NK-1R Antagonists

NK-1R antagonists possess antidepressant, anxiolytic, and antiemetic properties (Pojawa-Golab et al. 2019). A tripeptide named as QWF has a dual function as an antagonist of NK-1R and of MRGPRs (Azimi et al. 2016). QWF inhibits SP-induced activation of MrgprB2, MRGPRX2 as well as MrgprA1 in HeLa cells and itching in mice (Azimi et al. 2016). QWF also inhibits the degranulation induced by C48/80, atracurium, and ciprofloxacin in LAD2 cells (Azimi et al. 2016). NK-1R antagonists L733060 and aprepitant prevent SP-induced activation of MrgprB2 but not of MRGPRX2 or MrgprA1 (Azimi et al. 2016). These findings may promote the development process of several NK-1R antagonists in clinical trials for treatment of inflammatory skin disease, uremic itch, and cholestatic itch (Azimi et al. 2016).

Table 3 Antagonists of Mas-related G protein-coupled receptors in mast cell activation

Inhibitors	Stimulant	Inhibition for receptor	Inhibited cell types	References
PTx	C48/80, SP, Icatibant, PACAP (6–27), hBD3, hBD4, LL-37, MCDP, [Lys^{10}, Leu^{13}] mastoparan, AG-30/5C	Gαi proteins	LAD2, RPMC, MRGPRX2-RBL-2H3, MRGPRX2-HEK293	Barrocas et al. (1999), Chen et al. (2007), Mori et al. (2000b), Mukai et al. (2007), Niyonsaba et al. (2002), Roy et al. (2019a), Tatemoto et al. (2006)
Saikosaponin A	C48/80, ciprofloxacin, sisomicin, morphine	MRGPRX2	MSMC, LAD2, MRGPRX2-HEK293	Wang et al. (2018)
QWF	C48/80, SP, atracurium, ciprofloxacin	MrgprB2, MRGPRX2, MrgprA1, NK-1R	LAD2, NK-1R-HeLa, MrgprB2-HeLa, MrgprA1-HeLa, MRGPRX2-HEK293, MrgprB2-HEK293, MrgprA1-HEK293	Azimi et al. (2016)
L733060	SP	MrgprB2, NK-1R	MrgprB2-HeLa	Azimi et al. (2016)
Aprepitant	SP	MrgprB2, NK-1R	MrgprB2-HeLa	Azimi et al. (2016)
IL-33	C48/80, SP	MRGPRX2	HSMC	Wang et al. (2019b)
SCF	C48/80, SP	MRGPRX2	HSMC	Babina et al. (2018a)
Resveratrol	C48/80, AG-30/5C, Icatibant	MRGPRX2	LAD2, MRGPRX2-RBL-2H3	Roy et al. (2019a)
Quercetin	C48/80, SP	MRGPRX2	LAD2, MRGPRX2-HEK293	Ding et al. (2019)
Osthole	C48/80, SP, LL-37	MRGPRX2	LAD2, MRGPRX2-RBL-2H3	Callahan et al. (2020)
Paeoniflorin	C48/80	MRGPRX2	LAD2	Wang et al. (2020b)
Genistein	C48/80	MRGPRX2	LAD2, MRGPRX2-HTLA	Kumar et al. (2020)
Shikonin	C48/80	MRGPRX2	LAD2, MRGPRX2-HEK293	Wang et al. (2020a)

(continued)

Table 3 (continued)

Inhibitors	Stimulant	Inhibition for receptor	Inhibited cell types	References
Piperine	C48/80, LL-37	MRGPRX2	LAD2, MRGPRX2-HEK293	Qiao et al. (2020)
Paeoniflorin	C48/80	MRGPRX2	LAD2	Wang et al. (2020b)
Small-molecule MRGPRX2 antagonists	SP, cortistatin-14	MRGPRX2	MRGPRX2-HEK293, $HCMC_{CT}$	Ogasawara et al. (2019)
Aptamer-X35	C48/80, SP	MRGPRX2	MRGPRX2-RBL-2H3	Suzuki et al. (2020)

6.2 Cytokines

6.2.1 Interleukin-33

Interleukin-33 (IL-33) is a tissue-derived nuclear cytokine, which belongs to the IL-1 superfamily, expressed in endothelial cells, epithelial cells, and fibroblast-like cells, and acts as a crucial immune modulator with pleiotropic activities in immune responses, and an important role in allergic, fibrotic, infectious, and chronic inflammatory diseases (Cayrol and Girard 2018). IL-33 has also gained fame as a MC lineage-supportive and -modulating cytokine. A chronic exposure of skin MCs to IL-33 virtually eliminates expression of MRGPRX2 via c-Jun N-terminal kinase (JNK), erases MCs responsiveness to C48/80 and SP, and eliminated the pseudo-allergic/neurogenic route (Wang et al. 2019b). However, a short burst of IL-33 primes the pseudo-allergic/neurogenic (and also the allergic) effect and degranulation of skin-derived MCs by activating p38 (Wang et al. 2019b). IL-33 crucially regulates the MRGPRX2 cascade in skin MCs and displays a dual-direction regulation on the allergic and pseudo-allergic/neurogenic routes.

6.2.2 Stem Cell Factor

Stem cell factor (SCF) is a dimeric molecule and acts through the c-Kit receptor tyrosine kinase on cell survival, migration, and proliferation depending on the cell type (Lennartsson and Ronnstrand 2012). SCF dominates MCs biology. In addition to growth and survival, SCF also promotes degranulation via FcεRI when given acutely (Babina et al. 2018a). Conversely, short preincubation with SCF decreases the responsiveness of HSMC such as histamine release to C48/80 and SP and potently inhibits pseudo-allergic degranulation (Babina et al. 2018a). In addition, it not only acutely interferes with the MRGPRX2 initiated cascade, but also through restriction of receptor expression to perpetuate inhibition (Babina et al. 2018b). The

clinically meaningful MC degranulation pathways are independent from each other, and SCF can have selective dampening functions on MCs (Babina et al. 2018b).

6.3 *Herbal Extracts*

6.3.1 Saikosaponin A

Saikosaponin A is an important active compounds extracted from *Radix bupleuri* and has the effect of inflammation inhibition, relieving pain and anti-allergy (Fu et al. 2015; Park et al. 2002; Zhou et al. 2014). Saikosaponin A inhibits C48/80-induced calcium flux and degranulation in LAD2 cells and skin inflammation in vivo (Wang et al. 2018). This inhibition also exists in C48/80-induced Ca^{2+} influx in MRGPRX2-HEK293 cells (Wang et al. 2018). In addition to C48/80, saikosaponin A also restrains calcium flux and degranulation induced by ciprofloxacin hydrochloride, sisomicin, and morphine hydrochloride in LAD2 cells (Wang et al. 2018). Saikosaponin A acts as an inhibitor for MRGPRX2.

6.3.2 Resveratrol

Resveratrol, a polyphenolic phytoalexin found in a variety of plant species and berries, exhibits antioxidant, anti-inflammatory, anti-apoptotic, and anticancer effects (Jardim et al. 2018). Resveratrol inhibits degranulation induced by C48/80, AG-30/5C, and icatibant in MRGPRX2-RBL-2H3 cells and in LAD2 cells (Roy et al. 2019a). The inhibition of MCs degranulation by resveratrol has important clinical implication for modulating pseudo-allergic drug reactions.

6.3.3 Quercetin

Quercetin, a biological brass compound, was found in tea, apples, onions, apples, and grapes that have antitumor, antioxidant, anti-radiation, antiviral, and anti-cardiovascular diseases activity (Chu et al. 1992; Kandaswami et al. 1992; Sugihara et al. 1999). Quercetin also attenuates C48/80- and SP-triggered paw thickness in mice and inhibits PLCγ-IP_3R-associated calcium influx and the release of β-hexosaminidase, histamine, MCP-1, and IL-8 in MRGPRX2-HEK293 cells or LAD2 cells (Ding et al. 2019). Quercetin is a potential candidate to suppress MRGPRX2-induced pseudo-allergic reactions (Ding et al. 2019).

6.3.4 Osthole

Osthole, 7-methoxy-8-isopentenoxycoumarin, is a natural coumarin found in the fruits of *Cnidium monnieri (L.)* and has the effect of anti-inflammation, antitumor, antidiabetic properties and acts against allergic asthma (Chiang et al. 2017; Chou et al. 2007; Fan et al. 2019; Liang et al. 2009). Interestingly, recent reports demonstrated that osthole inhibits MRGPRX2 responses in MCs (Callahan et al. 2020). Osthole attenuates C48/80-, SP-, and LL-37-induced Ca^{2+} mobilization, degranulation, and chemokine/cytokine production in LAD2 cells or MRGPRX2-RBL-2H3 cells (Callahan et al. 2020). Moreover, osthole does not compete with the MRGPRX2 ligands for interaction with the receptor, but rather regulates MRGPRX2 activation via allosteric modifications and reduces both surface and intracellular expression levels of MRGPRX2 in MCs (Callahan et al. 2020). Osthole, a natural compound, is a potential and safer candidate for the treatment of pseudo-allergic reactions in humans (Callahan et al. 2020).

6.3.5 Genistein

Genistein is a non-steroidal polyphenol and possesses protective effects in inflammation, diabetes, cancer, and hypersensitivity reactions (Braxas et al. 2019; Liu et al. 2019; Sahin et al. 2019; Yeh et al. 2018). Genistein has also been reported for its anti-inflammatory activity on MCs (Kim et al. 2014). Genistein inhibits C48/80-triggered degranulation and Ca^{2+} mobilization in LAD2 cells or MRGPRX2-HTLA cells (Kumar et al. 2020). Moreover, genistein attenuates the blood vessel leakage and paw thickness in mice. Genistein binds at a different binding site compared with C48/80 and interacts with Cys258 and Cys95 via pi-sulfur bonds, Tyr89 and Asn85 hydrogen bonds, Pro262, Val88 pi-alkyl bond, and Tyr89 pi-pi T shaped bond, demonstrating a non-competitive antagonistic activity for MRGPRX2 (Kumar et al. 2020).

6.3.6 Shikonin

Shikonin, a natural naphthoquinone, is isolated from the root of *Lithospermum erythrorhizon Sieb. et Zucc, Arnebia euchroma (Royle) Johnst,* or *Arnebia guttata Bunge* that have an inhibitory effect on inflammation, virus infection, and cancer (Wang et al. 2019a). Shikonin also is a potential antagonist for MRGPRX2. Shikonin can suppress C48/80-induced pseudo-allergic reactions by inhibiting MCs activation. Shikonin suppresses C48/80-induced Ca^{2+} influx and degranulation in LAD2 cells via the PLCγ/PKC/IP_3 pathway (Wang et al. 2020a). Moreover, shikonin inhibits C48/80-induced increase of MRGPRX2 expression in HEK cells, displaying specific interactions with MRGPRX2 (Wang et al. 2020a). Shikonin

could be a potentially specific antagonist of MRGPRX2, inhibiting pseudo-allergic reactions.

6.3.7 Piperine

Piperine is a pungent nitrogenous alkaloid extracted from the fruit of *Piper longum L.*, *Piper nigrum L.,* and other *Piper* species that displays anti-inflammatory, antioxidant, hepatoprotective, and antiulcer activities (Chavarria et al. 2016; Srinivasan 2007). Piperine also has the antianaphylactoid effects. Piperine attenuated MCs degranulation induced by endogenous and exogenous substances, such as LL-37, C48/80, and ciprofloxacin. Piperine suppresses C48/80-induced PLCγ/IP_3R related intracellular Ca^{2+} mobilization and inhibits the release of histamine, PGD2, TNF-α, IL-31, IL-8, and MCP-1 in LAD2 cells or MRGPRX2-HEK293 cells (Qiao et al. 2020). Piperine can bind to MRGPRX2 as a specific antagonist and inhibit the stimulation by other agonists (Qiao et al. 2020).

6.3.8 Paeoniflorin

Paeoniflorin, a monoterpene glycoside, is extracted from the Chinese herb *Radix Paeoniae alba* and has antitumor and neuroprotective effects (Cong et al. 2019; Yang et al. 2018). Paeoniflorin also has inhibitory effects on C48/80-induced pseudo-allergic reactions both in vitro and in vivo. Paeoniflorin binds to the MRGPRX2 protein and downregulates the phosphorylation levels of key kinases in PLCγ-regulated calcium influx and MAPK or ERK-mediated cytokine synthesis pathways inhibiting MRGPRX2-induced pseudo-allergic reactions (Wang et al. 2020b). Paeoniflorin can serve as an MRGPRX2 antagonist in the treatment of pseudo-allergic and inflammatory diseases (Wang et al. 2020b).

6.4 Small-Molecule MRGPRX2 Antagonists

Hiroyuki Ogasawara found two novel small-molecule MRGPRX2 antagonists: compound 1 (1-(5H-10λ^2-phenazin-5-yl)ethan-1-one) and compound 2 (3-(pyridine-2-ylmethyl)-2-thioxo-2,3-dihydro-4H-1λ^2-quinazolin-4-one) (Ogasawara et al. 2019). Both compounds obstruct SP- and cortistatin-14-induced intracellular Ca^{2+} mobilization in MRGPRX2-HEK293 cells, inhibit the degranulation of human cord blood-derived MCs, and block the GTP-γl binding activities of a Gα protein just downstream of MRGPRX2, but did not block NK-1R or M2R-mediated intracellular Ca^{2+} mobilization (Ogasawara et al. 2019). These compounds serve as specific antagonists for MRGPRX2 without inhibitory activity to NK-1R or other Gi-coupled GPCRs such as M2R, opening up new avenues for the therapeutic

implications of human MRGPRX2 antagonists for the treatment of pseudo-allergic and inflammatory diseases.

6.5 MRGPRX2-Targeting Antagonistic DNA Aptamer

ssDNA aptamer drugs could be effective in the development of specific antagonists against targeted proteins and have many advantages over small molecules and antibody drugs. Aptamer-X35 inhibits C48/80- and SP-triggered histamine release of MRGPRX2-RBL-2H3 cells. Moreover, subcutaneous injection of aptamer-X35 inhibits an MRGPRX2-mediated anaphylactic shock in rats (Suzuki et al. 2020). Aptamer-X35, a ssDNA aptamer, has been identified as a potential new MRGPRX2 antagonist against pseudo-allergic reactions (Suzuki et al. 2020).

7 Balanced and Biased Ligands of MRGPRX2

GPCRs can signal via multiple transducers, including heterotrimeric G proteins, G protein receptor kinases, and β-arrestins. After ligand binding and G protein activation, the receptor is phosphorylated on its Ser/Thr residues at their carboxyl-terminus by GRKs and recruits adapter proteins β-arrestins, which have been implicated in the regulation of GPCR desensitization, endocytosis, and internalization (Benovic et al. 1986; Lohse et al. 1990). Most drugs that activate GPCRs are thought to "equally" target distinct signaling pathways mediated by different G proteins and β-arrestins. These agonists are known as balanced agonists (Smith et al. 2018). However, GPCR agonists that preferentially activate G proteins are known as G protein-biased and those which activate β-arrestin are known as β-arrestin-biased agonists (Smith et al. 2018; Urban et al. 2007). Ligands of MRGPRX2 are either balanced agonists or G protein-biased agonists. Lansu et al. used a novel high throughput β-arrestin activation assay to screen small molecules for MRGPRX2 activation. They found that C48/80, SP, cortistatin-14, PAMP (9–20), cetrorelix, complanadine A, and some opioids activate both β-arrestin-dependent gene expression and Gαq-mediated intracellular calcium release via MRGPRX2 and are balanced agonists. Some FDA-approved drugs such as mastoparan, kallidin, and icatibant only activate Gαq-mediated intracellular calcium release via MRGPRX2 resulting in injection-site pseudo-allergic drug reactions, but do not result in a β-arrestin expression (Lansu et al. 2017). AG-30/5C can induce Gαi-mediated Ca^{2+} mobilization and cause MCs degranulation without β-arrestin-mediated gene expression that serves as a G protein-biased agonist for MRGPRX2 in MCs and contributes to host defense. The molecular mechanism by which these ligands activate MRGPRX2 via G protein and β-arrestin signaling pathways is not clear. It is possible that balanced agonists (such as C48/80, SP, and cortistatin-14) and G protein-biased agonists (such as AG-30/5C and icatibant) induce different conformations of MRGPRX2 to activate different signaling pathways.

8 Summary

MRGPRs are very promising targets for MCs activation in host defense, immunomodulation, and the prevention and treatment of pseudo-allergic drug reactions and inflammatory diseases. Screening and classifying MRGPR agonists and antagonists and summarizing their signaling pathways would significantly improve our understating of MRGPR-mediated physiological and pathological effects on MCs. The MRGPRX2-mediated MCs activation induced by FDA-approved drugs, NPs, and MCDPs causes degranulation, releasing inflammatory mediators, and ultimately resulting in pseudo-allergic drug reactions, pain, itching, and inflammatory diseases. In addition to degranulation, AMP-mediated MCs activation induces the production of several chemokines and cytokines, contributing to host defense, immunomodulation, and wound healing. MRGPRX2 is also considered to be a therapeutic target for pseudo-allergic diseases. Some non-selective GPCR inhibitors, cytokines, herbal extracts, small-molecule MRGPRX2 antagonists, and ssDNA aptamer drugs are effective in inhibiting or blocking MRGPRX2-mediated signaling and have important clinical implication for modulating pseudo-allergic drug reactions and inflammatory diseases (Callahan et al. 2020; Chen et al. 2007; Ogasawara et al. 2019; Suzuki et al. 2020; Wang et al. 2020b).

Research into the mechanism of MRGPR-mediated MCs activation has made major strides in recent years. Diverse MRGPR ligands seem to utilize common sites on the receptors to induce MCs degranulation (Alkanfari et al. 2018). The activation of MRGPRX2 is both Gαq- and Gαi-coupled, and this coupling likely initiates phosphorylation cascades and induces rapid and transient Ca^{2+} mobilization, as well as MAPK and NF-κB pathway activation resulting in degranulation and generation of new inflammatory mediators. However, the signaling pathway of MRGPRX2-mediated MCs activation is not as clear as that of FcεRI-mediated MCs activation. Still, much work remains to be done to further explore the mechanism of MRGPRX2-mediated MCs activation and to perfect treatments and alleviate adverse reactions.

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Single-Cell Sequencing and Organoids: A Powerful Combination for Modelling Organ Development and Diseases

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Abstract The development and function of a particular organ and the pathogenesis of various diseases remain intimately linked to the features of each cell type in the organ. Conventional messenger RNA- or protein-based methodologies often fail to elucidate the contribution of rare cell types, including some subpopulations of stem cells, short-lived progenitors and circulating tumour cells, thus hampering their applications in studies regarding organ development and diseases. The scRNA-seq technique represents a new approach for determining gene expression variability at the single-cell level. Organoids are new preclinical models that recapitulate complete or partial features of their original organ and are thought to be superior to cell models in mimicking the sophisticated spatiotemporal processes of the development and regeneration and diseases. In this review, we highlight recent advances in the field of scRNA-seq, organoids and their current applications and summarize the advantages of using a combination of scRNA-seq and organoid technology to model diseases and organ development.

Keywords Development · Disease · Organoids · Single-cell sequencing

Abbreviations

3D	3 dimensional
APs	Apical progenitors
ASCs	Adult stem cells
BPs	Basal progenitors
CEL-seq1/2	Cell expression by linear amplification and sequencing
CF	Cystic fibrosis
ECs	Endothelial cells
FACS	Fluorescence-activated cell sorting
FGF-2	Fibroblast growth factor 2
hCOs	Cortical domain
hCSs	Human cerebral cortical spheroids
hMGEOs	Human brain organoids representing the MGE domain
IGF-1	Insulin-like growth factor 1
iPCs	Induced pluripotent stem cells
IVT	In vitro transcription
LCM	Laser capture microdissection
MARS-seq	Massively parallel RNA single-cell sequencing
MCs	Mesenchymal cells
MDS	Miller-Dieker syndrome

MoMLV RT	Moloney murine leukaemia virus reverse transcriptase
mRNA	Messenger RNA
NGC	Next-generation sequencing
O_2	Oxygen
OC	Ovarian cancer
PDAC	Patient-derived pancreatic ductal adenocarcinoma
PMA	Phi29 DNA polymerase-based mRNA transcriptome amplification
popRNA-seq	Population RNA sequencing
PSCs	Pluripotent stem cells
scRNA-seq	Single-cell messenger RNA sequencing
scRNA-seq	Single-cell sequencing
SSPCs	Somatic stem and progenitor cells
STRT-seq	Single-cell tagged reverse-transcription sequencing
SVZ	Subventricular zone
TGFB1	Transforming growth factor beta 1
TGFB2	Transforming growth factor beta 2
TTA	Total transcript amplification
VE	Vascular endothelial
VEGF	Vascular endothelial growth factor
VZ	Ventricular zone

1 Introduction

The identification and characterization of all cell types in an organ are crucial for understanding the development and function of the particular organ and the pathogenesis of various diseases, since individual cells may exert distinct effects, as exemplified, for instance, by different enteroendocrine cell types that secrete different hormones (Grun et al. 2015). Until recently, methods relying on messenger RNA (mRNA) or protein to identify and characterize subpopulations of cells mostly depended on a few known marker genes; nevertheless, these methods are less suitable for identifying rare cell types, for instance, certain stem cells, short-lived progenitors and circulating tumour cells (Grun et al. 2015). Recently, however, single-cell messenger RNA-sequencing (scRNA-seq) has emerged as a broadly applicable new approach to determining gene expression variability at the single-cell level (Hwang et al. 2018). From a unicellular perspective, scRNA-seq has been used for deconstructing tissue heterogeneity, identifying cells as intermediates of two different cell types, discovering rare and novel cell types and delineating cell differentiation pathways (Camp et al. 2015; Grun et al. 2015). Whereas most applications of scRNA-seq are tissue based and depend on the purity of cell clusters, the subsequent further isolation of discrete cell types by fluorescence-activated cell sorting (FACS) or other techniques can be used to characterize the functions of a cell type.

Both organoids cultured from pluripotent stem cells and adult stem cells are capable of regenerating most, if not all, cell types in the original organ and can be

used to study the complex spatiotemporal processes of development and regeneration and the molecular basis of diseases (Ayabe et al. 2018). Notably, organoids are suitable for studying certain aspects of the native organ that are virtually inaccessible in vivo; for instance, the response to specific hormones and growth factors, food components and microbiota-derived factors can be discerned and used to supplement culture medium (Ayabe et al. 2018; Beumer et al. 2018). Moreover, adult stem cell-derived organoids consist of pure epithelial cells and can be enriched for certain cell types by manipulating the composition of the culture medium, thereby avoiding challenges posed by the complexity of whole organs (Grun et al. 2015). Thus, organoids might circumvent some of the drawbacks of bodily tissues as cell resources for scRNA-seq.

In this review, we discuss the concepts, key features and current applications of scRNA-seq and organoid techniques and highlight and summarize the advantages of using a combination of scRNA-seq and organoid technology to model diseases and organ development.

2 Single-Cell Sequencing

2.1 Definition

What is scRNA-seq? Single-cell sequencing is an epoch-making technology used to obtain genetic information from single cells. Recently, single-cell sequencing has shown unprecedented accuracy for exploring biological processes and the pathogenesis of various diseases. In particular, scRNA-seq can be used to establish RNA expression profiles at the unicellular level and identify rare cell types in bulk cell populations or even in whole tissues. A variety of scRNA-seq types have been developed based on different platforms, and these scRAN-seq techniques and their applications were summarized in Table 1 (Boroviak et al. 2018; Chen et al. 2018; Chung et al. 2017; Dalerba et al. 2011; Hamza et al. 2019; Ho et al. 2018; Kim et al. 2015, 2016; Kumar et al. 2018; Lee et al. 2014; Messmer et al. 2019; Miyamoto et al. 2015; Suzuki et al. 2015; Tang et al. 2010a; Tirosh et al. 2016; Winterhoff et al. 2017; Wong et al. 2017; Wu et al. 2018; Yan et al. 2013).

2.2 The Advantage of scRNA-Seq

Owing to the spatiotemporal variations of epigenetic modifications and transcriptomes, different types of cells display features of functional diversity. For multicellular organisms, the heterogeneity of various cell populations can be easily observed and appreciated. However, in single cells of a similar type, determining the heterogeneity of gene expression and function has always been a challenge. In addition, technical limitations of gene chips or next-generation sequencing (NGS)

Table 1 Different types of single-cell RNA sequencing and their applications

Author	Year	Source	Platform	Significance
Circulating tumour cell				
Miyamoto et al.	2015	Prostate cancer	CTC-iChip and ABI SOLiD platform	Reveals the potential mechanism of drug resistance by separating CTC cells
Wong et al.	2017	Prostate cancer	CTC-iChip and SMARTer kit	New method of blood-based diagnostics
Hamza et al.	2019	Mouse small cell lung cancer	Microfluidic device and smart-Seq2	New method for collecting CTC cells
Embryogenesis				
Tang et al.	2010	Inner cell mass (ICM) cells	Tang method	Early genetic and epigenetic modification during embryogenesis
Yan et al.	2013	Human preimplantation embryos and human embryonic stem cells (hESCs)	Tang method	Transcriptome landscapes of early embryos and hESCs
Chen et al.	2018	Spermatogenic cells	FACS and smart-Seq2	Shows heterogeneity of mammalian spermatogenesis
Boroviak et al.	2018	Human, marmoset and mouse embryos	Manual and smart-Seq	Preimplantation development and early mammalian embryogenesis cross-species
Messmer et al.	2019	Naive and primed human pluripotent stem cells	FACS and smart-Seq2	Reveals the transcriptional landscape of human pluripotency of human ESCs
Drug resistance				
Kim et al.	2015	Lung adenocarcinoma	Fluidigm C1 and SMARTer kit	Identifies the rare cell population with drug resistance
Lee et al.	2014	Breast cancer	Micromanipulators and ovation RNA-Seq system	Demonstrates tumour heterogeneity of drug tolerance
Suzuki et al.	2015	Lung adenocarcinoma	Fluidigm C1 and SMARTer kit	Maps the transcriptome of individual cells after drug treatment
Kim et al.	2016	Metastatic renal cell carcinoma	Fluidigm C1 and SMARTer kit	To reduce transcriptional heterogeneity, design optimized the combination of targeted agents
Wu et al.	2018	Esophageal squamous cancer	Microscope and smart-Seq2	$KRT19^{high}$ subpopulation was found resistant to paclitaxel, combining with CFZ offers a novel method of treatment

(continued)

Table 1 (continued)

Author	Year	Source	Platform	Significance
Ho et al.	2018	Melanoma	Fluidigm C1 and 10x genomics	Studies tumour heterogeneity after drug treatment
Intra-tumour heterogeneity				
Dalerba	2011	Colon cancer	SINCE-PCR	Discovered novel markers and rare cell populations in colon tumors
Winterhoff et al.	2017	Ovarian cancer	Fluidigm C1 and SMARTer kit	Identified a rare population of cancer cells and enhances the understanding of drug resistance and progression of ovarian cancer
Tirosh et al.	2016	Metastatic melanoma	Smart-Seq2	Deals with tumour heterogeneity and immune cell status
Chung et al.	2016	Breast cancer	Fluidigm C1 and SMARTer kit	Demonstrates heterogeneous tumour cells and immune cells
Kumar et al.	2018	Syngeneic mouse tumour models	10x chromium controller (10x genomics)	Identifies communication networks by evaluating ligand receptor interactions

relate to the bulk quantities of DNA or RNA extracted from over 100,00 cells (Hwang et al. 2018). These techniques provide average global gene expression profiles, but cell-to-cell variations may be hidden. Intercellular heterogeneity is evident among different tissues but may also exist within the same tissue. For example, intestinal tissues harbour a large number of different cell types, and their lineages and differentiation states determine how the cell responses to surrounding cells and microenvironments. The application of scRNA-seq at an individual cell level provides crucial information about the origin, function, and individual variation of different cell types.

2.3 The Critical Steps of scRNA-Seq

2.3.1 Capturing Single Cells

Multiple steps are involved in scRNA-seq, including single-cell separation, reverse transcription, cDNA amplification and cDNA library preparation to prepare single-cell expression profiles (Haque et al. 2017). However, the most important steps are the effective isolation of single cells and cDNA amplification approaches. Although capturing single cells may seem like a trivial exercise, it is truly the main challenge to successful scRNA-seq and may include serial dilution (Reizel et al. 2011; Zhang et al. 2006), micromanipulation (Kvist et al. 2007), FACS (Stepanauskas and

Sieracki 2007), microfluidics (Marcy et al. 2007) and laser capture microdissection (LCM) (Frumkin et al. 2008; Yachida et al. 2010). FACS is a powerful technique that is used to separate individual cells or populations according to cell surface markers or other specific characteristics. This technique has the advantage of accurate cell sorting, being a mature technology, and offering standardized results. However, a major drawback results from single cells being easily contaminated during the sorting process, which has an impact on cell viability and status (Shapiro et al. 2013). Recently, the microfluidic technique has opened a new route for single-cell separation and analysis (Streets et al. 2014), as it combines multiple processes, including cell culture, single-cell capture, cDNA synthesis and sequence detection. Multiple platforms can handle this complicated task, including the Illumina Bio-Rad single-cell sequencing solution, BD Rhapsody single-cell analysis system, 10X Chromium single-cell gene expression solution, ICELL8 single-cell system and Fluidigm C1™ system. However, no perfect solutions are applicable for all cases, and the approach selected totally depends on the experimental design.

2.3.2 From Single Cells to a cDNA Library

After the capture, the single cells are lysed, and a droplet is formed. The first strand of cDNA is efficiently generated. Different approaches are used to synthesize the second strand of cDNA. These methods are roughly divided into two main methods: full-length amplification and strand-specific amplification.

According to the different amplification principles, full-length amplification is further divided into PCR-based amplification, in vitro transcription or rolling circle amplification.

PCR-Based Amplification

PCR is characterized by exponential amplification. After single-cell capture, lysis and mRNA reverse transcription, at the 3′ end of the first strand of cDNA, a 30-nt poly (A) tail is established prior to the initiation of second-strand cDNA synthesis (Lazinski and Camilli 2013). This method was first applied to scRNA-seq by Tang et al. (2009, 2010b). However, this approach has two main drawbacks. The first is premature transcription termination during the reverse transcription process, which leads to a partial loss of transcript information, resulting in 3′ coverage bias (Ramskold et al. 2012). To solve this problem, the template-switching mechanism is developed. Based on the intrinsic character of Moloney murine leukemia virus reverse transcriptase (MoMLV RT), 3–4 cytosines are added to the 3′ end of the first strand to serve as universal primers. Consequently, only full-length cDNA can be amplified, which guarantees transcript integrity. Based on this method, SMART-seq was developed and optimized in SMART-seq2 (Picelli et al. 2014). Single-cell tagged reverse-transcription sequencing (STRT-seq) is also based on this principle (Svensson et al. 2018).

T7-Based In Vitro Transcription (IVT) Amplification

Exponential cDNA amplification is prone to generate bias, while a linear amplification strategy solves this problem successfully. IVT amplification using T7 RNA polymerase in vitro creates linear DNA and is characterized by a relatively low error rate. Compared to the PCR-based amplification method, it is unnecessary to provide the second strand of cDNA with an anchor sequence. Within each round of amplification, the transcripts are inclined to become shorter than the original strands, increasing the risk of more intensive 3′ coverage bias. Recently, however, new optimized approaches have been developed, including cell expression by linear amplification and sequencing (CEL-seq1/2) (Hashimshony et al. 2012), massively parallel RNA single-cell sequencing (MARS-seq) (Picelli 2017) and Quartz-seq (Sasagawa et al. 2013).

Rolling Circle Amplification

Phi29 is a DNA polymerase cloned from the *Bacillus subtilis* phage Phi29. It is characterized by strand displacement and stability that allow efficient DNA synthesis for a relatively long time and is widely used for genome amplification. Pan et al. successfully amplified approximately 5,000 transcripts from single K562 cells (Pan et al. 2013). Interestingly, because of the few RNA sequences with poly(A) tails in prokaryotes, random primers may be used for cDNA amplification, which makes this approach appropriate for prokaryote genomes. In addition, other methods are based on Phi29 DNA polymerase-based mRNA transcriptome amplification (PMA) (Liu et al. 2014) and total transcript amplification (TTA) (Kang et al. 2011).

3 Organoids

The first organoid model was established in the Hubrecht Institute of the Royal Netherlands Academy of Arts and Sciences (Sato et al. 2009). Since then, various organoids of animal and human origin have been successfully cultured. Organoids are defined as in vitro models derived from stem cells and recapitulate most, if not all, characteristics of in vivo organs (Di Lullo and Kriegstein 2017). Two types of stem cells are used for organoid culture: pluripotent stem cells (PSCs) and adult stem cells (ASCs) (Clevers 2016). Organoid models are thought to be superior to cell lines since stable cell lines are tumour-based or immortalized, grow in 2 dimensions rather than in 3 dimensions and contain only a single cell type (Yin et al. 2019b). These immortalized cancer-derived cell cultures thus fail to represent the complicated multicellular structure of a native organ (In et al. 2016). Very recently, a report published in Nature Biotechnology stated that purportedly identical HeLa cell lines originating from different laboratories showed multiple variations, which may reduce the repeatability of results obtained from these cell lines (Liu et al. 2019).

In contrast, organoids were confirmed to contain most, if not all, of the cell types of the original organ, enable long-term culture, and grow as a three-dimensional (3D) structure, thus exquisitely mimicking the in vivo physiology (Sato and Clevers 2013). Notably, organoids are capable of maintaining genomic stability after long-term passaging (Sato et al. 2009). To date, a variety of organoid species have been established from different organs, including the intestine (Sato et al. 2009), liver (Hu et al. 2018), kidney (Schutgens et al. 2019), pancreas (Broutier et al. 2016) and brain (Qian et al. 2019), as well as their cancerous counterparts (Kopper et al. 2019).

Organoids have been broadly applied for disease modelling, drug testing, transcriptome/epigenome/proteome and metabolome analysis, targeted gene editing and host-microbiome interaction studies. The first successful application of organoids was in studies of cystic fibrosis (CF), for which the authors developed a high-throughput organoid swelling assay and applied it for diagnosis, functional investigations and personalized drug screening (Dekkers et al. 2013). Since then, many more applications of organoids have been reported, particularly in the fields of bacterial, viral and parasite infections, including for studying *Salmonella* (Yin and Zhou 2018; Zhang et al. 2014), *Escherichia coli* (Karve et al. 2017), *Shigella flexneri*, rotavirus (Yin et al. 2015), norovirus (Ettayebi et al. 2016), Middle East respiratory syndrome coronavirus (Zhou et al. 2017), Cryptosporidium (Heo et al. 2018) and *Toxoplasma gondii* (Delgado Betancourt et al. 2019). Intestinal organoids were reported to be suitable platforms for studying antiviral drugs and their mode of action (Yin et al. 2018a, b). Patient-derived organoids have been used for drug screening for multiple diseases (Saito et al. 2019). Intestinal organoids are also thought to be potential models for livestock studies (Yin et al. 2019c) and nutritional studies (Yin et al. 2019a). Importantly, organoids are widely used for modelling tumours, including colorectal cancer (Bolhaqueiro et al. 2019), ovarian cancer (Kopper et al. 2019), liver tumours (Tharehalli et al. 2019) and glioblastoma (Linkous et al. 2019). Interestingly, Kopper et al. cultured 56 organoid lines from 32 patients with ovarian cancer (OC) that covered all main subtypes of OC and harboured many histological and genomic characteristics of in vivo tumours, representing suitable resources for xenografts and a highly suitable preclinical models for testing drug sensitivity (Kopper et al. 2019). Collectively, organoid models open a new avenue to many biological and pharmacological studies.

4 The Combination of scRNA-Seq and Organoid Model Development, Regeneration and Diseases

Recently, a set of important studies using scRNA-seq and organoid technologies to model organ development, tissue regeneration and diseases has been published (Table 2), highlighting the potential importance of the combination of these two state-of-the-art techniques in this field.

Table 2 Applications of combination of organoids and scRNA-seq

Author	Year	Organoids	Finding
Discovery rare/novel cell type and gene markers			
Grün et al.	2015	Intestinal organoids	Identified Reg4 to be a gene marker of enteroendocrine cells and three novel subtypes of enteroendocrine cells
Beumer et al.	2018	Intestinal organoids	Intestinal organoids harboured a complete set of subtypes of enteroendocrine cells and identified certain unnoticed subtypes
Fujii et al.	2018	Intestinal organoids	The native cellular diversity of intestinal organoids was in accordance with in vivo intestinal epithelium at a single-cell stratification
Haber et al.	2017	Intestinal organoids	Illustrated unnoticed subtypes of intestinal epithelial cell and corresponding gene signatures and distinguished two novel subsets of tuft cells
Schwalie et al.	2017	Intestinal organoids	Two novel highly expressing genes (Lcp1 and Vgll4) in ISCs were identified
Lee et al.	2017	Lung organoids	Confirmed Lgr5+ and Lgr6+ as gene markers of mesenchymal cells in the adult lung
Wollny et al.	2016	Pancreas organoids	Identified a subtype of progenitor-like acinar cells expressing progenitor makers with self-renewal ability
Zepp et al.	2017	Lung organoids	Found that the Axin2-Pα lineage was able to generate more lung alveolar organoid and played a major role in homeostasis and regeneration of the lung
Recapitulate cellular heterogeneity of bodily organ			
Camp et al.	2015	Liver organoids	Illustrated the lineage relationships among cells, by sequencing 425 single-cell transcriptomes from distinct stages during differentiation of liver organoids
Peng et al.	2018	Liver organoids	Hepatocytes could be converted into 3D liver organoids, which recapitulated many aspects of bodily liver
Sloan et al.	2017	Brain oganoids	The hCSs contain astrocyte-lineage cells, indicating a highly similarity to bodily brain cells
Xiang et al.	2017	Brain oganoids	The hMGEOs and hCOs of human brain organoids could develop neuronal functions and display similar cellular structures in bodily MGE and cortex
Camp et al.	2015	Brain oganoids	The cortex-like domain of cerebral organoids recapitulated many features of in vivo fetal tissue
Delineate cell differentiation paths			
Camp et al.	2015	Brain oganoids	Brain organoids have a proliferative and self-renewing scenario that is exploited too during differentiation of radial glial types and the differentiation paths of AP- and BP-derived newborn neuron mirror those of homogeneous CP neurons in native brain
Czerniecki et al.	2018	Kidney organoids	VEGF was capable of promoting a tenfold increase of ECs expressing CD31 and VE-cadherin in kidney organoids

(continued)

Table 2 (continued)

Author	Year	Organoids	Finding
Identify gene expression variability at single-cell stratification			
Camp et al.	2015	Brain organoids	Parsed out similarities between organoids and fetal cerebral cortex and studies tissue heterogeneity by using expression profile of individual gene?
Ayabe et al.	2018	Liver organoids	The unicellular gene expression profile indicated that MCs of the liver express specifically TGFB2, while ECs and MCs express both TGFB1 and TGFB3
Modelling diseases			
Haber et al.	2017	Intestinal organoids	Salmonella preferentially caused accumulation of secretory cell types, while *Heligmosomoides polygyrus* led to accumulation of absorptive enterocytes and Paneth cells
Chen	2018	Human colorectal cancer organoids	Studies tumour heterogeneity and the response to chemotherapy
Romero-Calvo et al.	2019	PDAC organoids	scRNA-seq showed that patient-derived pancreatic ductal adenocarcinoma organoids harboured patient-specific genomic and transcriptomic features?
Bershteyn et al.	2017	Brain organoids	A mitotic defect was recognized in outer radial glia

4.1 Discovery of Rare/Novel Cell Types and Gene Markers

Several studies have demonstrated that the combination of organoid technology and scRNA-seq creates a powerful tool for discovering rare and novel cell types in an organ. For example, to better parse the cell composition of the intestinal epithelium, Gurn et al. sequenced 238 randomly selected cells from intestinal organoids harbouring 3,000 transcripts per cell and quantified more than five transcripts corresponding to 3,777 genes in a single cell (Grun et al. 2015). In this study, the authors developed a RaceID algorithm with reliable specificity and sensitivity, allowing them to identify Reg4 as a new and reliable gene marker of enteroendocrine cells and three novel subtypes of enteroendocrine cells (Grun et al. 2015). Importantly, in retrospect, they realized that these newly discovered subtypes of enterochromaffin cells also existed in the native intestine (Grun et al. 2015). scRNA-seq confirmed that the intestinal organoids harboured a complete set of enteroendocrine cells, including subtypes that had been previously unnoticed (Beumer et al. 2018). Clearly, the use of scRNA-seq and organoids is capable of simplifying and refining the taxonomy of enteroendocrine cells (Beumer et al. 2018). Further, by modifying the composition of the culture media of intestinal organoids, the composition of cell types can be altered, and novel cell types might be defined on the basis of scRNA-seq (Beumer et al. 2018). Fujii et al. refined the intestinal organoid culture medium by supplementing it with insulin-like growth factor 1 (IGF-1) and fibroblast growth factor 2 (FGF-2) (denoted as IF medium), which was capable of enhancing the clonogenic capacity and CRISPR-genome engineering efficiency of human

intestinal stem cells (Fujii et al. 2018). With cells from IF-cultured human small intestinal organoids, scRNA-seq data revealed that the native cellular diversity was well conserved and in accordance with the composition of native intestinal epithelium at the unicellular level (Fujii et al. 2018). Haber et al. parsed out 53,193 individual cells from the native small intestine and from intestinal organoids of mice using scRNA-seq, identifying previously unnoticed subtypes of intestinal epithelial cells with novel gene signatures (Haber et al. 2017). On this basis, two novel subsets of tuft cells were distinguished (Haber et al. 2017). Based on scRNA-seq, Schwalie et al. developed a computational model that can be used to accurately identify somatic stem and progenitor cells (SSPCs) from differentiated cells in many tissues (Schwalie et al. 2017). In this study, two novel genes highly expressed in ISCs were identified, Lcp1 and Vgll4, and the knockdown of these genes by shRNA suppressed the growth of intestinal organoids (Schwalie et al. 2017). Lee et al. used scRNA-seq and organoid cultures to confirm Lgr5+ and Lgr6+ as gene markers of mesenchymal cells in the adult lung (Lee et al. 2017). Wollny et al. combined multicolour lineage tracing, scRNA-seq, and pancreas organoid-formation assays to determine a subtype of progenitor-like acinar cells expressing progenitor markers and displaying self-renewal ability. These new cell types eventually differentiated into mature acinar cells and served as a pool of replenishing new acinar cells to maintain pancreas homeostasis (Wollny et al. 2016). To identify tissue-specific mesenchymal lineages and the molecular pathways, Zepp et al. performed scRNA-seq and population RNA sequencing (popRNA-seq) on 5,572 lung mesenchymal cells and partitioned them into five different clusters. Among these clusters, the Axin2-Pα lineage was able to generate more lung alveolar organoids compared to other lineages, indicating that this lineage may serve as a mesenchymal alveolar niche cell that contributes to homeostasis and the regeneration of lung tissue (Zepp et al. 2017). Thus, the combination of scRNA-seq and organoid technology has been shown to offer a powerful platform to identify rare/novel cell types and gene markers in an organ.

4.2 Recapitulation of the Cellular Heterogeneity of Bodily Organs

A proper understanding of the cellular heterogeneity of an organ is conditional for revealing the underlying development process and the pathogenesis of diseases. The dissection of the hierarchy and functional diversity of stem cells and their differentiated offspring is indispensable and a challenge in developmental biology (Tang et al. 2017). The 3D organoids provide an exquisite tool for probing the cellular heterogeneity of an organ, which circumvents the drawbacks of conventional in vitro 2D models that fail to mimic the cell interactions taking place at the organ level (Camp et al. 2015). The development of both organoid and scRNA-seq techniques rapidly advanced the investigation into the cellular heterogeneity of an organ. Camp

et al. cultured 3D liver bud organoids using induced pluripotent stem (iPS) cells and performed scRNA-seq to confirm the remarkable consistency between the 3D liver bud organoids and foetal liver cells, specifically showing that the 3D liver bud organoids were capable of recapitulating hepatic, stromal and endothelial interactions (Camp et al. 2015). By sequencing 425 single-cell transcriptomes from distinct stages during the differentiation of liver bud organoids, Camp et al. revealed the key lineage relationships among the cells (Camp et al. 2015). Strikingly, by applying scRNA-seq using the 10X Genomics Chromium system, it was found that TNFα, an injury-induced inflammatory cytokine, was capable of promoting the hepatocyte content of 3D liver organoids, indicating that they could recapitulate many aspects of in vivo liver regeneration after injury at the organoid level (Peng et al. 2018). Sloan and colleagues cultured human cerebral cortical spheroids (hCSs) containing astrocyte-lineage cells using pluripotent stem cells that were subjected to high-depth bulk and single-cell RNA sequencing and reported high similarity to native brain cells (Sloan et al. 2017). Moreover, to refine the identification of cell types and analyse the diversity of hCS-derived glial cells, hCSs were subjected to scRNA-seq at various time points, and the results showed that astrocytes changed from being in a predominantly foetal state to increasingly advancing towards a mature state (Sloan et al. 2017). To better profile the system heterogeneity and development process of the brain, human brain organoids representing the MGE domain (hMGEOs) and cortical domain (hCOs) were successfully cultured from human pluripotent stem cells. Using scRNA-seq, the hMGEOs and hCOs were shown to be capable of efficiently developing neuronal functions and displayed similar cellular structures as observed in MGE and the cortex in the body (Xiang et al. 2017). In a similar study, scRNA-seq showed that the cortex-like domain of cerebral organoids recapitulated many features of in vivo foetal tissue (Camp et al. 2015). Thus, the combination of organoid technology and scRNA-seq opens a new opportunities for studying the cellular heterogeneity of native organs.

4.3 Delineation of Cell Differentiation Pathways

The scRNA-seq technique is able to provide single-cell-based signatures of distinct cell types in an organ, which is useful for determining the intermediates and surrogates of cell types, thus realizing the computational delineation of differentiation pathways. A recent scRNA-seq study revealed the development process of human cerebral and cerebral organoids cultured from iPSCs and delineated cell differentiation pathways using Monocle (an algorithm for analyzing cell lineage relationships) to illustrate a path linking apical progenitors (APs) to neurons (e.g. indirect neurogenesis) through EOMES-expressing basal progenitors (BPs) (Camp et al. 2015). Nevertheless, Monocle failed to completely map the number of proliferative and self-renewing cell divisions within the progenitor pools. Thus, an

adjacency network based on pairwise correlations between cells was generated, which succeeded in mimicking the differentiation pathways of neurogenesis, a few cases of direct neurogenesis from APs to BPs and bidirectional paths between the APs in the ventricular zone (VZ) and BPs in the subventricular zone (SVZ). It accurately reflected the proliferative and self-renewing scenario to differentiate radial glial types and the differentiation pathways of AP- and BP-derived newborn neurons integrated into homogeneous CP neurons in the bodily brain (Camp et al. 2015). Thus, scRNA-seq demonstrated that cerebral organoids were capable of exquisitely mimicking in vivo neuron differentiation pathways. The scRNA-seq technique has also been applied to kidney organoids derived from human pluripotent stem cells. In this study, more than 10,000 cells were sequenced, revealing the spectrum of differentiation and maturation states of kidney organoids, which mainly consisted of early tubular cells and early podocytes (Czerniecki et al. 2018). Notably, the scRNA-seq technique confirmed that supplementation with vascular endothelial growth factor (VEGF) was capable of promoting a tenfold increase in endothelial cell (EC) expression of CD31 and vascular endothelial (VE) cell expression of cadherin in kidney organoids. Therefore, the combination of scRNA-seq and organoid technology may be used advantageously for discovering chemicals that can govern organoid differentiation pathways and build more sophisticated kidney organoids containing glomerular basement membranes (Czerniecki et al. 2018). Collectively, the combination of scRNA-seq and organoid technology has great promise as a powerful tool for modelling cell differentiation pathways in organ development and disease processes.

4.4 Identification of Gene Expression Variability at the Single-Cell Level

The analysis of gene expression at the single-cell level provides a powerful platform for ascertaining tissue heterogeneity and development (Camp et al. 2015). In a study, human cerebral organoids were subjected to scRNA-seq to obtain gene expression profiles of individual cells. This information was used to parse the similarities between organoids, the foetal cerebral cortex and native tissue, demonstrating that cerebral organoids can be used as powerful models to recapitulate the development and evolution of neocortical cell biology (Camp et al. 2015). In an attempt to dissect the effect of oxygen (O_2) on intercellular communication during hepatogenesis in the liver and in liver bud organoids derived from human pluripotent stem cells, a recent scRNA-seq-based study showed that hypoxia was able to enhance hepatic differentiation and concomitantly inhibit the expression of transforming growth factor beta 1 (TGFB1) and TGFB3; nevertheless, extensive hypoxia increased TGFBs and cholangiocyte marker expression (Ayabe et al. 2018). Notably, scRNA-seq captured the unicellular gene expression profile, indicating that the mesenchymal cells (MCs)

of the liver specifically express the transforming growth factor beta 2 (TGFB2) gene, whereas the endothelial cells (ECs), as well as the MCs, preferentially expressed both TGFB1 and TGFB3 (Ayabe et al. 2018). As exemplified by these studies, the combination of scRNA-seq and organoids provided a new way to discern the variability of gene expression at the single-cell level, which has deepened the understanding of organ development and disease states.

4.5 Modelling Diseases

Distinct cell subpopulations may exert particular functions in disease. Ascertaining the specific roles of individual cell types in diseases might lead to precision medicine. Strikingly, Haber et al. profiled Salmonella- and *Heligmosomoides polygyrus*-infected intestinal organoids with scRNA-seq and showed that Salmonella infection preferentially caused the accumulation of secretory cell types, while the *Heligmosomoides polygyrus* infection resulted in the accumulation of absorptive enterocytes and Paneth cells (Haber et al. 2017). In addition to organoids cultured from healthy adult stem cells or pluripotent stem cells, they can also be cultured from patient-derived stem cells, which accurately reflect the biological underpinnings of a particular disease. The combined utilization of patient-derived pancreatic ductal adenocarcinoma (PDAC) organoids and scRNA-seq showed that these organoids harbour patient-specific genomic and transcriptomic profiles (Romero-Calvo et al. 2019). Importantly, three clusters were classified based on the information obtained by scRNA-seq: cluster 1 comprised 97.2% of the total cell population, cluster 2 contained 0.9% of all cells and expressed genes (amphiregulin and epiregulin) involved in tumorigenesis and cluster 3 contained 1.9% of the cell population and expressed the pancreatic cancer stem cell gene marker Prominin-1 (Romero-Calvo et al. 2019). In another study, cerebral organoids were cultured from control and Miller-Dieker syndrome (MDS)-induced pluripotent stem cells (iPSCs) and subjected to scRNA-seq, revealing a mitotic defect in outer radial glia (Bershteyn et al. 2017). Notably, the correction of MDS-causing chromosomal deletions and severe apoptosis of founder neuroepithelial stem cells greatly alleviated the cell migration defect and augmented horizontal cell division in the cerebral organoids (Bershteyn et al. 2017). Collectively, the combination of organoid and scRNA-seq technology holds great promise for modelling diseases, precision medicine and preclinical drug treatment studies.

5 Limitations of Organoid Models

Organoids show profound advantages in modelling development, diseases, stem cell physiology and drug discovery. However, organoid models have several limitations: (1) Incomplete differentiation. For instance, it was reported that kidney organoids

cultured from human pluripotent stem cells contained immature tubules and vasculature, which weakened their ability to mimic the native kidney (Czerniecki et al. 2018). (2) Inaccessibility to the polarized layer. In intestinal organoids, cells polarize towards the inside of the lumen of the organoid, and this enclosed geometry generates a diffusion barrier that may require complicated operation (i.e. microinjection) to deliver drugs to the lumen, thus limiting throughput and their utility for drug development. (3) Furthermore, the information obtained from scRNA-seq may be noisy, as exemplified by the distinct expression of many genes in cells of foetal versus human cerebral organoids (Camp et al. 2015). (4) Certain genes may not be detected by scRNA-seq but remain identifiable by other sequencing methods, such as bulk RNA-seq (Czerniecki et al. 2018). (5) scRNA-seq lacks sufficient sensitivity for recognizing all expressed genes in a given cell; thus, other sequencing methods, such as popRNA-seq, may be needed to identify the unique lineage markers (Zepp et al. 2017). Therefore, more efforts should be invested in rendering more accurate scRNA-seq results and generating organoids that more closely reflect in vivo conditions.

6 Conclusions and Perspectives

A combination of state-of-the-art technologies, the culturing of organoids and scRNA-seq, has proven to be a powerful tool for modelling development, regeneration and diseases. The general process of executing this superior combination of techniques encompasses several steps: (1) culture of organoids from various types of stem cells; (2) separating organoids into good-quality single cells (cell viability $\geq$70%) and executing 10X Genomics scRNA-seq; and (3) applying a cyber-based algorithm to characterize gene expression at the single-cell level, which can eventually lead to the discovery of rare/novel cell types and gene markers, recapitulation of the cellular heterogeneity of native organs, delineation of cell differentiation pathways, identification of gene expression variability at the single-cell level and generation of disease models (Fig. 1).

With the development of organoid technology and scRNA-seq, the limitations of each technology can be compensated. For example, Transwell or organ-on-a-chip-based monolayers of intestinal organoids exposes the polarized epithelium side to drugs (Kozuka et al. 2017). Greater understanding of stem cell biology enables the accurate regulation of the differentiation pathways in organoids (Beumer and Clevers 2016). Advances in scRNA-seq techniques have increased analysis quality, efficiency and accuracy (Keren-Shaul et al. 2019). Therefore, the rapid increases in strong evidence supports the fact that the combination of organoids and scRNA-seq will provide a powerful avenue for studying organ development and diseases.

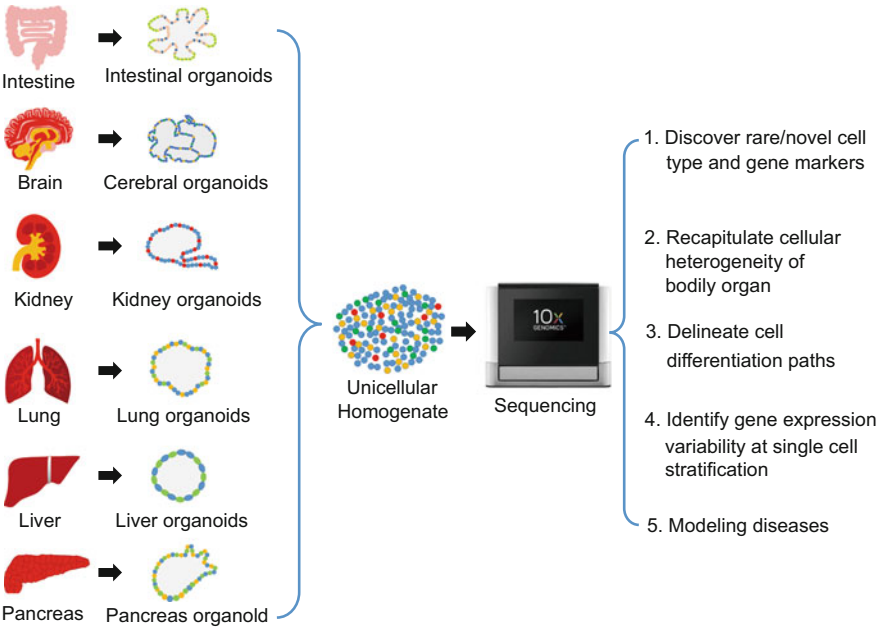

Fig. 1 A schematic diagram depicting the use of a combination of organoid technology and scRNA-seq in modelling organ development and diseases. Various types of organoids are cultured from distinct organs, dispersed into single cells and subjected to 10X Genomics scRNA-seq, followed by the application of cyber-based algorithms for several purposes, including (1) the discovery of rare/novel cell and gene markers, (2) analysis of cellular heterogeneity of native organs, (3) delineation of cell differentiation pathways, (4) identification of gene expression variability at the single-cell level and (5) modelling of diseases

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